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Development and Use of Chlorotetracycline Fluorescence as a Measurement Assay of Chloroplast Envelope-Bound Mg^{2+} [1]

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ABSTRACT

Experiments were conducted to develop chlorotetracycline (CTC) fluorescence as an assay of Mg^{2+} bound to the envelope of the intact chloroplast. This assay technique has been widely used to measure envelope associated divalent cations in animal cell and subcellular systems, but has not been used with chloroplasts. Chloroplast envelope-associated Mg^{2+} was altered by pretreatment with Mg^{2+} and divalent cation chelating agents and by additions of Mg^{2+} to the CTC assay medium. Results indicated that for a given chloroplast preparation, relative changes in envelope-associated Mg^{2+} can be effectively monitored with CTC fluorescence. It was concluded that the limitations of this assay system are: (a) chlorophyll strongly quenches CTC fluorescence signal, so a constant chlorophyll concentration must be maintained, (b) measurements must be made quickly, and (c) use of the technique to compare different chloroplast preparations may not be valid. Studies with $^{28}Mg^{2+}$ confirmed our interpretation of the fluorescence results, and also suggested that the chloroplast envelope is fairly impermeable to Mg^{2+}. It was concluded that changes in Mg^{2+} associated with the chloroplast due to incubation of plastids in solutions containing up to 5 millimolar Mg^{2+} may be exclusively due to increased envelope-associated Mg^{2+}. The CTC assay was used in experiments to demonstrate that increases in chloroplast envelope-associated Mg^{2+} inhibit photosynthetic capacity. This inhibition can be partially overcome by the presence of K^+ in the photosynthetic reaction media.

The fluorescence emission of CTC[2] has been used to measure the level of divalent cations (specifically Mg^{2+} and Ca^{2+}) associated with envelope membranes in a wide range of animal cells and subcellular particles such as erythrocytes (23), pancreatic acinar (6) and islet (24) cells, brain synaptosomes (3), liver mitochondria (4, 16), and sarcoplasmic reticulum vesicles from skeletal muscle (18). The utilization of CTC for these measurements is supported by the following conclusions. CTC preferentially forms a complex with Ca^{2+} or Mg^{2+} bound to the membrane phase (*cf.* 3). CTC complexes divalent cations in a 1:1 ratio (5). Also, the fluorescence quantum yield of the complex increases in apolar, in comparison to aqueous environments (16). These aspects of the physical interaction of CTC and cations results in an extremely sensitive probe which has been shown to increase in fluorescence emission by up to two orders of magnitude when CTC binds to membrane-associated Ca^{2+}, as compared to fluorescence of CTC alone (22).

The use of CTC as a probe in the aforementioned studies has contributed to the elucidation of the role of membrane-associated Ca^{2+} and Mg^{2+} in the regulation of metabolism and physiological function in a range of animal systems. The role of membrane-bound Ca^{2+} and Mg^{2+} in regulating physiological function is widespread in the animal literature. For example, pancreatic cell insulin secretion may involve membrane-associated Ca^{2+} (24). External Mg^{2+} interaction with the cell membrane has been associated with the maintenance of normal ion transport and electrical activity in the heart muscle (15). Membrane-associated Mg^{2+} has been found to have a regulatory role on liver mitochondrial membrane integrity (2). This last regulatory function may involve divalent cation regulation of a K^+/H^+ exchange mechanism in the mitochondrial membrane (13).

For well over a decade, the chloroplast envelope has been known to be fairly impermeable to Mg^{2+} (8, 11). However, external Mg^{2+} has been shown to have profound effects on the photosynthetic capacity of isolated chloroplasts (8, 12, 17). These studies have indicated that exogenous Mg^{2+} can facilitate K^+ efflux and H^+ influx into the stroma of the isolated chloroplast; photosynthesis is extremely sensitive to stromal pH. The mechanisms mediating this regulatory role of extra-chloroplastic Mg^{2+} are unknown. However, we speculate that high concentrations of external Mg^{2+} may result in an increased amount of chloroplast envelope-bound Mg^{2+}, and that it is the membrane associated Mg^{2+} that has a regulatory function.

In the research reported in this article, we investigated the usefulness of CTC fluorescence to probe Mg^{2+} associated with the chloroplast envelope. Several factors were considered at the outset which could limit the effectiveness of CTC as a probe of chloroplast envelope-associated Mg^{2+}. The quenching of the fluorescence signal by thylakoid pigment absorbance could diminish the membrane-associated increase in CTC-divalent cation complex fluorescence emission. As CTC fluorescence is substantially more sensitive to membrane-associated Ca^{2+} than Mg^{2+} (23), it was speculated that endog-

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[2] Abbreviation: CTC, chlorotetracycline.

enous envelope Ca^{2+} could mask changes induced by exogenous Mg^{2+} when it was bound to the envelope. Chloroplast envelope permeability to CTC could result in access to thylakoid membranes, and stromal and intrathylakoid pools of Mg^{2+}, thus masking changes in CTC fluorescence dependent on interaction with chloroplast envelope-associated Mg^{2+}. Research was undertaken to address these possibilities. Experiments were performed to characterize the usefulness of using CTC fluorescence to measure chloroplast envelope-associated Mg^{2+}. The CTC measurement technique was compared with measurements using $^{28}Mg^{2+}$. Finally, preliminary studies were undertaken to study envelope-associated Mg^{2+} effects on photosynthesis.

MATERIAL AND METHODS

Chloroplast Isolation

Intact (routinely greater than 90%) chloroplasts were isolated from spinach (*Spinacia oleracea* L. var 'Melody') plants grown in a growth chamber with a 10 h light (250 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at plant height) period maintained at 21°C (day)/16°C (night). Plants were grown in 1:1 peat/vermiculite potting mix. Pots were watered two times per week with water, and once per week with commercial (Peter's Geranium Special) fertilizer. Plants were generally used after 6 to 8 weeks of growth. Deribbed leaves (8–12 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 $MgCl_2$. After grinding twice for 3 s, the leaf homogenate was filtered through four layers of cheesecloth and two layers of Miracloth (Calbiochem) into two 50 mL centrifuge tubes, and centrifuged for 50 s at 750 g in an SS34 rotor (using a Sorvall RC5B centrifuge). Supernatants were decanted, and the pellets resuspended in a total of 15 mL of grind medium. In some experiments (as indicated in the text) this 15 mL resuspension was used to wash chloroplasts in grind medium with specific additions. In these "wash" experiments, the chloroplasts were incubated for 5 min, and recentrifuged at 700*g* for 50 s. After washing in 15 mL grind medium with appropriate additions and recentrifugation, the pellets were resuspended in 15 mL grind medium (with no additions). After the first resuspension in 15 mL grind medium (for standard isolation) or after the last resuspension in grind medium with no additions (in the wash experiments), the chloroplasts were layered on 15 mL of grind medium with 40% Percoll (Pharmacia) and 0.2% BSA in 50 mL centrifuge tubes. The tubes were then centrifuged in a swinging bucket (HB4) rotor at 2000*g* for 1 min. Supernatants were aspirated off, and the pellets were resuspended in a small amount of grind medium.

CTC Fluorescence Measurements

All measurements were made using a Aminco-Bowman spectrophotofluorometer. Excitation and emission wavelengths of 390 nm and 520 nm, respectively, were used for all studies. Schneider *et al.* (23) have previously found maximal emission wavelengths of 530 nm and 520 nm, for Ca^{2+}-CTC and Mg^{2+}-CTC in the presence of red blood cell membranes. Fluorescence dependent on quinine sulfate was regularly checked over the course of the study. These calibration curves all fell on the same line, indicating that the differences in CTC-Mg^{2+} fluorescence in the presence of chloroplasts recorded in different experiments were not due to varying sensitivity of the spectrophotofluorometer over the course of the study.

Unless otherwise noted, all fluorescence measurements were made within a period 15 s after addition of chloroplasts to a reaction mixture containing CTC. Fluorescence measurements were made in a reaction mixture (the standard medium used for photosynthesis assays in this laboratory) containing 50 mM Hepes-NaOH pH 7.6, 0.33 M sorbitol, 2 mM Na_2EDTA, 1 mM $MgCl_2$, and 1 mM $MnCl_2$. No attempt was made to alter the components of this reaction mixture to optimize relative fluorescence in the presence of other additions (such as Mg^{2+} or chloroplasts) to this mixture. Initial experiments also included 0.25 mM NaH_2PO_4, 1000 units/ml catalase, and 5 mM $NaHCO_3$. However, these last three components (used specifically for photosynthesis assays) had no effect on fluorescence measurements and were deleted from the reaction mixture used in later studies. Fluorescence measurements were made in a 1 cm × 1 cm cuvette containing a total of 3 mL reaction medium which included 50 μM CTC and chloroplasts equivalent to 15 μg Chl unless otherwise noted. The CTC was added to the cuvette from a 1 mM stock solution (in water) made fresh every day.

Photosynthesis

Photosynthetic rates of chloroplast preparations were ascertained by measuring O_2 evolution at 25°C and 1500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR with Hansatech DW1 O_2 electrodes (Decagon Devices, Pullman, WA). The fluorescence assay reaction medium was used, along with 5 mM $NaHCO_3$, 0.25 mM NaH_2PO_4, and 1000 units/mL catalase. Total volume was 1 mL, and chloroplasts equivalent to approximately 25 μg Chl were used for each assay.

$^{28}Mg^{2+}$ Studies

Treatment effects on Mg^{2+} associated with the chloroplast were monitored by incubating chloroplasts with $^{28}Mg^{2+}$, centrifuging chloroplasts through a Percoll cushion, and then resuspending the pellets in standard grind medium. Chloroplasts (200 μL/tube) were then separated from the suspending medium by centrifugation through silicone oil (100 μL) into 20 μL of 14% (v/v) perchloric acid in 0.4 mL microcentrifuge tubes. The silicone oil mixture was a ratio (by weight) of 0.1416:0.6917:0.1667 of 200 (2 cs):550:710 Dow Corning oils. $^{28}Mg^{2+}$ associated with the chloroplast pellet and incubation media was ascertained using a liquid scintillation spectrophotometer (Beckman 3801) assuming 100% counting efficiency. In all $^{28}Mg^{2+}$ experiments, $^{28}Mg^{2+}$ pelleted through the silicone oil in the sorbitol permeable space of chloroplasts was accounted for by assuming that supernatant $^{28}Mg^{2+}$ equivalent to 30 μL/mg Chl was associated with the chloroplast pellet. This value was never more than 8% of the total $^{28}Mg^{2+}$ in the chloroplast pellet. Corrections were made for radionuclide decay during the counting period.

Reagents

CTC was obtained from ICN Biochemicals (Cleveland, OH), silicone oils from William F. Nye Co. (New Bedford, MA), $^{28}MgCl_2$ from Brookhaven National Laboratory (through Martin Marietta Energy Systems, Memphis, TN), Hepes from Research Organics (Cleveland, OH), and A23187 from Calbiochem (San Diego, CA). All other reagents were obtained from Sigma (St. Louis, MO).

RESULTS AND DISCUSSION

CTC Fluorescence in the Presence of Chloroplasts

When CTC is added to a reaction mixture containing intact chloroplasts, fluorescence emission can be stable for as short as 20 to 40 s (Fig. 1). After this time, fluorescence increases dramatically. One possible explanation for this effect is that CTC rapidly penetrates the chloroplast envelope, and then slowly penetrates the thylakoid lamellae; with increasing fluorescence occurring due to CTC interaction with membrane sites in the thylakoid lumen. It should be noted that in some experiments, the stable phase of CTC fluorescence as shown in Figure 1 can be as long as several minutes (data not shown). Data presented in Figure 2 indicate that the phase of rapid increase in fluorescence as shown in Figure 1 is not coincident with CTC entry into the thylakoid lumen. In this experiment, CTC was added to chloroplasts which were hypotonically lysed by incubation in reaction medium lacking sorbitol. Therefore, the assay shown in Figure 2 exposed naked thylakoids immediately to CTC. No rapid increase (after a stable phase) of fluorescence was evident over the time course studied. Conversely, there was a slight, but steady decrease in fluorescence with time. When a comparison was made of CTC fluorescence from four separate chloroplast preparations in the presence or absence of sorbitol in the reaction medium, CTC fluorescence in the presence of the thylakoids was consistently somewhat lower than with intact plastids (data not shown). These data, along with the experiments shown in Figures 1 and 2 suggest that initial CTC fluorescence readings (during the 20–40 s phase of stable emission) in the presence of intact plastids allow for measurement of divalent cations associated with the chloroplast envelope. Therefore, in all subsequent experiments, CTC fluorescence measurements were made within 15 s of adding CTC to the reaction medium. The rapid rise in CTC fluorescence after the initial stable phase with intact chloroplasts (Fig. 1) likely is due to CTC entry into the stroma, and subsequent interaction with stromal Mg^{2+} and membrane systems within the chloroplast.

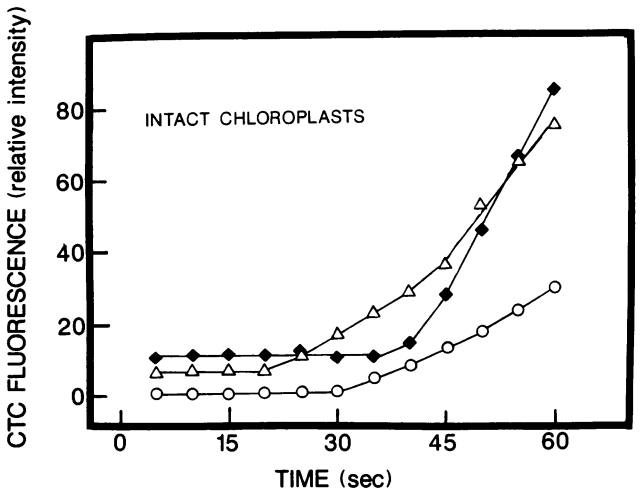

Figure 1. CTC (50 μM) fluorescence in the presence of intact chloroplasts (15 μg Chl/3 mL). Different symbols represent studies using different chloroplast preparations. CTC was added to the reaction media at 0 s. Each data point represents a single measurement.

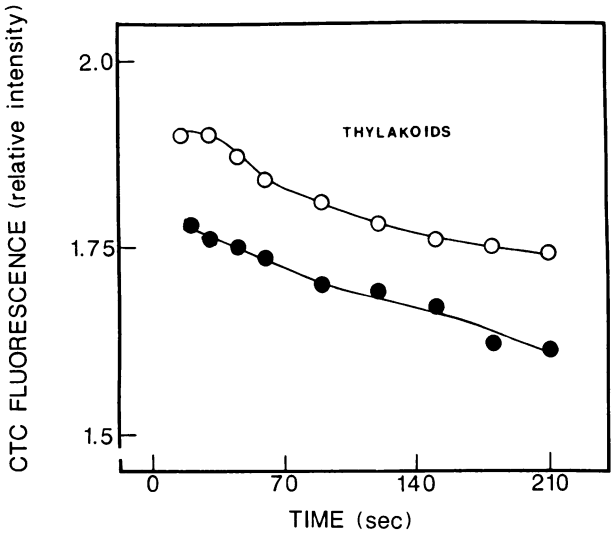

Figure 2. CTC (50 μM) fluorescence in the presence of free thylakoids. Chloroplasts (15 μg/3 mL) were incubated in reaction medium lacking sorbitol. CTC was added to the reaction media at 0 s. Results from two separate experiments (chloroplast preparations) are shown. Each data point represents a single measurement.

Table I. *Effect of Chloroplast Concentration on CTC Fluorescence*

Each value represents the mean of a single measurement taken in two separate experiments. For each experiment, all measurements were taken using aliquots from the same chloroplast preparation. CTC fluorescence was measured using the standard reaction medium, or the standard reaction medium with an additional 2 mM Mg^{2+}. CTC fluorescence was measured in the presence of chloroplasts equivalent to 7.5, 15, or 30 μg Chl/3 mL.

Treatment	CTC Fluorescence with Chloroplast Concentration (μg Chl/3 mL):		
	7.5	15	30
	relative intensity units/μg Chl		
Control	0.297	0.133	0.054
$+Mg^{2+}$	2.200	0.954	0.382

Data present in Table I indicate that the presence of chloroplasts in the reaction medium does result in some quenching of CTC fluorescence emission. Increasing chloroplast concentration in the reaction medium results in less fluorescence signal per unit Chl. This quenching of CTC fluorescence signal occurred in standard reaction medium, and also when 2 mM Mg^{2+} was added to the reaction medium (Table I). In all

subsequent experiments, Chl was maintained at 15 μg/3 mL for each assay.

Mg^{2+} Effects on CTC Fluorescence

In the absence of membranes (i.e. chloroplasts), fluorescence can be detected when CTC is added to reaction medium with Mg^{2+} (Fig. 3). In the reaction medium used in these studies a CTC concentration of 100 μM results in near-saturation, although fluorescence in the presence of 50 μM CTC is almost the magnitude found at 100 μM (Fig. 3). Despite the fact that Chl quenches CTC fluorescence (Table I), and CTC fluoresces in the presence of Mg^{2+} with no chloroplasts present (Fig. 3), external Mg^{2+} interaction with chloroplast envelopes can be detected by CTC fluorescence (Fig. 4). In these experiments, any fluorescence due to CTC interaction with native envelope-bound cations (*i.e.*, the Mg^{2+} and/or Ca^{2+} already on the chloroplast envelope) was blanked out at each CTC concentration. Therefore, at each CTC concentration, with no Mg^{2+} added to the reaction medium, fluorescence emission was zero. In the experiment shown in Figure 4, chloroplasts were incubated with Mg^{2+} at several CTC concentrations. At any Mg^{2+} concentration, increasing CTC resulted in increased fluorescence. We chose 50 μM CTC as a standard concentration for all subsequent studies. In previous studies with other organelles and cells, the CTC concentration used to measure membrane-associated Mg^{2+} and Ca^{2+} has varied from 10 μM (4, 24) up to 100 μM (6, 23).

The data shown in Figure 4 indicate that increasing reaction medium Mg^{2+} resulted in increased envelope-associated Mg^{2+}. The closed symbols in Figure 4 represent the fluorescence measured at 100, 50, and 12.5 μM CTC in the presence of 2 mM Mg^{2+} added to the reaction medium with no chloroplasts present. With 2 mM Mg^{2+} added to the reaction medium, fluorescence emission at each CTC concentration was more than an order of magnitude greater when chloroplasts were included in the reaction medium. It should be noted that these two values are not directly comparable; fluorescence in the presence of chloroplasts is substantially quenched (Table I). Therefore, the order of magnitude difference in measured CTC-Mg^{2+} fluorescence in the presence of chloroplasts represents an underestimation of the fluorescence emission of the CTC-Mg^{2+}-envelope membrane complex as compared to CTC-Mg^{2+} alone.

Data presented in Figure 5 suggest that Ca^{2+}-CTC-chloroplast envelope complex has a greater fluorescence than Mg^{2+}-CTC-envelope complex. With two separate chloroplast preparations, a given increase in reaction medium divalent cation

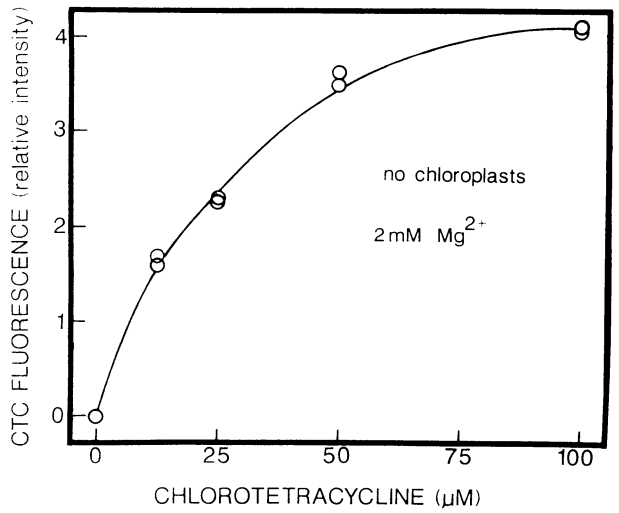

Figure 3. Fluorescence of varying concentrations of CTC in standard reaction medium with an additional 2 mM Mg^{2+} added. Two measurements were made at each CTC concentration.

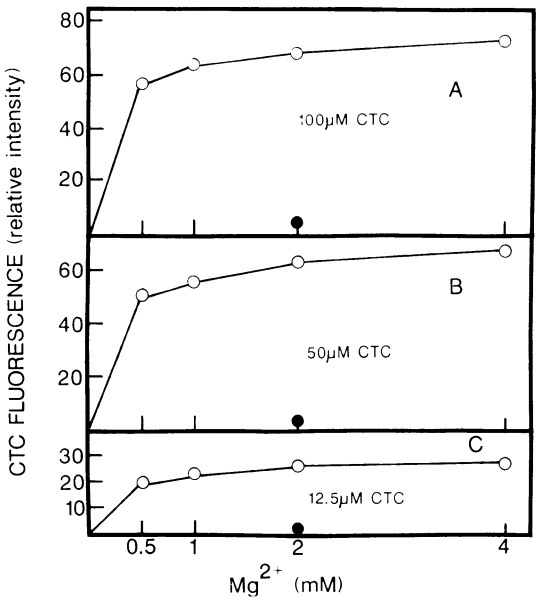

Figure 4. Fluorescence of CTC at 100 μM (A), 50 μM (B), or 12.5 μM (C) in the presence of chloroplasts (open symbols) at varying Mg^{2+} added to the standard reaction medium (the standard reaction medium already contains 1 mM Mg^{2+} and 1 mM Mn^{2+} chelated by 2 mM EDTA). The closed symbols represent measurements in the absence of chloroplasts. Each data point represents the mean of two replicate measurements.

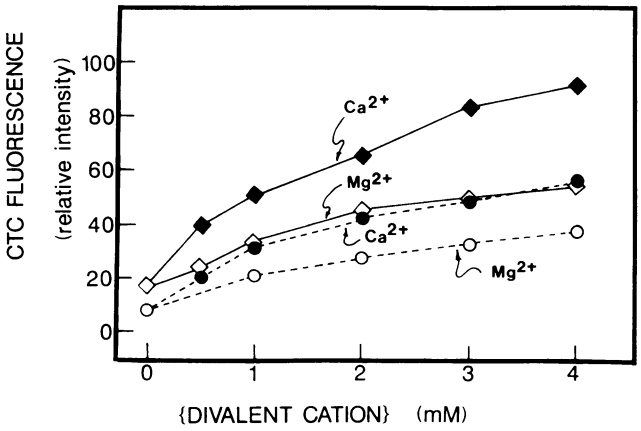

Figure 5. Effects of Ca^{2+} (closed symbols) and Mg^{2+} (open symbols) addition to the standard reaction medium on CTC fluorescence in the presence of chloroplasts. The two different symbols represent data from different chloroplast preparations. Each data point is the mean of two replications.

results in a greater increase in fluorescence when Ca^{2+}, as opposed to Mg^{2+}, is used. This has been shown previously with other reaction media and membrane systems (23). An alternative explanation for this effect is that the negatively charged sites on the chloroplast envelope have a greater affinity for Ca^{2+} than Mg^{2+}. This possibility is not ruled out by the experiments reported here.

In addition to the capability of measuring increased envelope membrane-associated Mg^{2+} or Ca^{2+} when chloroplasts are incubated with added divalent cation, CTC fluorescence emission can also be used to detect changes in envelope-associated divalent cations due to chloroplast pre-treatment. In Figure 6, CTC fluorescence measurements are shown for chloroplasts which were prepared using the standard isolation procedure, along with chloroplasts which were washed in EDTA or EGTA during isolation. All three preparations came from the same chloroplast pellet after the first centrifugation of the isolation procedure. With no Mg^{2+} added to the reaction medium, the values of CTC fluorescence are dependent on 'endogenous' divalent cations associated with the chloroplast envelope. Of course, the standard isolation medium does contain both EDTA and divalent cations. However, calculations of free Mg^{2+}, Mn^{2+}, and EDTA from the appropriate stability constants (8.7 for Mg^{2+}, and 14 for Mn^{2+}; 7) indicate that these values are extremely low, essentially zero for free Mn^{2+} and 44.7 nM for free EDTA and Mg^{2+}. Therefore, it can be deduced from the fluorescence values with no Mg^{2+} added to the reaction medium that EGTA and EDTA washes remove endogenous divalent cations from the chloroplast envelope (Fig. 6). Both EDTA- and EGTA-washed chloroplasts display less CTC fluorescence than chloroplasts prepared using the standard isolation procedure, when no Mg^{2+} is added to the reaction medium (Fig. 6). With no added Mg^{2+} in the reaction medium, the fluorescence of EDTA-washed chloroplasts is lower than that of EGTA-washed chloroplasts. The stability constants for Mg^{2+} and Ca^{2+} of EDTA are 8.7 and 10.6, respectively. For EGTA these values are 5.2 and 11.0 (7). EDTA chelates Mg^{2+} better than EGTA, and EGTA chelates Ca^{2+} better than EDTA. The fact that the EDTA wash lowered native fluorescence of chloroplasts more than EGTA suggests that Mg^{2+} contributes more to the native divalent cation pool associated with the chloroplast envelope than Ca^{2+}. Even though Ca^{2+}-CTC-membrane complex has a greater fluorescence than Mg^{2+}-CTC-membrane complex (Fig. 5), and EGTA removes relatively more Ca^{2+} than Mg^{2+} (as compared to EDTA), the native fluorescence of EGTA-washed plastids was greater than that of EDTA-washed plastids (Fig. 6).

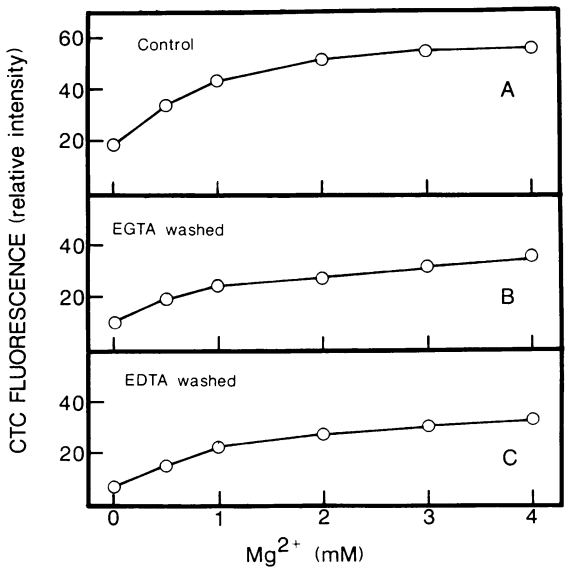

Figure 6. Effects of Mg^{2+} addition to the reaction medium on CTC fluorescence in the presence of chloroplasts isolated using the standard protocol (A), or with a 5 mM EGTA (B) or 5 mM EDTA (C) wash. Each data point represents the mean of two replicate measurements.

Adding Mg^{2+} to the reaction medium increases fluorescence with EGTA-washed (Fig. 6B) and EDTA-washed (Fig. 6C) chloroplasts. These data suggest that after removal of divalent cations from the envelope by chelate washes, Mg^{2+} can be added back to the membrane. It should be noted, however, that the same amount of reaction medium Mg^{2+} causes less increase in fluorescence with chelate-washed chloroplasts than with chloroplasts isolated using the standard media (compare Fig. 6, B and C, with A). It is not known why this occurs. It is doubtful that this effect is due to chelate carried in the isolation medium, as the chloroplasts were washed twice with standard medium, and then centrifuged through a Percoll cushion after the chelation treatment.

Data presented in Figure 7 further demonstrate that Mg^{2+}-washes increase, and EDTA-washes decrease chloroplast envelope-bound Mg^{2+}. In this experiment, chloroplasts were incubated in a high (10 mM) concentration of Mg^{2+} during isolation. After this treatment, they were subsequently washed in grind medium with various concentrations of EDTA. Increasing the concentration of EDTA in this chelation wash decreased the subsequent CTC fluorescence in these preparations (Fig. 7), with equimolar EDTA resulting in very low levels of envelope-associated Mg^{2+}. In a separate experiment, chloroplasts were washed successively in 10 mM Mg^{2+}, then in 10 mM EDTA, and then assayed for CTC fluorescence in the presence of increasing reaction medium Mg^{2+} (data not shown). With these chloroplasts, increasing Mg^{2+} in the reaction medium resulted in substantial increases in CTC fluorescence. This experiment, along with the data shown in Figures 6 and 7 indicate that Mg^{2+} can be added to, or removed from the chloroplast envelope in a fairly reversible manner.

Our investigation of the use of CTC fluorescence with chloroplasts indicates that with a given chloroplast preparation, relative effects of various treatments on chloroplast envelope-associated Mg^{2+} can be effectively monitored with CTC fluorescence. However, our initial studies with this technique suggest that it is not a good investigative tool for monitoring differences in chloroplast envelope-associated Mg^{2+} (and/or Ca^{2+}) in plastid preparations from different plants. The CTC fluorescence dependent on "native" divalent cations associated with chloroplasts (*i.e.* with plastids isolated using the standard media), and increases in fluorescence dependent on reaction medium Mg^{2+}, is quite variable when chloroplast preparations isolated from different plants are

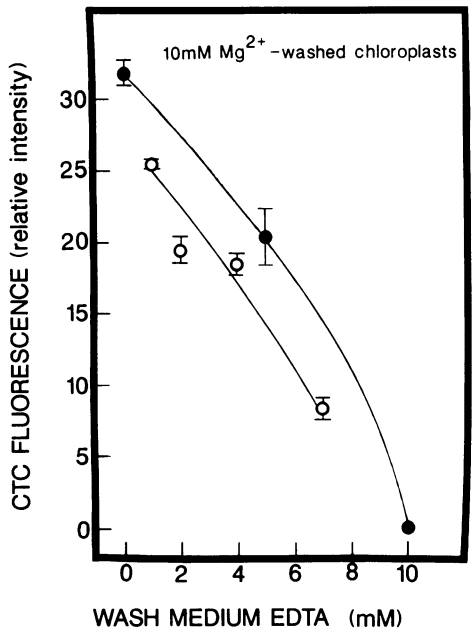

Figure 7. Effect of a 10 mM Mg^{2+}-wash, and subsequent EDTA-washes during chloroplast isolation on CTC fluorescence. After the last wash for each treatment in these experiments, chloroplasts were centrifuged through a 40% Percoll cushion made up in grind medium which lacked the typical additions of 2 mM EDTA, 1 mM $MgCl_2$, and 1 mM $MnCl_2$ ('low salt grind medium'). After isolation, chloroplasts were resuspended in low salt grind medium, and assayed in low salt reaction medium. Open and closed symbols represent data from two separate experiments. Each data point represents the mean ± SE (n=3).

compared. When six different preparations from six different spinach plants were compared, the CTC fluorescence with no Mg^{2+} added to the reaction medium varied from 10 to 32 relative intensity units (data not shown). With these same preparations, fluorescence emission with 4 mM Mg^{2+} added to the reaction medium varied between 24 and 94 relative intensity units (data not shown). In other studies, native fluorescence of chloroplasts was as low as 2 relative intensity units (Table II, also data not shown). It should also be noted that sometimes, plastid preparations displayed no CTC-associated fluorescence (data not shown); these preparations were not used in any of the studies shown in this report. It is not known why this variation occurs.

Substantiation of the CTC Assay: Studies with $^{28}Mg^{2+}$

Obviously, the use of radioactive Mg^{2+} (*i.e.* $^{28}Mg^{2+}$) would be a far more sensitive assay of Mg^{2+} associated with the chloroplast envelope than the use of CTC. Using labeled Mg^{2+} would also provide a more quantitative assay, as CTC measurement of chloroplast envelope-associated Mg^{2+} is only quantified as relative intensity units. However, $^{28}Mg^{2+}$ is a relatively expensive radionuclide, with a short half-life (20 h) which greatly limits its practical usefulness. Also, $^{28}Mg^{2+}$ is currently prepared for research use from only one source in the United States (the Brookhaven National Laboratory particle accelerator). The production schedule is erratic, and at most, $^{28}Mg^{2+}$ is available only several months per year.

Table II. *Effect of EDTA-Washes on Chloroplast Envelope Mg^{2+} as Determined with $^{28}Mg^{2+}$ Labeling and CTC Fluorescence*

For the labeling studies of experiment 1, chloroplasts were incubated in either standard grind medium with 0.4 Ci/mol $^{28}Mg^{2+}$ or grind medium with $^{28}Mg^{2+}$ and 5 mM additional EDTA for 5 min. Aliquots of the same chloroplast preparation were incubated in unlabeled medium for CTC fluorescence measurements. After incubation in the control, or EDTA-wash medium, chloroplasts were centrifuged through a Percoll cushion and resuspended in standard grind medium. Data are presented as means ± SE (n=4 for pellet Mg^{2+} and n=3 for CTC fluorescence). In the second experiment, chloroplasts were incubated in standard grind medium with 0.825 Ci/mol $^{28}Mg^{2+}$ for 5 min. After this labeling treatment and centrifugation through Percoll, chloroplasts were washed in either standard grind medium, or grind medium with 5 mM EDTA added. Chloroplasts were again centrifuged through a Percoll cushion, and resuspended in standard grind medium prior to determination of chloroplast-associated $^{28}Mg^{2+}$.

Treatment	Pellet Mg^{2+}	CTC Fluorescence
	μmol/mg Chl	*relative intensity*
Exp. 1		
Control	0.519 ± 0.003	1.81 ± 0.02
EDTA wash	0.310 ± 0.001	1.34 ± 0.03
Exp. 2		
Control	0.161 ± 0.005	
Post labeling EDTA wash	0.113 ± 0.004	

$^{28}Mg^{2+}$ was used in some experiments reported here with the goal of substantiating some of our hypotheses developed using the CTC assay. In the experiment shown in Figure 8, alterations in chloroplast-associated Mg^{2+} due to Mg^{2+} washes were assayed by including $^{28}Mg^{2+}$ in the wash medium. After incubation in grind medium with 0, 1, 2, and 5 mM Mg^{2+} added, chloroplasts were centrifuged through a Percoll cushion, resuspended in standard grind medium, and subsequently microcentrifuged through silicone oil cushions. As was deduced from the CTC fluorescence studies (Figs. 4–6), the data in Figure 8 suggest that increasing incubation medium Mg^{2+} results in an increase in chloroplast envelope-associated Mg^{2+}. In this experiment, $^{28}Mg^{2+}$ associated exclusively with the chloroplast envelope was not identified. However, theoretical considerations indicate that the Mg^{2+} pelleted with the chloroplasts through the silicone oil was likely not accumulated in the stroma. Assuming a stromal volume of 25 μl/mg Chl, the 3.5 μmol Mg^{2+}/mg Chl pelleted at 5 mM external Mg^{2+} (Fig. 8) would represent an accumulation of external Mg^{2+} in the chloroplast resulting in a 140 mM increase of the stromal Mg^{2+} concentration. Previous reports have indicated that the free stromal Mg^{2+} concentration in the dark is below 1 mM (20). Even assuming the chloroplast envelope to be freely permeable to Mg^{2+} (which is unlikely) (8, 11), Portis and Heldt's (20) analysis of stromal:external Mg^{2+} gradients indicates that accumulation in the stroma of the Mg^{2+} pelleted with chloroplasts in the experiment shown in Figure 8 would be electrochemically unfavorable. Thus, a far more likely explanation for the values of chloroplast-associated Mg^{2+} shown in Figure 8 is that the cation was bound by the chloroplast envelope.

Results of another experiment further support the conten-

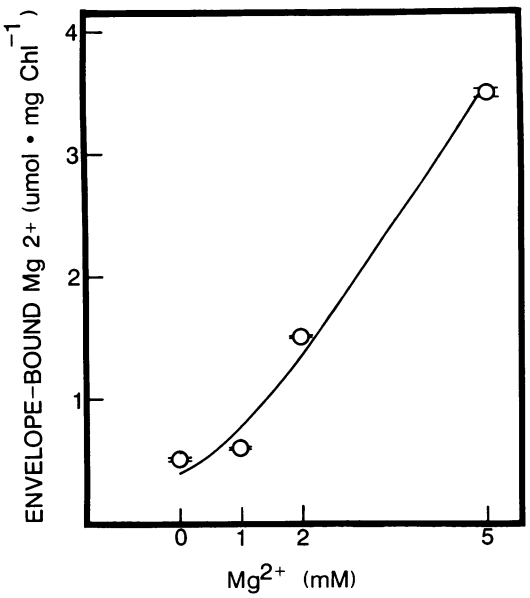

Figure 8. Effect of Mg^{2+} addition to the standard grind media as a wash treatment, on chloroplast envelope-bound Mg^{2+} as determined by silicone oil centrifugation of chloroplasts exposed to $^{28}Mg^{2+}$ washes. In this experiment, $^{28}Mg^{2+}$ (0.4 μCi/ml) was included in the wash media, along with different concentrations of unlabeled Mg^{2+}. Chloroplasts were incubated for 5 min in the wash media, centrifuged through a Percoll cushion, resuspended in standard grind medium, and then loaded onto silicone oil microcentrifugation tubes. Each data point represents the mean ± SE (n=4).

tion that Mg^{2+} pelleted with chloroplasts in the silicone oil studies was primarily associated with the chloroplast envelope. Chloroplasts were incubated with 2 mM $^{28}Mg^{2+}$ in the presence or absence of the divalent cation ionophore A23187 prior to silicone oil centrifugation. This ionophore has previously been used by Portis and Heldt (20) to allow for free movement of Mg^{2+} between incubation medium and the chloroplast stroma, resulting in essentially Mg^{2+} equilibrium in the dark. After incubation with 2 mM $^{28}Mg^{2+}$, 1.517±0.005 (n=4) μmol $^{28}Mg^{2+}$/mg Chl was associated with chloroplasts microcentrifuged through silicone oil. In the presence of 4 μM A23187, this value rose to 1.565±0.018 μmol $^{28}Mg^{2+}$/mg Chl. That portion of the pelleted Mg^{2+} which accumulated in the stroma under the control (*i.e.* no A23187) treatment in this experiment should have been released back into the incubation medium in the presence of A23187. There was no reduction of pelleted Mg^{2+} in the presence of A23187. In fact, there was a slight increase (of 48 nmol/mg Chl) in chloroplast-associated $^{28}Mg^{2+}$ in the presence of the ionophore. Again assuming 25 μL/mg Chl stromal volume, this increase would represent a 1.92 mM increase in stromal Mg^{2+}, if that was the site of the accumulation. As the incubation medium Mg^{2+} concentration was 2 mM (*i.e.* above that chelated by EDTA), it is interesting to speculate that the increase in pelleted Mg^{2+} in the presence of ionophore represents equilibration of a chloroplast site (*i.e.* the stroma) which is accessible to the radiolabeled incubation medium Mg^{2+} only in the presence of the ionophore.

Data presented in Table II show the effect of EDTA washes on chloroplast envelope-associated $^{28}Mg^{2+}$. In the first experiment, chloroplasts were incubated in standard grind medium, or grind medium with 5 mM EDTA added. $^{28}Mg^{2+}$ was included in the incubation medium of both treatments. Pellet $^{28}Mg^{2+}$ and CTC fluorescence were measured on the same chloroplast preparation. As has been shown previously (Fig. 6), the EDTA wash reduced CTC fluorescence. With the same chloroplast preparation, less $^{28}Mg^{2+}$ was found to bind to the chloroplast envelope (*i.e.* pelleted with the chloroplasts) (Table II). In the second experiment, chloroplasts were first incubated in $^{28}Mg^{2+}$, quantitatively separated from this medium (centrifuged through Percoll), and then incubated in unlabeled grind media with or without 5 mM EDTA. After quantitative separation again (centrifugation through Percoll), chloroplasts were resuspended in standard grind medium and microcentrifuged through silicone oil. The EDTA wash was found to reduce envelope-associated $^{28}Mg^{2+}$.

Collectively, the results of the $^{28}Mg^{2+}$ experiments (Figure 8, Table II, and the A23187 ionophore experiment mentioned in the text) support our interpretation of the CTC fluorescence studies. Both techniques suggest that; (a) adding Mg^{2+} to incubation medium (above that chelated by EDTA) results in increased chloroplast envelope-bound Mg^{2+}, and that (b) EDTA washes remove (at least a portion of) Mg^{2+} associated with the chloroplast envelope.

Effects of Chloroplast Envelope-Bound Mg^{2+} on Photosynthesis

Previous research has indicated that incubation of chloroplasts in medium with several millimolar Mg^{2+} can result in H^+ influx into the chloroplast resulting in stromal acidification and inhibition of photosynthesis (8, 17). Mg^{2+} inhibition of photosynthesis is characterized by a class of effects typical of other treatments which result in stromal acidification. These include an altered external PO_4^{3-} optima (12), relative insensitivity of photochemical reactions and 3-phosphoglycerate reduction (8), stimulation of photosynthesis by addition of K^+ and other monovalent cations (12, 17), and K^+ efflux from the stroma in the absence of high external K^+ (17). We hypothesized that these effects were facilitated not by the presence of free Mg^{2+} in the photosynthesis reaction medium, but rather by an increased degree of chloroplast envelope-bound Mg^{2+} resulting from exposure of chloroplasts to solutions with free Mg^{2+}.

Data presented in Figures 9 and 10 support this hypothesis. During isolation, chloroplasts were first washed in 2 mM EDTA, and then washed in grind medium with varying Mg^{2+} added. After exposure to the solutions with varying Mg^{2+}, the chloroplasts were centrifuged through Percoll cushions, insuring that there was no carryover of high Mg^{2+} media with the chloroplasts. CTC-fluorescence (Fig. 9A) and photosynthesis in the presence or absence of 40 mM K^+ in the reaction medium (Fig. 9B) were measured on these preparations. Increasing wash medium Mg^{2+} resulted in increased chloroplast envelope-bound Mg^{2+} (Fig. 9A), and inhibition of photosynthesis (Fig. 9B). However, when K^+ was added to the photosynthesis reaction medium, the inhibition of photosynthesis associated with increased envelope-bound Mg^{2+} was reduced. In two separate experiments, the Mg^{2+} washes resulted in different degrees of CTC fluorescence and inhibition of pho-

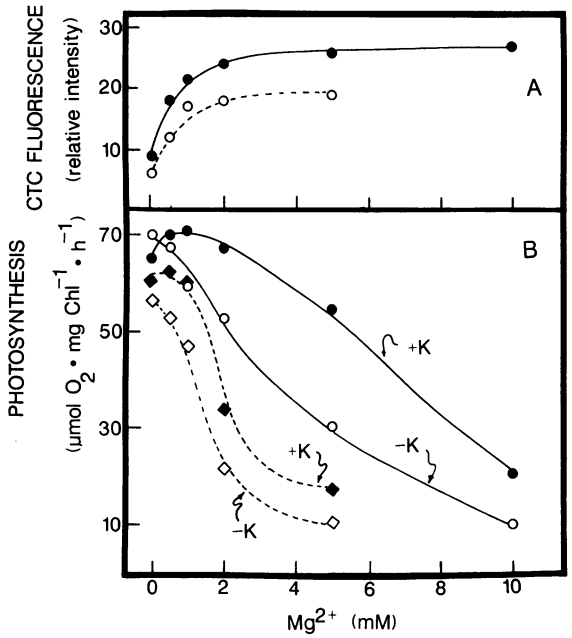

Figure 9. The effect of Mg^{2+}-washes on CTC fluorescence (A) and photosynthesis (B) of chloroplasts. Two experiments (broken and solid lines) with different chloroplast preparations are shown. Measurements were made in the presence or absence of 40 mM KCl added to the reaction medium. For CTC fluorescence, the presence of KCl had no effect; only results without KCl in the reaction medium are shown. For photosynthesis, closed symbols represent measurements made in the presence of KCl, open symbols represent measurements made in the absence of KCl. For CTC fluorescence measurements, each data point represents the mean of two replications. For photosynthesis, each data point represents a single measurement.

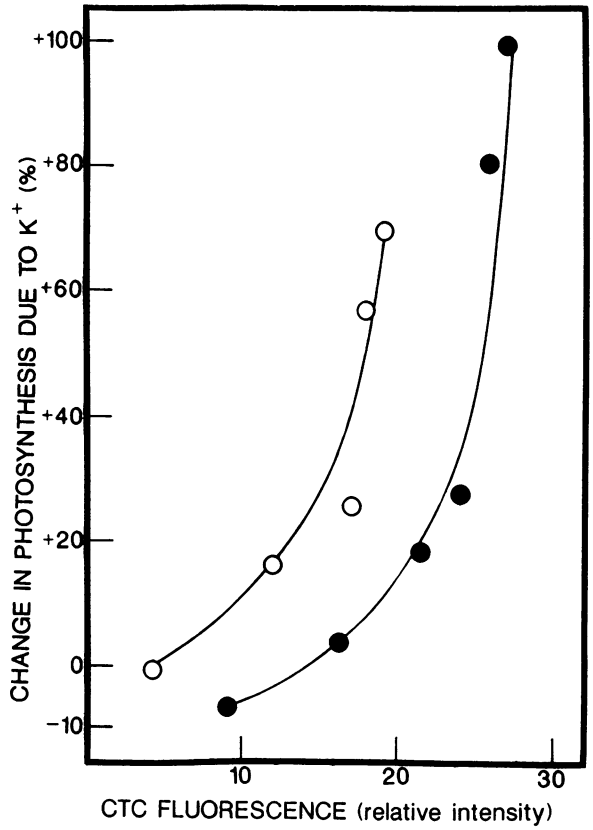

Figure 10. Relationship between relative degree of chloroplast envelope-bound Mg^{2+} as determined by CTC fluorescence, and effects of K^+ on photosynthesis. Data are recalculated from Figure 9. The values for changes in photosynthesis due to reaction medium K^+ were calculated by comparison of photosynthesis in the presence or absence of K^+ at a given Mg^{2+} concentration as shown in Figure 9. Open and closed symbols represent two different experiments.

tosynthesis. However, in both studies, the K^+ stimulation of photosynthesis in Mg^{2+}-washed chloroplasts was substantial. The relationship between chloroplast envelope-bound Mg^{2+} and sensitivity of photosynthesis to K^+ is more clearly shown in Figure 10. Increased CTC fluorescence was associated with increased stimulation of photosynthesis by K^+. It should be noted that this relationship, as shown in Figure 10, is not linear. However, the relationship shown in Figure 10 is likely influenced by the nature of the CTC assay, which is probably not linear (with regards to measurement of envelope-bound Mg^{2+}). As shown in Figures 4, 5, 6, and 9A, the relative increase in CTC fluorescence with increasing incubation medium Mg^{2+} is not linear, but typically hyperbolic. However, the $^{28}Mg^{2+}$ study shows a fairly linear relationship between incubation medium Mg^{2+} concentration, and degree of envelope-bound Mg^{2+} (Fig. 8). Nonetheless, the results of the experiments shown in Figures 9 and 10 clearly identify a regulatory role of chloroplast envelope-bound Mg^{2+} which can greatly affect photosynthesis.

Conclusion

When assays include a standard amount of Chl and CTC, and spectrofluorometric measurements are made within 15 s (our measurements were typically made within 5–10 s) of exposure of chloroplasts to CTC, the fluorescence measurement is an acceptable assay of chloroplast envelope-bound Mg^{2+} or Ca^{2+}. In this report the assay was used to demonstrate that endogenous Mg^{2+} associated with the chloroplast envelope can be altered by incubation of plastids in media with free Mg^{2+} or EDTA, in a fairly reversible manner. No attempt was made in this study to distinguish treatment effects on Mg^{2+} associated specifically with the inner or outer membrane of the chloroplast envelope. The data presented in this report indicate that the technique has the following limitations. Changes in envelope-associated Mg^{2+} can only be measured in relative terms. The relationship between changes in envelope-associated divalent cations and CTC fluorescence is likely non-linear. Also, the variation between chloroplast preparations in terms of measured CTC fluorescence response to the treatments used in this study suggest that comparison between preparations (and different plants) is not possible. The results of the studies with $^{28}Mg^{2+}$ support the interpretation of the fluorescence experiments; indicating that substantial changes in the amount of Mg^{2+} bound to the chloroplast envelope can be induced by *in vitro* treatments. Also, the $^{28}Mg^{2+}$ binding studies offer indirect support for the contention that the chloroplast limiting envelope is likely not very permeable to Mg^{2+}. In the most recent report in the literature

regarding external Mg^{2+} effects on chloroplast metabolism, Deshaies *et al.* (10) have concluded that Mg^{2+} moves rapidly from the external medium into the stroma. In their experiment, Deshaies *et al.* did not distinguish between changes in stromal and envelope-bound Mg^{2+} induced by incubating chloroplasts in media with high Mg^{2+}. The data we report with $^{28}Mg^{2+}$ in the presence of A23187 suggest that changes in envelope-bound Mg^{2+} should not be discounted with regards to affecting both the level of chloroplast-associated Mg^{2+}, and altered chloroplast metabolism as reported by Deshaies *et al.* (10)

The experiments relating altered chloroplast envelope-bound Mg^{2+} with photosynthetic capacity (Figs. 9 and 10) extend some hypotheses developed by Huber (12, 17) and Gimmler (8, 11). These experiments support the contention that Mg^{2+} bound to the chloroplast envelope, and not necessarily high external Mg^{2+}, may affect stromal pH and, hence, photosynthetic capacity.

The CTC fluorescence data, and photosynthesis experiments presented in this report provide the basis for some intriguing speculations regarding a discrepancy in the published literature concerning chloroplast photosynthesis. A review of the pertinent literature suggests that researchers working in different laboratories find very different effects of reaction medium K^+ on photosynthesis. Demmig and Gimmler (9), Kaiser *et al.* (14), and Robinson (21) have found that K^+ rapidly leaks out of chloroplasts, and that substantial (up to 100 mM in the absence of other monovalent cations) concentrations of K^+ are required in reaction media in order to obtain maximal photosynthesis with isolated chloroplasts. Other researchers (1, 17) (also see Fig. 9B, data points with no Mg^{2+} added to the reaction medium) find no substantial effect of added K^+ on photosynthesis. Also, the effect of exogenous Mg^{2+} on photosynthesis has been reported to be quite variable, with some researchers reporting differing sensitivity of photosynthesis to exogenous Mg^{2+} in the same publication (8, 19). S. C. Huber (personal communication) has speculated that growth conditions may greatly affect the level of endogenous Mg^{2+} bound to the envelope of isolated chloroplasts, and that this variation may be responsible for the varying sensitivity of photosynthesis to K^+ (as shown in Fig. 10). We have found large differences in CTC fluorescence (possibly dependent on different levels of endogenous envelope-bound Mg^{2+}) in chloroplasts isolated from different plants. These results suggest that future use of the CTC fluorescence assay developed in this report may resolve the basis for the variable response of chloroplast photosynthesis to exogenous K^+ and Mg^{2+}.

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