The Interaction of *N,N'*-Dicyclohexylcarbodiimide with the Energy Conservation Systems of the Spinach Chloroplast†

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ABSTRACT: The interaction of carbodiimides with chloroplast membranes to alter the expression of energy conversion is effected only by the lipid-soluble *N,N'*-dicyclohexylcarbodiimide (DCCD). The water soluble carbodiimides *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate are found to have little effect on energy conversion as expressed by electron transport, phosphorylation, or the light-mediated uptake of protons. The lipid-soluble DCCD interacts irreversibly with chloroplast membranes to inhibit phosphorylation and coupled electron transport. This interaction results in an enhanced capacity to maintain a proton gradient at pH regimes removed from the optimum of the proton pump. The interaction of DCCD with chloroplast fragments rendered completely devoid of energy conversion capability by EDTA extraction leads to the reconstitution of the proton pump. This effect is dependent on a period of illumination of the chloroplasts in the presence of the inhibitor and an electron carrier. The maximization of reconstitution is coincident with the maximization of the inhibition of electron transport. The interaction of DCCD with chloroplasts partially uncoupled by EDTA treatment results in a much more rapid reconstitution of proton uptake which is not dependent on electron transport. The interaction of DCCD with EDTA-treated chloroplast membranes under conditions which restore proton uptake also results in the stimulation of acid-induced ATP synthesis, a reaction dependent on a proton barrier. This effect depends on the presence of residual coupling factor functional in phosphorylation. These experiments show that the enhancement of proton uptake in control chloroplast fragments and the reconstitution of uptake in EDTA-uncoupled fragments depend on the inhibition of basal and EDTA-uncoupled electron transport and involves a marked increase in the capability of the chloroplast fragments to maintain a proton gradient at greatly reduced rates of electron flow. Such an interaction also allows the stimulation of ATP synthesis in a reaction which requires that the membrane serve as a proton barrier.

The light-driven translocation of protons by spinach chloroplast grana is an energy-linked process which requires the participation of the grana membrane as a proton barrier (Uribe and Jagendorf, 1968) and the participation of at least one membrane-bound protein. Proton translocation requires the physical presence of membrane-bound protein designated chloroplast coupling factor (CF_1) (Jagendorf and Smith, 1962; McCarty and Racker, 1966; Jagendorf and Neumann, 1965). The chloroplast coupling factor, which is the terminal enzyme of the phosphorylating, energy-transfer system, has a function in phosphorylation that is distinct from its role in nonphosphorylating energy conversion. The evidence for this derives from experiments with antibodies to CF_1 (McCarty and Racker, 1966, 1967) and energy-transfer inhibitors (Izawa *et al.*, 1966; Gross *et al.*, 1968; McCarty *et al.*, 1965; Izawa and Hind, 1967; Uribe, 1970, 1971).

The experiments of McCarty and Racker (1966) and Lynn and Straub (1969) have shown that proton translocation and phosphorylation in chloroplasts uncoupled by EDTA treatment can be reconstituted by the recombination of CF_1 with the grana membranes. EDTA treatment removes some 75% of the CF_1 from the grana membranes (McCarty and Racker, 1967). It was shown that the CF_1 reconstituted phosphorylation was further stimulated by addition of DCCD.[1] Treatment of EDTA-uncoupled chloroplasts with DCCD alone produced a slight stimulation of ATP synthesis and a complete reconstitution of proton uptake. These results implied that the restorative action of DCCD with respect to phosphorylation is dependent on the presence of a residual amount of membrane-bound CF_1.

Preliminary experiments (Uribe, 1971) have shown that the interaction of DCCD with energy conserving system of the chloroplast is complex and affects the energy conversion process at several levels. One interesting aspect is the inhibition of basal electron transport which is increased by illumination of chloroplasts in the presence of DCCD and an electron carrier. These reaction conditions are those which have been used to measure proton uptake and are similar to those used in demonstrating reconstitution of proton uptake by DCCD in EDTA-treated chloroplasts. Another important aspect is the apparently severe inhibition of electron flow by DCCD under conditions which lead to reconstitution of proton translocation.

The questions addressed in this paper are: (1) the relationship of the light-dependent interaction of DCCD with the grana membranes to phosphorylation, electron transport and proton translocation, (2) the relation of lipid solubility and chemical reactivity to the biological activity of the carbodiimides, (3) the relationship of the extent of uncoupling of proton translocation by EDTA to the reconstitution by DCCD, and (4) the effect of DCCD–membrane interaction on proton permeability of grana membranes.

Material and Methods

Chloroplast Preparation. Control chloroplasts were prepared from market spinach as previously described (Uribe, 1970) and resuspended in 0.35 M NaCl containing 10 mM (A) or 0.1 mM (B) Tricine (pH 8.0). Control chloroplast

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[1] Abbreviations used are: DCCD, *N,N'*-dicyclohexylcarbodiimide; EDAC, *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide; CMMT, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate; DCMU, dichlorophenyl-1,1-dimethylurea.

fragments (C) for use in the light-driven proton uptake and acid-induced ATP synthesis reactions were prepared as previously described (Uribe and Jagendorf, 1968) and resuspended in 10 mM NaCl containing 0.1 mM Tricine (pH 8.0). Chlorophyll was determined by Arnon's method (1949). EDTA treated chloroplasts were prepared by washing control chloroplasts at 0° with increasing concentrations of dilute EDTA at pH 8.5 at a chlorophyll concentration of 50 μg/ml and resuspending in the same solutions as the controls. Chloroplasts uncoupled in hypertonic medium were treated with 10^{-3} M EDTA in 0.4 M sucrose and resuspended in solution A listed for controls.

Reaction Mixtures and Assays. Ferricyanide reduction and photophosphorylation were measured as previously described (Uribe, 1970) in reaction mixtures which contained the following components in micromoles: Tricine (pH 8.0), 80; K_3FeCN_6, 2; $MgCl_2$, 10; $NaKHPO_4$, 10; ADP, 5; $^{32}PP_i$ containing approximately 5×10^5 cpm; and chloroplasts containing 50 μg of chlorophyll in a volume of 2.0 ml. Cyclic photophosphorylation was measured in reactions which contained the same components as those described for noncyclic phosphorylation with 50 μM pyocyanine substituted as an electron carrier. In certain experiments photophosphorylation with pyocyanine as the electron carrier was measured by the method of Nishimura *et al.* (1962) in a 6-ml thermostated Lucite cell fitted with a Thomas combination electrode. Reaction mixtures contained the following components in micromoles, Tricine 0.56; NaCl, 56; ADP, 10; $NaKHPO_4$, 10; $MgCl_2$, 10; pyocyanine, 0.05; chloroplasts containing 450 μg of chlorophyll. The light-induced uptake of protons and oxygen evolution accompanying *p*-benzoquinone reduction were measured as described above with the omission of ADP, $NaKHPO_4$, and $MgCl_2$. The Lucite cell was fitted with a Clark oxygen electrode and a Thomas combination pH electrode. Oxygen evolution and proton uptake were recorded as a function of time on a dual-channel recorder. Light intensity was approximately 5×10^5 ergs/cm² per sec. The phosphorylation and proton uptake resulting from these reactions were measured by adding aliquots of standard acid in the light. Acid-induced ATP synthesis reactions were run as previously described (Jagendorf and Uribe, 1966b). The reaction mixtures contained the following components in micromoles: for the base stage, Tricine (pH 8.4), 100; $NaKHPO_4$, 2; $MgCl_2$, 5; ADP, 0.2; NaOH, 20; and $^{32}PP_i$ with 5×10^5 cpm in a volume of 0.9 ml; for the acid stage, succinic acid (pH 4.0), 15; DCMU, 0.027; chloroplasts containing 150 μg of chlorophyll in a volume of 0.9 ml.

Addition of Carbodiimides. DCCD, EDAC, and CMMT were added with a plastic syringe as small aliquots of freshly prepared solutions in absolute ethanol with the final ethanol concentration being less than 1% in all cases.

Syntheses and Sources. Pyocyanine was synthesized by the method of McIllwain (1937) and Tricine by Good's method (1962). Benzoquinone was recrystallized from ethanol–water and stored in the dark in over desiccant. Fresh benzoquinone solutions were prepared prior to the beginning of each experiment. DCCD was purchased from the California Corp. for Biochemical Research; EDAC from K & K Laboratories and CMMT from Aldrich Chemical Co. All other reagents were the purest available commercial grades.

Results

Interaction of Carbodiimides with the Phosphorylating and Electron-Transport Systems. Previously reported experiments on the effect of DCCD on photophosphorylation by chloroplasts have not indicated if the inhibition of phosphorylation is reversible. Experiments on the inhibition of the red cell Mg^{2+}-ATPase by EDAC (Godin and Schrier, 1970) have indicated that the water soluble carbodiimide interacts irreversibly with the membrane, possibly forming a covalently bound inhibitor complex. An experiment to determine if the interaction of DCCD with the chloroplast system is reversible by washing was carried out as described in the legend of Table I. The results of this experiment show that the inhibition of photophosphorylation by a suboptimal concentration of DCCD is not reversed by washing of the chloroplasts. The data indicate that the lipid-soluble carbodiimide interacts with the phosphorylating system to yield a strongly inhibited irreversibly bound complex. Studies with labeled DCCD are under way to determine the chemical nature of the interaction.

The interaction of the lipid-soluble carbodiimide with the membrane localized energy conserving systems of the chloroplast is of interest as it alters both phosphorylating and nonphosphorylating energy conversion. Its action in this regard might be a consequence of its ability to penetrate the grana membranes or possibly due to the reactivity of the carbodiimide with the membrane components responsible for phosphorylating and nonphosphorylating energy conversion. It was of interest to compare the action of the lipid-soluble carbodiimide to that of a water-soluble compound of similar chemical and biological reactivity on several energy-linked reactions. The activity of EDAC is compared to that of DCCD relative to phosphorylation and electron transport (Table II). In contrast to DCCD, the water soluble carbodiimide is rel-

TABLE I: Effect of Washing of DCCD-Treated Chloroplasts on ATP Synthesis.

Treatment[a] No.	DCCD	ATP Formed[b] (μmoles/hr per mg of Chlorophyll)
1	−	350
2	−	247
3	+	199
4	+	140
5	+	115

[a] Chloroplasts containing 350 μg of chlorophyll in 2.5 ml were treated as follows. Treatments 1 and 3: 0.1 ml of ethanol or 0.6 mM DCCD in ethanol was added; the solution was allowed to remain at 0° while washing for treatments 2, 4, and 5 was carried out. Treatment 2: 0.1 ml of ethanol was added; chloroplasts were washed by centrifuging down and resuspending in original volume of resuspension mix; another 0.1 ml of ethanol was added. Treatment 4: 0.1 ml of 0.6 mM DCCD in ethanol was added; the solution was washed as in treatment 2 and 0.1 ml of ethanol was added. Treatment 5: 0.1 ml of ethanol was added; the solution was washed as in treatment 2 and then 0.1 ml of 0.6 mM DCCD was added. [b] The reaction mixtures contained the following components in micromoles: Tricine (pH 8.2), 80; $MgCl_2$, 10; $NaKHPO_4$, 10; ADP, 5; pyocyanine, 0.038; chloroplasts treated as above containing 70 μg of chlorophyll and $^{32}PP_i$ containing approximately 7×10^5 cpm in a total volume of 2.0 ml.

TABLE II: Effect of Lipid- and Water-Soluble Carbodiimides on Electron Transport and Phosphorylation.[a]

DCCD (μM)	Electron Flow		ATP Synthesis	EDAC (mM)	Electron Flow		ATP Synthesis
	Basal	Coupled			Basal	Coupled	
0	175	510	211	0	155	508	227
12.5	174	447	168	1	150	498	223
25	172	331	98	2	162	478	227
50	176	288	60	4	156	460	213
75	185	279	15	8	176	484	191
150		269	6	16	190	490	196

[a] The reactions were run at pH 8.2 as described in the Methods section using chloroplast preparation A. Values are μmoles of ferricyanide reduced and ATP formed per hr per mg of chlorophyll.

atively ineffective in the inhibition of either ATP synthesis or electron transport. Similar experiments have shown that the water-soluble CMMT, a carbodiimide more similar in structure to DCCD, is without effect on photophosphorylation and electron transport. The water-soluble carbodiimides do not undergo an interaction with the membrane-located components of the energy conversion system which is effective in altering these aspects of energy conservation.

DCCD has been shown to interact with the energy conversion system at several levels. It is a strong inhibitor of photophosphorylation and coupled electron flow (Table I and McCarty and Racker, 1967). It can also inhibit basal electron transport and the inhibition measured at submaximal concentrations can be shown to increase by the preillumination of the chloroplasts in the presence of DCCD and an electron carrier (Uribe, 1971). An illustration of this point in a coupled noncyclic system is shown in Figure 1. DCCD at a concentration of 75 μM effectively inhibits ATP synthesis and the cessation of ATP synthesis causes a 35% reduction in the rate of electron transport. Chloroplasts preincubated in darkness are not further inhibited with respect to electron transport. In contrast, the rate of electron transport continues to be inhibited as a function of time of preillumination in the presence of DCCD. The inhibition of nonphosphorylating electron flow in a coupled system is thus seen to be dependent on preillumination as is that reported for basal systems.

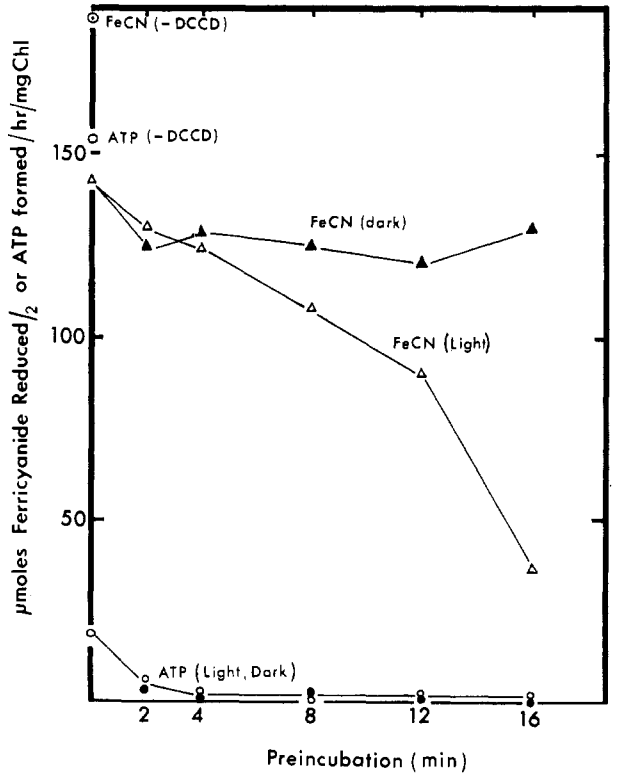

FIGURE 1: Effect of preincubation on phosphorylation and coupled electron flow. Reactions were run as described in Methods using chloroplasts (A). Preincubation in light or darkness was carried out by mixing the chloroplasts with the reaction mix *minus* ferricyanide for the preincubation period and then adding ferricyanide. Illumination of the complete reaction mixture was carried out for 1.5 min.

The interaction of DCCD with EDTA-treated chloroplasts is significantly different from that observed with whole chloroplasts. It causes a concentration-dependent inhibition of uncoupled electron flow which is accompanied by a stimulation of residual phosphorylating activity and can also result in a time-dependent reconstitution of the proton pump (McCarty and Racker, 1967). Experiments in this laboratory have shown that inhibition of EDTA uncoupled electron transport by a given concentration of DCCD is further increased by preillumination of the uncoupled chloroplasts in the presence of the inhibitor and an electron-transport cofactor (the conditions required for the reconstitution of the proton pump, Uribe, 1971). The treatment of chloroplasts with low concentrations of EDTA which results in the removal of coupling factor and the uncoupling of phosphorylation and proton uptake from electron flow also results in the osmotic disorganization of chloroplast structure; thus the differential response of the electron-transport system of EDTA-uncoupled chloroplasts to DCCD might be due to an altered accessibility of the reagent to the electron-transport components. The results of Table III show that chloroplasts which have been treated with an uncoupling concentration of EDTA in the presence of hypertonic sucrose have a similar concentration dependence for DCCD inhibition of electron transport. These data indicate that the altered reactivity of the EDTA-treated chloroplasts is specifically dependent on the removal of CF_1 from the grana.

Reconstitution of the Proton Pump. KINETICS. The finding that DCCD can restore phosphorylation slightly and the pH rise completely in EDTA-treated chloroplasts which are completely uncoupled with regard to these reactions yet have some 25% of their CF_1 remaining indicates that the uncoupling of the proton pump is not strictly a function of the loss

TABLE III: Effect of DCCD on EDTA-Uncoupled Electron Flow.[a]

DCCD (μM)	Ferricyanide Reduced (μmoles/hr per mg of Chlorophyll) EDTA	EDTA–Sucrose
0	346	302
12.5	344	296
25	280	253
50	194	174
75	141	146
150	61	97

[a] Reactions were run at pH 8.2 as described in the Methods section using chloroplasts treated with EDTA or EDTA–sucrose as described and resuspended in solution A.

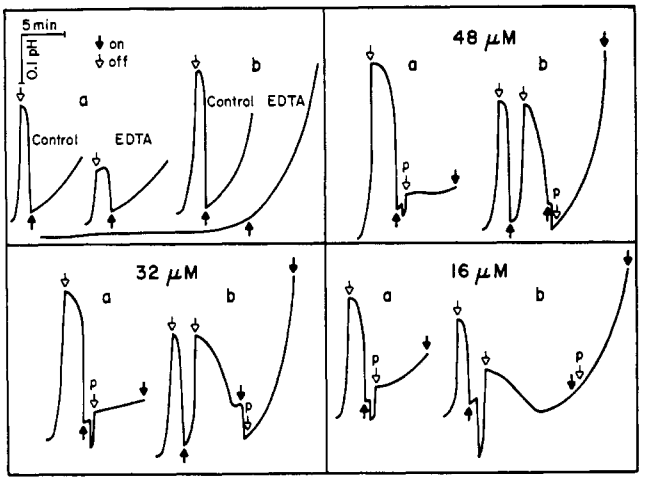

FIGURE 2: Kinetics of the reconstitution of the proton pump in chloroplasts totally or partially lacking energy conversion capability. Reconstitution experiments were carried out in reactions containing 450 μg of chlorophyll and the components described in Methods for the proton uptake reaction. The EDTA-treated chloroplasts used were prepared as described in Methods and were completely (b) or partially uncoupled (a) with respect to phosphorylation and proton uptake. Control chloroplasts (C) were prepared as described in Methods. EDTA-treated chloroplasts were illuminated in the presence of 16, 32, and 48 μM DCCD for 5 min; then the light was turned off and pyocyanine (P) added at an 8.3 μM concentration and the pH was adjusted to 7.1 $\pm$ 0.025. Illumination was then continued to a maximal reconstitution of proton uptake. The light was then turned off and after the efflux of H^+ was complete the pH was readjusted as above and a second cycle of proton uptake completed for the completely uncoupled reaction. Control and EDTA-treated reactions minus DCCD were carried out as described in Methods with illumination beginning after 5-min preincubation in the dark.

of membrane-bound coupling factor. It was important to this work to try to relate the extent of uncoupling as revealed by the loss of phosphorylating capacity to the loss of the capacity to generate a proton gradient. This was essential to study the relation of the extent of energy coupling to the reconstitution of the proton pump by DCCD.

It was desirable to be able to prepare EDTA-treated chloroplasts with a given degree of energy conversion capability. This proved to be difficult as the degree of uncoupling produced by a given concentration of EDTA was quite variable due to variability of carryover of ions in each preparation. Such carryover was sufficient to protect the chloroplasts from uncoupling as described by Avron (1963). Another difficulty was that the extent of uncoupling of phosphorylation measured varied with the type of reaction mixture and electron carrier or acceptor used. It was found that a good correlation between uncoupling of phosphorylation and loss of proton pump activity could be obtained if the residual phosphorylation was measured by the method of Nishimura *et al.* (1962) as described in Methods. When completely or partially uncoupled chloroplasts were required a chloroplast preparation was treated with a range of EDTA concentrations and immediately tested for residual activity with respect to the proton pump and phosphorylation by this method.

Our initial reconstitution experiments had shown that the interaction between DCCD and the chloroplast membranes responsible for reconstitution of proton uptake shares certain features in common with the light-dependent inhibition of basal electron flow. This common feature was a requirement for a period of illumination of the uncoupled chloroplasts in the presence of DCCD and an electron carrier. The data of Figure 2 illustrate the DCCD concentration dependence of the reconstitution in chloroplast fragments completely and partially uncoupled with respect to phosphorylation and the proton pump by EDTA treatment. The data show that when EDTA-uncoupled chloroplasts are illuminated in the presence of DCCD in the absence of an electron carrier there is no reconstitution of the proton pump. On addition of pyocyanine and continued illumination there is a time and concentration dependent reconstitution of the capacity to take up protons. The data also show that the final extent of the proton uptake reconstituted is concentration dependent as well.

Reconstitution of the proton uptake reaction in chloroplasts partially uncoupled by EDTA treatment studied using chloroplasts prepared as described in Methods is also shown. In contrast to the reconstitution of the completely uncoupled system, the reconstitution here proceeds to a maximal extent without the necessity of a period of electron transport. Again, the extent of the reconstituted proton uptake reaction is concentration dependent. The data of this experiment indicate that the interaction responsible for the restoration of nonphosphorylating energy conversion is enhanced by the presence of catalytically active CF_1 as is photophosphorylation. The increase in the proton uptake over the control seen in this experiment is significant as it can be related to the apparent decrease in proton permeability of the grana membranes which will be shown to cause a stimulation of acid-induced ATP synthesis.

Relationship of the Reconstitution of the Proton Pump to the Inhibition of Electron Flow. The preillumination requirement for the inhibition of noncyclic electron flow and the pyocyanine requirement for reconstitution of the pH rise reaction suggested that these processes might be related and perhaps interdependent. The class III acceptor, *p*-benzoquinone (Saha *et al.*, 1971), is an effective cofactor for electron transport and phosphorylation; it undergoes a photoreduction coupled to photophosphorylation which is extremely sensitive to DCCD (100% inhibition by 0.1 mM without preincubation, E. Uribe unpublished). It can also support an easily measurable proton uptake reaction. The reduction of *p*-benzoquinone and the proton uptake reaction were followed simultaneously as described in Methods. The effect of DCCD on electron transport and the proton pump in control chloroplasts is shown in the data of Table IV. The effect of DCCD on

TABLE IV: Effect of DCCD on Benzoquinone Reduction and Proton Translocation.[a]

Initial Reaction pH	*p*-Benzoquinone Reduction (μmoles/hr per mg of Chlorophyll) Control	Plus DCCD	Proton Uptake (nequiv/mg of Chlorophyll) Control	Plus DCCD
6.1	71	48	420	254
6.6	83	59	330	253
7.1	107	60	220	242
7.6	143	71	108	182
8.1	261	95	76	168

[a] Benzoquinone reduction and proton uptake were measured using control chloroplasts (B) prepared as described in Methods. Reaction mixtures contained the following components in μmoles: NaCl, 875; Tricine, 0.55; benzoquinone, 5; chloroplasts containing 300 μg of chlorophyll and 600 nmoles of DCCD where indicated. The values shown for electron transport represent initial rates of reaction.

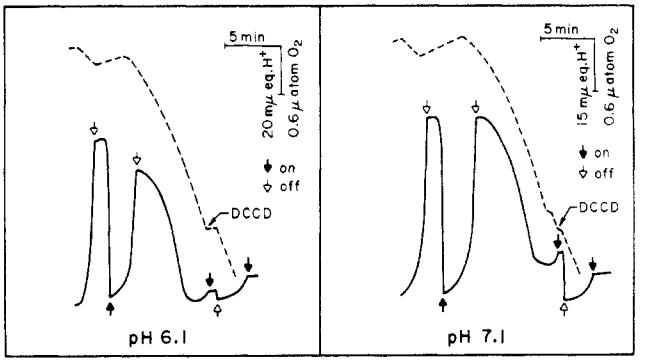

FIGURE 3: Kinetics of the reconstitution of proton uptake and inhibition of electron flow. Reactions contained the components described for Figure 2 with 0.83 mM *p*-benzoquinone being present as the electron acceptor. Measurement of reduction and proton uptake was carried out as described in Methods. The initial reaction pH was 6.1 ± 0.025 or 7.1 ± 0.025. Reconstitution of the pH rise in the EDTA-treated chloroplasts completely devoid of energy conversion capability by 100 μM DCCD was carried out as for Figure 2.

electron transport in this basal system is a concentration-dependent inhibition of electron flow. This inhibition, as that of the basal ferricyanide system, increases in severity as a function of time of electron transport, *i.e.*, longer reaction times (Uribe, 1971). The effect on the proton pump is similar to that reported for the pyocyanine-supported reaction. One contrasting point is the apparent inhibition of the proton pump near its pH optimum by 100 μM DCCD. At higher pH regimes there is still effective inhibition of electron flow; however, there is no inhibition of proton uptake, pH 7.1. At still higher pH's (7.6 and 8.1), a stimulation of the proton uptake over the control values is noted. The inhibition of the proton uptake at pH ranges close to the optimum for the proton pump was attributable to an inhibition of electron transport below a rate necessary to the support of the maximal proton uptake as similar results could be noted by the limitation of electron flow by addition of DCMU or by reducing the incident light intensity. Reduction of the rate of electron flow by these methods to the same extent as that given by 100 μM DCCD had only inhibitory effects on proton uptake at pH 7.1, 7.6, and 8.1.

Relationship of the Reconstitution of the Proton Pump to the Inhibition of EDTA-Uncoupled Electron Flow. The benzoquinone system allows the correlation of the electron-transport-enhanced inhibition of basal electron flow in this system (Table IV) with the electron-transport-dependent reconstitution of the proton pump in EDTA-treated chloroplasts (Figure 2). The experiment of Figure 3 shows that the inhibition of EDTA-uncoupled electron transport with benzoquinone as the electron acceptor is similar to that noted to the ferricyanide system. There is an initial inhibition of electron flow followed by an increased inhibition as a function of time of illumination. The pH rise reconstitution and oxygen evolution curves show the same time and concentration dependence as in the pyocyanine system indicating that the maximal reconstitution of the pH rise reaction in chloroplasts completely uncoupled by EDTA treatment is coincident with a maximal inhibition of electron flow.

Interaction of a Water-Soluble Carbodiimide with the Proton Translocation System. The experiments of Table II indicate that the water-soluble carbodiimide EDAC has little effect on either phosphorylation or electron transport in standard reaction mixtures. The effect of this compound on nonphosphorylating energy conversion was investigated as described for experiments with DCCD with benzoquinone as the electron acceptor.

These experiments showed that EDAC has no effect on either the proton uptake of control chloroplasts measured at the optimum of the proton uptake reaction or the rate of electron transport at pH 6.1. Its presence is also without effect on electron flow in EDTA-treated chloroplasts and was not effective in the reconstitution of the proton uptake reaction nor did it prevent or alter the rate or extent of reconstitution by DCCD (E. Uribe unpublished experiments).

Stimulation of Acid-Induced ATP Synthesis. The stimulation of phosphorylation and the reconstitution of the proton pump in EDTA-uncoupled chloroplasts by DCCD might be due to the protection of a nonphosphorylated high-energy intermediate required for either process. Another alternative is that the interaction reduced the proton permeability which is responsible for uncoupling. The apparent increase in the extent of the pH rise reaction of pH 7.6 and 8.1 on treatment of chloroplasts with DCCD indicates that proton permeability is decreased; thus it seemed possible to test the effect of DCCD on a reaction dependent on the capacity of membranes to maintain a proton gradient. The chloroplast fragments reported to be uncoupled by treatment with EDTA retain some 25–30% of their CF_1 even as they are almost completely uncoupled with respect to phosphorylating and nonphosphorylating energy conversion (McCarty and Racker, 1967; E. Uribe, unpublished). It would seem, then, that DCCD might make these fragments competent in acid-induced ATP synthesis by reducing proton permeability.

EDTA-treated chloroplast fragments prepared as described in Methods were treated with DCCD under conditions known to promote a reconstitution of the proton pump (Figure 4). The treatment of coupled control chloroplasts with DCCD causes a slight stimulation of ATP synthesis followed by an inhibition. As can be seen the stimulation is similar for all three cases; however, preincubation with DCCD causes a much more severe inhibition of ATP synthesis in this system. The addition of DCCD to fragments completely uncoupled by EDTA treatment is seen to be totally ineffective in causing

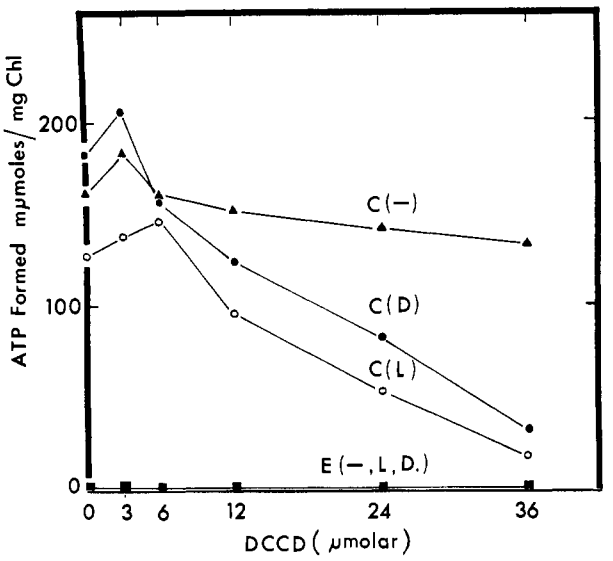

FIGURE 4: Effect of DCCD on acid-induced ATP synthesis in control and EDTA uncoupled chloroplasts. Reactions were carried out as described in Methods with control (C) and EDTA treated chloroplasts which were completely devoid of energy conversion capability. Chloroplasts were mixed with the indicated concentrations of DCCD and then immediately carried through the reaction procedure (−) or incubated in light (L) or darkness (D) for 5 min prior to running the reactions.

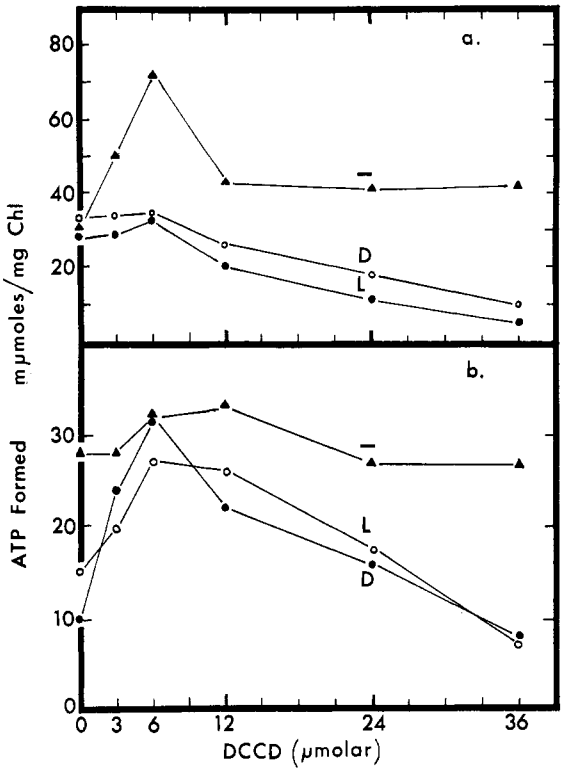

FIGURE 5: Stimulation of acid-induced ATP synthesis in partially uncoupled chloroplast fragments. Reactions were run as for Figure 4 using partially uncoupled chloroplasts prepared by treating with 2 mM EDTA as described in Methods. The residual phosphorylation at pH 8.2 and proton uptake at pH 7.1 were determined as described in Methods. The chloroplasts used in expt a had no measurable proton uptake or phosphorylation using this assay. The chloroplasts used in expt b retained 50 and 25% of the proton uptake and phosphorylation capacity respectively as compared to the controls. The control rate of phosphorylation was 47.6 μmoles/hr per mg of chlorophyll and the control proton uptake was 177.6 nequiv of H^+/mg of chlorophyll. The control acid-induced ATP synthesis in nmoles/mg of chlorophyll was 112 (−); 79 (D) and 62 (L) for expt a which had 4-min preincubations and 57 (−), 46 (D) and 23 (L) for expt b which had 2-min preincubation.

a recovery of capacity to synthesize ATP in a pH transition.

The experiments of Figure 2 indicated that when residual phosphorylation activity is present the reconstitution of proton uptake by DCCD is not time dependent; thus the effect of DCCD on the acid-induced ATP synthesis reaction in partially uncoupled chloroplast preparations was investigated. The curves of Figure 5 illustrate the concentration dependence of the DCCD effect in chloroplasts partially uncoupled by controlled EDTA treatment. In expt a chloroplasts which showed no observable proton uptake or light-driven phosphorylation but retain some 30% of the acid-induced ATP synthesis activity (EDTA treated; 0, DCCD) were used. Here DCCD causes a maximal stimulation in the absence of preincubation and has no inhibitory effect. Preincubation for 4 min in light or darkness results in a decrease in ATP synthesis which is slightly stimulated at low concentrations of DCCD and inhibited at higher levels. When fragments which retain 26% of their photophosphorylation capacity and 50% of their proton-uptake capacity, expt b, are used the addition of low concentrations of DCCD again causes stimulation in the absence of preincubation. Preincubation for 2 min in light or darkness with 3–12 μM DCCD causes a marked stimulation followed by inhibition. These data illustrate that the DCCD–membrane interaction required for enhancing ATP synthesis is time dependent and that this interaction is occurring as the phosphorylation function of the coupling factor is being impaired.

Discussion

The experiments reported in this communication illustrate some aspects of the interaction of the lipid-soluble carbodiimide DCCD with chloroplast grana membranes. The results have relevance to the understanding of how the interaction can result in the alteration of the expression of energy conversion by the photosynthetic system. The interaction of DCCD with control chloroplasts results in an immediate and effective inhibition of phosphorylation and the inhibition of coupled electron transport. This interaction is completely without effect on the proton-uptake reaction. Illumination of chloroplasts in the presence of DCCD and an electron carrier or acceptor results in the inhibition of basal electron flow at pH ranges favoring proton translocation or phosphorylation. High DCCD concentrations (100 μM) can inhibit proton uptake as well at pH ranges favoring the proton pump due to a severe limitation of electron flow (Table IV). At higher pH ranges the inhibition of electron flow is not accompanied by inhibition of proton uptake and indeed the presence of this compound stimulates the extent of the proton gradient measured. The experiments reported cannot distinguish if the effect on proton uptake is due to an increased rate of uptake or a decreased permeability of the membranes to protons; however the stimulation of the acid-induced ATP synthesis reaction by DCCD indicates that the latter is a more plausible explanation of the observations.

Comparison of the activity of lipid- and water-soluble carbodiimides toward several energy-linked chloroplast reactions has shown that the interaction of the carbodiimides

with the chloroplast system is dependent on lipid solubility. The lipid-soluble carbodiimide is a potent inhibitor of phosphorylation and electron transport and can function to reconstitute the proton pump while the water-soluble carbodiimides EDAC and CMMT have little effect on energy conversion in the chloroplast system.

Experiments on the inhibition of electron flow in EDTA-treated chloroplasts have indicated that the interaction of DCCD with this type of chloroplast is substantially different from that noted in coupled chloroplasts. The data of Table III indicate that the altered response of EDTA-uncoupled chloroplasts is specifically related to the removal of CF_1 by the EDTA wash and not a consequence of membrane disorganization induced by washing with low osmotic strength solutions. The data suggest that the removal of CF_1 specifically alters the accessibility of DCCD to the electron transport chain and this is responsible for the increased sensitivity of the uncoupled electron flow to this compound.

The interaction of DCCD with chloroplasts completely uncoupled with respect to photophosphorylation and proton uptake by EDTA treatment results in a reconstitution of the proton pump which is also dependent on electron transport. When reconstitution is determined as a function of electron flow the maximal reconstitution is seen to correspond to the maximal inhibition of electron transport. In chloroplasts partially uncoupled by EDTA treatment so that they retain some phosphorylation and proton pump activity the interaction responsible for reconstitution of the proton pump is not dependent on electron transport; thus, the electron-transport requirement is noted only when all catalytically active CF_1 is removed from the chloroplasts by EDTA extraction. These data indicate that catalytically active coupling factor facilitates the binding of DCCD to the site responsible for inhibition of electron flow and that CF_1 may have a direct role in the proton-uptake reaction. The electron-transport requirement in completely uncoupled chloroplast fragments might also indicate that the CF_1 remaining after EDTA extraction which may be effective in the reconstitution of the proton-uptake reaction is in a lipophilic environment which is made more accessible to DCCD by conformational changes accompanying electron flow. This is supported by that apparent specificity of the reconstitution for the lipid-soluble carbodiimide. The difference in activity between the lipid- and water-soluble carboxyl active reagents might be due to the difference in structure and not a function of solubility properties; thus, EDAC might be able to interact with but not bind to the energy conserving sites on the membrane. The experiments which have shown that EDAC does not interfere with the reconstitution of the proton pump by DCCD support the idea that lipid solubility as well as the chemical reactivity is essential to the reconstitution of this reaction.

The work of Jagendorf and Uribe (1966a) and Uribe and Jagendorf (1968) had shown that the chloroplast preparations used to demonstrate high proton pump and acid-induced ATP synthesis activities are highly disorganized grana membranes which are very weakly coupled with respect to photophosphorylation. The experiments of Kaplan *et al.* (1967) also indicated that the residual coupling factor activity was low, indicating a loss of CF_1 on hypotonic breakage during preparation. These data would be explained by the differential sensitivity of the various energy conversion reactions to removal of coupling factor as described herein. Preparation of chloroplast fragments active in the acid-induced ATP synthesis reaction results in chloroplasts competent in maintaining and utilizing a proton gradient yet presumably deficient in coupling factor. These are found to be stimulated in their acid-induced ATP synthesis activity by DCCD. This is expected from the findings of Racker and Horstmann (1967) and McCarty and Racker (1967) that stimulation of oxidative and photophosphorylation requires the presence of catalytically active coupling factor. In accord with this, note that addition of DCCD to EDTA-treated chloroplast fragments completely incapable of energy coupling does not cause a reconstitution of acid-induced ATP synthesis (Figure 4). The stimulation of energy coupling by DCCD is observed only when there is a residual energy coupling expressed as a capacity for photophosphorylation, acid-induced ATP synthesis or proton uptake capacity remaining after EDTA treatment. Preincubation with DCCD under conditions found to reconstitute the proton pump causes a stimulation of acid-induced ATP synthesis in chloroplasts with little residual phosphorylating activity again indicating the time and electron flow dependence of binding when little residual CF_1 remains. These results indicate that the coupling factor which is responsible for reconstitution of the proton pump in chloroplasts completely uncoupled by EDTA treatment is not functional in phosphorylation. An alternate explanation is that the EDTA treatment removes both coupling factor and another protein or coupling factor subunits required for the proton-uptake reaction. These possibilities are being investigated further.

Stimulation of the acid-induced ATP synthesis reaction in partially coupled chloroplasts and the stimulation of the proton pump at pH 7.5 and 8.1 strongly suggest that the DCCD–membrane interaction is promoted by CF_1 and results in a specific decrease in proton permeability of the grana membrane. Such a decrease results in an increased proton uptake in the pH range 7–8 where the proton pump is not favored and in an increased capacity to utilize at a proton gradient for the synthesis of ATP in the presence of catalytically active coupling factor.

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Synthesis of Bile Pigments in Plants. Formation of Carbon Monoxide and Phycocyanobilin in Wild-Type and Mutant Strains of the Alga, *Cyanidium caldarium*†

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ABSTRACT: Wild-type cells of the alga, *Cyanidium caldarium*, lack photosynthetic pigments when grown in darkness but produce phycocyanin and chlorophyll a when placed in the light. During synthesis of phycocyanobilin, the bile pigment prosthetic group of phycocyanin, wild-type cells evolved equimolar quantities of carbon monixide (CO) at identical rates. Cells of the wild-type, mutant III-D-2 (which makes more pigment per cell than the wild type), and mutant GGB strains (which makes phycocyanin but not chlorophyll a), administered δ-aminolevulinic acid-5-^{14}C (ALA), produced equimolar quantities of labeled CO phycocyanobilin in which the specific activity of CO was one-seventh that of phycocyanobilin. This suggests that CO and algal bile pigment are derived from the carbon skeleton of protoporphyrin IX. Mutants which are unable to make phycocyanin did not produce CO in the light. Wild-type cells incubated with unphysiologic concentrations of ALA-5-^{14}C in the dark excreted labeled phycocyanobilin and evolved labeled CO at comparable rates. The specific activity of evolved CO was one-seventh that of excreted phycocyanobilin. This shows convalent linkage of phycocyanobilin to phycocyanin apoprotein is not an essential step in production of algal bile pigment, and that the mechanism of porphyrin ring opening and CO formation appears to be similar whether phycocyanobilin is apoprotein bound or apoprotein free. The probable involvement of a metal complex of protoporphyrin IX as the direct precursor of phycocyanobilin is discussed, as is the relation of algal CO production to CO exhalation in rats, dogs, and man as a consequence of administered heme and hemoglobin conversion to mammalian bile pigment.

Phycocyanin and phycoerythrin are bile pigment–protein-complexes found in the photosynthetic apparatus of red, blue-green, and cryptomonad algae (O'hEocha, 1966). These algal biliproteins are thought to serve as accessory pigments by virtue of their promotion of photosynthetic oxygen evolution and a chlorophyll a fluorescence (Myers, 1971). Biliproteins exist *in vivo* as large aggregates called phycobilisomes, which appear in stained sections in the electron microscope as granules, 350–450 Å in diameter, located on the surfaces of thylakoid membranes in chloroplasts (Gantt and Conti, 1967). Biliprotein molecules are comprised of subunits which associate and dissociate in aqueous solution into larger or smaller molecular species depending on the pH, ionic strength of buffer, and pigment concentration (O'hEocha, 1965; Hattori *et al.*, 1965). The results of sodium dodecyl sulfate polyacrylamide gel electrophoresis of phycocyanin suggest that the molecule consists of two distinct subunits with molecular weights of 16,000 and 17,000, respectively (Bennett and Bogorad, 1971). Each subunit is believed to contain one residue of the bile pigment chromophore, phycocyanobilin. Phycocyanobilin is structurally related to bilirubin (Cole *et al.*, 1967; Crespi *et al.*, 1967), the principal mammalian bile pigment derived from hemoglobin in senescent erythrocytes (Lester and Troxler, 1969). Phycocyanobilin is covalently linked to phycocyanin apoprotein (Siegelman *et al.*, 1967), and comprises 3.6–4.0% (by weight) of the phycocyanin molecule (Troxler and Lester, 1967; Crespi *et al.*, 1968). The "biosynthetic unit" of phycocyanin, therefore, consists of a polypeptide chain in the 16,000 molecular weight range (*ca.* 130 amino acid residues) to which 1 residue of phycocyanobilin (molecular weight of the free acid = 586; Cole *et al.*, 1967; Crespi *et al.*, 1967) is covalently linked. It is not known whether phycocyanobilin and phycocyanin apoprotein are synthesized separately and then joined, or if a "biosynthetic unit" of phycocyanin is derived from conversion of a metalloporphyrin–protein complex to a phycocyanobilin–protein complex *via* porphyrin ring opening *in situ*, although either alternative is possible (Bogorad and Troxler, 1967). Studies on phycocyanin biosynthesis are complicated by the fact that (a) it is a conjugated protein whose moieties are end products of different metabolic pathways, (b) the nature of the covalent linkage between bile pig-

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