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INHIBITORY SITE OF CARBONYL CYANIDE *m*-CHLOROPHENYLHYDRAZONE IN THE ELECTRON TRANSFER SYSTEM OF THE CHLOROPLASTS

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SUMMARY

The inhibitory effect of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) on the electron transport system of chloroplasts was investigated. At concentrations higher than $1 \cdot 10^{-7}$ M, CCCP acted as an inhibitor of the Hill reaction. Two different processes of CCCP inhibition were discernible; the one which was accomplished very rapidly and independently of light, and the other which developed slowly and only in light. Both processes of inhibition were not reversed by washing the inhibited chloroplasts.

The rapid type of inhibition in the dark was observed not only in the Hill reaction in normal (untreated) chloroplasts, but also in ascorbate *plus* hydroquinone-supported $NADP^+$ photoreduction and manganese-supported 2,6-dichlorophenol-indophenol (DCIP) photoreduction in heated chloroplasts, whereas $NADP^+$ photoreduction supported by reduced DCIP was not inhibited.

CCCP inactivated the Hill reaction by affecting the quantum efficiency of the reaction without influencing the rate constant of the dark reaction. There was no loss in the manganese content in chloroplasts caused by incubation with CCCP in the dark.

The variable fluorescence in chloroplasts was decreased by CCCP at concentrations higher than $1 \cdot 10^{-7}$ M, with concomitant elimination of the induction phenomenon. However, on addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) to the CCCP-inhibited chloroplasts there occurred a recovery of the induction phase of fluorescence. The pool size of the electron acceptor of photosystem II decreased with increasing concentrations of CCCP, but the pool size of the primary electron acceptor determined in the presence of DCMU was not affected by the poison.

It is postulated that the site of CCCP inhibition is located between the endogenous redox substance (Y_1) which receives electrons from artificial electron donors such as ascorbate, and the primary electron donor of photosystem II (Y_2).

INTRODUCTION

A strong inhibitory action of ring-substituted phenylhydrazones of carbonyl cyanide on the oxidative phosphorylation in mitochondria and on the light-induced

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyurea; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

phosphorylation in chloroplasts has been described by HEYTLER AND PRICHARD[1], and HEYTLER[2]. Subsequent studies on the effects of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) on the photosynthetic reactions in chloroplasts have established that these substances are potent uncouplers of photophosphorylation, exerting, at considerably low concentrations ($1 \cdot 10^{-6}$ M), an inhibitory action on ATP formation with concomitant stimulation on rate of the non-cyclic electron transport[3-7]. DE KIEWIET *et al.*[7] and PLENGRIDHYA AND BURRIS[5] found that these substances at still higher concentrations (*e.g.* $1 \cdot 10^{-5}$ M) caused, besides their uncoupling action, an inhibition of the Hill reaction. DE KIEWIET *et al.*[7] proposed that the inhibitory site of CCCP in the Hill reaction was at the O_2-evolving process, since CCCP did not inhibit, but stimulated to some extent, the rate of $NADP^+$ photoreduction with reduced DCIP as electron donor, which was catalyzed solely by system I. Studies on the electron transport system of Euglena chloroplasts carried out by KATOH AND SAN PIETRO[8] confirmed the above observations and further showed that CCCP, as well as 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), was effective in suppressing the ascorbate-supported $NADP^+$ photoreduction in heated Euglena chloroplasts. This limits the location of the CCCP-sensitive site on the electron transport chain between a site where ascorbate, in place of water, donates its electrons to system II, and a site where reduced DCIP donates its electron to system I. A similar observation was later reported by ITOH *et al.*[9], who showed that CCCP suppressed the 2,6-dichlorophenol indophenol (DCIP) photoreduction in Tris-inhibited chloroplasts with $MnCl_2$ as the electron donor. RENGER[10] found, in an experiment using repetitive flashes of short duration and saturating intensity, that in the presence of CCCP, the yield of O_2 evolution per flash decreased when the duration of dark intervals was lengthened. He assumed, therefore, that CCCP reacts with the primary electron donor for photosystem II which had been oxidized by preceding flashes.

BANNISTER[11], and BANNISTER AND RICE[12] showed with algal cells that the elimination of photosynthetic O_2 evolution caused by 10 μM of FCCP, was accompanied by a marked decrease in the fluorescence yield and disappearance of the induction of fluorescence at the onset of illumination. Since then, effects of CCCP and FCCP on the yield and time-dependent changes of fluorescence in photosynthetic organisms and isolated chloroplasts have been studied, and the results obtained were discussed mainly in terms of the uncoupling action of the substances[13-17]. On the other hand, ITOH *et al.*[9] showed a CCCP-induced lowering of fluorescence yield of the chloroplasts and, on the basis of this and other observations, assumed that the inhibition site of CCCP was somewhere in the electron transport pathway on the water-side of photosystem II.

The present work was undertaken to investigate the mode and site of inhibition by CCCP on the photosynthetic electron transport system, through analyses of the effects of the poison on the electron transfer and fluorescence in spinach chloroplasts. Evidence was obtained indicating that CCCP inactivates the electron transport system on the way from water to photosystem II; after the site where the artificial electron donor such as ascorbate or ascorbate *plus* hydroquinone provides its electron to photosystem II, but before the site of an endogenous primary electron donor for photosystem II.

MATERIALS AND METHODS

Chloroplast preparation

Chloroplasts were isolated from market spinach leaves as described by KATOH *et al.*[18], using a solution containing 0.4 M sucrose, 0.05 M phosphate, 0.01 M NaCl (pH 7.8) as the preparation medium. Heat treatment of the chloroplasts was carried out by incubating a small test tube containing chloroplasts suspended in the preparation medium in a water bath at 45° for 3–5 min.

Measurements of the Hill reaction and $NADP^+$ photoreduction

The rates of DCIP and $NADP^+$ photoreduction were measured by following the absorbance changes at 580 and 350 nm, respectively, as described by KATOH *et al.*[18], using a modified type of Hitachi spectrophotometer, EPU 2A. Actinic light furnished from a 600-W quartz iodine lamp was filtered through a water filter of 7 cm thickness, a red cut-off filter (VR-66 and 63, Toshiba) and an infrared absorbing filter (HA 50, Hoya). Light intensity at the position of reaction mixture was $1.6 \cdot 10^5$ ergs·cm^{-2}·sec^{-1}. Reaction systems were also similar to those described elsewhere[18]. The compositions of the reaction mixtures are indicated in legends of respective figures. Chlorophyll was determined by the method of ARNON[19].

Fluorescence determination

The fluorescence from chloroplasts was measured by the method virtually identical with that described by MURATA *et al.*[20]. A chloroplast suspension in a cell (1.0 cm × 1.0 cm × 4.0 cm) with four transparent sides was illuminated by the exciting light which was provided from a 100-W tungsten lamp, and passed through a glass filter, (B 460, Hoya) and 480-nm interference filter. Fluorescence was measured at an angle of 90° to the exciting light beam using a photomultiplier (R-236), protected against the scattered exciting light with a 684-nm interference filter, and a cut-off filter (VR 65, Toshiba). The time-course of fluorescence was recorded by a strip chart servo recorder or a synchroscope (Toshiba Electric Co.). Chloroplasts were suspended in the preparation medium at concentrations corresponding to about 2 μg chlorophyll per ml.

Manganese determination

Manganese in chloroplasts was determined by the permanganate method described by SANDELL[21]. The chloroplast suspension was transferred into a Kjeldahl flask, with the addition of concentrated HNO_3 and H_2SO_4, and heated with the occasional addition of HNO_3 in small portions. To the colorless solution finally obtained, potassium periodate and $AgNO_3$ were added to oxidize manganese and the concentration of permanganate thus formed was determined by measuring the absorbance at 530 nm.

RESULTS

Effects of CCCP on the Hill reaction and photoreductions

Time-courses of the Hill reaction. CCCP, at lower concentrations, increases the rate of the Hill reaction as a result of uncouping of the photophosphorylation process,

whereas, at high concentrations, it inhibits the electron transport of photosynthesis[5,7]. Therefore, the effect of CCCP on the Hill reaction was determined in the presence of an uncoupler, methylamine. Fig. 1 shows the time-courses of the photoreduction of DCIP in the presence of various concentrations of CCCP which were added to the reaction mixture immediately before switching the light on. There was a marked suppression of the initial rates of DCIP photoreduction. In addition, it was found that the rate of photoreduction continued to decrease during the reaction, thereby indicating further progress of inhibition by CCCP. This is in marked contrast to the case with DCMU, with which the suppressed reaction proceeded linearly with time (Curve d, Fig. 1).

Reversibility of inhibition. The mode of time-dependent progress of CCCP-inhibition is indicated more clearly in Fig. 2, in which the reversibility of the inhibition was studied by measuring the initial rate of the DCIP Hill reaction with chloroplasts, which had been washed with the preparation medium after the pre-incubation with CCCP for a varied time and in the light and dark. An appreciable inhibition of the DCIP Hill reaction activity was observed with chloroplasts, to which 25 μM amounts of CCCP had been added, immediately followed by dilution with 10 vol. of chilled preparation medium, and collection by rapid centrifugation. There was no progress of inhibition during continued incubation in the dark for at least several minutes. On the other hand, further suppression of the activity was observed with chloroplasts

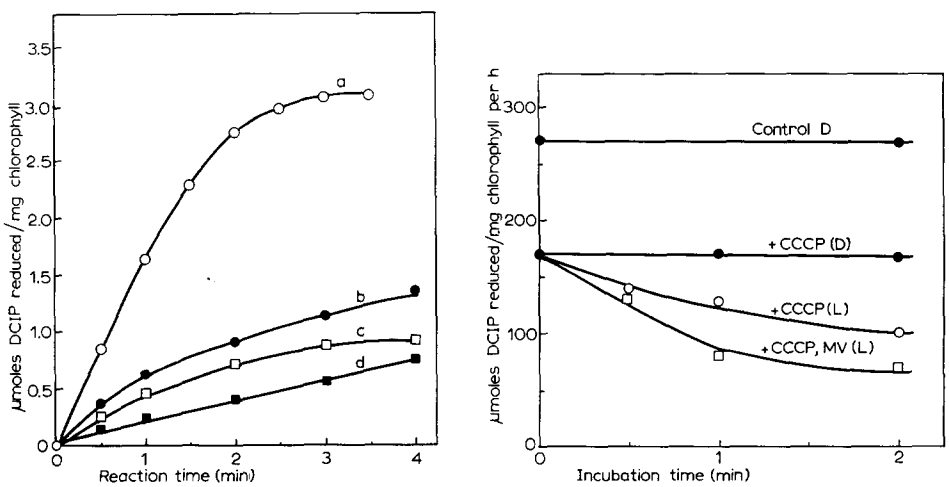

Fig. 1. Time-courses of the Hill reaction with DCIP as the electron acceptor in the presence of CCCP and DCMU. Reaction mixture contained, in a final volume of 3.0 ml, the following components, in mM; DCIP, 0.032; methylamine·HCl, 10; NaCl, 10; phosphate buffer pH 7.8, 50 and chloroplasts equivalent to 24.3 μg chlorophyll. Curve a, no inhibitor added; Curves b and c, in the presence of 12 and 23 μM CCCP; Curve d, in the presence of $1 \cdot 10^{-7}$ M DCMU.

Fig. 2. The activity of the DCIP Hill reaction in chloroplasts pre-incubated with CCCP for a varied time in the light and the dark. Chloroplasts suspended in the preparation medium were incubated at 25° for an indicated period of time with and without CCCP (25 μM) in the light (L) and the dak (D). Where indicated 35 μmoles of methyl viologen (MV) were also included in the pre-incubation mixture. After the pre-incubation, the chloroplasts were diluted with the chilled preparation medium, collected rapidly by centrifugation, and finally re-suspended in the reaction mixture. Final volume of reaction mixture was 0.7 ml, which contained the following components in mM: DCIP, 0.014; methylamine·HCl, 10; NaCl, 10; phosphate buffer (pH 7.5), 50. Chlorophyll concentration was 4–7 μg/0.7 ml reaction mixture.

preincubated with the poison in the light. It is concluded, therefore, that CCCP inhibits the Hill reaction by two different mechanisms; one is a very rapid inhibition which is independent of light, whereas the other proceeds slowly and only in the light. Both types of inhibition could not be reversed by washing the pre-incubated chloroplasts.

PLENGRIDHYA AND BURRIS[5] showed that, in contrast to the case of the uncoupling action of CCCP, the Hill reaction suppressed by CCCP could not be recovered by the addition of cysteine. This was confirmed in the present work. The addition of cysteine to the reaction mixture could not reverse either of the two types of CCCP-induced inhibition of the Hill reaction. It was found, however, that when cysteine or ascorbate was present in the medium during pre-incubation of chloroplasts with CCCP in the light, there was no slow progress of inhibition. On the other hand, the addition of methylviologen to the pre-incubation medium accelerated the progress of light-dependent inhibition (Fig. 2, bottom curve). ITOH *et al.*[9] showed that, on illumination, their CCCP-inhibited chloroplasts were subject to photobleaching of the carotenoids, which could be protected by the addition of reducing reagents, and promoted by the presence of Hill oxidant. Therefore, it seems reasonable to assume that the light-dependent inhibition is brought about secondarily through photodestruction of the pigment system of the chloroplasts in which the normal flow of electrons had been inhibited by the fast light-independent action of CCCP. In the following, therefore,

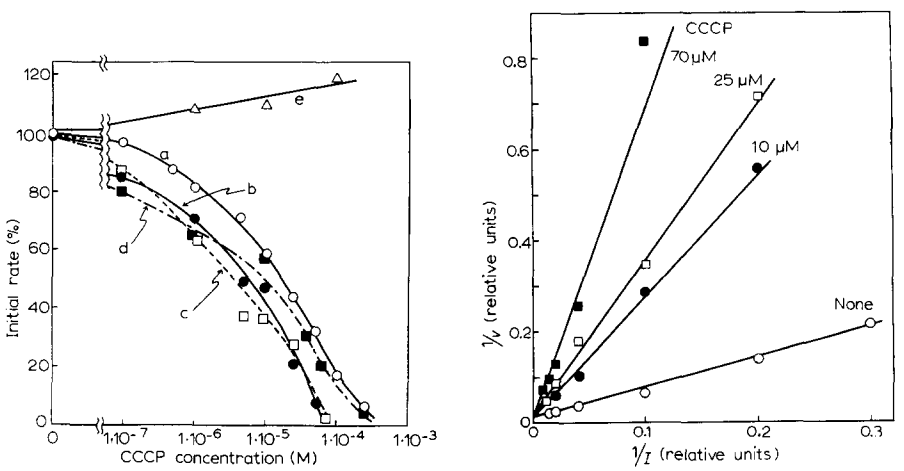

Fig. 3. Effects of varied concentrations of CCCP on the Hill reaction and the photoreduction with artificial electron donors. Curves a and b, Hill reaction with DCIP and $NADP^+$ as electron acceptor, respectively, in untreated chloroplasts. Curves c, d and e were determined with heat-inactivated chloroplasts. Curve c, $NADP^+$ photoreduction with 0.3 mM ascorbate *plus* 0.3 mM hydroquinone; Curve d, DCIP photoreduction with 150 μM $MnCl_2$; Curve e, $NADP^+$ photoreduction with 14 mM ascorbate *plus* 14 μM DCIP in the presence of 10 μM DCMU. Reaction mixture for DCIP photoreduction was the same as described in Fig. 2, except that 150 μM $MnCl_2$ were added in Curve d. For $NADP^+$ photoreduction, the reaction mixture contained 0.4 mM $NADP^+$, in place of DCIP, and a saturating amount of spinach ferredoxin together with indicated electron donors. Chlorophyll concentration was 2.5–5.5 μg/0.7 ml.

Fig. 4. Effect of light intensity on DCIP Hill reaction rate in the presence of various concentrations of CCCP. Experimental conditions were the same as described in Fig. 2, except that chlorophyll concentration was 3.4 μg/0.7 ml reaction mixture. Intensity (I) of actinic light (660–800 nm), $1.6 \cdot 10^{-5}$ ergs·cm^{-2}·sec^{-1} is expressed as 100 (*i.e.* $1/I = 0.01$). Light intensity was reduced with the use of neutral density filters.

the inhibition which takes place immediately on addition of CCCP in the dark was mainly studied.

Photoreduction with artificial electron donor. Fig. 3 shows the effects of varied concentrations of CCCP on the rate of the Hill reaction and the photoreduction with artificial electron donors. Since in this experiment chloroplasts had been uncoupled by the addition of methylamine·HCl, there was no possibility of any stimulation of the Hill reaction rate as a result of the uncoupling action of CCCP at any concentration of CCCP used.

In accord with the observation of KATOH AND SAN PIETRO[8] with Euglena chloroplasts, the ascorbate- or ascorbate *plus* hydroquinone-supported $NADP^+$ photoreduction in heated spinach chloroplasts was inhibited by CCCP. A similar inhibitory action of the poison was observed in the DCIP photoreduction in heated chloroplasts with manganese as electron donor. The concentration required for 50% inhibition was about $1 \cdot 10^{-5}$ M of CCCP both for the Hill reaction and the photoreduction in the presence of artificial electron donors. On the contrary, $NADP^+$ photoreduction supported by ascorbate *plus* DCIP was not suppressed, but rather stimulated by addition of the inhibitor.

Light intensity. Fig. 4 shows the effect of CCCP on the rate of the Hill reaction determined under varied light intensities. The double reciprocal plots of the rates of the Hill reaction, V, in the presence of various concentrations of the poison *versus* light intensity, I, gave a set of straight lines with the same intercept on the ordinate but with different slopes[22]. It can be concluded, therefore, that CCCP affects the quantum efficiency of the Hill reaction, leaving unchanged the maximum rate obtained under saturating light intensity.

Manganese content

The loss of the Hill activity caused by heat treatment or treatment with Tris or hydroxylamine of chloroplasts was shown to be accompanied by a release of manganese from the chloroplasts[9,23,24]. In order to see whether CCCP induced a release of manganese from the chloroplasts, the manganese was determined with the chloroplasts which had been incubated with $2.7 \cdot 10^{-4}$ M CCCP for 10 min in the dark and then washed with the preparation medium containing $1 \cdot 10^{-3}$ M EDTA. The results obtained are summarized in Table I. The manganese content of the untreated chloroplasts was found to be one atom of manganese to 46–73 molecules of chlorophyll, which

TABLE I

MANGANESE CONTENTS IN UNTREATED AND CCCP-TREATED CHLOROPLASTS

Details of treatment: see text. Determination of manganese was described in MATERIALS AND METHODS

Expt.	*Manganese content (molecules chlorophyll per atom Mn)*	
	Untreated chloroplasts	*CCCP-treated chloroplasts*
1	73	85
2	59	59
	59	57
3	46	59

is in agreement with the results of ANDERSON *et al.*[25] and CHENIAE *et al.*[26]. The CCCP treatment of chloroplasts, which resulted in a substantial loss of the Hill activity, caused almost no change in the manganese content in the chloroplasts. It was apparent, therefore, that the inhibitory action of CCCP was not due to a loss of manganese from the chloroplasts.

Effect of CCCP on the fluorescence of chloroplasts

Effect of CCCP on the time-course of fluorescence of the chloroplasts is shown in Fig. 5. At the onset of illumination, the intensity of fluorescence rises instantaneously to a level, F_i, and then increases gradually to reach a steady level, F_s (ref. 27). On addition of CCCP, the F_s level of fluorescence was lowered; with $6 \cdot 10^{-5}$ M of the poison, the transient rise of fluorescence from F_i to F_s was completely abolished. A decrease in the fluorescence yield in the presence of CCCP has been previously reported by BANNISTER[11] and ITOH *et al.*[9]. On the other hand, little change was observed in the level of F_i, in the presence of CCCP.

It was noted from the comparison of the results in Fig. 6 with those in Fig. 3 that the variable fluorescence ($F_s - F_i$) was much more sensitive to CCCP than the Hill reaction activity. Namely, the concentrations of CCCP required to decrease the variable fluorescence and the Hill activity by 50% were $1 \cdot 10^{-6}$ M and $1 \cdot 10^{-5}$ M, respectively. As is illustrated in Fig. 7, it was found that the decrease in the F_s level caused by CCCP was more marked at lower intensities of the exciting light, thereby resulting in an intensification of the "weak light effect" of MURATA *et al.*[20]. It appears, therefore, that the high sensitivity of fluorescence to CCCP observed in Fig. 6, was, at least partly, due to the light intensities used for fluorescence determination, which were significantly lower than that for the Hill reaction.

When $1 \cdot 10^{-5}$ M of DCMU was added to the CCCP-inhibited chloroplasts, the fluorescence intensity rose very rapidly from F_i level to F_s, as was observed with the chloroplasts to which only DCMU, but not CCCP, was added (Fig. 5). The heights

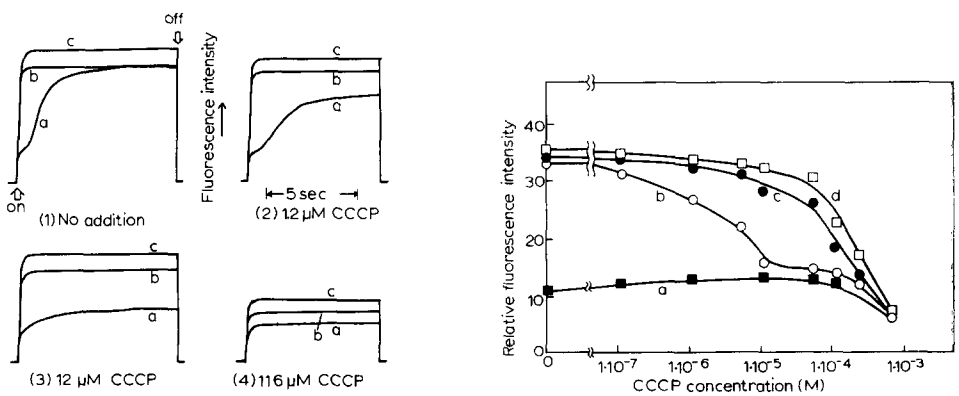

Fig. 5. Time-courses of fluorescence induction in the chloroplasts in the presence of varied concentrations of CCCP. Reaction mixture contained, in a final volume of 3 ml; 5.1 μg chlorophyll, 0.4 M sucrose, 10 mM NaCl, 10 mM methylamine·HCl, 50 mM phosphate buffer (pH 7.8). Following substances were added: Curve a, no addition; Curve b, 10 μM DCMU; Curve c, sodium dithionite.

Fig. 6. Effect of CCCP on the fluorescence yield at initial (Curve a) and steady state (Curve b) levels in the absence of any addition or in the presence of 10 μM DCMU (Curve c), or sodium dithionite (Curve d). Experimental conditions were the same as in Fig. 5.

of the F_S level attained in the concomitant presence of the two poisons decreased only slightly with increasing concentrations of CCCP up to $1 \cdot 10^{-4}$ M, and more markedly at concentrations higher than $1 \cdot 10^{-4}$ M. The addition of sodium dithionite to the CCCP-inhibited chloroplasts also caused a very rapid rise of fluorescence to the F_S level, which was significantly higher than that obtained with DCMU. 10 mM of hydroxylamine was also effective in increasing the fluorescence intensity, but to a much limited extent. 10 mM of ascorbate was entirely without effect.

Fig. 8 shows the effects of varied concentrations of CCCP on the size of the pool of electron acceptors on the reducing side of system II, expressed in terms of the "work integral" of MURATA *et al.*[27] (arbitral units). In the absence of DCMU, the relative size of the pool thus measured passed a maximum and then decreased with increasing concentrations of CCCP. On the other hand, the pool size of the primary electron acceptor which underwent photoreduction by photosystem II in the presence of DCMU was not altered by the addition of CCCP. It appears, therefore, that the

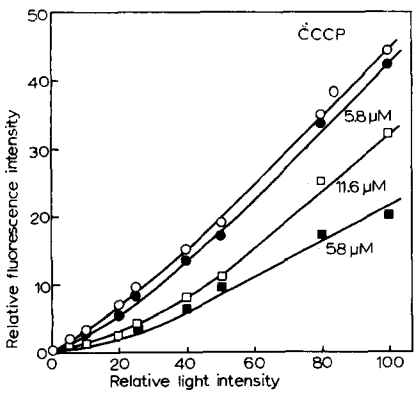

Fig. 7. Dependence of fluorescence yield, F_s, on the light intensity in the presence of various concentrations of CCCP. Experimental conditions were the same as described in Fig. 5, except that chlorophyll concentration was 4.2 μg/3 ml.

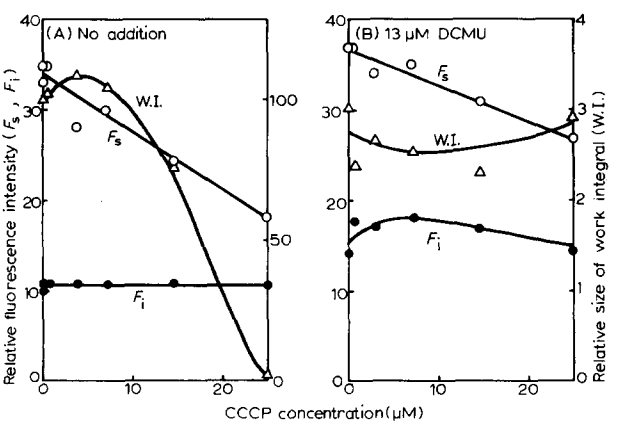

Fig. 8. Effect of CCCP on the work integral (W.I.), steady level (F_s) and initial level (F_i) of fluorescence in the absence (A) and in the presence (B) of 13 μM DCMU. Experimental conditions were as described in Fig. 5.

CCCP-inhibited chloroplasts still retain a pool of electron donor which rapidly provides electrons to the primary electron acceptor (see DISCUSSION).

DISCUSSION

The results obtained in the present study indicate that the mode of the inhibitory action of CCCP on the electron transport system of the chloroplasts was quite unique and differs from that of any inhibitor or inhibitory treatment of chloroplasts so far described. The fast development of the inhibition of the Hill reaction on the addition of CCCP resembles the mode of inhibition by DCMU, but is in marked contrast to the slow progress of the inhibition observed in the case of the hydroxylamine- and Tris treatments of chloroplasts. The inactivation of the Hill activitiy caused by treatment with hydroxylamine or Tris was shown to be accompanied by a concomitant release of manganese from the chloroplasts[9,23,24]. It was indicated in the present work, however, that CCCP inhibits the Hill activity without any significant loss of manganese from the chloroplasts. On the other hand, the irreversibility of the CCCP-induced inhibition suggests a mechanism different from that for the DCMU inhibition of the Hill activity, which was known to be readily reversed by washing the inhibited chloroplasts[28].

It was shown that CCCP decreased the quantum efficiency of the Hill reaction without affecting the dark rate constant, thereby suggesting the location of the inhibitory site of CCCP somewhere close to photosystem II. Of interest in this connection is the finding that the $NADP^+$ photoreduction with ascorbate or ascorbate–hydroquinone couple, or the DCIP photoreduction with manganese as the electron donor, was equally inhibited by CCCP. These facts suggest the CCCP inhibits the electron transfer system at a site which differs from that affected by heat or Tris treatment; more precisely stated, somewhere between an endogenous redox substance (Y_1), which receives electrons from ascorbate or other electron donors for photosystem II and at a site where reduced DCIP donates its electron to photosystem I.

Studies on the effects of CCCP on the fluorescence of the chloroplasts afforded more information concerning the site of action of CCCP, especially when comparison was made with the effects of other inhibitors. CCCP was found to decrease the fluorescence yield of the chloroplasts, a finding which is in agreement with the previous observation of BANNISTER[11]. It was shown in the present work that the decrease in the fluorescence yield caused by CCCP was due to the suppression of the variable portion of the fluorescence, whereas the F_i level was little affected by the poison.

According to the assumption of DUYSENS AND SWEERS[29], the fluorescence yield is regulated by a hypothetical redox substance, Q. When Q is in the oxidized state it quenches fluorescence, but on its reduction by photosystem II, it is converted into the non- or much less quenching form. The rise of the fluorescence intensity from F_i to F_s reflects the reduction of Q and the adjacent pool of endogenous oxidant, A, of the chloroplasts[27,30,31]. The observed decrease in the F_s level in the presence of higher concentrations of CCCP, therefore, indicates that Q remains in the oxidized and quenching state, most probably due to the inactivation of the photoreduction of Q by the photosystem II.

It was found that on addition of DCMU, the fluorescence intensity of the CCCP-inhibited chloroplasts showed a very rapid rise at the onset of illumination[27,29].

This effect of DCMU on the time-course of fluorescence induction was the same as that observed with the chloroplasts in the absence of CCCP, and can be explained by assuming an inhibition by DCMU of the re-oxidation of reduced Q through A (see Fig. 9). In fact, the pool size of the primary electron acceptor, Q, determined in the presence of DCMU was not affected by the addition of CCCP (Fig. 8). It is assumed, therefore, that the photoreducing activity of photosystem II is still operating in the presence of CCCP. In addition dithionite was found to be as effective in increasing the fluorescence yield in the presence of CCCP as in the absence of the poison. This supports the above assumption that Q remained oxidized in the light in the CCCP-inhibited chloroplasts.

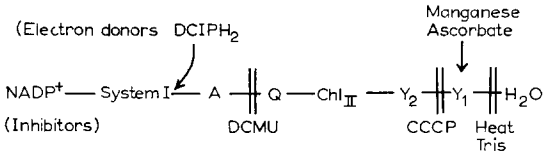

Fig. 9. A proposed scheme illustrating inhibitory sites of CCCP and other inhibitors in the electron transfer system of the chloroplast.

It is inferred that CCCP blocks the electron transfer from Y_1 which accepts electrons from the artificial electron donors for photosystem II, to Y_2, the primary electron donor for photosystem II. The size of the endogenous electron donor Y_2 must be comparable to, or larger than the pool size of Q, so that in the presence of DCMU, the reduction of Q took place rapidly in the CCCP-inhibited chloroplasts. In the absence of DCMU, however, Q is mostly kept in the oxidized state, since the limited resource of electrons in Y_2 will be exhausted before the pool of oxidant between the two photosystems (*i.e.* Q and A) becomes fully reduced. The marked decrease in transient fluorescence, as well as the apparent abolishment of the work integral in the presence of higher concentrations of CCCP reflects this circumstance. These considerations are schematically illustrated in Fig. 9.

At the closure of the present article, there appeared an abstract of a report read by Dr. Homann in the recent Annual Meeting of the American Society of Plant Physiologists[32]. Most of his experimental results and conclusions seem to be in agreement with ours. We are highly interested in a detailed comparison of the experimental results of these two independent research works.

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