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# Evidence for the Location of Divalent Cation Binding Sites on the Chloroplast Membrane

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*Summary.* We have used a combination of chemical labeling and detergent fractionation techniques to locate the divalent cation binding sites on the chloroplast membrane. We determined the $Ca^{2+}$-binding properties of Triton X-100 subchloroplast particles. Photosystem II (TSFII) particles showed one binding site with $n=8.4$ μmoles-mg chl$^{-1}$ and $k_d=20$ μM. Photosystem I (TSFI) particles contained two binding sites. The first had an $n=1.5$ μmoles-mg chl$^{-1}$ and $k_d=4$ μM. The second had an $n=9.6$ μmoles-mg chl$^{-1}$ and $k_d=160$ μM. We have previously shown (Prochaska & Gross, *Biochim. Biophys. Acta* **376**:126, 1975) that the divalent cation binding sites could be blocked using a water-soluble carbodiimide plus a nucleophile. Chlorophyll *a* fluorescence and light-scattering changes were affected at the same carbodiimide concentrations emphasizing the relationship between these processes. The carbodiimide-sensitive sites were found to be located on the Photosystem II particles. A direct correlation between the inhibition of calcium binding and the carbodiimide-mediated incorporation of a ($^{14}$C)-nucleophile was observed upon varying such parameters as carbodiimide concentration, nucleophile concentration, pH, and time of reaction. The presence of $CaCl_2$ during the carbodiimide plus nucleophile modification procedure decreased the incorporation of ($^{14}$C)-nucleophile, emphasizing the competition of the $CaCl_2$ and the modification reagents for some of the same sites. Sodium dodecylsulfate gel electrophoresis of chlorophyll protein aggregates suggested that the site of competition of the calcium chloride and the modification reagents was the light-harvesting chlorophyll *a/b* protein.

Divalent cations have been shown to regulate several important chloroplast processes including structural changes such as shrinkage and thylakoid stacking (Izawa & Good, 1966; Dilley & Rothstein, 1967; Gross & Packer, 1967; Murakami & Packer, 1971; Murata, 1971*b*; Gross & Prasher, 1974), the distribution of excitation energy (Homann, 1969; Murata, 1969, 1971*a*, *b*; Murata, Tashiro & Takamiya, 1970; Ben-Hayyim & Avron, 1971; Sun & Sauer, 1972; Gross & Hess, 1973; Mohanty, Braun & Govindjee, 1973; Briantais, Vernotte & Moya, 1973; Marsho & Kok, 1974; Jennings & Forti, 1974; Barber, Telfer, Mills &

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Nicolson, 1975; Butler & Kitajima, 1975; Li, 1975; Wydrzynski, Gross & Govindjee, 1975; Gross, Zimmerman & Hormats, 1976), $H^+$ ion uptake (Gross & Packer, *unpublished results*), and $CO_2$ fixation (Sugiyama, Nakayama & Akazawa, 1968; Jensen & Bahr, 1972; Walker, 1972). Gross and Hess (1974) showed that divalent cation binding to the chloroplast membrane is responsible for the first three effects cited above. The next logical step for these studies was to determine the chemical nature of the binding groups and to identify the binding moieties.

One method for determining this involves fractionation of the chloroplasts using nonionic detergents after which the cation-binding ability of each fraction is determined. One problem with this method is that new sites may be exposed upon fractionation of the chloroplast membranes. Therefore, we have combined this method with specific chemical labeling of the divalent cation binding sites. If the cation binding sites were labeled with a chemical modification reagent, we could then fractionate the chloroplasts and determine the location of a radioactively labeled chemical modifier. The first step in this procedure is to find a chemical modification reagent which can selectively block the divalent cation binding sites. Either carboxyl groups on proteins or phosphate groups on phospholipids are logical candidates for the location of the divalent cation binding sites due to their anionic character. Phospholipids can be ruled out since they are too few in number (Vernon, Ke, Mollehauer & Shaw, 1969; Allen, Good, Trosper & Park, 1972) to account for the total number of binding sites on the chloroplast membrane. Therefore, carboxyl groups on proteins are the best candidates for the binding sites. The method which we will use for blocking carboxyl groups involves carbodiimide-mediated amide bond formation between the carboxyl groups and a nucleophile such as glycine ethyl ester (Hoare & Koshland, 1967; Means & Feeney, 1971):

$$\begin{aligned} &\textcircled{P}-\overset{\overset{O}{\|}}{C}-O^- + R_1-N=C=N-R_2+R_3-NH_2 \\ &\rightarrow \textcircled{P}-\overset{\overset{O}{\|}}{C}-NH-R_3+R_1-NH-\overset{\overset{O}{\|}}{C}-NH-R_2. \end{aligned} \tag{1}$$

If a radioactively labeled nucleophile is used during the modification, the radioactive nucleophile can be covalently incorporated into the protein. This procedure has been used to block cation binding sites on the membranes from crab claws (Shrager & Profera, 1973). In a previous paper (Prochaska & Gross, 1975), we showed that this treatment inhibits

calcium binding and cation-dependent changes in chloroplast structure and chlorophyll *a* fluorescence. In this paper, we will show that the modification is specific for the divalent cation binding sites and we will use a radioactively labeled nucleophile such as glycine ethyl ester to locate the cation-binding sites.

## Materials and Methods

### *Chloroplast Isolation*

Chloroplasts were isolated in 50 mM Tris Cl(pH 7.5)+350 mM sucrose and washed once in 100 mM sucrose according to the method of Gross (1971). Chlorophyll concentrations were determined according to the method of Arnon (1949).

### *EDC-Mediated Incorporation of $^{14}$C-GEE into Chloroplasts*

Equal volumes of chloroplasts, appropriate concentrations of EDC[1] and $^{14}$C-GEE with a specific activity of 2 mC/mole were mixed and incubated at 23 °C for 60 min at pH 6.0±0.2. The reaction was quenched by addition of 0.5 M Tris-succinate buffer, pH 6.0. Excess $^{14}$C-GEE was removed by an additional wash of 0.5 M Tris-succinic acid-buffer, pH 4.25. The chloroplasts were than dialyzed against distilled water for 1 day with multiple changes. Little, if any, $^{14}$C-GEE was found to bind to the chloroplast membrane nonspecifically. Dialyzed chloroplasts were plated on planchets and counted on a Nuclear-Chicago Gas Flow Counter.

### *Preparation of Triton X-100 Subchloroplast Particles*

EDC-treated and control chloroplasts were solubilized at 4 °C for 2 hr in a medium containing 300 mM sucrose, 100 mM NaCl, 50 mM Tris-Cl (pH 7.5) and 1 g Triton X-100 per 40 mg chl. Control chloroplasts were pretreated in the same manner as the EDC-treated chloroplasts used for SDS electrophoresis (*see below*). except that the EDC and GEE were omitted. A centrifugation scheme similar to Vernon, Ke, Katoh, San Pietro and Shaw (1966) was followed. All fractions were assayed for chlorophyll concentration and chlorophyll *a/b* ratios. All pellets were suspended in 100 mM sucrose and stored at −20 °C.

### *$^{45}Ca^{2+}$ Binding to Triton X-100 Subchloroplast Particles*

Calcium binding was determined by equilibrium dialysis according to the method of Davis and Gross (1975). One ml samples containing 17 to 25 μg chl were dialized for 16–

1 *List of abbreviations:* EDC, 1-ethyl 3(3-dimethyl amino propyl) carbodiimide; GEE, glycine ethyl ester; PPO, 2,5 diphenyl oxazole; POPOP, *p*-bis-[2(5-phenyl-oxazolyl)] benzene; SDS, sodium dodecyl sulfate; TSFI, Photosystem I Triton X-100 subchloroplast particle; TSF II, Photosystem II Triton X-100 subchloroplast particle.

18 hr against 24 ml of a solution containing 100 mM sucrose +0.2 mM Tris base +4–800 μM $CaCl_2$ containing 0.08 μC $^{45}Ca^{2+}$. The amount of $Ca^{2+}$ bound was calculated from the difference in radioactivity between the inside and outside of the dialysis bags.

### *Lipid Extraction of Chemically Modified Chloroplasts*

Pigments and lipids were extracted according to the method of Dilley, Peters and Shaw (1972). ($^{14}C$)-labeled chloroplasts were washed 3 times in acetone for 10 min and the protein precipitate was collected by centrifugation at 20,000 × *g* for 15 min. The protein pellet was resuspended in chloroform-methanol (3:1 v/v) for a 30-min incubation period. The protein was collected by centrifugation at 20,000 × *g* for 15 min. All supernatants and the final protein pellet were assayed for radioactivity as described earlier.

### *SDS-Gel Electrophoresis $^{14}C$-GEE-Labeled Chloroplasts*

A two-step procedure was used for the chemical modification of the chloroplasts. First, the extraneous GEE-binding sites were blocked by reacting the chloroplasts at pH 6.0 for 40 min at room temperature in the presence of 0.01 M EDC and 0.13 M unlabeled GEE. The chloroplasts were collected by centrifugation and resuspended in 0.1 M sucrose. They were then reacted for 40 min at pH 6.0 with 0.1 M EDC+0.13 M GEE containing $^{14}C$-GEE with a specific activity of 1 C/mole in the presence and absence of 100 mM $CaCl_2$. This second modification step should block only the divalent cation binding sites. The chloroplasts were then dialyzed against distilled water for 24 hr after which they were solubilized in 50 mM Tris-Cl (pH 8.2)+0.5% SDS and electrophoresis carried out according to the method of Thornber, Gregory, Smith and Baily (1967*a*). The gels were scanned at 680 and 650 nm for chlorophyll *a* and *b*, respectively. The gels were then fixed in 10% TCA for 15 min, and stained for protein in 50% methanol, 10% acetic acid solution that was 0.25% in Coomassie brilliant blue. Destaining was accomplished by treating the gels with 50% methanol and 10% acetic acid solution and the gels were swelled to a normal size by the addition of a solution of 7.5% acetic acid and 5% methanol. Gels were then scanned for absorbance at 540 nm on a Guilford gel scanner, sliced to 1 mm thickness by a Joyce Loebl mechanical gel slicer, and solubilized by $H_2O_2$ at 50 °C for 5 hr (Ledinko, 1973). One ml of NCS tissue solubilizer was added to the gels and the resulting solution was allowed to stand for at least 5 hr. Ten ml of toulene scintillator containing 8 g of PPO and 0.1 g POPOP per liter of toulene was added. Vials were then counted in a Packard Liquid Scintillation Counter.

## Results

We used two methods for determining the location of the divalent cation binding sites. First, we determined $Ca^{2+}$ binding parameters for sub-chloroplast particles. Second, we blocked the $Ca^{2+}$ binding sites with a water soluble carbodiimide +$^{14}C$-glycine ethyl ester, after which we fractionated the chloroplasts and determined the location of the label.

*Divalent Cation Binding to Control Triton X-100 Subchloroplast Particles*

Divalent cation binding was determined as a function of divalent cation concentration for both Photosystem II and Photosystem I particles. Photosystem II (TSFII) (Fig. 1) particles possess one site which binds 8.4 μmoles $Ca^{2+}$/mg chl with dissociation constant of 20 μM (Table 1). The number of sites is much larger than that observed for unfractionated chloroplasts (Gross & Hess, 1974). This suggests that new sites were exposed when the chloroplasts were treated with detergent. Both the number of sites and the dissociation constant agree very well with those obtained by Davis and Gross (1975) for the light-harvesting chlorophyll *a/b* protein of Kung and Thornber (1971). Since this protein is part of the Photosystem II particle as isolated using Triton X-100, we suggest that the binding sites on the TSFII particle may be on the light-harvesting component.

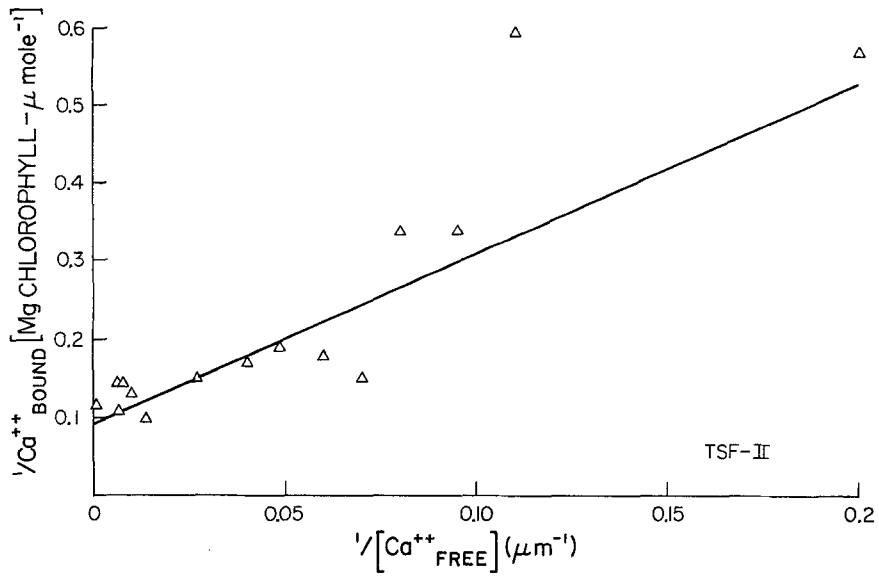


Fig. 1. Concentration dependence of [$^{45}Ca^{2+}$]-ion binding to TSFII subchloroplast particles. Photosystem II subchloroplast particles were isolated and equilibrium dialysis was performed as described in Materials and Methods. The data were plotted as a double reciprocal fraction as indicated below:

$$\frac{1}{[Ca^{2+}_{bound}]} = \frac{1}{n}\left\{1 + \frac{k_d}{[Ca^{2+}_{free}]}\right\}$$

where $n$ is the total number of binding sites and $k_d$ is the dissociation constant. [$Ca^{2+}$] represents the calcium ion concentration in the medium at equilibrium

Table 1. Divalent cation binding of Triton X-100 subchloroplast particles

| | $n$ (μmoles/mg chl) | $k_d$ (μM) |
|---|---|---|
| PSII | 8.4±0.7 | 18±1 |
| PSI site 1 | 1.5±0.3 | 4±2 |
| site 2 | 9.6±3.3 | 146±46 |

Divalent cation binding was measured as described for Figs. 1 and 2. $n$ (the number of binding sites) and $k_d$ (their dissociation constants) were calculated as described for Fig. 1.

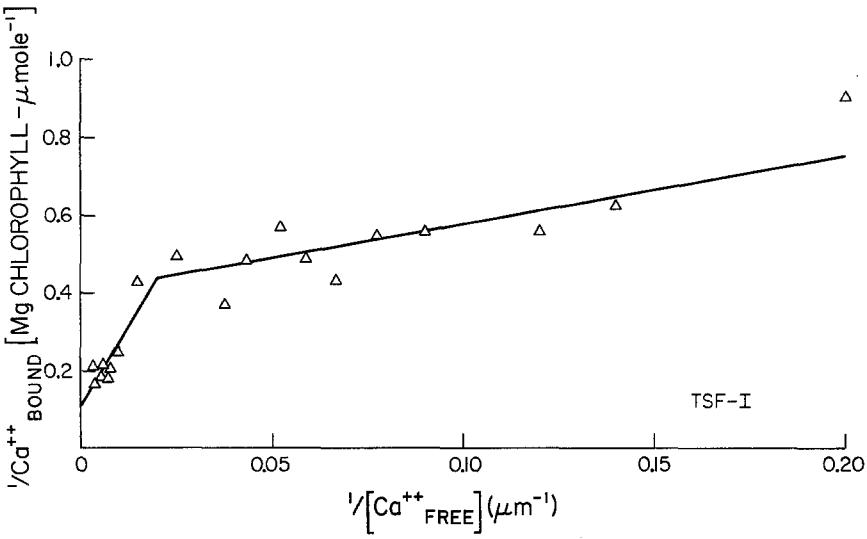


Fig. 2. Concentration dependence of [$^{45}$Ca]-ion binding to TSFI subchloroplast particles. All conditions were as described in Materials and Methods. The data were treated as described in Fig. 1

The Photosystem I (TSFI) (Fig. 2) particles contain two classes of divalent cation binding sites. The first or high affinity site binds 1.5 μmoles/mg chl with a dissociation constant of 4 μM. This may correspond to the high affinity site on the chloroplast membrane (Gross, 1972). This site is responsible for reversal of quaternary ammonium salt uncoupling in chloroplasts (Gross, 1971). The second class of sites which has a dissociation constant of 160 μM and binds 9.6 μmoles $Ca^{2+}$/mg chl may be responsible for regulation of excitation energy distribution within Photosystem I. Gross *et al.* (*in preparation*) and Prochaska and Gross (*in preparation*) have demonstrated that divalent cations promote excitation

energy transfer within Photosystem I according to both quantum yield and chlorophyll *a* fluorescence measurements. Again, as in the case of Photosystem II, we have the problem of determining which sites were exposed on the surface of intact chloroplast membranes.

### *Use of Chemical Modification to Distinguish the Divalent Cation Binding Sites*

Specific chemical modification can be used to distinguish between the original and newly exposed sites. If we react intact membranes with EDC + GEE, we will block the original binding sites (*see* Prochaska & Gross, 1975). If we then fractionate the chemically modified chloroplasts and determine the binding capabilities of the subchloroplast particles, we will detect only the newly exposed sites since the others are blocked. The number of original sites equals the total number of sites on the unmodified particles (which contain both original and newly exposed sites) minus the total number of sites on the chemically modified particles. We determined the distribution of the calcium binding sites in the various fractions of Triton X-100 subchloroplast particles obtained from control or chemically modified chloroplasts (Table 2). A concentration of 200 μM $CaCl_2$ was used in these experiments. This concentration should saturate

Table 2. Effects of 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide upon calcium binding in Triton X-100 subchloroplast particles

| Fraction | $Ca^{2+}$ Binding (μmoles-mg $chl^{-1}$) | | Δ Binding (control-reacted | % Total $Ca^{++}$ binding in fraction | % Total inhibited binding in fraction |
|---|---|---|---|---|---|
| | Control chloroplasts | EDC-reacted | | | |
| TSFII (PSII) | 4.5 | 2.1 | 2.5 | 28 | 49 |
| Intermediate fraction | 3.6 | 2.2 | 1.3 | 20 | 23 |
| TSFI (PSI) | 3.2 | 2.5 | 0.7 | 15 | 9 |
| 144,000 × *g* × 10 hr supernatant | 3.1 | 2.6 | 0.5 | 37 | 19 |

Chloroplast isolation, chemical modification and Triton X-100 fractionation were carried out as described in Materials and Methods. $Ca^{++}$ ion binding was carried out as described for Fig. 1 except that 200 μM $CaCl_2$ was used.

all of the divalent cation binding sites. Thus, the values reported should closely approximate the total number of cation binding sites. Control preparations of the 144,000 × *g* final supernatant contained 37% of the total binding sites. This supernatant contains phospholipids, sulfolipids and chlorophyll-Triton X-100 micelles. In comparison, the Photosystem II (TSFII) fraction contained only 28% of the total cation binding in the chloroplast membrane. TSFI particles contain only about 15% of the divalent cation binding capacity of the chloroplast membrane.

A different pattern emerges when we examine the difference between the particles obtained from control and carbodiimide-modified chloroplasts. In this case, the largest enrichment of carbodiimide-modified sites (50%), corresponding to a difference of 2.5 μmoles $Ca^{2+}$/mg chl, is found in the TSFII particles. In contrast, the Photosystem I (TSFI) particles bind only a net difference of 0.7 μmole $Ca^{2+}$/mg chl, accounting for 10% of the original binding sites. Also the difference in the binding in the 144,000 × *g* supernatant was 0.5 μmole/mg chl, accounting for 19% of the total carbodiimide-inhibitable binding. These results are consistent with the idea that the original binding sites are located primarily on the Photosystem II particle with a smaller amount on Photosystem I. These results also suggest that many new sites are exposed upon solubilization of the chloroplasts with detergent.

### *Incorporation of $^{14}$C-GEE into Chloroplast Membranes*

Another approach to the problem of the location of the divalent cation binding sites is to study the EDC-mediated incorporation of $^{14}$C-GEE into the chloroplasts. This approach will be of value only if the treatment blocks the cation binding sites alone and no others. We can be satisfied that this is true if the following criteria are met. The carbodiimide and nucleophile concentration dependence, pH dependence and kinetics of $^{14}$C-GEE incorporation must parallel inhibition of $Ca^{2+}$ ion binding. Also there must be a stoichiometric relationship between the amount of $^{14}$C-GEE incorporated and the number of divalent cation binding sites. A plot of the reciprocal of $^{14}$C-GEE incorporation *vs.* the reciprocal of EDC concentration (Fig. 3) shows two straight-line segments indicative of two sites of reaction of the carbodiimide. The first (high affinity site) shows a maximal incorporation of 4.2 μmoles $^{14}$C-GEE and is half-saturated at 3.2 mM EDC. The carbodiimide concentrations involved are too low for this site to be involved in calcium

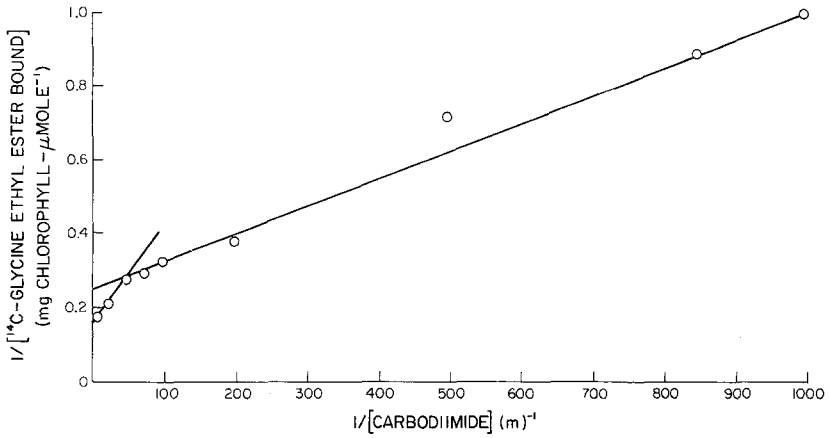


Fig. 3. Double reciprocal plot of the EDC concentration dependence of incorporation of [$^{14}$C]-GEE into chloroplast. Chloroplasts were incubated with 0.13 M $^{14}$C-GEE + various concentrations of EDC. Other conditions were as described in Materials and Methods. A double reciprocal plot was constructed according to the following relationship:

$$\frac{1}{[GEE_{bound}]} = \frac{1}{n}\left\{1 + \frac{k_d}{[EDC]}\right\}$$

where $n$ is the number of binding sites and $k_d$ is the dissociation constant. GEE bound refers to the amount of [$^{14}$C]-GEE bound at a specific EDC concentration

binding to the membranes. However, it may be responsible for inhibition of electron transport (*see* McCarty, 1974). The second site incorporates 2.9 μmoles $^{14}$C-GEE/mg chl and is half-saturated at 0.019 M EDC. This concentration is equal to that responsible for half-maximal inhibition of calcium binding and the incorporation of $^{14}$C-GEE is approximately twice the number of total binding sites (Site I and Site II) on the chloroplast membrane. Since the binding sites are thought to be divalent (Gross & Hess, 1974), a ratio of 2 $^{14}$C-GEE incorporated per divalent cation binding site is reasonable. Therefore, we can conclude that the second class of sites for carbodiimide interaction corresponds to the $Ca^{2+}$ binding sites. When we examine the GEE concentration and pH dependence of $^{14}$C-GEE incorporation into chloroplasts (Fig. 4 and Table 3), we see that there is a good correlation with inhibition of $Ca^{2+}$ ion binding. Also, the kinetics are the same for both the incorporation of $^{14}$C-GEE and the inhibition of $^{45}Ca^{2+}$ binding. These results reinforce the conclusion that the second class of sites of carbodiimide interaction corresponds to the $Ca^{2+}$ binding sites. We can eliminate interference by the first carbodiimide interaction site (observed at low concentrations of EDC) as follows: First, we react the chloroplasts with 10 mM EDC plus

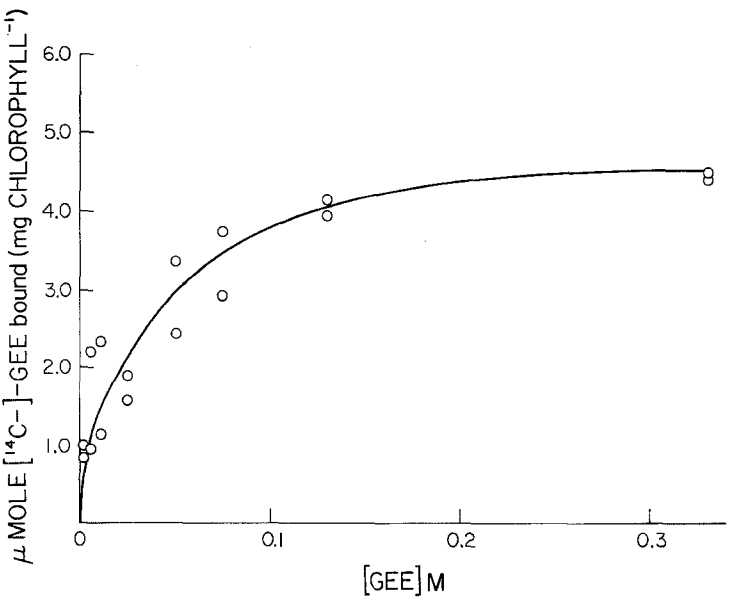


Fig. 4. GEE concentration dependence of EDC-mediated incorporation of [$^{14}$C]-GEE into chloroplasts. Chloroplasts were reacted with 0.1 M EDC and various $^{14}$C-GEE concentrations. Chloroplasts were washed, dialyzed, and counted for radioactivity as described in Materials and Methods

Table 3. Correlation between carbodiimide-mediated $^{14}$C-glycine ethyl ester incorporation and the inhibition of calcium ion binding by the carbodiimide plus nucleophile

| Variable | | Inhibition of calcium-ion binding by EDC+GEE | | Incorporation of $^{14}$C-GEE into chloroplasts | | $N=$ μmole GEE bound / mg chl |
|---|---|---|---|---|---|---|
| | | Effect: 1/2 maximal | maximal | Effect 1/2 maximal | maximal | |
| EDC | Site I | – | – | 0.0032 M | – | 4.2 |
| | Site II | 0.02 M | 0.05 M | 0.019 M | 0.05 M | 2.9 |
| GEE | | 0.05 M | 0.1 M | 0.04 M | 0.15 M | 4.25 |
| pH | | 6.5 | 5.0 | 6.5 | 4.0 | 9.0 |
| Time | | 20 min | 60 min | 15 min | 80 min | 5.0 |

The inhibition of calcium binding was performed according to the method of Prochaska and Gross (1975). For these studies, one parameter was varied while the other three were held constant. GEE, pH, and time were held constant in the case of varying EDC concentration, etc. Similar experiments were performed with $^{14}$C-labeled glycine ethyl ester. All other conditions were as described in Materials and Methods.

unlabeled GEE. This will block the first site. Then we raise the EDC concentration to 100 mM and add $^{14}$C-GEE. This will selectively label the second site of $^{14}$C-GEE incorporation, which corresponds to the divalent cation binding site.

### *Effect of $CaCl_2$ on EDC-Mediated Incorporation of GEE into Chloroplasts*

If GEE reacts with the $Ca^{2+}$ binding sites, addition of $Ca^{2+}$ ions should cause a decrease in the carbodiimide-mediated incorporation of $^{14}$C-GEE due to competition effects. The results presented in Fig. 5 show that this is correct. The effect is greatest at short times of incubation due to the fact that $Ca^{2+}$ ions bind reversibly whereas EDC+GEE reacts irreversibly. Furthermore $Ca^{2+}$ inhibition of $^{14}$C-GEE incorporation is greater at lower GEE concentrations (Table 4). This is also expected if GEE and $Ca^{2+}$ compete with each other. These results provide further evidence that EDC+GEE reacts with the $Ca^{2+}$ binding sites.

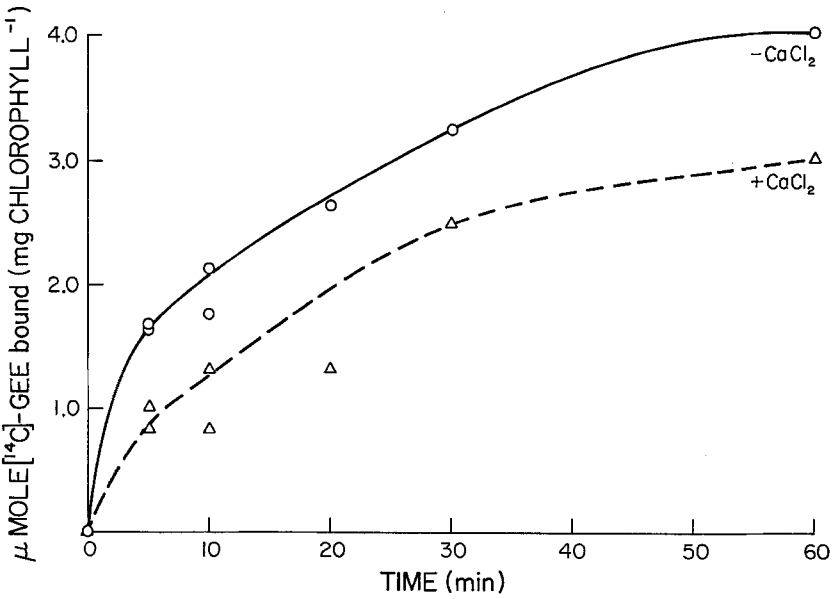


Fig. 5. Effect of calcium upon the EDC-mediated incorporation of $^{14}$C-GEE into chloroplasts. Chloroplasts were incubated in a medium that consisted of 0.1 M EDC, 0.1 M $^{14}$C-GEE, with and without 100 M $CaCl_2$. Other conditions were as described in Materials and Methods

Table 4. $CaCl_2$-induced inhibition of $^{14}C$-GEE incorporation into chloroplasts

| Conditions: GEE (mM) | $CaCl_2$ (mM) | $^{14}C$-GEE Bound (μmoles-mg chl$^{-1}$) | Inhibition of $^{14}C$-GEE incorporation (per cent) |
|---|---|---|---|
| 130 | 0 | 1.26 | – |
| 130 | 90 | 1.16 | 9 |
| 40 | 0 | 0.21 | – |
| 40 | 90 | 0.13 | 38 |

Chloroplasts were reached with 0.1 M EDC + the concentrations of GEE indicated. Other conditions were as described in Materials and Methods.

### *SDS-Gel Electrophoresis of $^{14}C$-GEE-Labeled Chloroplasts*

The calcium binding experiments reported above showed that most of the carbodiimide-sensitive $Ca^{2+}$ binding sites were located in the Photosystem II fraction. The Photosystem II fraction is composed of a Photosystem II core complex containing only chlorophyll *a* (this contains the reaction center for Photosystem II) and the light-harvesting chlorophyll *a/b* protein (Ogawa, Obata & Shibata, 1966; Thornber *et al.*, 1967*a*; Thornber, Stewart, Hatton & Baily, 1967*b*; Kung & Thornber, 1971; Thornber, 1975). The latter protein is a logical candidate for the binding sites since, when isolated, it binds divalent cations with dissociation constant similar to that for intact membranes. (Davis & Gross, 1975).

The light-harvesting chlorophyll *a/b* pigment protein can be separated by SDS gel electrophoresis according to the method of Thornber *et al.* (1967*a*). Three pigment-containing bands are obtained corresponding to the core complex for Photosystem I (CPI) (mol wt ~ 100 kD), the light-harvesting chlorophyll *a/b* protein (CPII) (mol wt ~ 30 kD) and a free pigment band. The first two bands stain with Coomassie brilliant blue.

To determine whether the light-harvesting chlorophyll *a/b* protein contained the $Ca^{2+}$ binding sites we did the following experiment. First, we reacted chloroplasts with 10 mM EDC + unlabeled GEE. This would block the sites which did not bind $Ca^{2+}$ ions (*see* Fig. 3). Second, we reacted the chloroplasts with 100 mM EDC + $^{14}C$-GEE. Since the other sites were already blocked, only the $Ca^{2+}$ binding sites should react with the $^{14}C$-GEE. Then we solubilized the membranes with SDS and performed gel electrophoresis according to the method of Thornber *et al.* (1967*a*). Fig. 5 shows both protein concentration (Coomassie brilliant blue stain) and $^{14}C$-GEE as a function of position on the gel. We see a

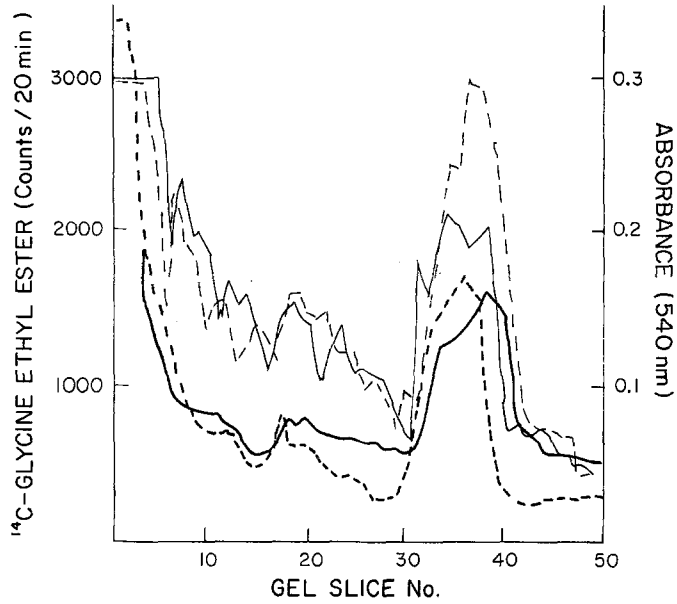


Fig. 6. SDS gel electrophoresis of $^{14}$C-GEE labeled chloroplasts. Chloroplasts were reacted in the presence or absence of 100 mM $CaCl_2$ and washed and dialyzed as described in Materials and Methods. SDS gel electrophoresis was performed according to the method of Thornber *et al.* (1967*a*). The gels were stained for protein, optically scanned, sliced, and counted for radioactivity as described in Materials and Methods. (- - - -) Absorbance at 540 nm − $CaCl_2$; (——) Absorbance at 540 nm + $CaCl_2$; (– – –) $^{14}$C-GEE − $CaCl_2$; (——) $^{14}$C-GEE + $CaCl_2$

large amount of incorporation of $^{14}$C-GEE into the light-harvesting chlorophyll *a/b* protein. However, there is also incorporation of $^{14}$C-GEE into the Photosystem I P700 chlorophyll *a* protein and the material at the top of the gel. We did the following experiment to distinguish the $Ca^{2+}$ binding material from that nonspecifically labeled with $^{14}$C-GEE. We have shown (Fig. 5 and Table 4) the $^{14}$C-GEE incorporation which corresponds to the real $Ca^{2+}$ binding sites is decreased if the chemical modification reaction is run in the presence of $Ca^{2+}$ ions. Therefore, if we react the chloroplasts with GEE + EDC in the presence of $Ca^{2+}$ prior to SDS treatment, those fractions which contain the divalent cation binding sites should show a decrease in $^{14}$C-GEE labeling. When we did the experiment (Fig. 6), we observed a significant decrease in labeling for the light-harvesting chlorophyll *a/b* protein with almost no change in any other lipo-protein band. We can conclude that the most probable location of the divalent cation binding sites is on the light-harvesting chlorophyll *a/b* protein.

Table 6. Lipid depletion of EDC + $^{14}$C-GEE-modified chloroplast membranes

| Treatment | % $^{14}$C-GEE in fraction | % Chlorophyll in fraction |
|---|---|---|
| 1st acetone wash | 9.5 | 90 |
| 2nd acetone wash | 0.6 | 5 |
| 3rd acetone wash | 0.6 | 5 |
| Chloroform methanol | 2.8 | 0 |
| Protein | 86.4 | 0 |

Chloroplasts were reacted with EDC + GEE as described in Materials and Methods. Lipids were extracted according to the method of Dilley *et al.* (1972).

### *Lipid-Protein Fractionation of $^{14}$C-GEE-Labeled Chloroplasts*

Although the chemical modification technique which we used is primarily directed toward carboxyl groups on proteins, amide bonds can also be formed with phosphate groups on phospholipids (for a further discussion *see* Prochaska and Gross, 1975). Therefore, it was important to know whether the binding sites which reacted with the $^{14}$C-GEE were located on the proteins. To determine this, we extracted the pigments and lipids from $^{14}$C-GEE-treated chloroplasts according to the scheme presented in Table 5 and determined the amount of $^{14}$C-GEE bound to each fraction. We found that 86% of the label was bound to the protein fraction. Therefore, we can conclude that the binding sites are protein in nature. This is consistent with the idea that cation-mediated changes in protein-protein interactions regulate chloroplast structure and excitation energy transfer.

Membranes appear to differ with respect to the nature of the divalent cation binding sites. Red blood cell membranes are like chloroplasts in that the majority of the binding sites are protein in nature (Forstner & Mannery, 1971) whereas in mitochondria they are phospholipid in nature (Jacobus & Brierly, 1969).

### *Effect of Illumination on EDC-Mediated Incorporation of $^{14}$C-GEE into Chloroplast Membranes*

We have previously shown that illuminating the chloroplasts in the presence of pyocyanin, a co-factor for cyclic electron flow, caused a

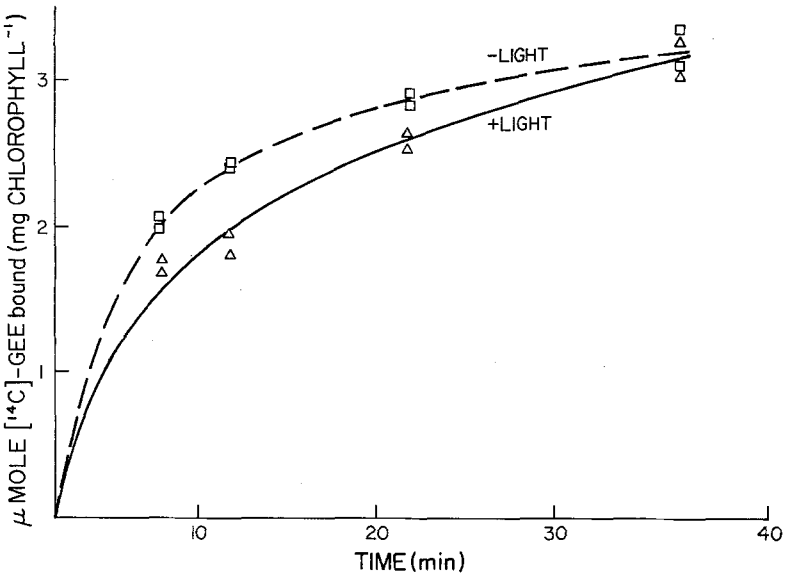


Fig. 7. Effect of light on the EDC-mediated incorporation of $^{14}$C-GEE. Chloroplasts were reacted at 15 °C with 0.1 EDC plus 0.13 [$^{14}$C]-GEE at pH 6.0 for the time period indicated. 45 μM pyocyanin was present to facilitate cyclic electron flow. The intensity of white light was $2.2 \times 10^5$ erg/cm$^2$ sec. Other conditions were as described in Materials and Methods

decrease in the extent of inhibition of $Ca^{2+}$ ion binding caused by EDC +GEE (Prochaska & Gross, 1975). This is parallel by a decrease in the incorporation of $^{14}$C-labeled GEE (Fig. 7) as expected. One possible explanation is that light causes a structural change which decreases the number of sites which can react with the carbodiimide. Light-induced conformational changes have been observed in chloroplasts but are thought to be associated with ion movements (Dilley & Vernon, 1965; Crofts, Deamer & Packer, 1967; Dilley *et al.*, 1972), or with electron transport through Photosystem II.

## Discussion

A combination of $Ca^{2+}$ binding and chemical-labeling techniques has allowed us to elucidate the chemical nature and location of the

divalent cation-binding sites on the chloroplast membrane.[2] We have found that the binding sites are on proteins rather than phospholipids and that the protein involved is the light-harvesting chlorophyll *a/b* protein described by Thornber *et al.* (1967*a*, *b*; 1975) and Ogawa *et al.* (1966). This conclusion is supported by the work of Davis, Armond and Gross (1976) who showed a parallel increase in the number of $Ca^{2+}$ binding sites and the amount of the chlorophyll *a/b* protein during the greening of pea seedlings. Moreover, Davis and Gross (1975) showed that the isolated light-harvesting chlorophyll *a/b* protein binds divalent cations with a dissociation constant similar to that for intact membranes.

However, both the Photosystem II particles and the light-harvesting chlorophyll *a/b* protein contain a greater number of binding sites than are exposed on the surface of intact chloroplast membranes. In addition, Gross and Hess (1974) showed that washed chloroplast membranes retain large amounts of tightly bound mono- and divalent cations (particularly, $Na^+Ca^{2+}$ and $Mg^{2+}$ ions). We propose that cation binding to the buried sites in intact membranes promotes interactions between membrane proteins in vivo and that these interactions are important in maintaining membrane integrity. In addition, there are additional sites which are exposed on the surface of intact membranes and which serve to regulate excitation energy transfer, grana stacking and $H^+$ ion uptake.

The light-harvesting chlorophyll *a/b* protein is a logical choice for a regulatory protein because its primary role is one of absorbing light energy and transferring it to a particular photosystem. It has been shown to bind to both Photosystem II and Photosystem I core complexes (Vernon, Shaw, Ogawa & Raveed, 1971; Klein & Vernon, 1974; Arntzen, *personal communication*; Gross *et al.*, *in preparation*). Slight changes in its interactions with either of the two core complexes could alter the extent and direction of excitation energy transfer. This is because energy transfer is dependent upon the distance between and mutual orientation

2 Barber and Mills (1976) have suggested that the cation concentration in the diffuse double layer rather than direct ion binding is responsible for the divalent cation control of excitation energy transfer. The attraction of a divalent cation by the electrical field generated by a negative charge is equivalent to binding. Binding of ions to specific sites can occur without showing ion selectivity. Moreover, the concentration of cations within the double layer does not invalidate the concept of a dissociation constant which represents the concentration of cations which must be added to the medium to cause half-saturation of the sites. The concentration of free cations in the stroma is lower than previously estimated due to the large number of cation binding sites on both the thylakoid membranes and stroma proteins. We estimate that the concentrations of $Mg^{2+}$ and $Ca^{2+}$ are approximately 0.1 mM and that the free internal $K^+$ ion concentration is approximately 10 mM (*see* Gross *et al.*, 1976).

of pigment molecules (Förster, 1965; Hoch & Knox, 1968). Seely (1973) constructed a computer model of the two photosynthetic units in which as few as four chlorophyll molecules play a pivotal role in the transfer of excitation energy from Photosystem II to Photosystem I. We envision a fraction of the light-harvesting chlorophyll *a/b* protein playing this important role. The cation concentration of the stroma would determine whether the molecules in question would be more tightly bound to the Photosystem II or Photosystem I core complexes. This, in turn, would channel energy transfer to a particular photosystem.

Davis and Gross (1975) have shown that divalent cations cause the light-harvesting chlorophyll *a/b* protein to aggregate. The aggregation may also serve to regulate energy transfer.

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## References

Allen, C.F., Good, P., Trosper, T., Park, R.B. 1972. Chlorophyll, glycolipid and protein ratios in spinach chloroplast grana and stroma lamellae. *Biochem. Biophys. Res. Commun.* **48**:907

Arnon, D.I. 1949. Copper enzymes in isolated chloroplast polyphenol oxidase in *beta vulgaris*. *Plant Physiol.* **24**:1

Barber, J., Mills, J. 1976. Control of chlorophyll fluorescence by diffuse double layer. *Febs Lett.* **68**:288

Barber, J., Telfer, A., Mills, J., Nicolson, J. 1975. Slow chlorophyll fluorescence changes in intact chloroplasts. Evidence for cation control. *Proc. Int. Congr. Photosyn. Res.* M. Avron, editor. Vol. I, p. 53. Elsevier, Amsterdam

Ben-Hayyim, G., Avron, M. 1971. Light distribution and electron donation in the "Z" scheme. *Photochem. Photobiol.* **74**:389

Briantais, J.-M., Vernotte, C., Moya, J. 1973. Intersystem exciton transfer in isolated chloroplasts. *Biochim. Biophys. Acta* **325**:530

Butler, W.L., Kitajima, M. 1975. Fluorescence quenching PSII of Chloroplasts. *Biochim. Biophys. Acta* **376**:116

Crofts, A.R., Deamer, D.W., Packer, L. 1967. Mechanism of light-induced structural changes in chloroplasts. II. The role of ion movements in volume changes. *Biochim. Biophys. Acta* **131**:97

Davis, D.J., Armond, P.A., Gross, E.L. 1976. Differentiation of chloroplast lamellae: Onset of cation regulation of excitation energy distribution. *Arch. Biochem. Biophys.* **175**:64

Davis, D.J., Gross, E.L. 1975. Protein-protein interactions of light-harvesting pigment protein from spinach chloroplasts. I. $Ca^+$ binding and its relation to protein association. *Biochim. Biophys. Acta* **387**:551

Dilley, R.A., Peters, G.A., Shaw, E.R. 1972. A test of binary chloroplast membrane hypothesis by using a non-penetrating chemical probe, *p*-(diazonium)-benzene sulfonic acid. *J. Membrane Biol.* **8**:163

Dilley, R.A., Rothstein, A. 1967. Chloroplast membrane characteristics. *Biochim. Biophys. Acta* **135**:427

Dilley, R.A., Vernon, L.P. 1965. Ion and water transport processes related to the light dependent shrinkage of chloroplasts. *Arch. Biochem. Biophys.* **111**:365

Förster, T.H. 1965. Modern quantum chemistry. *In*: Istambul International School of Quantum Chemistry Lectures. O. Sinanoglu, editor. Academic Press Inc., New York

Forstner, J., Mannery, J.F. 1971. Calcium binding by human erythrocyte membranes: Significance of carboxyl, amino, and thiol groups. *Biochem. J.* **125**:343

Gross, E.L. 1971. Uncoupling of photo phosphorylation: Inhibition by quaternary ammonium salts and zwitterionic buffers. *Arch. Biochem. Biophys.* **147**:77

Gross, E. 1972. Divalent cation binding to chloroplast membranes and its relationship to reversal of quaternary ammonium salt uncoupling of phosphorylation. *Arch. Biochem. Biophys.* **150**:324

Gross, E.L., Hess, S.C. 1973. Monovalent cation-induced inhibition of chlorophyll *a* fluorescence: Antagonism by divalent cations. *Arch. Biochem. Biophys.* **159**:833

Gross, E.L., Hess, S.C. 1974. Correlation between calcium ion binding to chloroplast membranes and divalent cation-induced structural changes and changes in chlorophyll *a* fluorescence. *Biochim. Biophys. Acta* **339**:334

Gross, E.L., Packer, L. 1967. Ion transport and conformational changes in spinach chloroplast grana, osmotic properties and divalent cation-induced volume changes. *Arch. Biochem. Biophys.* **121**:779

Gross, E.L., Prasher, S.H. 1974. Correlation between monovalent cation-induced decreases in chlorophyll *a* fluorescence and chloroplast structural changes. *Arch. Biochem. Biophys.* **164**:460

Gross, E.L., Zimmerman, R.J., Hormats, G.F. 1976. The effect of mono and divalent cations on the quantum yields for electron transport in chloroplasts. *Biochim. Biophys. Acta* **440**:59

Hoare, D.G., Koshland, D.E. 1967. A method for the quantitative modification and estimation of carboxylic acid groups in proteins. *J. Biol. Chem.* **242**:2447

Hoch, G., Knox, R.S. 1968. Photophysiol. A. Giese, editor. Vol. 3, p. 93. Academic Press Inc., New York

Homann, P. 1969. Cation effects on the fluorescence of isolated chloroplasts. *Plant Physiol.* **44**:932

Izawa, S., Good, N.E. 1966. Effect of salts and electron transport on the conformation of isolated chloroplasts. II. Electron microscopy. *Plant Physiol.* **41**:544

Jacobus, W.E., Brierly, G.P. 1969. Ion transport by heart mitochondria. XVI. Cation binding by submitochondrial particles. *J. Biol. Chem.* **244**:5004

Jennings, R.C., Forti, G. 1974. The influence of magnesium on the chlorophyll fluorescence yield of isolated chloroplasts. *Biochim. Biophys. Acta* **347**:299

Jensen, R.G., Bahr, J.T. 1972. Regulation of $CO_2$ fixation by $Mg^{2+}$ in isolated spinach chloroplasts. *Proc. 2nd Int. Congr. Photosyn. Res.* G. Forti, M. Avron and A. Melandri, editors. p. 789. Elsevier, Amsterdam

Klein, S.M., Vernon, L.P. 1974. Protein composition of spinach chloroplasts and their photosystem I and photosystem II subfragments. *Photochem. Photobiol.* **19**:43

Kung, S.D., Thornber, J.P. 1971. Photosystem I and II chlorophyll-protein complexes of higher plant chloroplasts. *Biochim. Biophys. Acta* **253**:285

Ledinko, N. 1973. Nuclear acidic protein changes in adenovirus-infected human embryo kidney cultures. *Virology* **54**:294

Li, Y.-S. 1975. Salts and chloroplast fluorescence. *Biochim. Biophys. Acta* **376**:180

Marsho, T.V., Kok, B. 1974. Photosynthetic regulation by cations in spinach chloroplasts. *Biochim. Biophys. Acta* **333**:353

McCarty, R.E. 1974. Inhibition of electron transport in chloroplasts between the two photosystems by a water-soluble carbodiimide. *Arch Biochem. Biophys.* **161**:93

Means, G.E., Feeney, R.E. 1971. Chemical Modification of Proteins. Holden-Day, San Francisco.

Mohanty, P., Braun, B.Z., Govindjee. 1973. Light-induced slow changes in chlorophyll *a* fluorescence in isolated chloroplasts; Effects of magnesium and phenazine methosulfate. *Biochim. Biophys. Acta* **292**:459

Murakami, K., Packer, L. 1971. The role of cations in the organization of chloroplast membranes. *Arch. Biochem. Biophys.* **146**:887

Murata, N. 1969. Control of excitation transfer in photosynthesis. II. Magnesium ion-dependent distribution of excitation energy between two pigment systems in spinach chloroplasts. *Biochim. Biophys. Acta* **189**:171

Murata, N., Tashiro, H., Takamiya, A. 1970. Effects of divalent metal ions on chlorophyll *a* fluorescence in isolated spinach chloroplasts. *Biochim. Biophys. Acta* **97**:250

Murata, N. 1971*a*. Effects of monovalent cations on light-energy distribution between two pigment systems of photosynthesis in isolated spinach chloroplasts. *Biochim. Biophys. Acta* **226**:422

Murata, N. 1971*b*. Control of excitation transfer in photosynthesis. V. Excitation transfer between two pigment systems in isolated spinach chloroplasts. *Biochim. Biophys. Acta* **245**:365

Ogawa, T., Obata, R., Shibata, K. 1966. Two pigment proteins in spinach chloroplasts. *Biochim. Biophys. Acta* **112**:223

Prochaska, L.J., Gross, E.L. 1975. The effect of 1-ethyl-3(3-dimethyl aminopropyl) carboxdiimide on calcium binding and associated changes in chloroplast structure and chlorophyll *a* fluorescence in spinach chloroplasts. *Biochim. Biophys. Acta* **376**:126

Seely, G.R. 1973. Energy transfer in a model of the photosynthetic unit of green plants. *J. Theor. Biol.* **40**:189

Shrager, P., Profera, C. 1973. Inhibition of the receptor for tetrodotoxin in nerve membranes by reagents which modify carboxyl groups. *Biochim. Biophys. Acta* **318**:141

Sugiyama, T., Nakayama, N., Akazawa, T. 1968. Structure and function of chloroplast proteins. V. Homotropic effect of bicarbonate in RUDP carboxylase reactions and the mechanism of activation by magnesium ions. *Arch. Biochem. Biophys.* **126**:737

Sun, A.S.K., Sauer, K. 1972. Pigment systems and electron transport in chloroplasts. Emerson enhancement in broken spinach chloroplasts. *Biochim. Biophys. Acta* **256**:409

Thornber, J.P. 1975. Chlorophyll proteins; Light harvesting and reaction center components of plants. *Annu. Rev. Plant Physiol.* **26**:127

Thornber, J.P., Gregory, R.P.F., Smith, C.A., Baily, J.L. 1967*a*. Studies on the nature of chloroplast lamellae. I. Preparation and some properties of two chlorophyll-protein complexes. *Biochemistry* **6**:391

Thornber, J.P., Stewart, J.C., Hatton, M.W., Baily, J.L. 1967*b*. Studies on the nature of chloroplast lamellae. II. Chemical composition and further physical properties of two chlorophyll-protein complexes. *Biochemistry* **6**:2006

Vernon, L.P., Ke, B., Katoh, S., San Pietro, A., Shaw, E.R. 1966. Properties of subchloroplast particles prepared by the action of digitonin, Triton X-100 and sonication. *Brookhaven Symp. Biol.* **19**:102

Vernon, L.P., Ke, B., Mollehauer, H.H., Shaw, E.R. 1969. Composition and structure of spinach subchloroplast fragments obtained by the action of triton X-100. *In*: Progress in Photosynthesis Research. H. Metzner, editor. Vol. I, p. 137. Internation Union of Biological Sciences, Tubingen

Vernon, L.P., Shaw, E.R., Ogawa, T., Raveed, D. 1971. Structure of photosystem I and photosystem II of plant chloroplasts. *Photochem. Photobiol.* **14**:343

Walker, D.A. 1972. The affinity of ribulose diphosphate carboxylase for $CO_2$ bicarbonate. *Proc. 2nd Int. Congr. Photosyn. Res.* G. Forti, M. Avon, and A. Melandri, editors. p. 1773. Elsevier, Amsterdam

Wydrzynski, T., Gross, E.L., Govindjee. 1975. Effects of sodium and magnesium cations on the "dark" and "light"-induced chlorophyll *a* fluorescence yields in sucrose washed chloroplasts. *Biochim. Biophys. Acta* **376**:151