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STUDIES ON ELECTRON TRANSPORT ASSOCIATED WITH PHOTOSYSTEM I

III. THE REDUCTION SITES OF VARIOUS HILL OXIDANTS IN THE PHOTOSYNTHETIC ELECTRON TRANSPORT SYSTEM

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SUMMARY

1. $HgCl_2$ treatment of chloroplasts was effective in inducing an inhibition of 2,6-dichlorophenolindophenol (DCIP)-Hill activity in chloroplasts from several higher plants.

2. The Hill reaction with various electron acceptors having a relatively high redox potential was severely, but not completely, inhibited by the $HgCl_2$ treatment of spinach chloroplasts. The surviving Hill activity in $HgCl_2$-treated chloroplasts was somewhat higher with lipophilic electron acceptors (*e.g.* 2,5-dimethylbenzoquinone) than with hydrophilic acceptors (*e.g.* DCIP).

3. DCIP photoreduction in *Euglena* chloroplasts, sonicated spinach chloroplasts, or Photosystem II particles was highly resistant to the $HgCl_2$ treatment.

4. The $HgCl_2$-insensitive part of the Hill activity showed an optimum at a more acidic pH and required higher concentrations of the electron acceptors for the maximum activity, as compared with the $HgCl_2$-sensitive part of the activity.

5. A high concentration of dibromothymoquinone was effective in restoring the DCIP-Hill reaction activity in $HgCl_2$-treated chloroplasts.

6. The results obtained indicate that the electron acceptors tested predominantly receive electrons at the reducing side of Photosystem I.

INTRODUCTION

A wide variety of electron acceptors can be utilized in the Hill reaction in isolated chloroplasts. Besides $NADP^+$, which serves as a physiological electron acceptor in photosynthesis, ferricyanide, indophenol dyes, quinones and viologen dyes are usually employed as electron acceptors. Among these, $NADP^+$ and viologen dyes are reduced at the reducing end of Photosystem I, since only Photosystem I can produce a reductant strong enough to reduce these electron acceptors having

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; DBTQ, dibromothymoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

low redox potentials[1,2]. On the other hand, there are still significant controversies among the results published with respect to the reduction site of Hill oxidants having a relatively high redox potential, such as ferricyanide and 2.6-dichlorophenolindophenol (DCIP)[3–11]. They may be reduced by a weak reductant produced by Photosystem II, as well as by the reductant produced by Photosystem I.

In a previous paper[12] we showed that, when chloroplasts were treated with $HgCl_2$, plastocyanin *in situ* was inactivated and consequently the electron transport from cytochrome *f* to P_{700} was specifically inhibited. Electron transfer from water to cytochrome *f* was not affected by the $HgCl_2$ treatment of chloroplasts[12]. Since its inhibition site is very close to Photosystem I, $HgCl_2$ would be especially suitable for the determination of the reduction sites of electron acceptors. It was, therefore, decided to investigate the effects of $HgCl_2$ on the Hill reaction with various electron acceptors. The results obtained indicate that in chloroplasts, all the electron acceptors are predominantly reduced at the reducing side of Photosystem I.

MATERIALS AND METHODS

Spinach, *Brassica campestris* and *Allium odorum* were obtained from a local market. Chloroplasts were prepared as described previously for spinach chloroplasts[13]. The methods of culturing and chloroplast preparation for *Euglena gracilis*, strain Z, were as described in a previous paper[7]. Sonication of spinach chloroplasts was previously described[6]. Photosystem II particles were prepared according to the method of Ohki and Takamiya[14]. Concentrations of chlorophyll were determined as described by Arnon[15].

Photoreductions of DCIP and ferricyanide were measured by following the absorbance changes at 580 and 420 nm, respectively, with a Hitachi EPU 2A Spectrophotometer modified as described previously[13]. Photoreduction of oxidized *p*-phenylenediamine was determined as described by Saha *et al.*[11]. The Hill reaction was also followed with a Clark-type oxygen electrode[13].

Dibromothymoquinone (DBTQ) is a gift from Professor A. Trebst of Ruhr University (Bochum, Germany).

RESULTS

Fig. 1 shows the effects of $HgCl_2$ treatment of chloroplasts from three plant materials on the Hill reaction with DCIP as electron acceptor. As described in the previous paper[12], the degree of inhibition depends on the ratio of $HgCl_2$ to chlorophyll rather than on the concentration of $HgCl_2$ alone. DCIP-Hill activity in chloroplasts prepared from spinach, *Brassica campestris* and *Allium odorum* was largely inhibited by the treatment of chloroplasts with $HgCl_2$ at a molar ratio of $HgCl_2$ to chlorophyll of about unity.

Fig. 2 shows the effect of $HgCl_2$ on the Hill activity with various electron acceptors having a relatively high redox potential. It is seen that $HgCl_2$ was strongly inhibitory on activities of the Hill reactions not only with an ionic electron acceptor such as ferricyanide but also with lipophilic electron acceptors such as *p*-benzoquinone, 2,5-dimethylbenzoquinone and the oxidized form of *p*-phenylenediamine. With the all electron acceptors tested, maximum inhibitions were attained at the $HgCl_2$ to

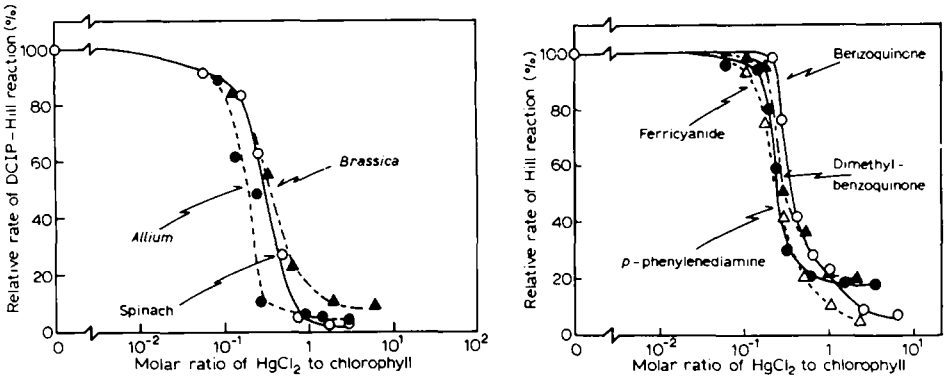


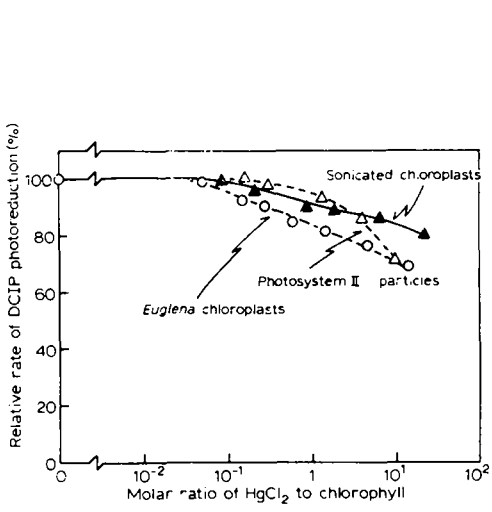
Fig. 1. Effects of $HgCl_2$ on the DCIP-Hill reaction in chloroplasts from various plants. The reaction mixture contained, in a final volume of 2 ml: 50 mM phosphate buffer (pH 7.5), 10 mM NaCl, 10 mM methylamine·HCl and 20 μM DCIP. Chlorophyll concentrations were 1.8, 6.8 and 3.5 μg/ml for spinach, *Brassica campestris* and *Allium odorum* chloroplasts, respectively. Chloroplasts were preincubated at 0 °C for about 1 h with $HgCl_2$ at the indicated molar ratios of $HgCl_2$ to chlorophyll. Red light (660–800 nm) of $4.1 \cdot 10^5$ erg·cm^{-2}·s^{-1} was used as actinic light. Photoreduction rates of the control were 706, 314 and 403 μequiv·mg chlorophyll^{-1}·h^{-1} for spinach, *Brassica* and *Allium* chloroplasts, respectively.

Fig. 2. Effects of $HgCl_2$ on the Hill reaction with various electron acceptors. Reaction conditions for photoreduction of oxidized *p*-phenylenediamine were the same as described in Fig. 1, except that DCIP was replaced by 1 mM *p*-phenylenediamine *plus* 2 mM ferricyanide[11]. Chlorophyll concentration was 2.9 μg/ml. The Hill reaction with ferricyanide, benzoquinone and dimethylbenzoquinone was determined with a Clark-type oxygen electrode. The reaction mixture contained, in a final volume of 4 ml, 50 mM phosphate buffer (pH 7.5), 10 mM NaCl, 10 mM methylamine·HCl. Either 2.5 mM ferricyanide, 2.5 mM benzoquinone or 0.5 mM 2,5-dimethylbenzoquinone was added as the electron acceptor. Chlorophyll concentrations were 12 μg/ml for ferricyanide and benzoquinone-Hill reaction and 10 μg/ml for dimethylbenzoquinone-Hill reaction, respectively. Rates of the Hill reaction of the control (100%) were 334, 408, 288 and 440 μequiv·mg chlorophyll^{-1}·h^{-1} for *p*-phenylenediamine, ferricyanide, benzoquinone and dimethylbenzoquinone-Hill reaction, respectively.

chlorophyll ratios of about unity. The inhibition was, however, not complete and there was always a residual activity that survived the $HgCl_2$ treatment of chloroplasts even at a $HgCl_2$ to chlorophyll ratio higher than one. This is in contrast to the case of methylviologen photoreduction, with which a complete inhibition was readily attained[12]. It is also noted that the surviving activity of the Hill reaction with oxidized *p*-phenylenediamine and dimethylbenzoquinone was always higher than that with DCIP or ferricyanide as electron acceptor. Addition of 3-(3,4-dichlorophenyl)-1,1--dimethylurea (DCMU, 10^{-5} M) completely eliminated the surviving activity.

The strong inhibitory effects of $HgCl_2$ on the Hill reactions observed above indicate participation of plastocyanin in the electron transfer from water to the electron acceptors tested. It follows that in chloroplasts, the acceptors are predominantly reduced at the reducing side of Photosystem I. The residual activity observed in $HgCl_2$-treated chloroplasts is interpreted to be due to a leakage of electrons to the acceptor from the intermediary electron carriers located between Photosystem I and II. In accordance with this hypothesis, DCIP photoreduction in *Euglena* chloroplasts, sonicated spinach chloroplasts and Photosystem II particles

prepared from digitonin-treated spinach chloroplasts was highly resistant to $HgCl_2$ (Fig. 3). In these preparations, DCIP is reduced by Photosystem II alone, since the electron transfer from Photosystem II to PI is interrupted by a release of cytochrome *f* or plastocyanin[6,7,14].



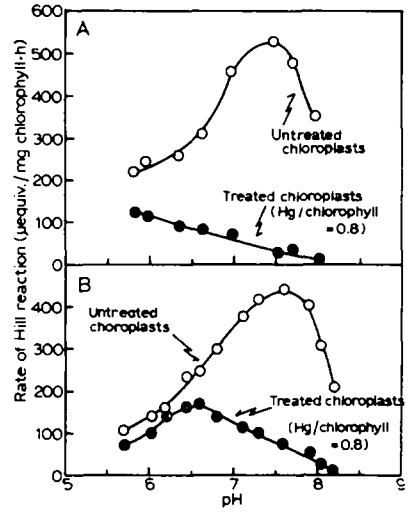


Fig. 3. Effects of $HgCl_2$ on the DCIP photoreduction in *Euglena* chloroplasts, sonicated spinach chloroplasts and Photosystem II particles. Experimental conditions were the same as in Fig. 1, except that the pH of phosphate buffer was 6.3. 1 mM of diphenylcarbazide was added. Chlorophyll concentrations were 12, 2.9 and 3.0 μg/ml for *Euglena* chloroplasts, sonicated spinach chloroplasts and Photosystem II particles, respectively. Chlorophyll *a* to *b* ratio of the particles was 2.0. Photoreduction rates of the control were 80, 98 and 94 $\mu equiv \cdot mg\ chlorophyll^{-1} \cdot h^{-1}$ for *Euglena* chloroplasts, sonicated spinach chloroplasts and Photosystem II particles, respectively.

Fig. 4. pH-dependency of the Hill reaction in untreated and $HgCl_2$-treated spinach chloroplasts. (A) DCIP-Hill reaction. (B) dimethylbenzoquinone-Hill reaction. Reaction conditions for DCIP and dimethylbenzoquinone-Hill reaction were the same as described in Figs 1 and 2, respectively, except that pH of the reaction medium was varied. ○—○, untreated chloroplasts. ●—●, chloroplasts preincubated with $HgCl_2$ at a molar ratio of $HgCl_2$ to chlorophyll of 0.8. Chlorophyll concentrations were 2.8 and 12.6 μg/ml, for DCIP- and dimethylbenzoquinone-Hill reaction, respectively.

Further evidence supporting the above view was obtained from experiments in which dependencies on reaction conditions of the $HgCl_2$-sensitive and -insensitive parts of the Hill activity were compared. Figs 4A and 4B indicate that in untreated chloroplasts, the Hill activity, with DCIP or dimethylbenzoquinone as electron acceptor showed a maximum at pH 7.5. In contrast, the Hill activity in $HgCl_2$-treated chloroplasts was very low at pH 7.5 but increased gradually as the pH decreased. A maximum at pH 6.5 was observed in the case of dimethylbenzoquinone-Hill reaction: the surviving activity at this optimum pH amounted to about 35% of the maximum activity in the untreated chloroplasts. With DCIP as electron acceptor, the activity at pH 6.0 in the treated chloroplasts was 20% of the maximum activity in the untreated chloroplasts at pH 7.5. The difference in the pH-dependency of the Hill reaction is compatible with the above assumption that the reduction site of the electron acceptor is different between untreated and $HgCl_2$-treated chloroplasts.

Fig. 5 compares the Hill activities in the untreated and $HgCl_2$-treated chloroplasts in the presence of various concentrations of electron acceptors. At pH 7.5, determination of the DCIP-Hill reaction was obscured at high concentrations of DCIP because of the deep colour of the dye. Nevertheless, it can be seen in Fig. 5A that the rates of the photoreduction increased markedly at the low range of dye concentrations but tended to saturation at concentrations higher than 10 μM in untreated chloroplasts. Difference between the untreated and treated chloroplasts was more clearly seen with DCIP-Hill reaction at pH 6.3, where the colour of DCIP was much less intense than that at pH 7.5 (Fig. 5B), and with the dimethylbenzoquinone-Hill reaction at pH 7.5 (Fig. 5C). In untreated chloroplasts there are two distinct phases in the concentration dependency of the Hill reaction; the activities increased markedly with increasing concentration of DCIP and dimethylbenzoquinone up to 5 μM and 0.2 mM, respectively. Further increase in concentrations of the acceptors induced little or only a slight increase in the activity. After treatment of chloroplasts with $HgCl_2$, the first phase of concentration dependency disappeared and only a second phase of gradual increase was observed. This is interpreted as indicating that photoreduction at the reducing side of Photosystem I was saturated at a low concentration of the electron acceptor, whereas the $HgCl_2$-insensitive photoreduction, which occurs between Photosystem I and II, requires high acceptor

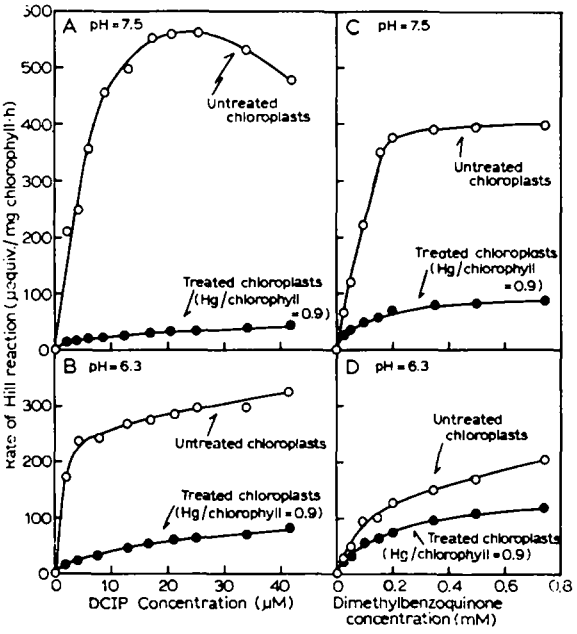


Fig. 5. Effects of concentrations of electron acceptor on Hill activity in untreated and $HgCl_2$-treated spinach chloroplasts. A and B, DCIP-Hill reaction at pH 7.5 and 6.3, respectively. C and D, dimethylbenzoquinone-Hill reaction at pH 7.5 and 6.3, respectively. Experimental conditions for DCIP- and dimethylbenzoquinone-Hill reaction were the same as described in Figs 1 and 2, respectively, except that varied concentrations of the electron acceptors were added. ○—○, untreated chloroplasts. ●—●, chloroplasts preincubated with $HgCl_2$ at a molar ratio of $HgCl_2$ to chlorophyll of 0.9. Chlorophyll concentrations were 5.4 and 13.1 μg/ml, for DCIP and dimethylbenzoquinone-Hill reaction, respectively.

concentrations for the maximum activity. The difference in concentration dependency between untreated and $HgCl_2$-treated chloroplasts was less marked with dimethylbenzoquinone-Hill reaction at pH 6.3 (Fig. 5D). This may be an indication that at this pH, a significant portion of quinone photoreduction is mediated by Photosystem II.

The inhibitory effects of $HgCl_2$ on the Hill reaction so far studied are in accord with those of DBTQ described by Trebst *et al.*[9,16], in that the Hill reaction with high potential acceptors is markedly but incompletely inhibited by the inhibitor. However, magnitudes of the residual activities with DCIP- and ferricyanide-Hill reactions in DBTQ-inhibited chloroplasts were significantly higher than those in $HgCl_2$-treated chloroplasts (*cf.* ref. 16).

We confirmed the observations of Böhme *et al.*[16] that DBTQ is less inhibitory than $HgCl_2$ towards the DCIP-Hill reaction. Further addition of $HgCl_2$ to DBTQ-inhibited chloroplasts failed to reduce the remaining activity. Quite unexpectedly, however, addition of a high concentration of DBTQ was found to recover the lost activity of $HgCl_2$-treated chloroplasts to a significant extent (Fig. 6). Time course of DCIP photoreduction recovered now shows a lag at the onset of the illumination and a post-illumination reduction of the dye which persists for a while in the dark. A high concentration of DBTQ was also effective in overcoming the inhibition induced by low concentration of DBTQ (10^{-7} to 10^{-6} M). The maximum stimulation of the steady rate and the maximum extent of the post-illumination reduction were attained at a similar concentration of DBTQ (about $5 \cdot 10^{-5}$ M). We interpret these observations to indicate that DBTQ at higher concentrations serves as an electron acceptor for the Hill reaction, and in turn reduces DCIP rather slowly. Since the

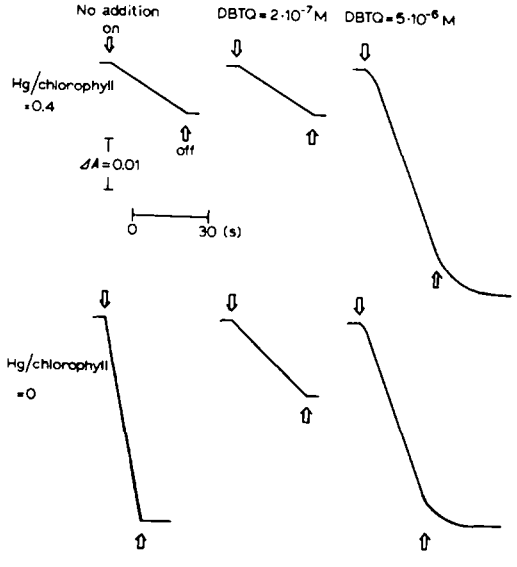


Fig. 6. Effects of DBTQ on time course of DCIP-Hill reaction in untreated and $HgCl_2$-treated spinach chloroplasts. Reaction conditions were the same as described in Fig. 1. Chloroplasts were treated with $HgCl_2$ at a $HgCl_2$ to chlorophyll ratio of 0.4. Chlorophyll concentration was 3.2 μg/ml.

DBTQ-induced stimulation of DCIP photoreduction occurred both in DBTQ- and $HgCl_2$-inhibited chloroplasts, DBTQ must be reduced at a site between Photosystem II and the DBTQ-sensitive site. A similar partial recovery of the DCIP photoreduction was observed on addition of 10^{-4} M of dimethylbenzoquinone to $HgCl_2$-treated chloroplasts.

DISCUSSION

The results obtained in the present work with $HgCl_2$ as an inhibitor of plastocyanin strongly indicate that in a steady state of the Hill reaction under a saturating intensity of light, various electron acceptors are predominantly reduced at the reducing side of Photosystem I in Class II chloroplasts.

Presumably, the intermediary electron carriers of the photosynthetic electron transport chain connecting Photosystems I and II are located inside the thylakoid membrane and are not exposed to the external reaction medium, whereas, the strong reductant produced by Photosystem I is readily accessible to the exogenous electron donors added.

This hypothesis is in accord with the observed fact that the $HgCl_2$-insensitive portion of the photoreduction, which represents the leakage of electrons at certain sites between the two photosystems, was larger with a lipophilic electron acceptor than with a hydrophilic acceptor; a lipophilic electron acceptor can more easily reach the intermediary electron carriers inside the thylakoid membrane than an ionic acceptor. A similar situation has been discussed by Saha *et al.*[16] and Trebst (personal communication). This view is also compatible with the observation of Kok *et al.*[5], Ke[8] and Lien and Bannister[10] that DCIP is rapidly reduced by Photosystem I and slowly by Photosystem II.

Photosystem II-mediated photoreduction of DCIP in *Euglena* chloroplasts, sonicated spinach chloroplasts or Photosystem II particles are largely insensitive to $HgCl_2$. This confirms our conclusion in the previous paper[12] that $HgCl_2$ is not inhibitory on the electron transport associated with Photosystem II. It was shown previously that the Hill reaction in *Euglena* chloroplasts and sonicated spinach chloroplasts required relatively acidic pH and high concentration of the electron acceptor for the maximum activity[6,7,17,18]. This was also found to be the case with the $HgCl_2$-resistant part of the Hill activity. It appears, therefore, that the Photosystem II-mediated photoreduction in three chloroplast preparations shares a common rate-limiting step. The rate-limiting reaction in the photoreduction in $HgCl_2$-treated chloroplasts is most possibly the reaction between the electron acceptor added and the endogenous reductants produced by Photosystem II. It may be that even in sonicated chloroplasts or *Euglena* chloroplasts, the electron transfer from the endogenous reductants to the acceptor added is a rate-limiting step of the overall reaction.

The effect of Hg^{2+} on electron transport in chloroplasts has previously been studied by Izawa and Good[19]. They concluded that Hg^{2+} is an energy transfer inhibitor, since Hg^{2+} partially inhibited photophosphorylation and the coupled electron transport, whereas the basal and uncoupled electron transports were completely insensitive to Hg^{2+}. However, the present and previous[12] studies clearly demonstrated that Hg^{2+} acts as a potent inhibitor of electron transport in chloro-

plasts in the presence of an uncoupler (methylamine·HCl). We consistently observed a significant inhibition of the Hill reaction at the higher range of Hg^{2+} concentration (corresponding to the Hg^{2+} to chlorophyll ratios of 0.6 to 0.9 (Fig. 6 to Fig. 8 in ref. 19)) employed by them. In the present work, $HgCl_2$ was shown to be inhibitory on the Hill reaction in chloroplasts from various higher plants and with a variety of electron acceptors. These further show the usefulness of $HgCl_2$ as a specific inhibitor of electron transport associated with Photosystem I.

ADDENDUM

Three papers, published after the manuscript of this paper had been completed, by Ouitrakul and Izawa, Izawa *et al.* and Trebst and Reimer [*Biochim. Biophys. Acta* 305 (1973) 105, 119 and 129, respectively] contain some informations similar to that presented here.

ACKNOWLEDGEMENTS

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