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ACTIONS OF CARBONYLCYANIDE *m*-CHLOROPHENYLHYDRAZONE ON ELECTRON TRANSPORT AND FLUORESCENCE OF ISOLATED CHLOROPLASTS

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SUMMARY

The actions of carbonylcyanide *m*-chlorophenylhydrazone (CCCP) on photosynthetic electron transport and fluorescence have been studied using isolated pokeweed (*Phytolacca americana*) chloroplasts which often had been uncoupled by EDTA washing or by addition of methylamine.

The presence of CCCP markedly enhanced the photoreduction of oxygen by System 1 with or without added electron donors. It is concluded that the decreased steady state level of chloroplast fluorescence after an addition of CCCP was largely due to an increased rate of electron flow through System 1 to oxygen, rather than to an inhibition of System 2.

The inhibition of oxygen evolution by moderate amounts of CCCP was only slight initially, but became more severe during illumination. CCCP appeared to divert oxidizing equivalents from System 2 into destructive side reactions.

CCCP strongly inhibited the reoxidation of the reduced primary acceptor Q in 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)-poisoned chloroplasts. A comparative study of similar actions by various electron donors to System 2, and by inhibitors of phosphorylation, failed to reveal a simple mechanism. One has to take into account not only a direct reduction of the oxidized reaction partner of Q^- by the added agent, but also its effect on the membrane integrity, and on the interactions between the electron carriers in System 2.

INTRODUCTION

The carbonylcyanide phenylhydrazones represent a class of metabolic poisons which interfere with the orderly progress of membrane-dependent reactions, such as phosphorylations, interactions of electron carriers, and transport processes[1,2]. Halogen substituted members of this group, *e.g.* the chloro-derivative carbonylcyanide *m*-chlorophenylhydrazone (CCCP) are widely used as uncouplers of photophosphorylation reactions in chloroplasts *in vitro* as well as *in situ*[2,3]. While the mechanism of their

Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol; TMPD, *N,N,N',N''*-tetramethyl-*p*-phenylenediamine; Q, "quencher", the primary electron acceptor of System 2; F_0, the invariable fluorescence yield; F_{DCMU}, the fluorescence yield in the presence of 10 μM DCMU.

uncoupling action has been studied extensively in many laboratories, other effects of the carbonylcyanide phenylhydrazones on electron transport processes have received much less attention. It has been observed several years ago, for example, that at concentrations about 10 times higher than needed for uncoupling, CCCP would inhibit photosynthetic oxygen evolution[4,5]. Recent data[6-8] suggest that this inhibition occurs on the oxidizing side of Photosystem 2 rather on the reducing side which is the site of action of the "classical" inhibitors of photosynthetic oxygen evolution, like *o*-phenanthroline and the substituted ureas[9].

RENGER[7] determined the oxygen yield in two light flashes which were separated by dark times of various lengths. In the presence of CCCP, the yield in the second flash declined if the dark time had been longer than 25 msec. The author concluded that CCCP had reduced a primary oxidation product which had been formed in the first flash. Additional evidence for an action of CCCP on the oxidizing side of Photosystem 2 was provided by ITOH *et al.* [8]. These investigators have shown that the decreased fluorescence yield of chloroplasts in the presence of CCCP can be restored to a higher level by addition of substances which serve as artificial electron donors to System 2. On the basis of the widely accepted theory of DUYSENS AND SWEERS[10], such fluorescence data can be explained by assuming that CCCP is capable of inhibiting the electron flow between water and some electron carrier close to the reaction center of System 2. CCCP, therefore, appeared to affect Photosystem 2 in a way quite similar to an extraction of chloroplast manganese[11-13], chloride deficiency[14], and ultraviolet irradiation[15,16].

In this present study we investigated the actions of CCCP on fluorescence properties and electron transport processes of isolated chloroplasts. Simply because of its uncoupling activity, CCCP was expected to increase the rates of electron flow whenever it was limited by passage through a coupling site. To exclude such effects, the electron transport chain was uncoupled from ATP synthesis by other means prior to an addition of CCCP. Our results suggest that CCCP interferes with an uncoupled photosynthetic electron transport at two points: on the reducing side of System 1, and on the oxidizing side of System 2. At both sites it appears to facilitate a diversion of redox equivalents from their normal path into side reactions with added or native molecules.

MATERIALS AND METHODS

Preparation of chloroplasts

Initial studies on the action of CCCP were done with spinach (*Spinacea oleracea*) chloroplasts, but a continuous supply of such material is not easily available in Florida. All the quantitative data which are reported in this paper, have been obtained with chloroplasts isolated at 0 to 5° from field grown pokeweed (*Phytolacca americana*). During the hot summer months, cut stems were kept at room temperature in tap water under dim light for at least 24 h before use. Leaves were ground for 10 sec with a Waring blendor in 0.4 M sucrose containing 50 mM Tricine–NaOH buffer at pH 7.4, and 2 mM $MgSO_4$. The slurry was squeezed through 8 layers of cheese cloth, the filtrate spun in a clinical centrifuge at full speed for 10 min, and the resulting pellet was resuspended in the same medium. After another centrifugation, the chloroplasts were suspended in the above mentioned solution supplemented with 10 mM NaCl at a

chlorophyll concentration of about 1.5 mg/ml. This final suspension was freed of most of the starch grains and of large chloroplast aggregates by centrifugation in the clinical centrifuge for 1 min, including time of acceleration. When stored in ice and darkness, these chloroplast preparations did not significantly change their fluorescence characteristics for sometimes up to 20 h quite in contrast to similar preparations from tobacco.

$MgSO_4$ was omitted from the grinding medium if part of the chloroplasts was to be subjected to an "EDTA washing" in order to uncouple their electron transport chain from ATP synthesis[17]. The EDTA-washed chloroplast pellet[18] was suspended in a medium identical to that used for the normal preparations. EDTA-washed chloroplasts were considered to be satisfactorily uncoupled when, at saturating light intensities, the addition of 15 mM methylamine enhanced the rate of their ferricyanide Hill reaction less than 10%. Under the same conditions, the rate of the Hill reaction of normal, coupled chloroplasts from pokeweed was increased at least 5 fold.

Sometimes, an addition of 15 mM methylamine–HCl at pH 7.4 was used to uncouple phosphorylation in untreated chloroplasts. As far as the observed effects of CCCP were concerned, no significant difference was ever noted between chloroplasts uncoupled by EDTA washing, or by addition of methylamine.

"Tris-washed" chloroplasts with an inactivated Photosystem 2 (ref. 13) were obtained by suspending the pellet from the first centrifugation in 0.8 M Tris–HCl at pH 8.0, and sedimenting it after 5 to 10 min at 0° by a 5-min centrifugation at 5000 $\times$ *g*. The final suspension medium was the same as that described above for the other chloroplast preparations.

Measurements and reaction mixtures

All chloroplast reactions were performed at 25° in solutions identical to the suspension medium. When in the reaction oxygen served as final electron acceptor, 0.2 mg catalase (Sigma Chem. Comp.) per 3 ml and 3% ethanol were included in order to prevent any interference from native catalase or a possible accumulation of H_2O_2. Photoreductions of artificial electron acceptors were measured as described previously[19]. Changes in oxygen concentration were monitored using a Clark type oxygen sensor (Yellow Springs Instrument Comp.) which was incorporated into a thermostated plastic chamber (volume 3 ml) with an airtight lid. "Red" light was obtained from a projection lamp equipped with a red plastic filter, and its intensity was determined between 600 and 700 nm[19] with an ISCO Spectroradiometer (Instruments Specialty Comp.).

Fluorescence measurements were done with a setup quite similar to that described earlier[20]. Green actinic light (λ_{max} = 530 nm), separated from the light of a projection lamp by a series of plastic filters, was admitted to the sample chamber by an electric shutter (opening time approx. 20 msec). Two light intensities were selected with a variable transformer: a "high" intensity with 22000 ergs·sec^{-1}·cm^{-2} (equivalent to about 10^{-8} Einstein), and a "low" intensity which was 1/10 of the high one. The fluorescence emission at 683 nm was separated at a right angle to the incident light with a Bausch and Lomb monochromator, and detected by an EMI 9558B photomultiplier tube. The signal was displayed either on a Honeywell X–Y recorder (model 530T), or on a storage oscilloscope (Tektronix 564B) equipped with a camera. Far-red illumination could be provided from the side opposite to the actinic beam

by use of a General Electric Co. "Sungun" and a Rohm and Haas plastic filter FRF-700. Its intensity was about 0.5 nEinstein·sec^{-1}·cm^{-2} between 700 and 730 nm.

The rise time of the fluorescence in the absence or in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was measured as area over the fluorescence induction curve according to MALKIN AND KOK[21], using either a planimeter, or cutting out the appropriate sections and weighing them on an analytical balance.

Repeated evacuation of chloroplast suspensions in Thunberg cuvettes, or bubbling nitrogen through, was found to result in some irreversible damage to the chloroplasts as reflected by lowered electron transport rates and increased rise times of the fluorescence after readdition of air. This had to be taken into account when anaerobic experiments were evaluated.

Chlorophyll was determined according to MCKINNEY[22]. CCCP (Calbiochem) was added from ethanolic solutions, never allowing the final alcohol content in the chloroplast suspension to exceed 3%. DCMU was pipetted from an aqueous solution the concentration of which was determined using an extinction coefficient of $1.85 \cdot 10^4$ $l \cdot cm^{-1} \cdot mole^{-1}$ at 249 nm. All chemicals used in this study were of reagent grade.

RESULTS

The quenching of chloroplast fluorescence by CCCP

As mentioned above in the INTRODUCTION, ITOH *et al.*[8] have shown that a CCCP addition decreases the steady-state fluorescence yield $F_\infty - F_0$ of isolated chloroplasts. According to the theory of DUYSENS AND SWEERS[10], the level of the "variable" fluorescence emission is controlled by the degree of reduction of the total pool of primary electron acceptors Q in Photosystem 2. Under steady state conditions, therefore, the fluorescence intensity must depend on the rate of electron delivery through Photosystem 2 as well as on the rate of electron utilization by System 1, and by autoxidations[21,23]. It follows that the CCCP induced decrease in the fluorescence yield has to be attributed to a low rate of photoreduction and/or a high rate of reoxidation of Q.

An inhibited photoreduction of Q is known from chloroplasts in which the water oxidizing system has been impaired by an extraction of chloroplast manganese[11–13], by chloride deficiency[14], or by ultraviolet irradiation[15,16]. In all of these cases, a high rate of electron flow to Q was restored by addition of compounds acting as electron donors to intermediates on the oxidizing side of Photosystem 2. This could be deduced easily from a donor dependent regeneration of the fluorescence rise[11–16]. ITOH *et al.*[8] have reported that an addition of the electron donor couple ascorbate-*N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) reversed the CCCP-induced decrease of the fluorescence yield. Evidently, CCCP had inhibited normal electron flow at a site located on the oxygen evolving side of System 2. Although we could confirm the fluorescence quenching by CCCP, we have been unable to observe any significant recovery of the fluorescence yield after addition of electron donors like ascorbate–TMPD, ascorbate–*p*-phenylenediamine, hydroxylamine, or H_2O_2 (Fig. 1). As already noted by ITOH *et al.*, Mn^{2+} was also ineffective.

These results made it doubtful whether the fluorescence decrease was due to any inhibition by CCCP of the electron flow on the oxidizing side of System 2. One possible other explanation for the effect of CCCP on the fluorescence level was that

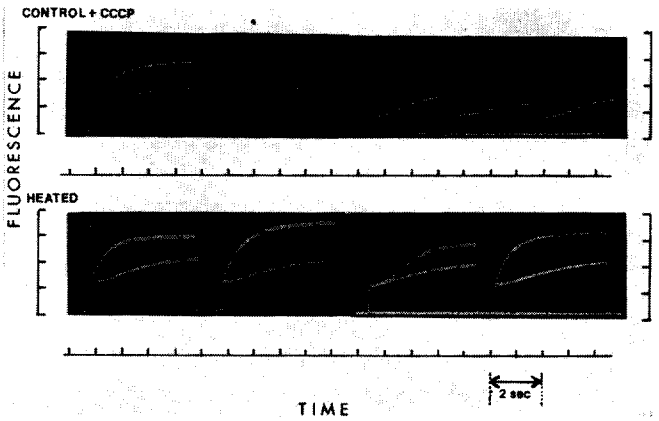

Fig. 1. Effect of electron donors on the fluorescence rise in chloroplasts. Upper series: normal chloroplasts, 8 μM chlorophyll; 10 μM CCCP added except where indicated. Lower series: chloroplasts with a Photosystem 2 impaired by heat treatment (see ref. 9), 8 μM chlorophyll. A, + 0.05 mM TMPD + 0.5 mM ascorbate; B, + 1.5 mM hydroxylamine; C, + 30 mM H_2O_2; D, + 0.5 mM $MnSO_4$. Low yield traces in lower series: no addition.

TABLE I

EFFECT OF 15 μM CCCP ON THE PHOTOREDUCTION OF OXYGEN BY ISOLATED CHLOROPLASTS UNDER VARIOUS CONDITIONS

Data represent uptake of μmoles $O_2 \cdot mg^{-1}$ chlorophyll $\cdot h^{-1}$; reaction medium was 80% air saturated sucrose–tricine–$MgSO_4$–NaCl medium as described in MATERIALS AND METHODS *plus* 0.2 mg catalase and 3% ethanol. Further additions as indicated: 50 μM methyl viologen; 40 μM DCIP + 8 mM ascorbate; 3 mM NH_2OH; 15 mM methylamine–HCl pH 7.4; 10 μM DCMU. Chlorophyll concentration 25 μM, but 2.5 μM for the ascorbate–DCIP–methyl viologen system. Light intensity = 100 corresponds to 125000 erg $\cdot sec^{-1} \cdot cm^{-2}$ red light. The data are corrected for any oxygen uptake in the dark. They are taken from one representative experiment. Addition of 10 μM DCMU to reaction mixtures marked * reduced light dependent oxygen uptake to less than 2 μmoles $O_2 \cdot mg^{-1}$ chlorophyll $\cdot h^{-1}$ at light intensity = 20.

Chloroplasts	*Additions*	*Light intensity:*					
		100		*20*		*2*	
		−CCCP	*+CCCP*	*−CCCP*	*+CCCP*	*−CCCP*	*+CCCP*
EDTA washed	None*	—	—	8.3	16.5	4.5	7.7
	NH_2OH*	—	—	18	26	—	—
	Ascorbate, DCIP, DCMU	31	55	26	43	10	17
	Ascorbate, DCIP, methyl viologen, DCMU	590	640	—	—	82	78
	Ascorbate, DCIP, methyl viologen, methylamine–HCl, DCMU	690	730	—	—	76	79
Coupled chloroplasts	None*	—	—	7.5	16.5	5.0	7.5
	Methylamine–HCl*	—	—	7.8	16.5	4.5	7.0
	Same, no ethanol	—	—	2.0	3.2	—	—

this compound could act as an artificial electron acceptor on the reducing side in the manner of a Hill oxidant. Such an action was expected to have the following consequences: (1) CCCP should support rather than inhibit oxygen evolution; (2) CCCP should be rapidly inactivated by illuminated chloroplasts; (3) during continued illumination, the fluorescence intensity should slowly approach that of an untreated control as all CCCP becomes photoreduced. However, none of these predictions could be supported experimentally.

While investigating the gas exchange of illuminated chloroplasts, we made the interesting observation that the light dependent oxygen uptake of chloroplast suspensions was enhanced in the presence of CCCP. As shown in Table I, this occurred in weak as well as strong light, and 10 μM DCMU inhibited the uptake more than 80%. Similarly, CCCP stimulated the DCMU-sensitive photoxidation of hydroxylamine. It also increased the rate of oxygen uptake in the photoxidation of the ascorbate-2,6-dichlorophenolindophenol (DCIP) couple by Photosystem 1 in the presence of DCMU. However, when the photoreduction of oxygen by Photosystem 1 was mediated by methyl viologen, an addition of CCCP had only a small influence (Table I). The effects on DCMU-poisoned chloroplasts can be explained by assuming that CCCP enhanced the autoxidation rate of an electron carrier on the reducing side of System 1. But a second site for a CCCP-facilitated autoxidation might exist between the two photosystems. This alternative was ruled out by an analysis of the fluorescence curves.

An oxidation of Q^- can be achieved *via* the slow autoxidation of adjacent electron acceptors[21], or by an illumination of Photosystem 1 with far-red light[21,23]. The degree of reoxidation of Q^- can be easily estimated by determining the relative time needed after onset of an actinic illumination to attain the steady state level of the fluorescence emission. The area over the fluorescence curve is a convenient measure of this "rise time"[21]. If CCCP increased the rate of autoxidation of an electron carrier between the two photosystems, the restoration of the fluorescence rise in the dark should be accelerated. This was not observed (Fig. 2).

Fig. 2 shows, however, that the reoxidation of Q^- by far-red light was hastened in the presence of CCCP. This observation can be accounted for in two very different ways; the more obvious being that CCCP accelerated the oxidation of Q^- by System 1, in accordance with the oxygen exchange data presented above. Alternately, a significant portion of the far-red light might have been absorbed by System 2 which would tend to maintain Q in the reduced state and slow the net rate of reoxidation by System 1. If CCCP were able to inhibit System 2, it would block this reducing effect of far-red light and seemingly speed the oxidation of Q^-. It was possible to decide in favor of the first, simpler, explanation by measuring the rate of reoxidation of Q^- in Tris-washed chloroplasts. Tris washing unquestionably inhibits System 2 (ref. 13) yet, as can be seen from Fig. 2, it had no effect on the time course of the regeneration of the fluorescence induction in far-red light.

It can be concluded from the results presented in this section that CCCP quenches the fluorescence of chloroplasts because it accelerates the System 1 mediated oxidation of Q^- by molecular oxygen.

Effects of CCCP on the oxidizing side of Photosystem 2

In the previous section it was shown that CCCP acts on Photosystem 1. We

shall now turn to the well known inhibitory interference of CCCP with the oxygen evolving process[4–6]. In an investigation of the mechanism of this inhibition it was advisable to exclude any distortion of the data by effects of CCCP outside System 2, *e.g.* those on System 1. This was done by adding DCMU which blocks electron transport between the primary acceptor Q and the pool of secondary acceptors A[10,21].

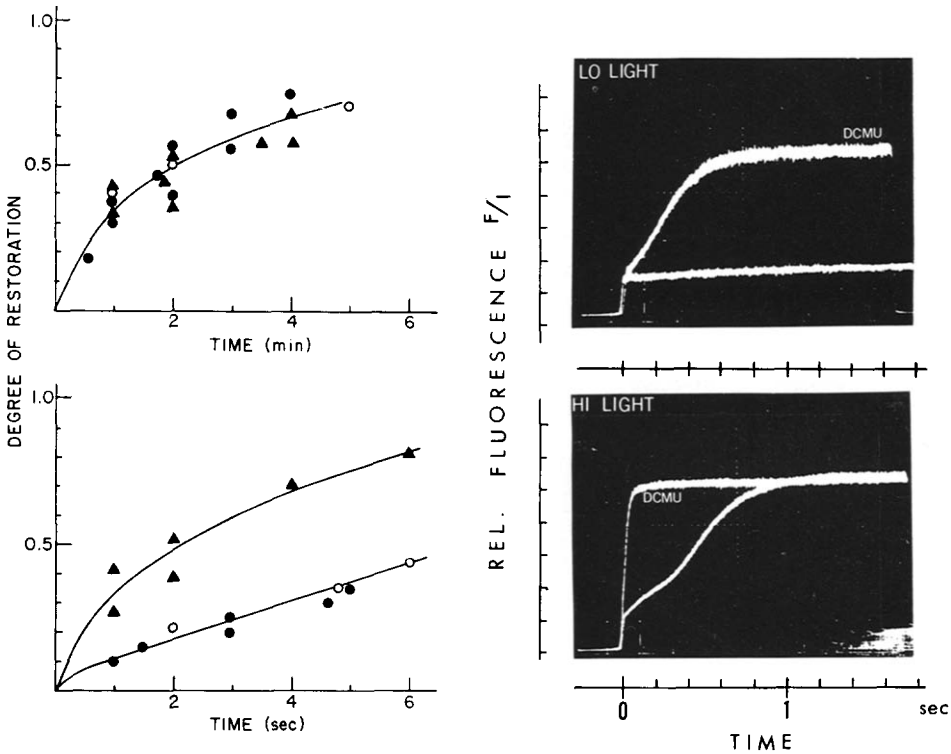

Fig. 2. Time course of restoration of chloroplast fluorescence under various conditions. 10 μM chlorophyll; restoration given as relative area over the fluorescence curve; area for complete restoration equals unity; recorded with 10 nEinstein·sec^{-1}·cm^{-2} green actinic light. Upper figure: restoration in darkness. Lower figure: restoration in far-red light. ●, one preparation normal chloroplasts + 15 mM methylamine–HCl, pH 7.4, and one preparation EDTA-washed chloroplasts; ▲, the same preparations + 5 μM CCCP; ○, Tris-washed chloroplasts with 50% of the Hill activity of the untreated control.

Fig. 3. Fluorescence rise at two light intensities. "HI" light intensity: 10 nEinstein·sec^{-1}·cm^{-2} green light; "LO" light intensity: 1 nEinstein·sec^{-1}·cm^{-2} green light. Normal chloroplasts, 8 μM chlorophyll; 10 μM DCMU present where indicated.

Since the concentration of A in chloroplasts is about 20 times that of Q[24], reduced Q accumulates very rapidly in illuminated, DCMU-poisoned chloroplasts. This is reflected by a fast rise of the fluorescence emission to its steady state level. In order to slow down the photoreactions, we chose a relatively low light intensity in our fluorescence studies. This facilitated the analyses of the fluorescence rise curves.

In Fig. 3, traces of the fluorescence induction of uninhibited, and DCMU-poisoned chloroplasts are shown for "high" and "low" exciting light intensities. Just as in the uninhibited chloroplasts, the fluorescence rise in the presence of DCMU

followed a nonlogarithmic time course. This phenomenon was already seen several years ago by DELOSME[25], who explained it by postulating some excitation transfer between the subunits of Photosystem 2.

As had been shown earlier by HEBER AND FRENCH[26] for chloroplasts *in vivo*, the induction curve of the fluorescence in the presence of DCMU was restored during a dark time of less than 0.5 min (Fig. 4). In accordance with these authors, this oxygen independent restoration of the fluorescence rise will be interpreted as representing a

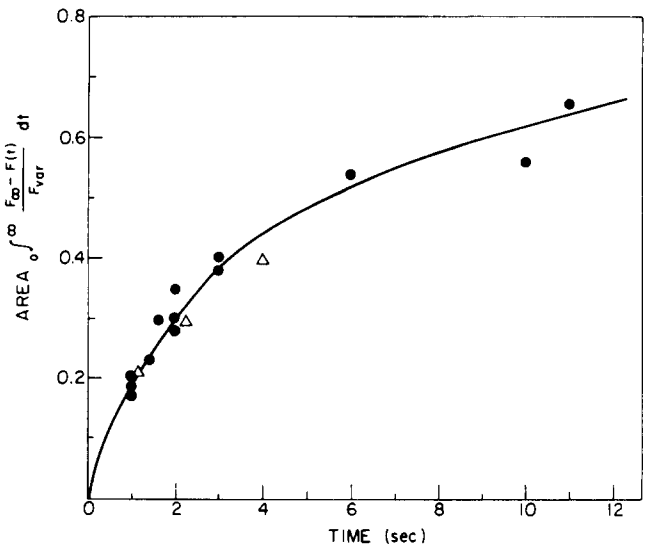

Fig. 4. Restoration of fluorescence rise in the presence of DCMU in darkness. 8 μM chlorophyll; 1 nEinstein·sec^{-1}·cm^{-2} green actinic light; ●, normal coupled chloroplasts; △, EDTA-washed chloroplasts.

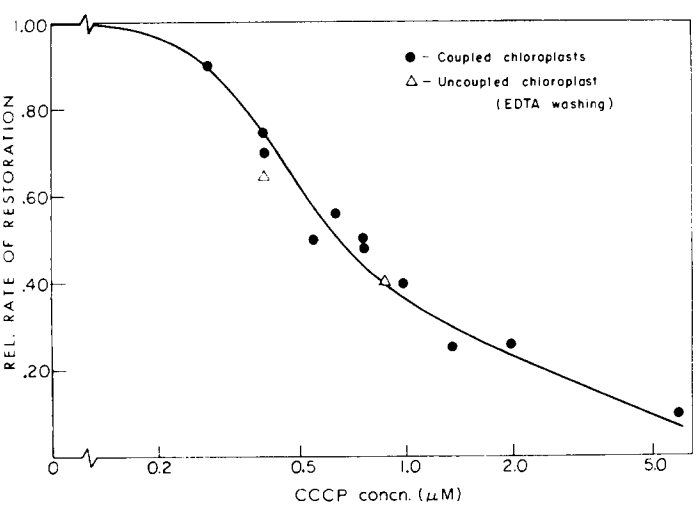

Fig. 5. Inhibition by CCCP of the fluorescence restoration in DCMU-poisoned chloroplasts. Degree of restoration after 2 sec darkness in absence of CCCP is set equal unity. Experimental conditions as in Fig. 4.

reaction between Q^- and an oxidized electron carrier on the water splitting side of System 2.

Interestingly, this back reaction was observed to be effectively retarded by low concentrations of CCCP (Fig. 5). In agreement with RENGER[7], our discovery could be explained by assuming that CCCP is able to reduce an oxidized intermediate located close to the site of oxygen evolution. Artificial electron donors to System 2 should act

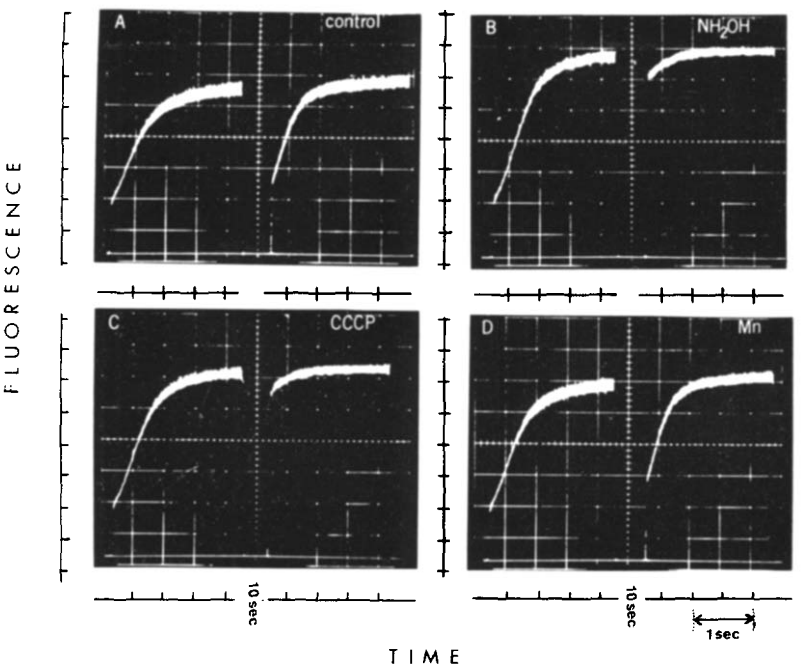

Fig. 6. Effect of various compounds on the restoration of chloroplast fluorescence in the presence of 10 μM DCMU. 1 nEinstein·sec^{-1}·cm^{-2} green light; EDTA-washed chloroplasts, 8 μM chlorophyll. The two recordings in each case were separated by 10 sec darkness. A: no addition. B: + 3 mM NH_2OH. C: + 8 μM CCCP. D: + 1 mM $MnSO_4$.

in identical fashion. As shown in Figs. 6 and 7, the restoration of the fluorescence induction in the presence of DCMU was indeed inhibited by established donors like hydroxylamine, H_2O_2, ascorbate–*p*-phenylenediamine and ascorbate–TMPD (see also Fig. 1). Surprisingly, Mn^{2+} was nearly ineffective although it has been shown to serve as a relatively efficient electron donor to Photosystem 2[11,27]. While this present work was in progress, similar observations concerning the inhibitory action of hydroxylamine on the back reaction in System 2 have been reported by BENNOUN[28].

The unexpected action of CCCP created a problem. If it were true that CCCP could act as an electron donor to System 2, one would expect it to support photoreductions of Hill oxidants while suppressing oxygen evolution. This was never observed. Furthermore, CCCP should become oxidized and possibly inactivated while serving as reductant. In experiments with living algae it has indeed been observed that a normal photosynthetic gas exchange reappeared during a prolonged illumination of CCCP-poisoned cells[29,30]. It would have been interesting to find out whether

System 2 activity in isolated chloroplasts would also recover from inhibitory effects of CCCP during a long light period. Unfortunately, the oxygen evolving apparatus became rapidly inactivated under such conditions. When the time course of the ferricyanide Hill reaction in uncoupled chloroplasts was followed in the presence of

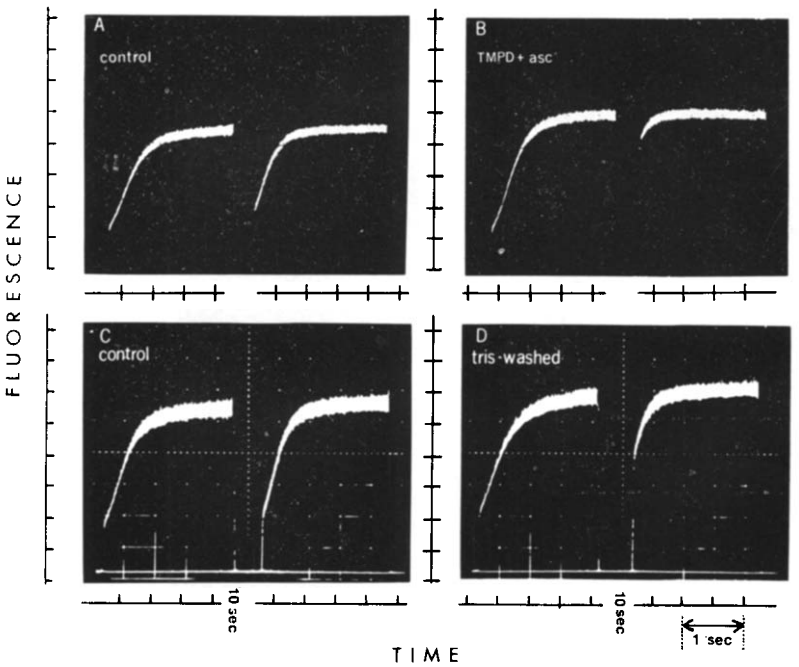

Fig. 7. Effect of TMPD–ascorbate and of Tris washing on the restoration of chloroplast fluorescence in the presence of 10 μM DCMU. A and B: normal chloroplasts + 15 mM methylamine–HCl, pH 7.4, in absence or presence of 0.05 mM TMPD + 0.5 mM ascorbate respectively. C and D: chloroplasts from different preparation + 15 mM methylamine–HCl, pH 7.4, untreated (C) or Tris washed (D), the latter having only 20 % of the control's Hill activity (for relevance of Fig. 7D see DISCUSSION). In each case 8 μM chlorophyll, illumination with 1 nEinstein·sec^{-1}·cm^{-2} green light.

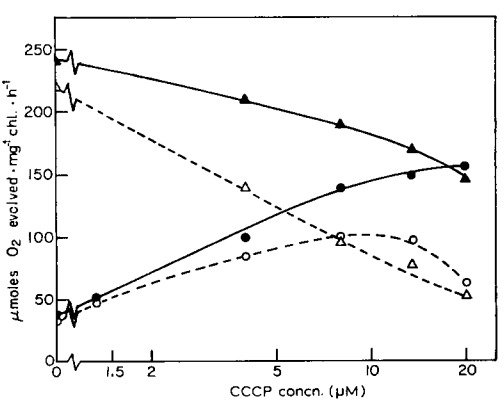

Fig. 8. Effect of CCCP on the ferricyanide Hill reaction. Solid lines, initial rate; broken lines, rate after 3 min. ●, ○, normal, coupled chloroplasts, 4 μM chlorophyll; ▲, △, normal chloroplasts + 15 mM methylamine–HCl, pH 7.4, 3 μM chlorophyll. Ferricyanide concentration 0.5 mM; 120000 erg·sec^{-1}·cm^{-2} red light.

TABLE II

CHANGE WITH TIME OF THE DCIP HILL REACTION IN THE PRESENCE OF 15 μM CCCP

Rates are given in μequiv reduced·mg^{-1} chlorophyll·h^{-1} for one representative experiment with EDTA-washed chloroplasts (3.5 μM chlorophyll). The "low oxygen" samples were illuminated in Thunberg cuvettes which had been evacuated after the buffer had been deaerated by bubbling nitrogen for 15 min. The buffer used was that described in MATERIALS AND METHODS. Light intensity = 100 corresponds to 120000 erg·sec^{-1}·cm^{-2} red light.

	Light intensity:					
	100				*8*	
	Air saturated		*Low oxygen content*		*Air saturated*	
CCCP addition:	−	+	−	+	−	+
Initial rate	660	540	550	340	190	140
Rate after 3 min	620	220	530	90	190	25

various amounts of CCCP (Fig. 8), a continuously declining rate was noticed. It looked as if, during the course of the reaction, the initially slight inhibition by CCCP became progressively stronger. This had been observed earlier by HIND *et al.*[31]. With coupled chloroplasts, the electron transport was accelerated by CCCP due to its uncoupling action, and inhibitory effects became evident only at rather high concentrations of this agent (Fig. 8). Preincubation of chloroplasts with CCCP in the dark did not affect the initial degree of inhibition. Table II reveals that similar results were obtained with DCIP as electron acceptor, even when atmospheric oxygen was excluded from the reaction mixture.

YAMASHITA *et al.*[32] have found that carotenoids in Tris-washed, and in CCCP-poisoned chloroplasts, became rapidly oxidized in the light. A photodestruction of the pigment system might have been the reason for the decrease in the electron transport rate which was just described. In such an event the fluorescence yield

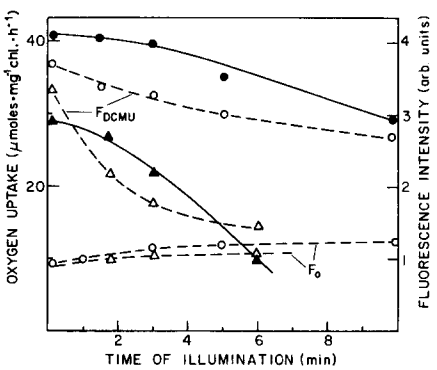

Fig. 9. Decrease of electron transport rate and of the variable fluorescence during illumination of EDTA-washed chloroplasts. Samples for fluorescence measurements in 10 nEinstein·sec^{-1}·cm^{-2} green light were taken before and after an illumination with 7500 erg·sec^{-1}·cm^{-2} red light. After recording the fluorescence in absence of DCMU for measurement of F_0, 10 μM DCMU was added to record the emission in its presence. Closed symbols, oxygen uptake; open symbols, fluorescence. Reaction mixture: 8 μM chlorophyll, 0.1 mM methyl viologen, 0.2 mg catalase, 3% ethanol, with 10 μM CCCP present (▲, △) or absent (●, ○).

F_{DCMU} might be expected to become smaller. From the experiment depicted in Fig. 9 it can be seen that the light dependent decrease in the rate of a Hill reaction was indeed accompanied by a loss of the variable fluorescence emission. As with the photobleaching of the carotenoids[31], this effect of CCCP and light on the chloroplast fluorescence was retarded by DCMU (Table III). The fluorescence yield could not be restored to normal by a chemical reduction of Q with hydrosulfite (not shown). It appeared, therefore, that the photoinhibition of CCCP-poisoned chloroplasts had resulted in a destruction of the reaction center complex of Photosystem 2.

TABLE III

CHANGE OF THE VARIABLE FLUORESCENCE YIELD DURING AN ILLUMINATION OF ISOLATED CHLOROPLASTS

Prior to the measurement of the fluorescence emission in 10 nEinstein·sec^{-1}·cm^{-2} green light (see MATERIALS AND METHODS) the chloroplasts (8 μM chlorophyll) had been illuminated for 3 min with 7500 erg·sec^{-1}·cm^{-2} red light in an sucrose–tricine–$MgSO_4$–NaCl medium as described in MATERIALS AND METHODS, supplemented with 0.2 mg catalase and 3% ethanol. The variable fluorescence $F_{DCMU} - F_0$ of samples illuminated in the presence of DCMU was estimated by assuming the same F_0 as was determined for the samples illuminated in the absence of DCMU. The variable fluorescence yield of the unilluminated control was set equal to 100.

	No addition	*+ 10 μM DCMU*	*+ 10 μM CCCP*	*+ 10 μM CCCP + 10 μM DCMU*
EDTA-washed chloroplasts	90	110	75	90
Tris-washed chloroplasts	60	90	20	65

DISCUSSION

The data presented in this paper reflect the complexity of the interactions of CCCP with the electron transport chain of isolated chloroplasts. The well documented activity of this compound as an uncoupler reveals its tendency to disrupt the integrity of organized reaction systems on biological membranes. Specifically, it has been shown that CCCP and related compounds inhibit conformational changes of membranes[33], and prevent the formation of pH gradients across membranes by increasing their permeability to protons[34].

At present it is difficult to see how these particular effects of CCCP can be related to the observations described in this paper. At this stage of investigation we can possibly rule out such actions of CCCP as being responsible for the decrease of the fluorescence emission from chloroplasts in the absence of DCMU. Yet, it may be that CCCP-induced structural changes were responsible for the apparent facilitated autoxidation of some electron carrier on the reducing side of System 1. It may be argued that the concentration of the reduced carrier, and hence the rate of its autoxidation, might be elevated if CCCP were capable of inhibiting cyclic electron flow in System 1. However, the relative effect of such back reactions in unpoisoned chloroplasts should become insignificant at high light intensities. Yet, the degree of the CCCP-induced acceleration of the oxygen consumption did not change when the light intensity was increased (Table I).

It is important to note that the faster electron flow to oxygen cannot simply be attributed to the uncoupling action of CCCP. The effect was the same for coupled chloroplasts, and for chloroplasts uncoupled by an EDTA washing, or an addition of methylamine. The rate of the oxygen uptake by illuminated chloroplasts was not limited by the electron flow through a coupling site since an addition of methylamine had no effect (Table I). Furthermore, neither CCCP nor methylamine changed significantly the rate of electron transport from ascorbate–DCIP to oxygen *via* methyl viologen when EDTA washed chloroplasts were used.

Obviously, the CCCP-induced decrease of the fluorescence level ought to be diminished at low oxygen concentrations. Anaerobiosis actually had such an effect, but no additional conclusions can be drawn: The same result would be expected, and was found, when the fluorescence level had been low due to an inhibition on the oxidizing side of System 2, *e.g.* as the result of Tris washing.

Unfortunately, the mechanism of action of CCCP on System 2 is even less clear. It has been reported in this paper that CCCP retarded the restoration of the fluorescence induction in DCMU-poisoned chloroplasts. This was interpreted as an inhibition of the reoxidation of Q^- by an intermediate on the oxidizing side of System 2. In a very recent publication, KRAAN *et al.*[34] have suggested that proton gradients may influence the rate of interactions between electron carriers of the oxygen evolving system. This prompted us to conduct a survey of various uncouplers and inhibitors of photophosphorylation as to their effects on the dark restoration of the fluorescence rise in the presence of DCMU. Among these, antimycin A was found to slightly retard the restoration, while desaspidin proved to be an inhibitor which was even more powerful than CCCP. The observation by HIND[35] that desaspidin is destroyed by illuminated chloroplasts, reminds us of the recovery of CCCP-poisoned algae during a prolonged light period[29,30]. The similar effect of desaspidin and CCCP on the chloroplast fluorescence, therefore, is possibly a consequence of the susceptibility of these inhibitors to destruction by an illuminated photosynthetic apparatus. The lack of effect of all other uncouplers (with the exception of antimycin A) certainly ruled out any direct influence of permeability changes in the chloroplast membranes.

The alleged destruction of desaspidin and of CCCP might conceivably be an oxidation, since various electron donors to Photosystem 2 were found to act in identical manner on the fluorescence kinetics of DCMU-inhibited chloroplasts. Such reductants supposedly react with accumulated oxygen precursors, *e.g.* the "S_2 state" of KOK *et al.*[36].

The stability of the high fluorescence state in the presence of electron donors, and of CCCP or desaspidin, may simply reflect some disruption of the native array of components in the oxygen evolving system. We have assumed that the underlying cause is an inhibited reoxidation of Q^- by an oxidized intermediate on the other side of the reaction center. The rate of this reaction would depend on the concentration of these oxidants, and on the spatial arrangement of prospective reaction partners on the membrane. While the former condition must vary with the availability of accessible artificial or native electron donors, the latter one would be expected to change whenever the membrane structure was altered.

In addition to directly participating in a redox reaction, CCCP might disturb the interaction between membrane bound electron carriers[37], thus diverting oxidizing equivalents into various side reactions. It is noteworthy that an inactivation of System

2 by Tris washing led to some inhibition of the backreaction, too (Fig. 7). Hydroxylamine, another agent which retards the reoxidation of Q^-, has been found not only to act as inhibitor of oxygen evolution and as electron donor to System 2, but also to extract the tightly bound chloroplast manganese from the thylakoids[12]. In contrast, added Mn^{2+} ions did not significantly affect the restoration of the fluorescence induction (Fig. 6), but they did so to some extent in Tris-washed chloroplasts. Since electron donation by Mn^{2+} ions to System 2 has been shown to override water oxidation[27], the failure of Mn^{2+} to retard the reoxidation of Q^- cannot simply be an accessibility problem. It may be that manganese interacts with a site different from that utilized by reductants like ascorbate–TMPD. But the question remains whether electron donation alone is sufficient for an inhibition of cyclic electron flow from Q^- in System 2.

Except for the easily demonstrable inhibition of the dark restoration of the fluorescence rise in DCMU-poisoned algae, it is difficult to verify the other observed effects of CCCP for chloroplasts *in vivo*. The peculiar changes of the fluorescence kinetics in living algae after addition of a CCCP analogue, which were described by BANNISTER[38], may be at least in part related to our findings with isolated chloroplasts. The problem with algae is the difficulty to separate the uncoupling effects of CCCP from its other actions on electron transport. While chloroplasts *in situ* may be better protected against photodestructions than isolated chloroplasts[29], *e.g.* by diverting photoxidative reactions to simple metabolites, the sensitivity of chloroplasts *in vitro* to CCCP is increased by trypsin treatment[6] or by Tris washing (Table III).

A final comment concerns the relatively weak decline of the fluorescence yield when CCCP-poisoned chloroplasts were illuminated in the absence of a Hill oxidant (compare Table III with Fig. 9). We must assume that the ensuing accumulation of reducing equivalents between the two photosystems prevented the destructive utilization of oxidized intermediates in System 2. Since CCCP was shown to inhibit the reoxidation of Q^-, there must be other cyclic electron transfer reactions to the oxidizing side of System 2, for example *via* cytochrome *b*-559 as proposed by KNAFF AND ARNON[39].

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