BBA 46414

STUDIES ON ELECTRON TRANSPORT ASSOCIATED WITH PHOTOSYSTEM I

I. FUNCTIONAL SITE OF PLASTOCYANIN; INHIBITORY EFFECTS OF $HgCl_2$ ON ELECTRON TRANSPORT AND PLASTOCYANIN IN CHLOROPLASTS

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SUMMARY

1. Incubation of chloroplasts with $HgCl_2$ at a molar ratio of $HgCl_2$ to chlorophyll of about unity, induced a complete inhibition of the methyl viologen Hill reaction, as well as methyl viologen photoreduction with reduced 2,6-dichlorophenolindophenol (DCIP) as electron donor. Photooxidation of cytochrome *f* was similarly sensitive towards $HgCl_2$, whereas photooxidation of P700 was resistant to the poison. Photoreduction of cytochrome *f* and light-induced increase in fluorescence yield were enhanced by the $HgCl_2$ treatment of chloroplasts.

2. Photooxidation of reduced yeast cytochrome *c* catalyzed by Photosystem I particles in the presence of plastocyanin was also suppressed by preincubation with $HgCl_2$. The inhibition was recovered by adding more plastocyanin, but not by addition of the particles, to the reaction mixture.

3. Incubation of reduced plastocyanin with $HgCl_2$ caused a bleaching of the blue color of the protein, whereas the oxidized protein was resistant to $HgCl_2$. A similar contrasting difference in sensitivity towards $HgCl_2$ was found in the Hill reaction in the absence and presence of ferricyanide.

4. Sonic treatment released plastocyanin from the untreated chloroplasts, whereas no plastocyanin was detected in the extract of the $HgCl_2$-treated chloroplasts. However, plastocyanin could be reconstructed by dialyzing the extract of the $HgCl_2$-treated chloroplasts against cysteine solution and then $CuSO_4$ solution.

5. The role of plastocyanin in the photosynthetic electron transport is discussed.

INTRODUCTION

Plastocyanin was first discovered in *Chlorella ellipsoidea* and subsequently in a wide variety of higher plants and algae[1,2]. The protein was shown to be localized

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PMS, phenazine methosulfate.

in the chloroplasts in the cells at a concentration comparable to those of cytochrome *f* and P700 (ref. 2). The first indication of a possible role of plastocyanin as an electron carrier in the photosynthetic electron transport system was the finding that sonic treatment of chloroplasts gave rise to an inactivation of $NADP^+$ photoreduction with water or reduced 2,6-dichlorophenolindophenol (DCIP) as electron donor, in parallel with solubilization of plastocyanin from the chloroplasts[3,4]. The lost activity was largely restored by the addition of plastocyanin to the treated chloroplasts[3,4]. Similar requirement for plastocyanin in the $NADP^+$ photoreduction has been repeatedly observed with chloroplasts, from which the protein was removed by detergent or mechanical treatments of chloroplasts[5-8]. These observations, together with the redox potential of the protein[9] comparable to that of cytochrome *f*, indicate that plastocyanin is functioning in the electron transport chain at the electron donating side of Photosystem I.

De Kouchkovsky and Fork[10] found a light-induced absorbance change at about 600 nm in *Ulva*, and also in other plants, which they ascribed to a photooxidation of plastocyanin. On a basis of the observed inhibition of the absorbance change by salicylaldoxime, Fork and Urbach[11] suggested that the reaction site of plastocyanin is located between Photosystem II and cytochrome *f*. Salicylaldoxime, however, was shown later to be an inhibitor of Photosystem II but not of plastocyanin[12,13].

Kok *et al.*[6,14] have compared kinetics of photooxidation of plastocyanin and *Euglena* cytochrome *f* added to chloroplasts which had been sonically treated in the presence of a detergent and proposed that plastocyanin and cytochrome *f* were functioning in two parallel pathways of electron transfer to Photosystem I. Similar parallel mode of action of plastocyanin and cytochrome *f* was also suggested by Witt *et al.*[15] from spectrophotometric determination of photooxidation of the two proteins, *in situ*.

A third possibility that plastocyanin mediates the electron transfer from cytochrome *f* to P700 was first proposed by Davenport[16], who found that sonically treated chloroplasts which still retained cytochrome *f* were inactive in $NADP^+$ photoreduction unless plastocyanin was provided externally. Wessels[17] found that in the small particles prepared by the digitonin treatment of chloroplasts, $NADP^+$ photoreduction was restored by the addition of plastocyanin but not of cytochrome *f*.

More convincing evidence for this possibility was presented by Gorman and Levine[18] through their experiments with *Chlamydomonas* mutants which were deficient either in plastocyanin or cytochrome *f*. They showed that plastocyanin was necessary for $NADP^+$ photoreduction, cytochrome *f* photooxidation and phenazine methosulfate (PMS)-catalyzed cyclic electron transfer coupled with phosphorylation. Hind[19], and Avron and Shneyour[20] showed that the addition of plastocyanin markedly accelerated the rates of cytochrome *f* photooxidation in chloroplasts which had been treated with detergent or sonicated, respectively.

More recently, the results of studies carried out by various investigators on the Photosystem I-enriched particles, prepared by the method of Michel and Michel-Wolwertz[21] using the French press, seems to add more confusion with respect to the role of plastocyanin in Photosystem I. Arnon *et al.*[22] reported that the particles were free of the protein but still active in mediating, at a substantial rate, $NADP^+$ photoreduction with reduced DCIP as electron donor. In their unique scheme of electron transport in chloroplasts, plastocyanin is located in the chain connecting

Photosystems IIa and IIb, whereas cytochrome *f* is placed in a cyclic electron transport chain including Photosystem I[23,24]. Murata and Fork[25] showed that chloroplast fragments prepared by the French press treatment of chloroplasts were capable of photooxidizing cytochrome *f* at a rapid rate comparable to that in the intact chloroplasts. Since they could not detect a significant amount of plastocyanin in the fragments, they concluded that plastocyanin did not mediate electron transfer between cytochrome *f* and P700[25]. On the contrary, Baszynski *et al.*[26] and Arntzen *et al.*[27], using a sensitive method of Plesničar and Bendall[28] for the determination of plastocyanin, showed that chloroplast fragments prepared by a similar technique retained a substantial amount of plastocyanin.

In this paper, we report the effects of $HgCl_2$ on the photochemical activities of spinach chloroplasts, including methyl viologen photoreduction, fluorescence induction, photooxidation and reduction of cytochrome *f* and photooxidation of P700. It will be shown that $HgCl_2$, at a concentration similar to that of chlorophyll, inhibits specifically the photooxidation of cytochrome *f* without any inhibition, but a slight acceleration of the photoreduction of the cytochrome and light-induced increase in fluorescence yield. Photooxidation of P700 is unaffected by $HgCl_2$. Evidence will be presented to indicate that $HgCl_2$ inactivates plastocyanin by replacing copper in the protein both *in vitro* and *in vivo*. It will be concluded that plastocyanin is functioning in the electron transfer from cytochrome *f* to the reaction centre of Photosystem I.

MATERIALS AND METHODS

Chloroplast preparation

Chloroplasts were isolated from market spinach leaves as described previously by Katoh *et al*[29] using a preparation medium containing 0.4 M sucrose, 0.05 M phosphate buffer (pH 7.8) and 0.01 M NaCl. Photosystem I particles were prepared from digitonin-treated chloroplasts according to the method of Ohki and Takamiya[30].

Concentrations of chlorophyll were determined by the method of Arnon[31]. Plastocyanin was prepared from spinach leaves according to the method described by Katoh *et al.*[9] with a slight modification. Purified yeast cytochrome *c* was obtained from Sankyo Co.

Measurements of the photochemical activities

The photoreduction of DCIP and ferricyanide and the photooxidation of cytochrome *c* were measured by following the absorbance changes at 580, 420 and 550 nm, respectively, using a Hitachi Spectrophotometer, EPU 2A, as described by Katoh *et al.*[29].

Oxygen uptake was determined with a Clark-type oxygen electrode as described previously[29]. The compositions of the reaction mixtures are indicated in the legends of respective figures.

Light-induced changes of cytochrome *f* and P700 were measured with an Aminco-Chance dual wavelength spectrophotometer. Actinic light, obtained from a 650-W tungsten iodine lamp (Ushio Co.), was passed through a water filter of 7 cm thickness and appropriate color filters or interference filters. The actinic light was blocked from the photomultiplier by inserting a guard filter between the sample

cuvette and the phototube: the filters used were bandpass filters, Toshiba VG-57 and Corning 4-96, for 554 nm light and for measurement in the Soret region, respectively.

Fluorescence of chlorophyll *a* emitted from chloroplasts was determined at 685 nm as described previously[29].

RESULTS

Methyl viologen photoreduction

Fig. 1 shows the effect of various concentrations of $HgCl_2$ on the activities of methyl viologen photoreduction in spinach chloroplasts with water or reduced DCIP as electron donor. The reaction was estimated by measuring the rates of light-induced O_2 uptake. In this reaction system reduced methyl viologen was quickly reoxidized by molecular oxygen and the decomposition of H_2O_2 formed was prevented by addition of KCN, as inhibitor of catalase. Low concentrations of Hg^{2+} have been shown by Izawa *et al.*[32] to act as an energy transfer inhibitor and suppress partly phosphorylating electron transport. In this experiment, therefore, methylamine, a potent uncoupler, was added to the reaction mixture. It was also found that the inhibition of the photoreduction was not instantaneous but gradually developed with time of incubation of chloroplasts in the presence of the poison. The increase in the degree of inhibition became negligible after 10 min of incubation of chloroplasts with $HgCl_2$ at 0 °C (see Fig. 9). The activities, therefore, were determined with chloroplasts which had been preincubated with $HgCl_2$ for at least 30 min. The inhibition, once developed, was not reversed by washing the chloroplasts with the preparation medium, or by addition of cysteine or EDTA. There was no difference in the progress of inhibition between light and dark incubation.

The magnitude of inhibition thus attained was found to depend on the molar ratio of the poison to chlorophyll rather than on the concentration of $HgCl_2$ alone.

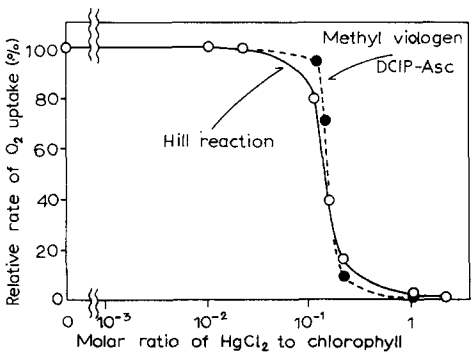


Fig. 1. Effects of $HgCl_2$ concentration on methyl viologen photoreduction in spinach chloroplasts. ○—○, methyl viologen Hill reaction. The reaction mixture contained 40 μM methyl viologen, 10 mM NaCl, 10 mM methylamine·HCl, 1 mM KCN, 50 mM phosphate buffer (pH 7.8) and chloroplasts equivalent to 38.2 μg chlorophyll in a final volume of 4 ml. ●---●, photoreduction with DCIP–ascorbate couple as electron donor. 0.1 mM DCIP, 2 mM ascorbate and 13 μM DCMU were added to the above mixture. Chloroplasts were preincubated with the indicated amounts of $HgCl_2$ at 0 °C for 30 min. Duration of reaction, 2 to 4 min. Rates of methyl viologen photoreduction in untreated chloroplasts with water or reduced DCIP as electron donor were 408 and 950 μmoles O_2/mg chlorophyll per h, respectively.

In the following, therefore, the effectiveness of $HgCl_2$ is expressed in terms of the molar ratios of $HgCl_2$ to chlorophyll.

As is shown in Fig. 1, inhibition of methyl viologen photoreduction with water as electron donor became apparent by the addition of Hg^{2+} at the poison to chlorophyll ratios higher than 0.1 and a complete inactivation was observed at a ratio of around unity. The Hill reaction with DCIP or ferricyanide as electron acceptor was also inhibited by $HgCl_2$ (see Figs 7 and 9).

Methyl viologen photoreduction mediated by Photosystem I with the ascorbate–DCIP couple as electron donor and in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), showed similar sensitivity to $HgCl_2$ as the Hill reaction (Fig. 1). This finding suggests that the two photoreductions were inhibited by $HgCl_2$ at the same site of electron transport, which was close to Photosystem I. Evidence in support of this assumption was obtained in the following experiments, in which the effect of $HgCl_2$ on the fluorescence of chlorophyll *a* in chloroplasts was studied.

Fluorescence induction

Fig. 2 shows time courses of changes in fluorescence intensity during illumination of the chloroplasts which had been kept in the dark for several hours. In the absence of any addition, the fluorescence time course showed a typical induction; *i.e.* an initial rapid rise and the subsequent slow rise to attain a steady level of emission. In chloroplasts treated with $HgCl_2$, the rate of fluorescence increase were accelerated and the steady state yield of fluorescence was increased. Somewhat similar effects on the fluorescence induction were observed in the presence of DCMU, which blocks the electron transfer from the primary electron acceptor of Photosystem II to adjacent electron carriers. However, the magnitudes of the changes induced by $HgCl_2$ were much smaller than those with DCMU. These observations suggest that $HgCl_2$ has

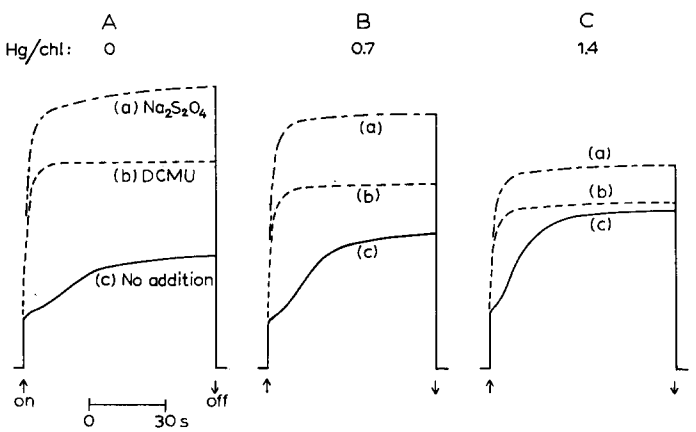


Fig. 2. Effect of $HgCl_2$ on the time course of fluorescence intensity during the induction period. Actinic light; blue light with maximum intensity at 480 nm, $2.5 \cdot 10^2$ ergs/cm^2 per s. Fluorescence emitted from the illuminated chloroplasts was measured at 685 nm (half band width, 12 nm). Chloroplasts were suspended in the preparation medium. Where indicated, 13 μM DCMU (b) or a few grains of $Na_2S_2O_4$ (a) were added. Chlorophyll concentration in the fluorescence measurement was 1.6 μg/ml. In B and C, chloroplasts were preincubated at 0 °C for 30 min with $HgCl_2$ at the $HgCl_2$/chlorophyll ratios of 0.7 and 1.4, respectively.

no significant effect on Photosystem II, but inhibits electron transport at a site closer to Photosystem I than the DCMU-sensitive site.

It was also noted that the fluorescence yield determined in the presence of DCMU and dithionite decreased at higher ratios of $HgCl_2$ to chlorophyll. Presumably, high concentration of $HgCl_2$ exerted an additional deleterious effect on the activity of Photosystem II.

Photooxidation and photoreduction of cytochrome f

Fig. 3 shows the effects of $HgCl_2$ on the light-induced absorbance changes of chloroplasts at 554 nm (reference wavelength, 540 nm). In the presence of ascorbate and DCMU, illumination induced a rapid absorbance decrease (Curve a). When chloroplasts were incubated with $HgCl_2$, the rapid absorbance change was completely eliminated and the rate of the remaining absorbance change, if any, became markedly sluggish (Curve b). Similar experiments were carried out at various wavelengths and the absorbance differences between the light steady state and the dark original level of absorbance were plotted against wavelengths. Light-*minus*-dark difference spectra thus obtained are shown in Fig. 4A. A negative peak with a maxi-

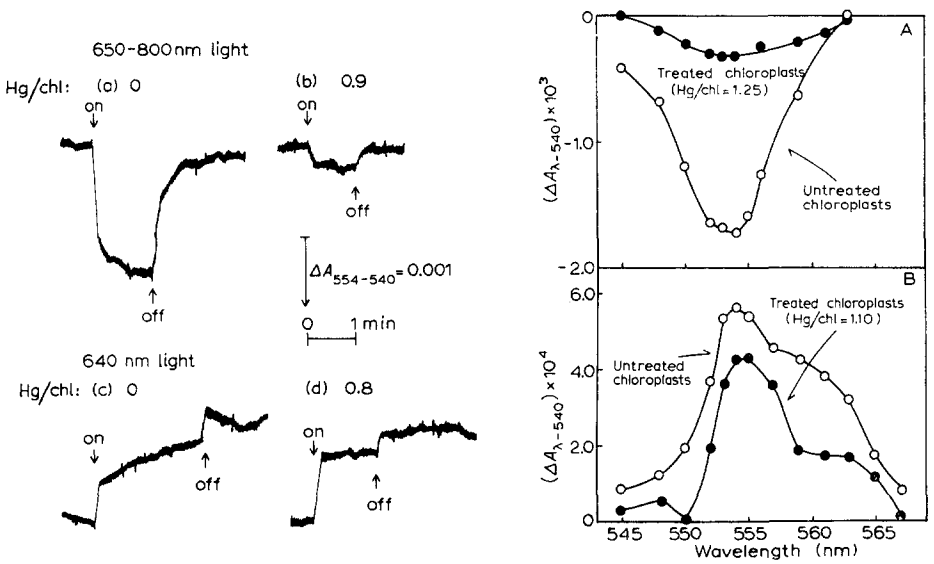


Fig. 3. Effects of $HgCl_2$ on light-induced absorbance changes of cytochrome *f*. Absorbance changes were determined at 554 nm (reference, 540 nm). The basal reaction mixture contained, in a final volume of 2 ml, 0.4 M sucrose, 0.05 M phosphate buffer, pH 7.8, 0.01 M NaCl, 0.01 M methylamine·HCl. In a and b, 2 mM ascorbate, 2 μM methyl viologen, 6 μM DCMU and chloroplasts containing 76 μg chlorophyll were added. Red light (650–800 nm, $8 \cdot 10^5$ ergs/cm^2 per s) was used as actinic light. In c and d, ferricyanide (50 μM) was added 5 min prior to the illumination, to the basal mixture which contained chloroplasts equivalent to 120 μg chlorophyll. Actinic light; 640 nm (half band width, 12 nm, $3.5 \cdot 10^4$ ergs/cm^2 per s). In b and d, chloroplasts were preincubated with $HgCl_2$ at the $HgCl_2$/chlorophyll ratios of 0.9 and 0.8, respectively.

Fig. 4. Light-*minus*-dark difference spectra of untreated and $HgCl_2$-treated chloroplasts. The reactions were carried out as described in Fig. 3, Curves a and b (A) and Curves c and d (B), but at various wavelengths with a fixed reference wavelengths (540 nm). Extents of the light-induced absorbance decrease (A) or increase (B) were plotted against wavelengths. Chlorophyll concentrations in the reaction mixture were 81 μg/ml (A) and 49 μg/ml (B). Chloroplasts were preincubated with $HgCl_2$ at a ratio of $HgCl_2$ to chlorophyll of 1.25 (A) and 1.10 (B), respectively.

mum at 554 nm in the spectrum of the untreated chloroplasts indicated that the absorbance change observed was due to the photooxidation of cytochrome *f*. No absorbance change which could be ascribed to redox changes of the *b*-type cytochromes was observed. It is apparent that the $HgCl_2$ treatment eliminated the rapid photooxidation of cytochrome *f*, leaving only a slow reaction with a diminished extent.

Fresh chloroplasts usually contain cytochrome *f* mostly in the reduced state. When ferricyanide was added in the dark to oxidize cytochrome *f*, subsequent illumination with 640 nm light induced a rapid absorbance increase in 554 nm followed by a much slower change of the same sign (Fig. 3, Curve c). On turning off the light, a small, rapid absorbance increase occurred but there was no return to the original level. Similar time courses of the cytochrome *f* change induced by System II light were previously observed by Cramer and Butler[33], and Avron and Chance[34]. Similar absorbance changes were observed in $HgCl_2$-treated chloroplasts, except that there was no slower phase in the light-induced absorbance increase, after the treatment. Light-*minus*-dark difference spectrum for the absorbance change in the untreated chloroplasts shows a peak at 554 nm and a shoulder at 560 nm, indicating that cytochrome *f* and *b*-type cytochrome(s) underwent photoreduction (Fig. 4B). Inhibitory effect of $HgCl_2$ treatment was apparent on the photoreduction of *b*-type cytochrome (possibly, cytochrome *b*-559), but not on that of cytochrome *f*. The observed stimulation of cytochrome *f* photoreduction results from the inhibition of photooxidation of the cytochrome. These results obtained from the methyl viologen photoreduction, fluorescence and cytochrome *f* changes are compatible with each other in indicating that $HgCl_2$ inhibits electron transport at a site close to Photosystem I.

Photooxidation of P700

The spectrophotometric experiment was extended further to the Soret region (400–450 nm) to investigate the photooxidation of cytochrome *f* and P700. Fig. 5

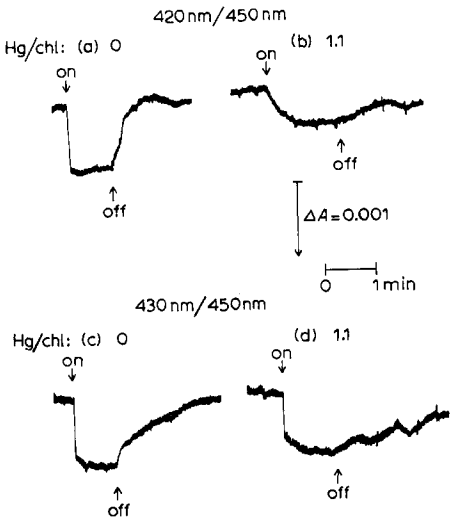


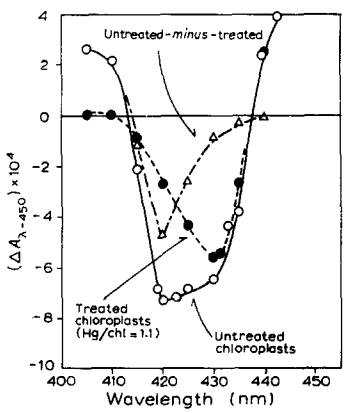
Fig. 5. Effects of $HgCl_2$ on light-induced absorbance changes at 420 nm (a, b) and 430 nm (c, d). Reaction conditions were the same with those for a and b in Fig. 3. Concentration of chlorophyll was 11.7 μg/ml. Chloroplasts preincubated with $HgCl_2$ were used in b and d. ($HgCl_2$ to chlorophyll ratio, 1.1.)

shows time courses of light-induced absorbance changes at 420 nm and 430 nm observed in the presence of methyl viologen, ascorbate and DCMU. Reference wavelength was 450 nm. Illumination induced rapid absorbance decreases at 420 nm and 430 nm, which were recovered to the initial absorbance levels on turning off the light.

In $HgCl_2$-treated chloroplasts, the rapid absorbance decrease at 430 nm was almost completely abolished. In contrast, the absorbance change at 430 nm was only slightly affected both in rate and extent. It is noted that the rate of recovery of absorbance at 430 nm in the subsequent dark period was considerably retarded after the $HgCl_2$ treatment (Curve d).

Light-*minus*-dark difference spectra in the Soret region (405–450 nm) determined with untreated and $HgCl_2$-treated chloroplasts are shown in Fig. 6. In untreated chloroplasts, the difference spectrum had a broad trough with a maximum at 420 nm and a shoulder near 430 nm. In $HgCl_2$-treated chloroplasts, the trough at 420 nm disappeared and the spectrum now showed a single trough at 430 nm. The difference between these two difference spectra showed a trough at 420 nm. The troughs at 420 nm and at 430 nm agree well with the absorption peaks of cytochrome *f*[35] and P700[36,37], respectively. It is thus apparent that $HgCl_2$ inhibited the photooxidation of cytochrome *f* but not the photooxidation of P700 in the chloroplasts.

Fig. 7 shows the extent of $HgCl_2$ inhibition of cytochrome *f* photooxidation determined at 554 nm, and the DCIP Hill reaction in chloroplasts. For the determination of the sensitivity of P700 photooxidation to $HgCl_2$, Photosystem I-enriched



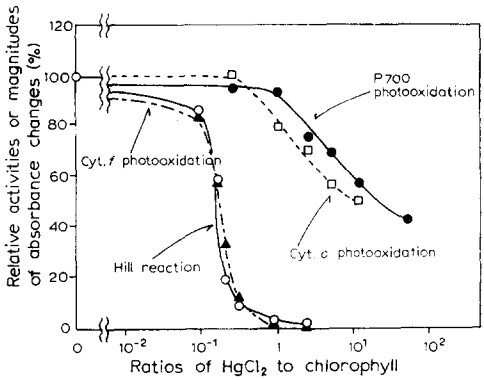


Fig. 6. Light-*minus*-dark difference spectra for the Soret region in untreated and $HgCl_2$-treated chloroplasts. Reaction conditions were the same as described in Fig. 5. Chloroplasts were preincubated with $HgCl_2$ at a ratio of $HgCl_2$ to chlorophyll of 1.1.

Fig. 7. Difference in sensitivities of the DCIP Hill reaction and cytochrome *f* photooxidation in chloroplasts and the P700 photooxidation and cytochrome *c* photooxidation in Photosystem I particles toward $HgCl_2$ treatment. Extents of photooxidation of cytochrome *f* were determined at 554 nm as described in a and b in Fig. 3. The rates of DCIP Hill reaction were determined spectrophotometrically as described previously[29]. The reaction mixture contained, in a final volume of 2 ml, 20 μM DCIP, 10 mM NaCl, 10 mM methylamine·HCl, 50 mM phosphate buffer (pH 7.5) and chloroplasts containing 15.6 μg chlorophyll. Extents of photooxidation of P700 were determined at 430 nm as described in Fig. 5, except that, in place of chloroplasts, Photosystem I particles were employed. Rates of photooxidation of cytochrome *c* were determined at 550 nm as described in Table I, except that plastocyanin was omitted from the preincubation mixture, and added just before the determination of the activity.

particles prepared by digitonin treatment of chloroplasts were employed to eliminate the contribution of cytochrome *f* to the absorbance change at 430 nm. Both chloroplasts and Photosystem I particles were preincubated at various ratios of $HgCl_2$ to chlorophyll. The sensitivity of cytochrome *f* photooxidation to $HgCl_2$ was comparable with that of the Hill reaction, whereas much higher ratios of $HgCl_2$ to chlorophyll were required to suppress the P700 photooxidation. Thus, the inhibition of cytochrome *f* photooxidation induced by $HgCl_2$ cannot be ascribed to an inactivation of P700. There are two possible explanations for the above findings. The first one is that P700 is not necessary for the activity of Photosystem I, as suggested recently by Rurainski *et al.*[38]. The second is that a substance which mediates electron transfer from cytochrome *f* to P700 is sensitive to $HgCl_2$.

Cytochrome c photooxidation by Photosystem I particles

The first possibility described above was studied by determining the effect of $HgCl_2$ on the photooxidation of reduced yeast cytochrome *c* which was catalyzed, in the presence of plastocyanin, by the Photosystem I-enriched particles obtained by digitonin treatment of chloroplasts. The results obtained are summarized in Table I.

TABLE I

EFFECTS OF $HgCl_2$ ON PHOTOOXIDATION OF CYTOCHROME *c* BY PHOTOSYSTEM I PARTICLES IN THE PRESENCE OF PLASTOCYANIN

"Complete" reaction mixture contained, in a final volume of 0.7 ml, 57 μM methyl viologen, 50 mM phosphate buffer (pH 7.8), 7.6 μM plastocyanin, 25 μM reduced cytochrome *c* and Photosystem I particles equivalent to 0.98 μM chlorophyll. The reaction mixtures were preincubated with 1.0 μM $HgCl_2$ for 30 min at 0 °C in the dark. ($HgCl_2$/chlorophyll ratio was 1.02.)

Expt No.	*Preincubation*	*Addition*	*Rate (μmoles cytochrome c reduced/mg chlorophyll per h)*
1	Complete	—	334
	Complete + $HgCl_2$	—	0
	− Photosystem I particles, + $HgCl_2$	+ Photosystem I particles	0
	− plastocyanin, + $HgCl_2$	+ plastocyanin	262
2	Complete + $HgCl_2$	—	18
	Complete + $HgCl_2$	+ plastocyanin	377
	Complete + $HgCl_2$	+ Photosystem I particles	73

The Photosystem I particles showed a high rate of photooxidation of reduced cytochrome *c* in the presence of methyl viologen and plastocyanin, as indicated in the table. When the complete assay mixture was incubated with $HgCl_2$ for 30 min, the capacity for photooxidation was completely abolished. In order to determine which component of the reaction had been affected by the $HgCl_2$ treatment, the assay mixture, from which either Photosystem I particles or plastocyanin had been omitted, was preincubated with $HgCl_2$. The particles or plastocyanin was added later, just before determination of the activity. Inactivation of the photooxidation occurred only when plastocyanin was present during preincubation with $HgCl_2$.

It was evident that plastocyanin, but not the Photosystem I particles, was the component which was inactivated by $HgCl_2$. This was further confirmed by the results of another set of experiments, which are shown in the lower part of the table. The inhibition caused by preincubation of the complete reaction mixture with $HgCl_2$ was recovered by subsequent addition of plastocyanin, but not by addition of untreated Photosystem I particles. This shows that the activity of Photosystem I is resistant to the action of $HgCl_2$. Fig. 7 also shows similar sensitivity of the photochemical activity and the P700 photooxidation of the Photosystem I particles to the $HgCl_2$ treatment. This favours the view that photooxidation of P700 is the primary photoact of Photosystem I, thereby excluding the first possibility noted above.

Interaction between plastocyanin and $HgCl_2$

The finding obtained above *in vitro* (*i.e.* with Photosystem I particles) that plastocyanin lost its catalytic activity when incubated with $HgCl_2$ suggests that a similar mechanism may operate in the $HgCl_2$-induced inhibition of cytochrome *f* photooxidation in chloroplasts: in other words, $HgCl_2$ may affect plastocyanin which participates in the electron transfer between cytochrome *f* and P700. A prerequisite for this hypothesis is that $HgCl_2$ reacts with plastocyanin fairly rapidly. This is apparently in contradiction to the previous observation of Katoh and Takamiya[3] that the reaction of $HgCl_2$ with the oxidized plastocyanin was very slow unless a high concentration of urea was added. It was found in the present work, however, that the addition of 10^{-4} M $HgCl_2$ to plastocyanin in the reduced state caused a rapid decrease in the amount of intact plastocyanin as detected by the blue color (absorbance maximum at 597 nm) developed by subsequent addition of ferricyanide: a 50% decrease took place after 3 min of incubation with $HgCl_2$ (Fig. 8). The rate of the $HgCl_2$-induced change was much slower with the oxidized plastocyanin, which is in accord with the previous observation[39]. It follows that the development of the inhibition should be very slow when plastocyanin in the chloroplasts was kept in the oxidized state during incubation with $HgCl_2$. This was actually found to be the case. As shown in Fig. 9, the development of inhibition of the Hill reaction was comparable to that of the above-observed reaction of $HgCl_2$ with reduced plastocyanin (Fig. 8). However, when chloroplasts had been preincubated with ferricyanide (2 mM) prior to addition of $HgCl_2$, the development of the inhibition during the subsequent $HgCl_2$ treatment was extremely slow. These facts clearly indicate that $HgCl_2$ caused the inhibition of electron transport in chloroplasts by inactivating the endogenous plastocyanin in the reduced state.

More direct evidence was obtained for the reaction of $HgCl_2$ with plastocyanin in chloroplasts. Chloroplasts equivalent to $1.7 \cdot 10^{-4}$ mole of chlorophyll were incubated with $1.8 \cdot 10^{-4}$ mole of $HgCl_2$ for 30 min and then washed with the preparation medium containing 10^{-3} M EDTA to remove free Hg^{2+}. The $HgCl_2$-treated chloroplasts were suspended in 0.15 M NaCl containing 0.01 M phosphate buffer (pH 7.8) and sonicated for 10 min. The homogenate obtained was centrifuged successively at $20000 \times g$ for 10 min, at $50000 \times g$ for 30 min and finally at $130000 \times g$ for 60 min. A control run was made with chloroplasts which were treated similarly except that incubation with $HgCl_2$ was omitted. Fig. 10 shows the ferricyanide oxidized-*minus*-ascorbate reduced difference spectra of the supernatants derived from the untreated and $HgCl_2$-treated chloroplasts. The spectrum for the extract of the un-

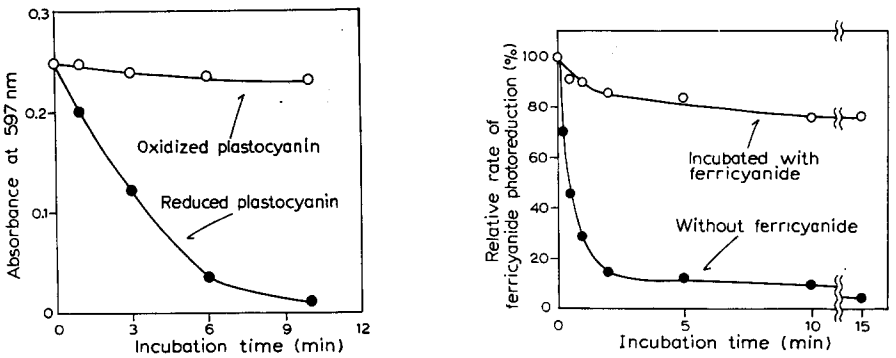


Fig. 8. Time courses of reaction of $HgCl_2$ with oxidized and reduced forms of plastocyanin. $2.1 \cdot 10^{-6}$ M plastocyanin was incubated with 10^{-4} M $HgCl_2$ in 0.6 ml of phosphate buffer (pH 7.5). In experiments with the reduced plastocyanin, the absorbance at 597 nm that appeared on subsequent addition of a few grains of ferricyanide, was determined after the indicated time of incubation.

Fig. 9. Effect of ferricyanide on the development of $HgCl_2$-induced inhibition of the Hill reaction. The activities of the Hill reaction with ferricyanide as electron acceptor were determined spectrophotometrically as described previously[29]. The reaction conditions were the same with that in the DCIP Hill reaction described in Fig. 7, except that 0.4 mM ferricyanide was used as the Hill oxidant. Chlorophyll concentration was 12.5 μg/ml. ●—●, chloroplasts were incubated with $HgCl_2$ in the absence of ferricyanide; the Hill reaction activity was determined immediately after addition of ferricyanide. ○—○, chloroplasts were preincubated with ferricyanide in the dark for 5 min and after addition of $HgCl_2$ ($HgCl_2$ to chlorophyll ratio was 0.8), incubated further for indicated times.

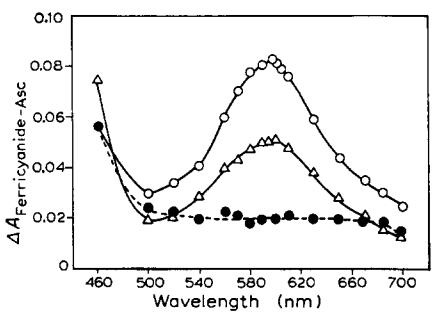


Fig. 10. Oxidized-*minus*-reduced difference spectra of the extracts obtained from untreated and $HgCl_2$-treated chloroplasts. The spectra were obtained by subtracting the absorption spectrum determined in the presence of ascorbate from that determined in the presence of ferricyanide. ○—○, extracts of untreated chloroplasts; ●---●, extracts of $HgCl_2$-treated chloroplasts; △—△, extracts of $HgCl_2$-treated chloroplasts, which was dialyzed against cysteine and then $CuSO_4$ solution.

treated chloroplasts showed a characteristic absorption of the oxidized plastocyanin, corresponding to a molar ratio of chlorophyll to plastocyanin of about 900. On the other hand, there was no absorbance peak showing the presence of plastocyanin in the difference spectrum of the supernatant from the $HgCl_2$-treated chloroplasts. The following experiment further indicated that the supernatant from the $HgCl_2$-treated chloroplasts contained plastocyanin in a Hg-bound form. The supernatant was dialyzed overnight against 0.01 M phosphate buffer (pH 7.8) containing 5 mM

of cysteine to remove Hg^{2+} from the protein: and then against the phosphate buffer containing $4 \cdot 10^{-5}$ M $CuSO_4$ for 3 h to reconstitute plastocyanin from the apoprotein. The oxidized-*minus*-reduced difference spectrum of the dialyzate now showed a band with a maximum at 597 nm. Plastocyanin thus recovered amounted to more than one-half that of plastocyanin extracted from the untreated chloroplasts. Since in similar experiments with the isolated and purified sample of plastocyanin, the yields of the recovery are 60 to 70 %, the above experimental result indicates that plastocyanin was solubilized by sonic treatment of the $HgCl_2$-treated chloroplasts in a form containing an Hg–sulfhydryl linkage.

DISCUSSION

Inhibition site of $HgCl_2$

The results obtained in the present work can be explained by a scheme of photosynthetic electron transport presented in Fig. 11. It was clearly indicated in the present study that $HgCl_2$ at a molar ratio of $HgCl_2$ to chlorophyll of unity inhibits specifically the electron transfer from cytochrome *f* to the reaction center of Photosystem I, P700. Photooxidation of cytochrome *f* was highly sensitive towards $HgCl_2$, whereas the effects of $HgCl_2$ on the electron transport reactions associated with Photosystem II were only moderate, if any. In fact, the photoreduction of cytochrome *f* and the induction of fluorescence were not inhibited, but stimulated to some extent in the $HgCl_2$-treated chloroplasts. This is what would be expected when the electron transport through Photosystem I was blocked. Similarly, the photooxidation of P700 and the activity of Photosystem I particles in mediating electron transfer from reduced plastocyanin to methyl viologen were equally resistant to $HgCl_2$. The inhibition site of $HgCl_2$ must, therefore, be between cytochrome *f* and P700.

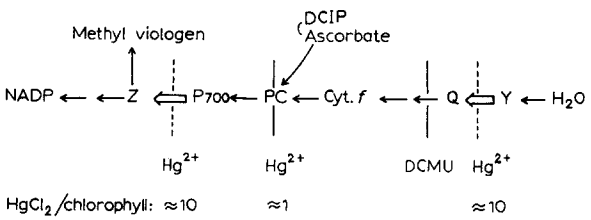


Fig. 11. A scheme for the photochemical electron transport system in chloroplasts showing the inhibitory site of Hg^{2+}. PC, plastocyanin.

In contrast to methyl viologen, ferricyanide and DCIP with rather high redox potentials, may be reduced by the reductant produced by Photosystem II, as has been demonstrated with sonicated spinach chloroplasts[4,40] or *Euglena* chloroplasts[41]. It was noted, however, that the activity of the Hill reaction with ferricyanide or DCIP as electron acceptor was also largely inhibited by $HgCl_2$ (*e.g.* $HgCl_2$/chlorophyll ratio, unity). It appears, therefore, that these oxidants accept electrons mainly at the reducing side of Photosystem I in intact chloroplasts. Details of the experimental results in this respect will be described elsewhere.

At higher $HgCl_2$ to chlorophyll ratios, $HgCl_2$ showed additional inhibitory actions on Photosystem I and II, as revealed by a suppression of photooxidation

of P700 and by decreased steady state fluorescence yield in the presence of DCMU or dithionite, respectively (Fig. 2). Presumably, $HgCl_2$ at higher concentration affects the pigment system or the reaction center of the two photosystems.

Role of plastocyanin

It was also clearly indicated that $HgCl_2$ reacts with reduced plastocyanin either in solution or in the membrane-bound state in chloroplasts. A finding that plastocyanin could be reconstituted by dialyzing the apoprotein prepared from the extracts of the $HgCl_2$-treated chloroplasts against a solution of $CuSO_4$ indicates that $HgCl_2$ attacked plastocyanin at the cupro-sulfhydryl linkage and replaced copper with mercury.

On the other hand, oxidized plastocyanin was highly resistant towards $HgCl_2$. A far higher $HgCl_2$ resistance of the Hill reaction in chloroplasts pretreated with ferricyanide supports the conclusion that the modification of endogenous plastocyanin by $HgCl_2$ is the actual mechanism of the inhibition of electron transport induced by $HgCl_2$. From all these findings, we may conclude that plastocyanin functions as an electron carrier between cytochrome *f* and P700.

This conclusion concerning the reaction site of plastocyanin is in agreement with those of Davenport[16], Wessels[17], Gorman and Levine[18], Hind[19], Vernon *et al.*[42], Avron and Shneyour[20], Baszynski *et al.*[26] and Arntzen *et al.*[27]. On the other hand, it is incompatible with the model of electron transport system proposed by Knaff and Arnon[23], in which plastocyanin and cytochrome *f* function in different electron transport chains, and with that of Murata and Fork[25], in which plastocyanin is excluded from the electron transfer from cytochrome *f* to P700. Further, it will be shown in the next following paper that chloroplast fragments prepared by a French press treatment require plastocyanin for Photosystem I activity.

In conclusion, we would suggest that $HgCl_2$ may serve as a useful tool in the study of electron transport around Photosystem I. A number of specific inhibitors are known which inhibit, each in a specific way, various sites of Photosystem II[13]. However, few inhibitors of electron transport associated with Photosystem I have been reported so far. Trebst and Burba[43] found that disulfodisalicylidenepropanediamine inhibits ferredoxin-dependent reactions of isolated chloroplasts. Antibodies against ferredoxin–$NADP^+$ oxidoreductase[44,45] and a water-soluble factor[45,46] have been shown to be effective in inhibiting $NADP^+$ photoreduction in the chloroplasts. $HgCl_2$, having a definite site and mode of inhibition, appears to be a better inhibitor. Another favorable feature of this inhibitor is that it inhibits the electron transport irrespective of the nature of the electron acceptor of Photosystem I.

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