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THE EFFECT OF 1-ETHYL-3(3-DIMETHYLAMINOPROPYL)CARBODIIMIDE ON CALCIUM BINDING AND ASSOCIATED CHANGES IN CHLOROPLAST STRUCTURE AND CHLOROPHYLL *a* FLUORESCENCE IN SPINACH CHLOROPLASTS

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SUMMARY

1. Chemical modification of carboxyl groups on the chloroplast membrane with a water-soluble carbodiimide plus a nucleophile caused inhibition of Ca^{2+} binding.

2. Both binding sites were affected and showed a decrease in the number of binding sites and an increase in the dissociation constant.

3. Cation-induced changes in chlorophyll *a* fluorescence and structural changes (ΔA_{540}) were inhibited at the same carbodiimide concentrations as Ca^{2+} binding, emphasizing the relationship between these processes.

4. Chloroplasts that were illuminated with high intensity light for short time periods showed a decrease in the carbodiimide-mediated inhibition of Ca^{2+} binding.

INTRODUCTION

Divalent cations, such as Ca^{2+} and Mn^{2+}, have been shown by Murata et al. [1–3] and Homann [4] to inhibit spillover of excitation energy from Photosystem II to Photosystem I in green plant photosynthesis. An increase in chlorophyll *a* fluorescence can be used to monitor the spillover effect. Murata [3], Murakami and Packer [5], and Mohanty et al. [6] have indicated that structural changes accompany the divalent cation-induced chlorophyll *a* fluorescence changes. Gross and Hess [7, 8] showed that divalent cation binding was responsible for both the structural and fluorescence changes. Gross and Hess [7, 8] also showed that there are two divalent cation binding sites. The first site (Site I) binds divalent cations to the extent of 0.65μ mole/mg chlorophyll with a dissociation constant of 8μ M. This site

Abbreviations: DCMU, 2,4-dichlorophenyl-1,1-dimethyl urea; DCC, dicyclohexyl carbodiimide; EDC, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride; CMC, 1-cyclohexyl-3-(2-morpholino-4-ethyl)carbodiimide metho-*p*-toluene sulfonate; Gly-OEt, glycine ethyl ester.

is responsible for the reversal of quaternary ammonium salt uncoupling [9]. The second site (Site II) binds divalent cations to the extent of 0.5 μmole/mg chlorophyll and has a dissociation constant of 51 μM [8]. This site is responsible for divalent cation-induced structural changes and chlorophyll *a* fluorescence changes [8]. In this paper, we will study the chemical nature of the binding sites. The identification of the binding sites and isolation of the binding entities is necessary to elucidate the mechanism by which divalent cations cause changes in chloroplast structure and chlorophyll *a* fluorescence.

One way to do this is to block the binding sites with a chemical modification reagent. The first step is to determine the conditions for blocking the binding sites, after which the selectivity of the reaction can be studied. If the modification is selective for the binding sites, the modifying reagent can be radioactively labelled and used as a marker to isolate the binding entities. Negatively charged groups such as phospholipids, sulfolipids and proteins are the most likely candidates for divalent cation binding.*

Water soluble carbodiimides have been used to modify carboxyl groups of proteins (13, 14). Carboxyl groups of a protein can be converted into amides by a two-step process. The first step involves the reaction of the carbodiimide with the carboxyl groups of the protein and the second step involves the nucleophilic attack of an amine upon the *o*-acylisourea (13). Upon amidation, the reactive carboxyl groups will thus be blocked as potential calcium binding sites. In this paper, the effects of three different carbodiimides upon the calcium binding in chloroplasts will be shown. The fact that these reagents affect chlorophyll *a* fluorescence changes will also be demonstrated. Future studies will involve the isolation and characterization of the calcium binding moiety.

MATERIALS AND METHODS

Chloroplast isolation

Chloroplasts were isolated from market spinach in 50 mM Tris–HCl (pH 7.5) and 350 mM sucrose. The chloroplasts were then washed in 100 mM sucrose as previously described [9].

Chlorophyll determinations

Chlorophyll concentrations were determined by the method of Arnon (15).

Carbodiimide reaction procedure

Equal volumes of chloroplasts, appropriate carbodiimide and appropriate nucleophile were mixed and allowed to react. The reaction mixture was immediately

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* The role of sulfolipids and phospholipids may be questioned for two reasons. First, there may not be sufficient phospholipid or sulfolipid in the membranes to account for binding. The estimates of Lichlenthaler and Park [10] of 0.5 μmole total phospholipid mg chlorophyll would allow a phospholipid binding site whereas those of Allen et al. [11] and Vernon et al. [12] of 0.33 μmole of phospholipid mg chlorophyll would not. Allen et al. [11] also indicate that there is only 0.23 μmole sulfolipid per mg chlorophyll in thylakoid membranes. The second reason is that when chloroplasts are extracted with chloroform–methanol (2 : 1, v/v) following $^{45}Ca^{2+}$-labeling, 81 % of the label appears in the protein fraction (Davis, D. J. and Gross, E. L., unpublished results). Thus, carboxyl groups are better candidates for the Ca^{2+} binding sites than the phospholipids.

titrated to pH 6.0 ($\pm$0.2) and maintained at that pH by either a Sargent recording pH stat or addition of HCl as needed. Time studies indicated the halftime of the reaction to be 20 min, therefore the reaction was allowed to proceed for 1 h to insure completion of the reaction. The reaction was stopped by addition of a 0.5 M Tris–succinic acid buffer, pH 6.0. The chloroplasts were then washed three times in 0.4 mM Tris and 100 mM sucrose.

Chlorophyll a fluorescence

Chlorophyll *a* fluorescence changes were measured as previously described [7]. Chloroplasts (at 4–7 μg/ml chlorophyll) were incubated at pH 8.0 in a medium consisting of 100 mM sucrose, 0.2 mM Tris, and 20 μM DCMU to insure that the Photosystem II traps were closed. The excitation and emission wavelengths were 470 and 680 nm, respectively.

$^{45}Ca^{2+}$ binding

Chloroplasts (from 10–20 μg/ml chlorophyll) were suspended in 30 ml of a medium containing 100 mM sucrose–0.2 mM Tris–0.2 mM $^{45}CaCl_2$ at pH 8.0. The chloroplasts were collected by centrifugation for 15 min at 10 000$\times g$. The chloroplasts were then suspended in distilled water, plated on planchets, and counted on a Nuclear-Chicago Geiger–Muller Counter.

Chemicals

DCC, EDC and Trizma base were purchased from Calbiochem, Ott-Story and Sigma Chemical Companies, respectively. Gly-OEt, CMC and methylamine were obtained from Aldrich Chemical Company. $^{45}CaCl_2$ was obtained from New England Nuclear. Other chemicals were of reagent grade. EDC and CMC were assayed for their purity by their melting points, 112.5–114.5 °C and 113–115 °C, respectively.

RESULTS

Inhibition of Ca^{2+} binding by a water-soluble carbodiimide plus a nucleophile

The chemical nature of the calcium binding site can be studied by the use of specific chemical modification reagents. The effect of a carbodiimide plus a nucleophile (Gly-O-Et) on Ca^{2+} binding was studied as a function of carbodiimide and nucleophile concentrations, pH and time of incubation. The results are presented below. Fig. 1 indicates the effect of varying EDC concentration on calcium binding to chloroplast membranes. Half-maximal inhibition occurred at 0.02 M EDC and maximum inhibition occurred at 0.05 M EDC. The inhibition of Ca^{2+}-induced structural changes occurred at the same concentrations, emphasizing that the two processes are related. A concentration of 0.1 M EDC was chosen for the subsequent experiments. When the glycine ethyl ester concentration was varied, half-maximal inhibition occurred at 0.05 M Gly-OEt and maximal inhibition at 0.1 M Gly-OEt. Moreover, no inhibition was observed in the absence of the nucleophile. This indicates that peptide bond formation occurred between the carboxyl groups and an exogeneous nucleophile (Gly-OEt) in the inhibited state, rather than with ε-amino groups of lysine on the chloroplast membrane. An absolute requirement for the nucleophile is necessary if we wish to locate the binding sites by means of a radioac-

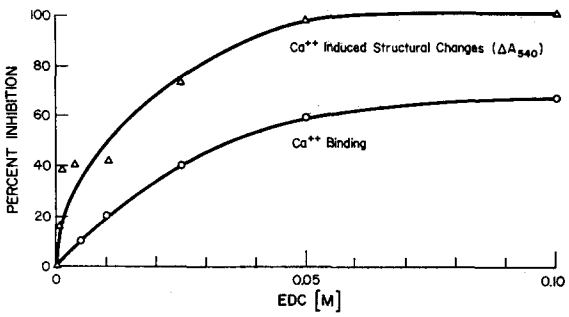

Fig. 1. The EDC concentration dependence of inhibition of both calcium ion binding and changes in chloroplast structure. Chloroplasts were reacted with concentrations of EDC in the presence of 0.13 M Gly-OEt. Both the control and reacted chloroplasts were washed once with 0.5 M Tris–succinic acid buffer, pH 6.0, and three times with 100 mM sucrose, 0.2 mM Tris. Calcium binding was performed as described in the Materials and Methods section. Control chloroplasts bound 1.03 μmoles Ca^{2+}/mg chlorophyll. For the structural changes, 0.67 mM $CaCl_2$ was added to modified and control chloroplasts and absorbance changes were monitored at 540 nm.

tively labelled nucleophile. Also, no inhibition was observed with Gly-OEt in the absence of carbodiimide or with the carbodiimide plus 0.13 M KCl rather than Gly-OEt. Again, using 0.1 M EDC, maximum inhibition of calcium binding occurs at 1 h and the halftime for the reaction was approximately 20 min. Identical results were obtained with 0.13 M Gly-OEt or 1.33 M Gly-OEt. Fig. 2 shows the effect of varying the pH of reaction on the extent of inhibition of both calcium binding and chlorophyll *a* fluorescence. Maximum inhibition of calcium binding occurs between pH 4.0 and 5.0 and decreases as the pH is raised. Interestingly, some inhibition is still obtained at pH 8.0. Fig. 2 also shows the effects of various pH values of the EDC reaction upon initial fluorescence levels. Maximum inhibition occurred at low pH values, with half-maximal inhibition at pH 6.0. The similarity of these results to those shown above suggests that inhibition of calcium binding and decreasing the initial fluorescence levels may be related.

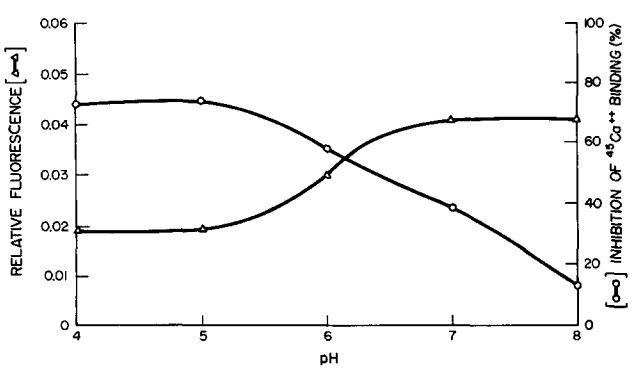

Fig. 2. The dependence of inhibition of calcium binding and initial chlorophyll *a* fluorescence on pH. Chloroplasts were reacted with 0.1 M EDC plus 0.13 M Gly-OEt at pH values of 4.0, 5.0, 6.0, 7.0 and 8.0. Other conditions were as described for Fig. 1. Control values bound 1.12 μmole Ca^{2+}/mg chlorophyll. The initial fluorescence was measured as described in the Materials and Methods section.

The question arose whether one or both of the calcium binding sites was blocked by the reagent. It was essential to determine whether the reagents were blocking the sites themselves, in which case we would expect a decrease in the number of binding sites (n) or whether it was preventing access to the sites which might be reflected as an increase in the dissociation constant (K_D). To answer this question, a complete Ca^{2+} concentration curve in the presence and absence of saturating concentrations of the modification reagents (Fig. 3) was determined. Fig. 3 shows that both n, the number of sites, and K_D, the dissociation constant, are affected by the reagents. Control values for the number of sites are 0.85 for Site I and 1.52 for Site I and Site II leaving 0.67 μmoles/mg chlorophyll for Site II alone. Dissociation constants for the control are 5 μM for Site I and 50 μM for Site II. In the EDC-reacted chloroplasts, n equals 0.16 for Site I and 0.62 for Sites I and II leaving 0.46 for Site II. The K_D values are 17 μM for Site I and 250 μM for Site II. It may be seen that both K_D and n are affected by the reagents. The number of sites is reduced while K_D values are increased. Therefore, this indicates that the effect is unlikely to be overcome by saturation. This means that both blocking of the sites and restriction of access to the sites occurs. If blocking access is the case, K_D would be affected, rather than both n and K_D. Thus, chemical modification blocks the binding sites. Also, both calcium binding sites are affected by this treatment.

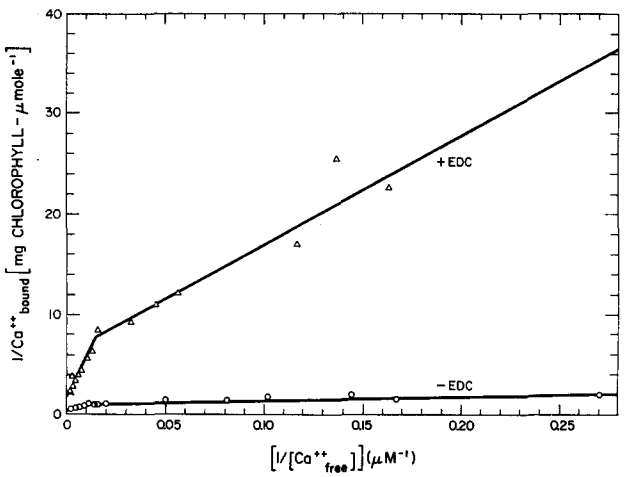

Fig. 3. The effect of chemical modification on the concentration dependence of Ca^{2+} binding to chloroplasts. Ca^{2+} binding was determined as a function of Ca^{2+} concentration in the medium for both control and reacted chloroplasts. Other concentrations were as described in the Materials and Methods section. A double reciprocal plot was constructed according to the following formula:

$$1/Ca^{2+}_{bound} = \frac{1}{n}\left(1+\frac{K_D}{Ca^{2+}_{free}}\right)$$

where n is the total number of binding sites and K_D is the dissociation constant. Ca^{2+}_{free} refers to the calcium ion concentration in the medium at equilibrium. Chloroplasts were reacted with 0.1 M EDC plus 0.13 M Gly-OEt for 1 h at pH 6.0. Both the control and reacted chloroplasts were washed as described in Fig. 1.

The effect of various carbodiimides and nucleophiles on inhibition of Ca^{2+} binding

Table I shows a comparison between different carbodiimides and nucleophiles on calcium binding in chloroplasts. Using CMC, the half-maximum concentration of the inhibition of calcium binding was similar in the presence and absence of a nucleophile. CMC has both cyclohexyl and morpholino moieties on the molecule. Due to this steric bulk, it can be argued that the CMC reaction involves cross linking of a carboxyl and an amino group on the exterior of the membrane. This process could possibly prevent access of the calcium to the binding sites. For CMC, the inhibition of calcium binding data shows a sigmoidal curve, thus indicating a cooperative effect (not shown). As one CMC molecule reacts in the membrane, more potential reactive sites are exposed to the reagent. DCC, which is sparingly soluble in water, inhibited some of the sites at much lower concentrations. This result indicates that some of the calcium binding sites might be in a lipophilic area of the membrane. EDC may be more effective than CMC because it is smaller in size and not a quaternary ammonium salt. Thus it is possible that EDC would penetrate the membrane in its uncharged form. For DCC and EDC, the nucleophile was varied. Since methylamine is a small molecule, it might be expected to be more efficient as a nucleophile for the inhibition of calcium binding. Table I indicates that Gly-OEt and methylamine do not differ significantly as nucleophiles in the inhibition of calcium binding.

Recently, McCarty [16] has found that EDC and light inhibits the following: (1) both coupled and uncoupled electron transport between plastoquinone and cytochrome *f*; (2) photophosphorylation; (3) proton uptake; (4) 518 nm absorbance change. Giaquinta et al. [17] found that light-stimulated CMC inhibition of Photosystem II activity. Therefore, we decided to examine the role of light in our EDC-

TABLE I

THE EFFECTS OF VARIOUS CARBODIIMIDES AND NUCLEOPHILES UPON CALCIUM ION BINDING

Calcium ion binding was done as indicated in the Materials and Methods section. All reactions were run at pH 6.0 for 1 h. The chloroplasts were washed as described in Fig. 1. The following nucleophile concentrations were used. For the CMC plus Gly-OEt reaction, 1.33 M Gly-OEt was used. For DCC, either 1.33 M methylamine or 0.13 M Gly-OEt was used. For EDC, both 1.33 M Gly-OEt and 0.13 M Gly-OEt or 0.13 M methylamine was used. CMC and EDC reactions were performed as described in Materials and Methods section. DCC in methanol comprised only 10 % of the reaction mixture. Control chloroplasts bound 1.2 ± 0.2 μmole Ca^{2+}/mg chlorophyll.

Carbodiimide	Nucleophile	Maximum % inhibition of calcium binding	Half-maximal concentration of inhibition
CMC	Gly-OEt	80	0.12
	0	55	0.11
DCC	Methylamine	30	<0.003
	Gly-OEt	30	0.003
EDC	Gly-OEt	70	0.015
	Methylamine	75	0.015
	0	0	0

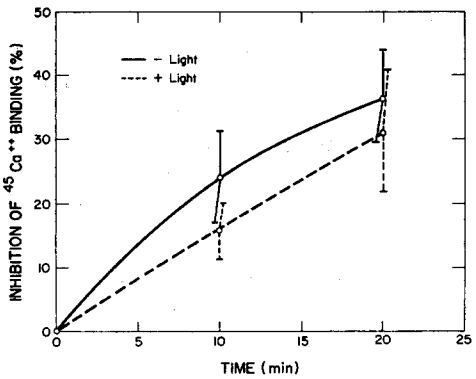

Fig. 4. The effect of light on the inhibition of calcium binding by EDC plus Gly-OEt. Chloroplasts were reacted at 15 °C with 0.1 M EDC plus 0.13 M Gly-OEt at pH 6.0 for the time period indicated. The light intensity was $1.8 \cdot 10^5$ ergs/cm^2 as measured by a Yellow Springs Instrument Co. Model 65 radiometer. Pyocyanin was present at a concentration of 45 μM. Control chloroplasts were washed as described in Fig. 1. Ca^{2+} binding was performed as described in the Materials and Methods section. Control chloroplasts bound 1.2 ± 0.2 μmole Ca^{2+}/mg chloroplasts.

mediated inhibition of calcium binding. Light caused a decrease in the inhibition of calcium binding at short times of illumination (see Fig. 4). However, the effect was overcome by longer times of illumination. This effect may be explained by some type of light-induced conformation change which decreases the accessibility of the reactive sites.

The effect of chemical modification on chlorophyll a fluorescence

It has been shown that a carbodiimide plus a nucleophile inhibit calcium binding in chloroplast membranes. However, it was important to determine what the reagents did to other reactions associated with calcium binding, e.g. cation-induced structural changes and changes in chlorophyll *a* fluorescence. Fig. 5 shows the effect of Gly-OEt and EDC+Gly-OEt on Na^+ and Ca^{2+}ion-induced chlorophyll *a* fluorescence changes. It can be seen that the EDC and Gly-OEt-treated chloroplasts lack a response to the cation-induced fluorescence changes. This effect might be caused by rigid orientation of the membranes in a certain conformation, thus inhibiting any conformational or structural changes [18]. Also, Gly-OEt inhibits the Na^+-induced fluorescence changes alone. This effect could be explained by the Gly-OEt being a monovalent cation at the pH of fluorescence measurements, thus interfering with the the monovalent cation-induced response. However, the effect of Gly-OEt on the Ca^{2+}-induced fluorescence changes is a decrease in chlorophyll *a* fluorescence, which indicates an increase in spillover. Control chloroplasts show the opposite effect, a decrease in spillover upon the addition of calcium. This treatment also decreases the initial fluorescence level (Fig. 6). Maximal decrease occurred at 0.05 M EDC. The half-maximal concentration of inhibition is 0.02 M EDC. This is a concentration similar to the one found for the inhibition of calcium binding Ca^{2+}-induced structural changes (Fig. 1).

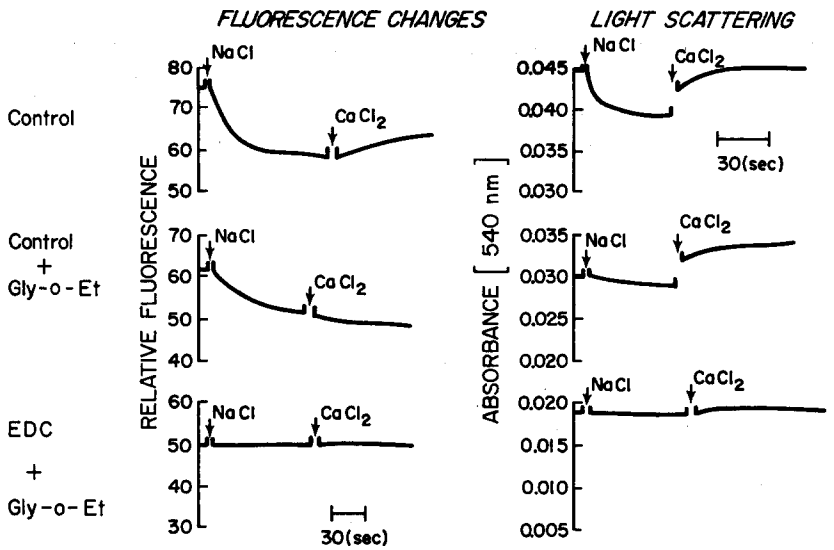

Fig. 5. The effect of EDC and Gly-OEt upon cation-induced chlorophyll *a* fluorescence and structural changes. Chloroplasts were reacted with 0.1 M EDC plus 0.13 M Gly-OEt or 0.13 M Gly-OEt alone at pH 6.0 for 2 h at 4 °C. Control chloroplasts were also kept at 4 °C for 2 h. Both control and reacted chloroplasts were washed with 0.5 M Tris–succinic acid buffer, pH 6.0 and 0.2 mM Tris plus 100 mM sucrose. Fluorescence changes were measured as in Materials and Methods section. 3.3 mM NaCl and 0.67 mM $CaCl_2$ were added step-wise to the reaction mixture and fluorescence changes were measured. Structural changes were measured at 540 nm in 100 mM sucrose plus 0.2 mM Tris plus 20 μM DCMU. 3.3 mM NaCl and 0.67 mM $CaCl_2$ were added step-wise to the reaction mixture and ΔA_{540} was measured. Calcium binding was determined on control and reacted chloroplasts. The inhibition of calcium binding for reacted chloroplasts was about 40 %. Control chloroplasts bound 1.27 μmoles Ca^{2+}/mg chlorophyll.

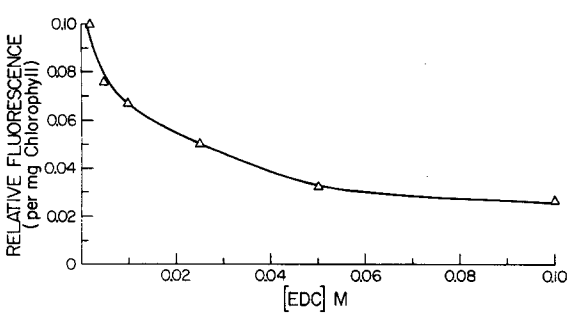

Fig. 6. The effect of varying EDC concentration on the initial fluorescence levels in chloroplasts. Chloroplasts were reacted and washed as described in Fig. 1. Initial fluorescence levels were determined as in Materials and Methods section.

The effect of chemical modification on Ca^{2+}-induced structural changes

Fig. 5 indicates the effect of Gly-OEt and EDC plus Gly-OEt on cation-induced structural changes in chloroplasts. It can be seen that both Na^+ plus Ca^{2+}-induced changes are inhibited almost entirely by EDC plus Gly-OEt. This is more

evidence for the restriction of conformational changes in the membranes. Fig. 5 also shows that Gly-OEt alone strongly inhibits Na^+-induced structuralchanges. The explanation for this effect is unknown. However, it is known that the Na^+-induced response is more sensitive, thus the positively charged Gly-OEt might have an effect upon this response. The Ca^{2+}-induced structural changes are unaffected by Gly-OEt treatment.

DISCUSSION

The results shown above indicate that the reaction of the chloroplasts with a water-soluble carbodiimide (EDC) plus a nucleophile inhibit divalent cation binding to the chloroplast membranes and related processes such as changes in a chloroplast structure and chlorophyll *a* fluorescence. The requirement for a nucleophile in the case of EDC indicates that calcium binding is inhibited due to peptide bond formation between Gly-OEt and the reactive groups on the chloroplast membrane. CMC, on the other hand, may inhibit Ca^{2+}ion binding by causing cross-linking between ε-amino groups and carboxyl groups since a nucleophile is not required. DCC, due to its lipophilic nature, may react with Ca^{2+} binding sites buried in the hydrophobic matrix of the membrane.

Recently Berg et al. [18] have studied the effects of CMC plus glycine methyl ester upon chloroplast membrane association. They indicate that charge neutralization of the carboxyl groups of the membrane induces membrane pairing. It is important that not only charge neutralization, but increased membrane hydrophobicity play an important role in this membrane pairing. The increased membrane hydrobicity would multiply the effects of hydrophobic interactions upon membrane structure.

Another interesting effect which is the result of this investigation is that the EDC plus Gly-OEt mediated inhibition of calcium binding is decreased by high intensity light. This suggests that light-induced structural changes limit the accessibility of EDC plus-Gly-OEt to potential calcium binding sites. It also indicates that in the light, EDC is not taken up to a great extent.

Finally, the possibility of the carbodiimide and nucleophile reacting with phospholipids instead of carboxyl groups arises. The chemistry of carbodiimides has been reviewed by Khorana [19] and Kurzer and Douraghi-Zadeh [20]. They both indicate that carbodiimides can react with phosphate esters or diesters to form the corresponding pyrophosphates. In the chloroplast lamellae, the major phospholipid is phosphatidylglycerol. If this phospholipid is presumed to be accessible to the solvent and can react with a carbodiimide, two possible reactions can occur. The first reaction in the presence of a nucleophile would be the formation of a diglycerol phosphoramidate which is labile to hydrolysis at acid pH [21]. This would result in an intact phosphoplipid or no inhibition of Ca^{2+} binding. The second reaction would involve cross linking of two phosphatidylglycerol molecules without a nucleophile requirement. The net result would be at tetraglycerol pyrophosphate. This compound would be extremely labile due to the absence of anionic stability [21]. Therefore, the result would again be phospholipid or no inhibition of calcium binding. In this system, a nucleophile is required for the inhibition of calcium binding.

ACKNOWLEDGEMENTS

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