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MERCURY-INDUCED ALTERATIONS OF CHLOROPHYLL a FLUORESCENCE KINETICS IN ISOLATED BARLEY (*Hordeum vulgare* L. cv. ALBORI) CHLOROPLASTS

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Abstract—Effects of $HgCl_2$-treatment on electron transport, chlorophyll a fluorescence and its quenching were studied using isolated barley (*Hordeum vulgare* L. cv. Albori) chloroplasts. Depending on the concentration of $HgCl_2$, photosynthetic oxygen-evolving activities of photosystem II (PS II) were greatly inhibited, whereas those of photosystem I (PS I) were slightly decreased. The inhibitory effects of $HgCl_2$ on the oxygen-evolving activity was partially restored by the addition of hydroxyamine, suggesting the primary inhibition site by $HgCl_2$2-treatment is close to the oxidizing site of PS II associated with water-splitting complex. Addition of 50 μM $HgCl_2$ decreased both photochemical and nonphotochemical quenching of chlorophyll fluorescence. Especially, energy dependent quenching (qE) was completely disappeared by $HgCl_2$-treatment as observed by NH_4Cl treatment. In the presence of $HgCl_2$, F'o level during illumination was also increased. These results suggest that pH gradient across thylakoid membrane can not be formed in the presence of 0 μM $HgCl_2$. In addition, antenna pigment composition might be altered by $HgCl_2$-treatment.

INTRODUCTION

It is well known that plants uptake heavy metals easily, which exert multiple inhibitory effects on photosynthesis at several stuctural and metabolic levels[1]. Mercury-containing compounds have been shown to inhibit photosyntesis in algae and isolated chloroplasts[2–5]. Some early works on the inhibtion of photosynthetic electron transport by mercury-treatment has revealed that the Hill reaction was mostly affected.[3,6] Miles *et al.*[7] suggested that mercuric chloride functions as a direct electron acceptor of the quencher with fluorescence in photosystem II (PS II).† Recently, Samson and Popovic[5] reported the inhibitory effect of mercury on PS II photochemistry associated with water-splitting system. It has been also proposed that mercury exerts multiple changes in the fluorescence of chlorophyll a in cyanobacteria.[4,8] Nevertheless, the mechanism of mercury inhibition on photosynthesis is not yet clear and its acting sites are only partially known.

Chlorophyll a fluorescence provides information on the absorption, distribution and utilization of light energy in photosynthesis. At room temperature, chlorophyll a fluorescence is predominantly associated with PS II. The yield of chlorophyll fluorescence observed during photosynthesis is quenched by photochemical (qQ) and non-photochemical (qNP) process.[9] At high light intensity, it has been assumed that energy-dependent quenching (qE), which is known to arise when a ΔpH is present across the thylakoid membranes[10] is the major qNP component.[11] The uncouplers such as nigericin and NH_4Cl induce rapid relaxation of fluorescence with the dissipation of the transthylakoidal ΔpH.[11,12]

In this report, the effects of $HgCl_2$ on the electron transport and changes in the fluorescence yield of chlorophyll, especially qE formation, are studied with isolated barley chloroplasts.

MATERIALS AND METHODS

Plant materials and chloroplast isolation. Barley (*Hordeum vulgare* L. cv. Albori) plants were grown at 25°C under continuous white light provided by banks of fluorescent lamps interspersed with incandescent light bulbs giving photosynthetic active radiation (PAR) of 60 μmol m^{-2} s^{-1} as previously described by Chun and Lee.[13] Chloroplast isolation was done as described previously.[14]

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† *Abbreviations:* qQ, photochemical quenching; qNP, non-photochemical quenching; qE, energy-dependent quenching; PS I, II, photosystem I, II; Fo, initial fluorescence; F'o, Fo recorded after induction of quenching; Fm, maximum level of chlorophyll yield; (Fv)m, variable fluorescence; (Fv)t, variable fluorescence at any time; DTT, dithiothreitol; PBQ, phenyl-p-benzoquinone; DCPIP, 2,6-dichlorophenolindophenol; MV, methylviologen; PAR, photosynthetically active radiation.

Isolated chloroplasts were suspended in 50 m*M* Hepes KOH (pH 7.6) buffer containing 0.33 *M* sorbitol, 5 m*M* $MgCl_2$ and 1 m*M* $MnCl_2$. Chl concentration was determined according to Arnon.[15]

Measurement of O_2 evolution and chl fluorescence. Photosynthetic O_2 evolution and chlorophyll fluorescence were measured at the same time using 50 μg Chl of chloroplasts and 0.1 m*M* methylviologen (MV) as electron acceptor at 25°C with Hansatech and Walz fluorometer according to Horton and Hague.[11] The initial rate was used to calculate oxygen-evolving activity. Actinic light was provided by a Schott KL1500-T light source, through a RG610 glass filter and an Ealing 680 nm short pass interference filter giving intensities between 330 and 1330 mol m^{-2} s^{-1} PAR, which was adjusted using neutral density filters.

RESULTS AND DISCUSSION

The photosynthetic oxygen-evolving activities of isolated barley chloroplasts has been studied. To compare the relative sensitivity of the PS II and PS I reaction to $HgCl_2$ treatment, chloroplasts were treated with various concentrations of $HgCl_2$. The whole electron transport system was measured using MV as an electron acceptor. The whole photosynthetic oxygen-evolving activity was inversely decreased with increased concentrations of $HgCl_2$ up to 100 μM (Fig. 1). PS II-dependent reaction was measured using p-benzoquinone (PBQ) as an electron acceptor. PBQ-supported O_2 evolution exhibited a similar pattern of inhibition by $HgCl_2$ treatment as whole chain electron transport (Fig. 1). Both whole chain electron transport and PS II supported O_2 evolution were inhibited by more than 50% in the 50 μM of $HgCl_2$-treated chloroplasts compared with control and 100 μM $HgCl_2$ was sufficient to induce complete inhibition of oxygen evolution. The effect of $HgCl_2$ on the PS-mediated electron transport from reduced dichlorophenolindophenol (DCPIP) to MV was also monitored polarographically by O_2 uptake. In contrast to PS II supported reaction, the PS I mediated reaction of barley chloroplasts showed only 10 and 30% inhibition with 50 μM and 100 μM of $HgCl_2$-treatment, respectively (Fig. 1). These results show that PS II supported electron transport is more susceptible to $HgCl_2$ inhibition than PS I mediated electron transport.

Table 1 shows the effect of hydroxylamine on the recover of oxygen uptake and fluorescence yield after $HgCl_2$ treatment. Hydroxylamine acts as an electron donor for PS II reaction center and inhibits oxygen evolution. $HgCl_2$ treatment inhibited oxygen uptake from H_2O to MV and decreased (Fv)m/Fm. However, the addition of hydroxylamine in the presence of $HgCl_2$ recovered the activity of oxygen uptake and fluorescence yield completely (Table 1).

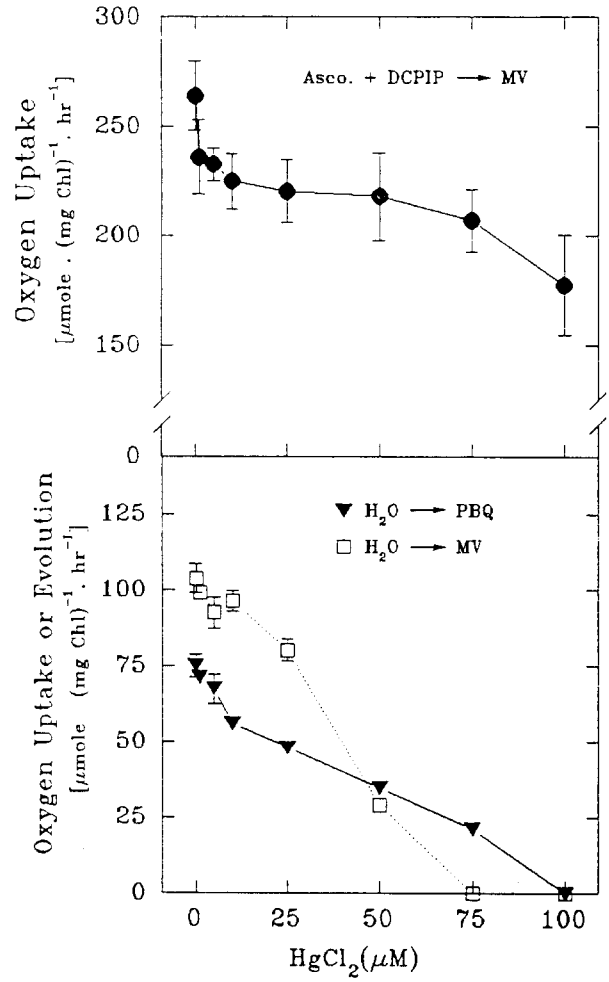

Figure 1. Electron transport activity of isolated barley chloroplasts treated with various concentrations of $HgCl_2$. The activity of PS II + I(□), the activity of PS II (▼), and the activity of PS I (●).

Table 1. The effect of $HgCl_2$ and hydroxylamine on oxygen uptake. Methylviologen was used as an electron acceptor

Treatment	Oxygen Uptake*	(Fv)m/Fm
No addition	118.4	0.776
50 μM $HgCl_2$	34.2	0.715
50 μM $HgCl_2$ + 10 m*M* NH_2OH	132.1	0.785

* μ mol O_2 (mg Chl)$^{-1}$ h^{-1}

These results suggest that inhibitory site of mercury in PS II is located at the oxygen-evolving complex. This is inaccordance with earlier findings that PS II activity inhibited by phenylmercuric acetate was restored by the addition of hydroxylamine in spinach chloroplasts[6] and the fluorescence yield is almost completely restored in barley leaf

slices[16] and in *Dunaliella tertiolecta* when hydroxylamine is added after $HgCl_2$ treatment.[5] Figure 2 shows $HgCl_2$ effect on the fluorescence induction in barley chloroplasts. Figure 2A is the chlorophyll fluorescence signal when barley chloroplasts are illuminated with saturating concentration (0.1 m*M*) of MV. The measuring beam and a saturating light pulse represents the initial fluorescence (Fo) and maximum fluorescence (Fm), respectively; the average (Fv)m/Fm ratio was 0.78. Illumination with actinic light resulted in rapid quenching of fluorescence, most of which was qNP (Fig. 2A).

When 50 μM of $HgCl_2$ was present in reaction mixture, The variable fluorescence [(Fv)m] was quenched and Fo level was almost constant (Fig. 2B) as previously observed by Samson and Popovic.[5] On the other hand, addition of 50 μM $HgCl_2$ increased variable fluorescence in a quenched state [(Fv)t] markedly in the continuous actinic light. When illumination was stopped, the relaxation of chlorophyll fluorescence quenching was not observed in the presence of $HgCl_2$ while F'o level was also markedly elevated. The increase F'o level compared with Fo level in the dark incubation with $HgCl_2$ suggests that chlorophyll a pigment system might be altered by 50 μM of $HgCl_2$ during illumination of actinic light (Fig. 2B). In Figure 2C, $HgCl_2$ was added into the incubation medium approximately 4 minutes after illumination. (Fv)t was increased by $HgCl_2$ addition, which means the decrease of fluorescence quenching. When illumination was off, saturating light pulses did not induce further relaxation of fluorescence quenching, suggesting $HgCl_2$ inhibits the formation of qNP.

Figure 3 shows the relaxation of fluorescence quenching by addition of DCMU in the absence (Fig. 3A) or in the presence of $HgCl_2$ (Fig. 3B). The treatment of $HgCl_2$ showed the decrease of qQ and qNP, especially qE (Fig. 3B). The decrease of qQ is due to the decrease of the electron transport activity (Fig. 1). Illumination with actinic light resulted in rapid quenching of fluorescence, most of which was qNP. However, (Fv)t was markedly increased in the presence of $HgCl_2$ while qNP was decreased. The addition of DCMU after illumination of actinic light induced a increased chlorophyll fluorescence level.[17] The initial fast rise reflects the closure of "Q traps", an effect similar to that of light doubling.[18] The fast rise of fluorescence is followed by a slower increase which is attributed to the decrease in the proton gradient.[17] Addition of 10 μM DCMU at steady state confirmed that both qE and qQ were decreased by $HgCl_2$ (Fig. 3B).

Figure 4 shows the relaxation of fluorescence quenching by addition of an uncoupler, NH_4Cl, immediately after turning off the actinic light.

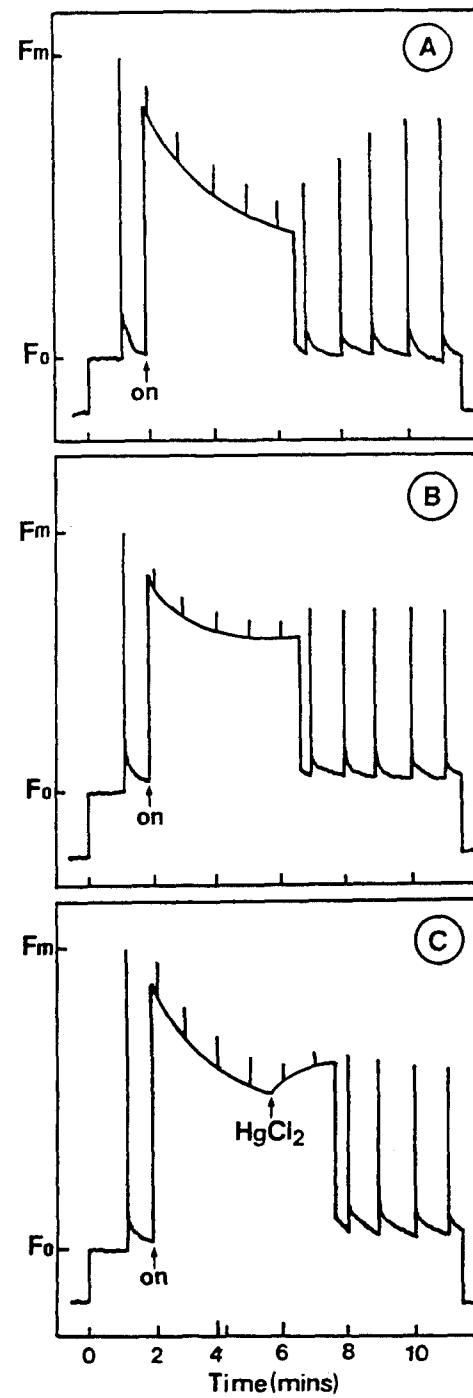

Figure 2. The effects of $HgCl_2$ on chlorophyll fluorescence induction trasients of isolated barley chloroplasts. Control (A); chloroplasts treated with 50 μM of $HgCl_2$ prior to illumination (B); treated 4 min after illumination (C). Saturating light intensity was 4,000 m^{-2} s^{-1} and actinic light intensity was 960 μmol m^{-2} s^{-1} Arrows of on and off indicate the turning on and off of actinic light each.

Addition of NH_4Cl caused complete relaxation of qE[12] (Fig. 4A). A relationship between quenching of chlorophyll fluorescence and the high energy state of the thlakoid membranes was first observed by

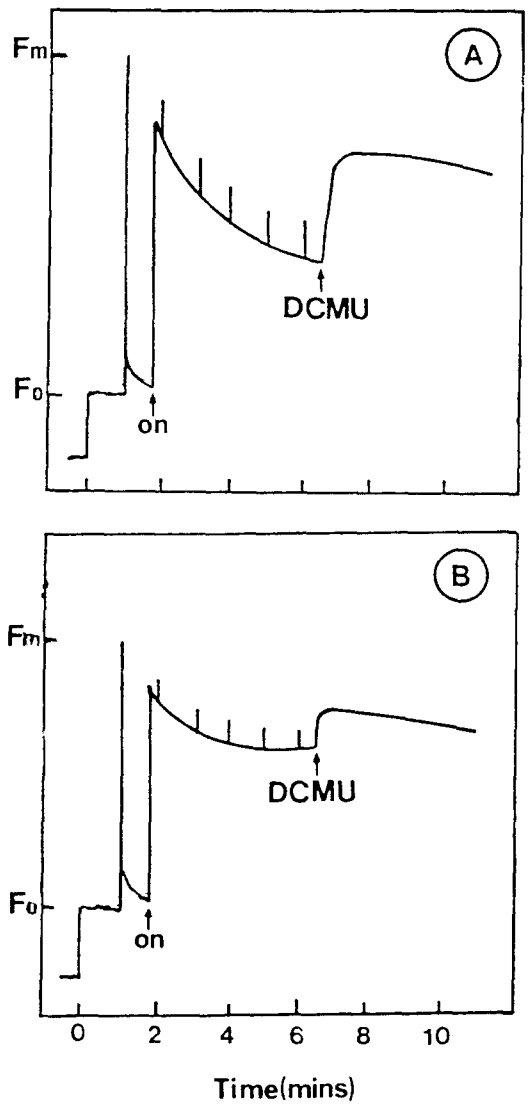

Figure 3. The relaxation of fluorescence quenching by the addition of DCMU in the absence or in the presence of $HgCl_2$. Control (A); 50 μM of $HgCl_2$-treated chloroplasts (B). Other conditions are the same as in Fig. 2.

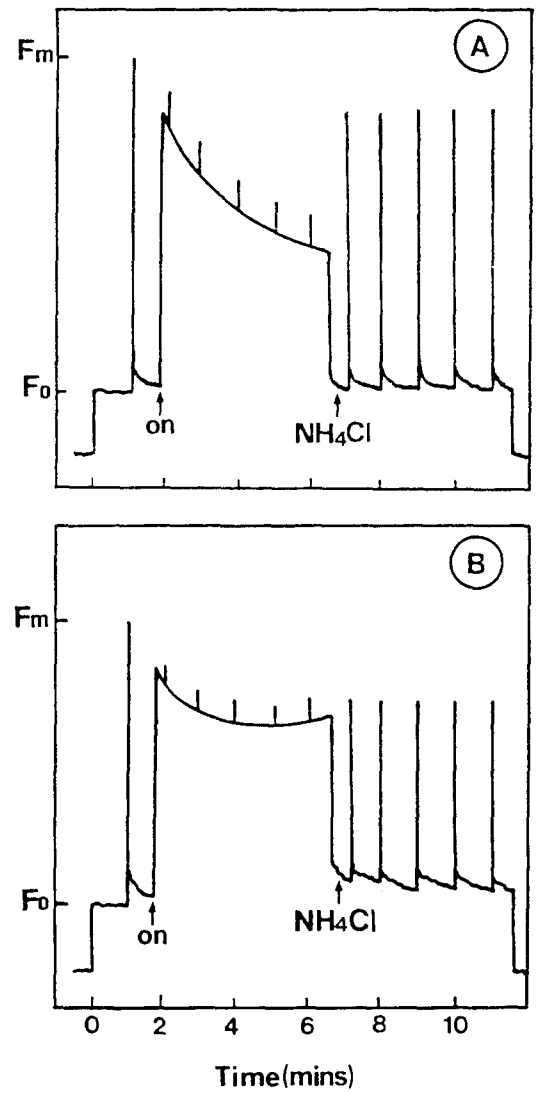

Figure 4. The relaxation of fluorescence quenching by the addition of an uncoupler, NH_4Cl, in the absence or in the presence of $HgCl_2$. Control (A); 50 μM of $HgCl_2$-treated chloroplasts (B). Other conditions are the same as in Fig. 2.

Murata and Sugahara.[19] While linearity between qE and ΔpH has been demonstrated,[10] the level of chlorophyll fluorescence quenching relative to ΔpH was diminished under certain circumstances. Addition of NH_4Cl after illumination showed rapid relaxation of fluorescence quenching (Fig. 4A). It has been reported previously that the mercury inhibition measured by the quenching of fluorescence was predominantly linked to PS II photochemistry and not related to the light-induced proton gradient causing the "*energy quenching*".[20] Nevertheless, addition of $HgCl_2$ did not cause further relaxation of fluorescence quenching (Fig. 4B), suggesting $HgCl_2$ inhibited qE by affecting the transthylakoidal ΔpH.

Antimycin A is a potent inhibitor of qE formation without affecting the transthylakoid ΔpH in spinach chloroplasts[21] and has effect on qE relaxation in *Dunaliella*.[12] Figure 5 shows the effect of antimycin A on qE relaxation in the presence or absence of $HgCl_2$. Addition of antimycin A prior to the actinic light illumination greatly reduced chlorophyll fluorescence quenching in comparison with Figure 3, without affecting qQ (Fig. 5A). In contrast, it was not affected by the addition of antimycin A when $HgCl_2$ was present (Fig. 5B). As shown in Fig. 5C and 5D, some relaxation of chlorophyll fluorescence quenching was observed if antimycin A added 2 minutes after illumination. On the other hand qE,

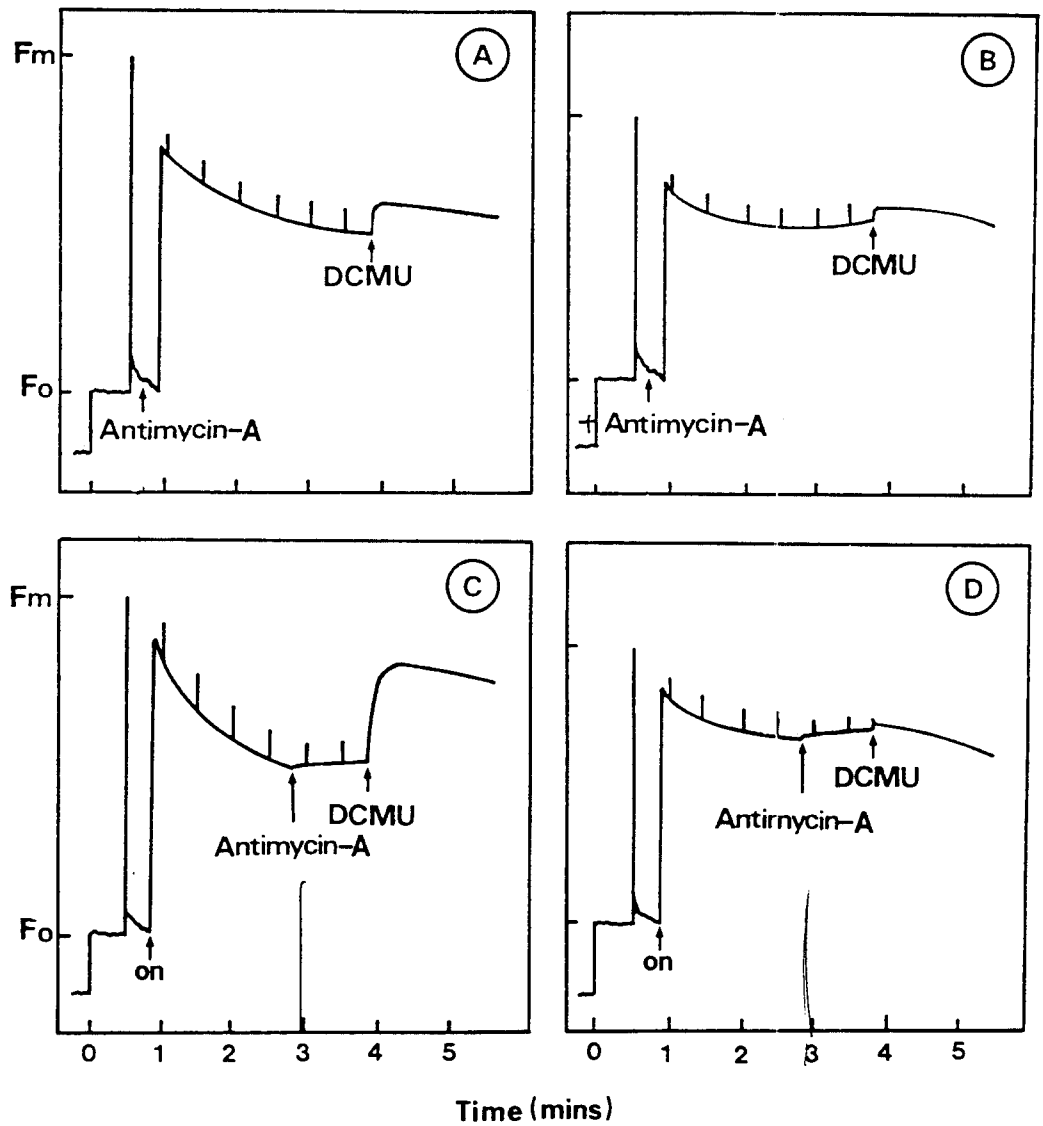

Figure 5. The relaxation of fluorescence quenching by the addition of antimycin A in the absence or in the presence of $HgCl_2$. Chloroplasts with antimycin A added prior to illumination (A); 50 μM of $HgCl_2$ treated chloroplasts with antimycin A added prior to illumination (B); Chloroplasts with antimycin A added after 2 min illumination (C); 50 μM of $HgCl_2$ treated chloroplasts with antimycin A addedd after 2 min illumination (D). Other conditions are the same as in Fig. 2.

which is already formed was not reversed (Fig. 5C). In the presence of $HgCl_2$, the relaxation of its quenching was completly inhibited (Fig. 5D).

These results indicate that $HgCl_2$ inhibits qE formation by the transthylakoid ΔpH. It is also possible that $HgCl_2$ may inhibit de-epoxidation of violaxanthin to zeaxanthin completly. The qE formation is dependent on the conversion of violaxanthin to zeaxanthin under light.[23,24] Demmig-Adams *et al.*[24] recently showed that an inhibitor of violaxanthin de-epoxidase, dithiothreitol (DTT), increased (Fv)t due to the relaxation of a portion of qE. At the present time, we do not know the effect of $HgCl_2$ on zeaxanthin level in the thylakoids and experiments to examine the level of zeaxanthin and violaxanthin in the thylakoids when $HgCl_2$ are treated are presently being performed.

In summary, our results showed that $HgCl_2$ inhibited the oxygen-evolving activity, especially PS II rather than PS I and consequently a simultanous loss of variable fluorescence which is restored by addition of hydroxylamine. It seems that the inhibitory effect of $HgCl_2$ is mainly associated with oxygenevolving complex in PS II. The inhibitory effect of $HgCl_2$ on qE formation is due to the inhibition of transthylkoid ΔpH formation. It is possibly that the inhibition of de-epoxidation of violaxanthin to zeaxanthin may be involved as well.

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