Effects of Lead Ions on Photosystem I in Isolated Chloroplasts: Studies on the Reaction Center $P700$*

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Abstract

Direct inhibition of the reaction center $P700$ of photosystem (PS) I is observed in isolated maize chloroplasts when lead salts are added. The latter diminish the steady-state absorbance change at 703 nm, as well as the electron spin resonance (ESR) signal I associated with $P700$. Lead ions change the kinetics of the dark re-reduction of photooxidized $P700$ ($P700^+$) from first to second order in normal and 3-(3,4 dichlorophenyl) 1,1-dimethylurea (DCMU) treated samples. Samples using artificial electron donors, *e. g.*, reduced phenazine methosulfate ($PMSH_2$) or reduced 2,6-dichlorophenol indophenol ($DCPIPH_2$), both of which have rate constants for re-reduction reaction an order of magnitude greater than in the normal chloroplast samples, show only altered first-order rate constants. These changes in the dark decay rates of $P700^+$ are explained on the basis of a lead-altered rate of electron transfer to $P700^+$. A kinetic analysis of the $P700$ absorbance change suggests that a diminished steady-state oxidation of $P700$ in light is not a consequence of the increased rate of electron donation to $P700^+$. Hence the presence of lead ions in chloroplast samples causes two simultaneous effects: (*1*) an inhibition of $P700$ photooxidation, and (*2*) an alteration of the kinetics of re-reduction of $P700^+$.

The influence of various cations on the primary processes of photosynthesis is of current interest. Most of these studies have been on the physiological cations, *e.g.*, Na^+, Mg^{++}, and Ca^{++} (Murata 1971a, b; Mohanty *et al.* 1972; Briantais *et al.* 1973; Gross and Hess 1973; Vander Meulen and Govindjee 1974; Wydrzynski *et al.* 1975), although some heavy metals, *e.g.*, Pb^{++}, Hg^{++}, and Cd^{++} have also been used in photosynthesis research (Miles *et al.* 1972, 1973; Bazzaz and Govindjee 1974a, b; Bradeen and Winget 1974; Hampp and Lendzian 1974). The increasing presence of lead in the environment is of concern to the pollution problem. Additionally, Pb^{++} ions are of interest for comparative investigations with other cations. Lead salts have been shown to definitely inhibit a site on the water oxidizing side of PS II (Miles et al. 1972; Bazzaz and Govindjee 1974a), but evidence supporting its influence on PS I has not been reported. An indirect approach [reduction of methyl viologen (MV) by $DCPIPH_2$] has led to the conclusion that Pb^{++} has no effect on PS I (Miles *et al.* 1972). We have examined this problem by looking directly at the oxidation and reduction changes of the reaction center of PS I by two methods: The absorbance change at 703 nm, first discovered by Kok (1956, 1957), and the change in the magnitude of ESR signal I (Kohl 1972; Warden and Bolton 1974). In ESR spectroscopy on photosynthetic materials, two signals labelled as signal I (associated with $P700^+$ of

242 photosystem I) and signal II (ascribed to PS II — Kohl *et al.* 1969; Babcock and Sauer 1973) have been extensively investigated. Signal I is observed only in light, has a "*g*" value of 2.002 5 $\pm$ 0.001 (Kohl 1972; Warden and Bolton 1974) and a half-bandwidth of 7.5 to 10.0 gauss (becoming broader with "contamination" with signal II) and is associated with photoreaction I.

In this paper we present data on the effects of lead ions on the steady-state absorbance change at 703 nm, the kinetics of the dark re-reduction of $P700^+$, and ESR signal I. Our results show that the effects of lead ions on PS I are twofold: (*1*) inhibition of the photooxidation of $P700$, and (*2*) alteration of the rate of electron transfer to $P700^+$ or its natural electron donor.

MATERIALS AND METHODS

Bundle sheath and mesophyll chloroplasts from maize (*Zea mays* L.) were prepared by a method modified after Woo *et al.* (1970) as described by Bakri (1972) and Bazzaz and Govindjee (1973, 1974a). It was found that the effects of lead on the optical $P700$ changes in mesophyll chloroplasts were unaltered by the presence or absence of 10 mM NaCl and 1 mM $MgCl_2$ both in the homogenizing and the suspension media. Consequently, both salts were excluded in most experiments. Chlorophyll concentrations were determined according to Arnon (1949).

Light-induced absorbance decrease of $P700$ was measured with the split-beam difference spectrophotometer of Sybesma and Fowler (1968), with the measuring monochromator set at 703 nm (half bandwidth, 9.9 nm) and a 703 nm interference filter (half- bandwidth, 12.5 nm) placed before the photomultiplier. Interference filters were used to provide the blue (481 nm; half-bandwidth, 8 nm; incident radiant flux density, 5.4 mW cm^{-2}) or the far red (729 nm; half-bandwidth, 11 nm; 2.0 mW cm^{-2}) actinic beam. Radiant flux densities were measured with a *Yellow Springs* radiometer (Model *No. 63*).

ESR signals were measured as described by Beinfeld (1973) by a *Varian ESR* Spectrometer, model *E-3*. For most measurements, the field was set at 3 360 gauss, the scan range $\pm$ 25 gauss, the scan time 4 min, and the time constant 1.0 s. The modulation amplitude was 4—6.3 $\times$ gauss, the microwave frequency 9.55 GHz, and the microwave power 16.0 mW.

The reaction mixtures were prepared by dilution of the chloroplast suspensions with 50 mM Tricine-NaOH buffer (pH 6.5 or 7.8) with or without various concentrations of $PbCl_2$ or $Pb(NO_3)_2$. Final chlorophyll concentrations of 1—1.5 mg ml^{-1} were used for ESR studies, and 100—200 µg ml^{-1} for optical work. 20—30 min of dark incubation with or without lead ions were allowed prior to making measurements.

All measurements reported were made at room temperature.

RESULTS AND DISCUSSION

Steady-State Photooxidation and Kinetics of Subsequent Dark Recovery of $P700$ as Detected by the Absorbance Change at 703 nm

Effect of lead salts on normal and DCMU treated chloroplasts

Isolated mesophyll chloroplasts treated with either $PbCl_2$ or $Pb(NO_3)_2$ showed a reduced light-minus-dark $P700$ signal, that is, a smaller absolute value for the absorbance change at 703 nm

Table 1

Relative steady-state absorbance decrease (ΔA) at 703 nm in normal isolated mesophyll chloroplasts upon treatment with $PbCl_2$. Chloroplast suspensions were prepared in white room light to final chlorophyll concentrations of 100—160 µg ml^{-1} in 50 mM Tricine — NaOH buffer ($\pm$ lead salt). The final lead ion concentrations were 7.2—8.6 mM. Samples were taken in a 0.5 ml cuvette of 1 mm path length, and given 15 min dark incubation prior to all experiments. Results reported were the average values of 3—11 repetitions each for pH 6.5 and 7.8 from 4 and 2 different chloroplast preparations, respectively.

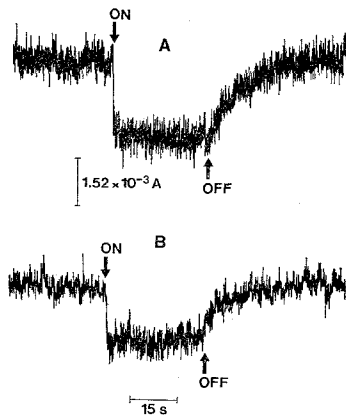| Concentration ratio lead/chlorophyll	pH	Excitation wavelength [nm]	$\dfrac{\Delta A \text{ (lead)}}{\Delta A \text{ (control)}}$
62 $\pm$ 13	6.5	481	0.66 $\pm$ 0.12
42 $\pm$ 11	6.5	729	0.75 $\pm$ 0.03
46	7.8	481	0.72 $\pm$ 0.08
46	7.8	729	0.72 $\pm$ 0.07

Fig. 1. Absorbance changes at 703 nm obtained from the optical $P700$ experiments for a normal untreated sample (A), and a lead-treated sample (B). The excitation wavelength was 481 nm, and the final chlorophyll concentration was 117 µg ml^{-1}. The total volume for each sample was 0.5 ml, and the lead concentration was 7.2 mM.

(Table 1; Fig. 1). This diminished negative absorbance change reflects the lowered steady-state equilibrium concentration of oxidized $P700$. The important features in the table and their implications are briefly discussed below.

(*1*) With reference to the widely accepted Z-scheme of photosynthesis (HILL and BENDALL 1960) the decreased steady-state $P700^+$ signal, in the presence of lead ions, may be a consequence of one or a combination of the following possible effects: (*a*) an accelerated re-reduction of $P700^+$ by natural or lead-induced cyclic or non-cyclic electron flow, and (*b*) an inhibition of the light-induced oxidation of $P700$. The slow re-reduction rate of $P700^+$ in the dark (as evidenced by the trace in Fig. 1 and the results plotted in Fig. 2A) suggests that the alternatives in (*a*) could not cause any significant change in the steady-state level of $P700^+$. Further support for this argument comes from the observation that a 40 % reduction of the irradiance, using a neutral density filter, only affected the onset kinetics of $P700$ photooxidation, so that it took a longer time for the system to reach steady-state. However, the final steady-state signal was the same as that when 100 % irradiance was used. We therefore consider that (*b*) is the predominant mechanism for the lowered steady-state $P700^+$ signal.

244 (2) The inhibition of the $P700^+$ signal in normal chloroplast systems was independent of pH at the two pH's (6.5 and 7.8) used. RUMBERG (1964) has shown that the absorbance change at 704 nm is relatively independent of pH from pH = 3 to 11, and our results imply that the action of lead on PS I, by whatever mechanism, is independent of pH in the range used here. This finding enables us to report results at one pH.

(3) The inhibition of the 703 nm absorbance decrease in chloroplasts was also independent of the wavelength (729 nm absorbed mainly by PS I, or 481 nm absorbed by PS I and PS II) of the exciting light at saturating radiant flux densities. The rapid establishment of steady-state upon illumination (Fig. 1) suggests that in saturating radiant flux densities, the rate of $P700$ oxidation far exceeds the rate of re-reduction of $P700^+$ from any natural electron donors. This results in an observed photo-induced steady-state $P700^+$ signal which is independent of the rates of the re-reduction reactions (argument against (1a) above). If the PS II site is the only site of action of lead, one would not expect any difference in the steady-state $P700^+$ signal between normal and lead-treated chloroplast samples. Thus, the observed inhibition of the $P700^+$ signal is taken to imply the existence of at least one other effect of lead ions different from the inhibition of electron flow on the oxidizing side of PS II reported by MILES et al. (1972) and BAZZAZ and GOVINDJEE (1974a).

(4) Comparison of the results in the first row with any of the other rows in Table 1 suggests that the absorbance change at 703 nm was dependent on the relative concentrations of lead ions to chlorophyll. Further investigations yield results confirming this point (Table 2).

Table 2

Relation of relative lead:chlorophyll concentration to the inhibition of the $P700$ signal in isolated mesophyll chloroplasts. Dilutions of stock suspensions of chloroplasts with 50 mM Tricine-NaOH buffer ($\pm$ lead salt) at pH 6.5 were carried out in white room light; each sample was further exposed to light for 3 more min before a 20 min dark incubation. All other details were as in Table 1. The results were the average of five repetitions each. Far-red light for excitation of th samples was produced by passing white light through a *Baird Atomic* interference filter with a peak at 729 nm and a half-band width of 11 nm. The incident radiant flux density was 2.0 mW cm^{-2}.

Lead [mM]	Chlorophyll [μg ml^{-1}]	[Lead]/[chlorophyll]	Inhibition by lead salt [%]
4	128	28	28 ± 9
24	128	167	52 ± 5
36	188	171	48 ± 4

(5) The direct effect of lead ions on $P700$ is further confirmed by the DCMU-insensitivity of the lead-induced inhibition of the extent of the steady-state 703 nm absorbance change. Similar results are again seen for both 481 nm and 729 nm actinic illumination (Table 3).

Decay kinetics of $P700^+$

It should be mentioned that while the extent of the steady-state absorbance change at 703 nm can readily monitor the amount of active $P700$, it provides very little information regarding the rate of electron flow from donors to $P700^+$. In our attempt to learn something of these latter processes, we have analyzed the kinetics of $P700^+$ re-reduction in the dark. We have obtained an estimate of the steady-state rate constant for electron flow between $P700^+$ and its physiological electron do-

Table 3

Ratios of absorbance change at 703 nm in the presence and in the absence of lead ions in normal and DCMU treated chloroplasts of maize. Samples were suspended in 50 mM Tricine-NaOH buffer ($\pm$ lead salt), pH 6.5, at the chlorophyll concentration of 117 μg ml^{-1}. A dark incubation of 20 min was given prior to all measurements. DCMU, where used, was 50 μM (final concentration). The reported errors reflect the precision of the average of three measurements.

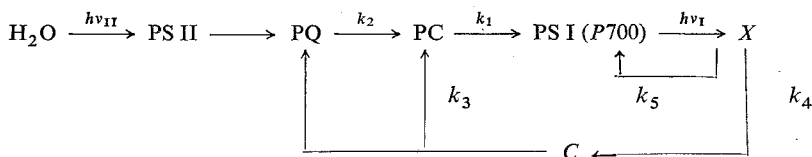| Sample [Lead]/[chlorophyll] = 55	Excitation wavelength [nm]	$\dfrac{\Delta A\,(\pm\,\text{DCMU}\,+\,\text{lead})}{\Delta A\,(\pm\,\text{DCMU}\,-\,\text{lead})}$
Normal	481	0.79 ± 0.11
	729	0.77 ± 0.07
DCMU- treated	481	0.77 ± 0.08
	729	0.75 ± 0.07

nor. The kinetics of the dark decay of the $P700^+$ signal, unlike its steady-state value in light, is an indicator of the total rate of electron transfer to the oxidized reaction center chlorophyll of PS I. Fig. 2 shows that (1) the dark decay of $P700^+$ to $P700$ in the seconds time region obeys first-order kinetics, and (2) in the presence of lead ions, this dark decay to $P700$ follows second-order kinetics.

On the basis of the reaction order obtained from kinetics measurements, one may think of $P700$ and its primary electron acceptor, X, as a single complex, $P700 \cdot X$, so that light absorpon and charge separation would result in the entity $P700^+ \cdot X^-$, its decay back to $P700 \cdot X$ would show first-order kinetics. Addition of lead ions may induce a physical separation of $P700$ from X, causing them to act as separate entities. Such a mechanism would explain (a) the lowered $P700^+$ signal, and (b) the change from first- to second-order decay kinetics of the signal, in samples in which lead ions are present.

The major problem with this explanation is that it predicts first-order kinetics for the dark re-reduction of $P700^+$, whereas HIYAMA and KE (1971), using flash illumination, reported a second-order dependence with the rate constant 3×10^9 M^{-1} s^{-1}, in normal chloroplasts.

The slow re-reduction of $P700^+$ following continuous excitation by far-red light has also been reported by other workers (MALKIN 1969; MARSHO and KOK 1970). MARSHO and KOK (1970) suggested that this DCMU-insensitive slow re-reduction may be *via* plastocyanin, since their observation precludes the involvement of cytochrome *f*. This suggestion also supports the view that plastocyanin is the electron donor to $P700^+$ (see TREBST 1974) as used in Eq. (1).

A simple kinetic model, based on the following scheme for electron flow in the two photosystems, explains our results:

$$\text{H}_2\text{O} \xrightarrow{h\nu_{II}} \text{PS II} \longrightarrow \text{PQ} \xrightarrow{k_2} \text{PC} \xrightarrow{k_1} \text{PS I }(P700) \xrightarrow{h\nu_I} X$$

with k_3, k_5, k_4, and C

where PS II = photosystem II, PQ = plastoquinone, PC = plastocyanin, $X =$ primary electron acceptor, and $C =$ an electron carrier in the cyclic electron pathway around photosystem I (PS I), and the symbols k are the rate constants for various reactions:
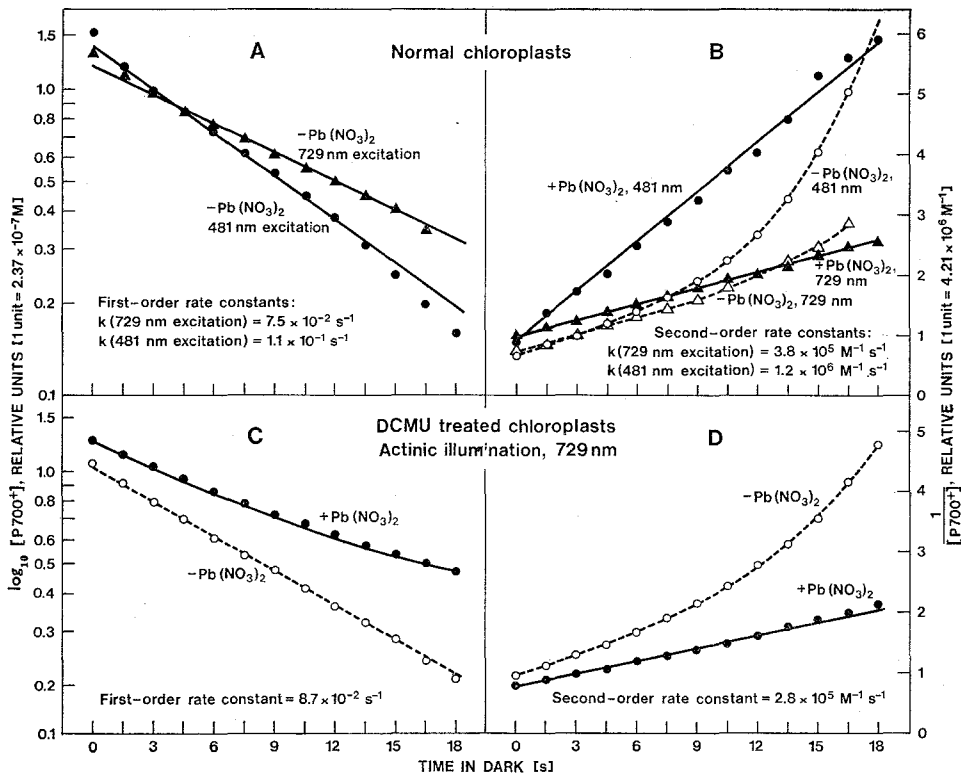
Fig. 2. (A) Plots of $\log_{10} [P700^+]$ *vs.* time for normal (A) or DCMU treated (C) chloroplast samples, $\pm$ Pb(NO$_3$)$_2$. (B) Plots of $[P700^+]^{-1}$ *vs.* time for the dark re-reduction of $P700^+$ for lead-treated chloroplasts (solid lines). The data from (A) were plotted for comparison (dashed lines). (D) Plots of $[P700^+]^{-1}$ *vs.* time for the results in (C). The samples of (C) and (D) contained: 117 μg ml^{-1} chlorophyll, 30 μM DCMU, 0.05 M Tricine-NaOH at pH 6.5, and 7.2 MB Pb(NO$_3$)$_2$, if present; the samples (A) and (B) contained the same except that DCMU was absent.

$$P700 + X \xrightarrow{h\nu_I} P700^+ + X^- \tag{1a}$$

(primary photochemical reaction of PS I)

$$PC + P700^+ \xrightarrow{k_1} PC^+ + P700 \tag{1b}$$
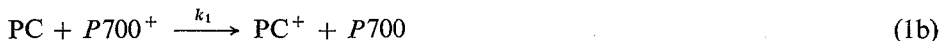
(oxidation of plastocyanin and reduction of $P700^+$)

$$PQ^{2-} + 2PC^+ \xrightarrow{k_2} PQ + 2PC \tag{1c}$$

(reduction of plastocyanin and oxidation of plastoquinone — reduced by PS II)

$$PC^+ + C \xrightarrow{k_3} PC + C^+ \tag{1d}$$

(reduction of plastocyanin by the electron carrier C)

$$X^- + C^+ \xrightarrow{\ k_4\ } X + C \tag{1e}$$

(oxidation of reduced primary electron acceptor by oxidized C)

$$P700^+ + X^- \xrightarrow{\ k_5\ } P700 + X \tag{1f}$$

(primary back reaction of PS I)

We note that the cyclic pathway (1e) plays an important part here because we do not have an external electron acceptor to accept electrons from X^-. From reactions (1b) and (1f), the rate of change of $[P700^+]$ is given by:

$$\frac{d[P700^+]}{dt} = -(k_1[PC][P700^+] + k_5[X^-][P700^+]) \tag{2}$$

An order of magnitude comparison of published results reveals that the half-life time for $P700^+$ reduction by reaction (1b) is approximately 10 μs (HAEHNEL *et al.* 1971; HAEHNEL and WITT 1972), while the half-life time for reaction (1f) is approximately 40 ms (HIYAMA and KE 1971). Thus, $k_5 \ll k_1$, and the second term on the right-hand side of Eq. (2) can be neglected to give:

$$\frac{d[P700^+]}{dt} = -k_1[PC][P700^+] \tag{3}$$

Our experimental results in lead-free samples in Fig. 2A and 2C require an exponential-type decay of $P700^+$ in the dark. This can be satisfied by a *pseudo*-first-order reaction in which [PC] in Eq. (2) is assumed to be constant, that is, $d[PC]/dt = 0$.

From reactions (1b) to (1d), the rate of change of [PC] is given by:

$$\frac{d[PC]}{dt} = -k_1[PC][P700^+] + k_2[PQ^{2-}][PC^+]^2 + k_3[C][PC^+] \tag{4}$$

For $d[PC]/dt$ to be zero,

$$-k_1[PC][P700^+] + k_2[PQ^{2-}][PC^+]^2 + k_3[C][PC^+] = 0 \tag{5}$$

This is the necessary condition for Eq. (3) to describe a *pseudo*-first-order reaction for the dark reduction of $P700^+$ in lead-free samples. However, with the addition of lead ions, the kinetics of the dark reduction of $P700^+$ is observed to have a second-order dependence on $[P700^+]$. (This is demonstrated by the occurrence of a linear relationship between $[P700^+]^{-1}$ and t in Fig. 2B and 2D.) This finding requires that [PC] in Eq. (3) be time-dependent, so that $d[PC]/dt \neq 0$ and Eq. (5) is no longer valid. This last condition could result from an unbalanced lead-induced increase in the first term and/or decrease in the third term in Eq. (5). (As reaction (1c) is known to be the rate limiting step in electron transport, the second term in Eq. (5) is assumed to be insignificant and is ignored.) Also, it is necessary to assume that, in the presence of lead ions, a stoichiometry exists between [PC] and $[P700^+]$, that is,

$$[PC] = n \cdot [P700^+], \tag{6}$$

where n is a constant, and Eq. (3) becomes

$$\frac{d[P700^+]_{+Pb}}{dt} = k_1 \cdot n \cdot [P700^+]^2_{+Pb} \tag{7}$$

This is the equation that gives the linear relationship between $[P700^+]^{-1}$ and t.

We determined the value of n in Eq. (6) as follows. Consider the instant when the illumination was discontinued ($t' = 0$). We know the value of $[P700^+]$ from direct measurements of the steady-state absorbance change at 703 nm; the second-order rate constant (k_1) is obtained from the $[P700^+]$ vs. time plot of the type in Fig. 2D. Finally, we estimate the initial rate of decay of $P700^+$ by estimating the initial slope of the $\log_{10}[P700^+]$ vs. time plot and multiplying it by 2.3 . $\cdot \log_{10}[P700^+]_{t'=0}$. Substituting the values of $d[P700^+]/_{dt}$, k_1, and $[P700^+]_{t'=0}$ in Eq. (3), the initial concentration of [PC] is obtained. The ratio of [PC] to $[P700^+]$, both taken at $t' = 0$, gave the value of n, the stoichiometric ratio between [PC] and $[P700^+]$. From Fig. 2C and 2D, $d[P700^+]/_{dt'}$ $= 2.6 \times 10^{-8}$ M s^{-1}, $[P700^+]_{t'=0} = 3 \times 10^{-7}$ M, and $k_1 = 2.8 \times 10^5$ M^{-1} s^{-1}. Using the above values in Eq. (3), [PC] is found to be 3.1×10^{-7} M $\simeq [P700^+]_{t'=0}$; that is, $n \simeq 1.0$. (The average value of n from the data for the three cases shown in Fig. 2 is 1.0 ± 0.1.) This agrees with the results of MARSHO and KOK (1970) who estimated relative pool sizes of 1 : 1 : 1 electron equivalents for cytochrome f : PC : $P700$.

Effects of lead on artificially provided pathways of electron flow

Phenazine methosulfate (PMS) induced cyclic flow. We studied the influence of lead ions on the steady-state photooxidation level and the dark re-reduction kinetics of $P700^+$ in the presence of PMS which induces an accelerated cyclic electron flow around PS I.

Table 4 confirms the earlier findings in this paper of the effect of lead on the steady-state $P700^+$ signal. The kinetics of the dark re-reduction of $P700^+$, however, were first-order irrespective of the presence or absence of lead. These results are not in conflict with the kinetics of re-reduction of $P700^+$ for chloroplast samples in the absence of PMS, which show second-order kinetics on the addition of lead salts. This is because the rate of re-reduction of $P700^+$ in the presence of 30 μM PMS exceeds an order of magnitude greater than in normal chloroplasts ($t_{1/2} = 450$ ms vs. 10 s), so that an effect on any slower reaction will not be apparent. Thus, in this system, an enhanced rate of decay of the $P700^+$ signal in the dark has to be attributed to an increase in the rate of flow of electrons; lead ions must further enhance by about 22 % the rate of electron flow around the PMS induced cyclic pathway ($t_{1/2} = 350$ ms vs. 450 ms).

Table 4

Effect of lead salts on the $P700$ signal in the presence of PMS (cyclic electron flow). Final concentrations in the 0.5 ml samples were 112—138 μg (chlorophyll) ml^{-1} suspension, 30—50 μM PMS, and 50 mM Tricine-NaOH buffer ($\pm$ lead salts) at pH 6.5. DCMU, where used, was present at the concentration of 30 μM. Preparation of samples was done in white room light. All measurements represent the average of 4—11 repetitions.

Sample	$\dfrac{[\text{Lead}]}{[\text{Chlorophyll}]}$	Excitation wavelength [nm]	$\dfrac{\Delta A \text{ (PMS} \pm \text{DCMU+lead)}}{\Delta A \text{ (PMS} \pm \text{DCMU}-\text{lead)}}$	Half-time, $t_{1/2}$ [ms]	
				$-$ lead	$+$ lead
−DCMU } +PMS	62	481 729	0.68 ± 0.07 0.65 ± 0.09	— —	— —
+DCMU } +PMS	46 ± 1	481 729	0.78 ± 0.04 0.76 ± 0.05	— 456 ± 15	— 353 ± 51

It may seem that the 20 fold increase in the rate of $P700^+$ re-reduction may significantly inhibit the steady-state absorbance decrease at 703 nm, and invalidate the above deductions. We are assured that this is not the case from the following observations: (*1*) The rapid photooxidation

of $P700$ to $P700^+$ (the rapid attainment of steady-state conditions) in the PMS system when light is turned on (see Fig. 3, *left*) shows that the rate of photooxidation of $P700$ greatly exceeds the re-reduction of $P700^+$ by reduced PMS. (2) Samples of chloroplasts from the same stock, and with identical chlorophyll content and pH, provide two additional pieces of evidence: (a) The presence of 30 µM PMS, instead of lowering the steady-state $P700^+$ signal, as should be the case if the higher rate of $P700^+$ re-reduction becomes comparable to the photooxidation rate of $P700$ at the existing radiant flux densities, gave a two-fold increase in its magnitude when compared to normal chloroplast samples. (This confirms very similar results reported by BORISOV and
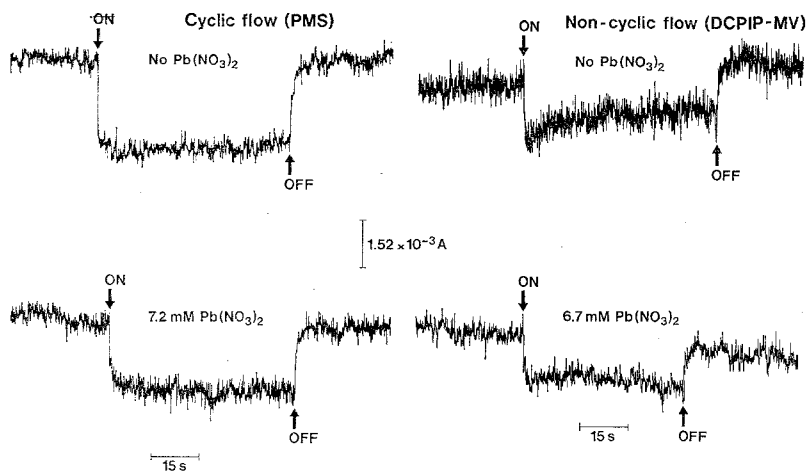

ig. 3. Recorder tracings of absorbance changes at 703 nm when actinic light is turned ON anp OFF. PMS-mediated cyclic flow: 140 µg (chlorophyll) ml^{-1}, 30 µM DCMU, 30 µM PMS, with or without Pb(NO$_3$)$_2$, pH 6.5. The DCPIPH$_2$ to MV non-cyclic electron transport system: 133 µg (chlorophyll) ml^{-1}, 30 µM DCMU, 23 µM DCPIP, 1 mM ascorbate, 100 µM methyl viologen, with or without Pb(NO$_3$)$_2$, pH 6.5.

Table 5

Effect of lead salts on the oxidation and reduction of $P700$ in non-cyclic electron flow from reduced DCPIP to methyl viologen. Final concentrations in the samples were about 135 µg (chlorophyll) ml^{-1} suspension, 1 mM sodium ascorbate, 23 µM DCPIP, 100 µM methyl viologen, 30 µM DCMU, and 50 mM Tricine-NaOH ($\pm$ lead salt). Samples were prepared in white room light. All measurements represent the average of 8—10 measurements.

pH	$\dfrac{[\text{Lead}]}{[\text{Chlorophyll}]}$	Excitation wavelength [nm]	$\dfrac{\Delta A\ (+\ \text{lead})}{\Delta A\ (-\ \text{lead})}$	Half-time, $t_{1/2}$ [ms]	
				— lead	+ lead
		481	0.70 ± 0.09	—	—
6.5	45	729	0.72 ± 0.06	350 ± 40	255 ± 15
7.8	40	729	0.70 ± 0.07	212 ± 19	255 ± 26

IL'INA (1973) using ascorbate-reduced N, N, N', N'-tetramethyl-phenylenediamine as electron donor and methyl viologen as electron acceptor.) (b) In the presence of DCMU, chloroplast samples with electron flow from $DCPIPH_2$ to methyl viologen gave the same $P700^+$ signal as samples with PMS-mediated cyclic flow, although the half-times for re-reduction of $P700^+$ were 350 and 450 ms, respectively. But the $DCPIPH_2$/methyl viologen system without lead treatment has the same half-time (350 ms) for dark $P700^+$ reduction as the lead-treated PMS system (see Tables 4 and 5). This suggests that the lead-treated samples showing 22% enhancement of the rate of re-reduction of $P700^+$ by reduced PMS (change of $t_{1/2}$ from 450 ms to 350 ms) should have the same steady-state $P700^+$ signal as untreated samples. The last two rows in Table 4 show an average of 23% inhibition of the steady-state $P700^+$ signal by lead. We attribute these results to the occurrence of fewer active $P700$ centers in the lead-treated samples.

Non-cyclic electron flow from reduced DCPIP to methyl viologen. Fig. 3, *right*, and Table 5 show the effect of lead on $P700$ when the electron flow is non-cyclic — from ascorbate-reduced DCPIP to methyl viologen. The recovery kinetics (Fig. 4) were distinctly first-order both in the absence and presence of lead. This observation raises a question as to whether the enhanced rate of re-reduction of $P700^+$ would result in a diminished light-induced $P700^+$ signal (see above). We believe that similar arguments as those given for the PMS system above are valid, and that the diminished absorbance changes detected are the result of inactivation of active $P700$ centers. However, the sole inactivation of a fraction of the active $P700$ pool would explain a lowered $P700^+$ signal, but not an altered $P700^+$ decay half-time, since the half-time of a first-order reaction is independent of the initial concentration of the reacting species. Hence, we again favor the inference that the effect of lead on the artificially induced systems are two fold: (a) it decreases the total amount of active $P700$; and (b) it alters the rate of electron donation from ascorbate-reduced DCPIP to $P700^+$.
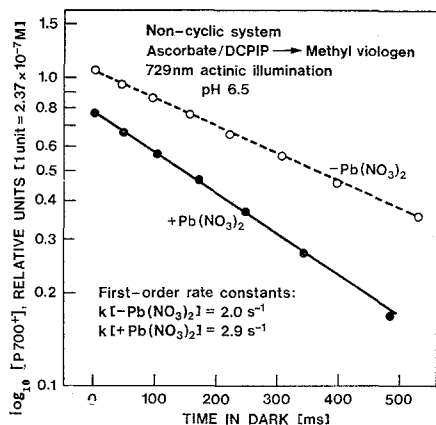

Fig. 4. $Log_{10}[P700^+]$ *vs.* time of the re-reduction of $P700^+$ in the system using electron flow from $DCPIPH_2$ to MV. Other details were as given for the same system in Fig. 3.

We now draw attention to two sets of results, reported by MILES *et al.* (1972), which we consider as providing further experimental support to our proposed mechanism of lead action. First, MILES *et al.* (1972) measured the rate of oxygen uptake resulting from the autooxidation of reduced methyl viologen, using ascorbate-reduced DCPIP as electron donor to PS I. At the highest lead ion concentration used ([lead]/[chlorophyll] ratio was 53), they observed a 10 % inhibition in lead treated (30-min) samples. The above-mentioned conditions are similar to those used in our experiments. Our proposal that lead ions inhibit the photooxidation of $P700$ directly implies a lowered concentration of X^-. Since the oxidation of X^- (or $P430^-$) by methyl viologen obeys *pseudo*-first-order kinetics (see HIYAMA and KE 1971), the presence of lead ions would cause a lower rate

of methyl viologen reduction. Secondly, MILES *et al.* (1972) also observed a lead-induced inhibition of PMS-mediated light-driven proton translocation. We suggest that this, again, is a direct consequence of lead ion inhibition of *P*700 photooxidation.

Electron Spin Resonance Signal I

As ESR signal I is another way to look at $P700^+$ (BEINERT *et al.* 1962; WARDEN and BOLTON 1972, 1973), we examined the effect of lead salts on its intensity in mesophyll and bundle sheath chloroplasts (Fig. 5; Table 6).
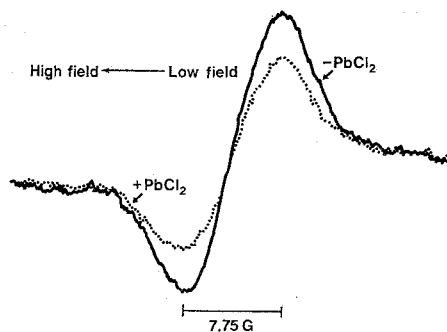

Fig. 5. Relative ESR signal I intensities from mesophyll chloroplast samples $\pm$ 6 mM $PbCl_2$. The medium contained 50 mM Tricine-NaOH buffer at pH 6.4, and the final chlorophyll concentration was 1.1 mg ml^{-1}. The flux density of exciting radiation was 5 W cm^{-2}.

Table 6

Electron spin resonance signal I intensities in mesophyll and bundle sheath chloroplasts of maize. Final chlorophyll concentrations in the samples were 1.1 mg ml^{-1} for mesophyll chloroplasts and 0.25 mg ml^{-1} for bundle sheath chloroplasts. All measurements were the average of three repetitions.

Conditions	Mesophyll chloroplasts		Bundle sheath chloroplasts	
	pH 6.4	pH 7.8	pH 6.4	pH 7.8
Control*	100 ± 6	100 ± 4	100 ± 5	100 ± 5
+ 6 mM lead**	80 ± 12	86 ± 6	86 ± 4	70 ± 3

* Signal I intensities in each column in this row have separately been normalized to 100.

** [Lead]/[chlorophyll] = 5 for mesophyll chloroplast samples and 21 for bundle sheath chloroplast samples. Radiant flux density for excitation was 5 W cm^{-2} as measured with a Model *65 Yellow Springs* Radiometer.

Signal I was 2—3 times larger in bundle sheath than in mesophyll chloroplasts at the same concentrations, in agreement with a greater amount of PS I in the former than in the latter (ANDERSON *et al.* 1971; MAYNE *et al.* 1971; BAZZAZ and GOVINDJEE 1973). The peak-to-peak width (ΔH) of this signal was 7.6—8 gauss. No significant difference in the amplitude of the signal II was observed in the two types of chloroplasts; its ΔH ranged from 26—28 gauss. The ratio of signal I/signal II was 3.7 and 11.5 in mesophyll and bundle sheath chloroplasts, respectively. Again, this confirms a substantial enrichment of signal I in bundle sheath chloroplasts.

Addition of $PbCl_2$ caused a decrease in the intensity of signal I, suggesting a lower concentration of $P700^+$ in the lead-treated samples. Similar results as those in Table 6 were observed at pH 7.8, when the ratios of lead to chlorophyll were raised to 11 in the case of mesophyll chloroplasts, and 17 in the case of bundle sheath chloroplasts. Since these experiments were done in continuous light, they are comparable with the ΔA changes in Tables 1 and 2. The argument that the absorbance change at 703 nm in steady-state represented most of the active $P700$ in the sample could also be applied here. Thus, the lower steady-state concentration of ESR signal I implies a lower concentration of total active $P700$.

CONCLUDING REMARKS

We have studied the steady-state absorbance change at 703 nm, using one minute illumination and the subsequent dark kinetics under a variety of conditions of electron flow. Our conclusions are based on the following assumptions: (*1*) the final steady-state absorbance change in saturating light represents most of the total concentration of active $P700$ in the sample; and (*2*) the dark kinetics, following illumination, represent the rate of electron donation to $P700^+$ by its natural electron donor (PC).

Addition of lead ions to chloroplast suspensions resulted in an apparent inactivation of $P700$. For example, in chloroplast samples where the lead to chlorophyll molar concentration ratios were approximately 45 : 1, a 28 % inhibition of the $P700^+$ signal was obtained. This result was found for normal, PMS-treated, and reduced DCPIP/methyl viologen treated chloroplasts (see Tables 1, 4 and 5 respectively).

Kinetic studies showed that introduction of lead ions into chloroplast samples altered the kinetics of reduction of $P700^+$. (*a*) In the case of normal and DCMU-treated chloroplasts, the reaction bringing about the reduction of $P700^+$ changed from first- to second-order (Fig. 2). This was simply explained as a higher rate of electron transfer from PC to $P700^+$ and/or a slower rate of electron transfer from a component C to PC^+ so that in the reaction: $\text{PC} + P700^+ \xrightarrow{k_1} PC^+ + P700$ the concentration of PC was time dependent and second-order kinetics was observed. (*b*) Systems ($PMSH_2$ or $DCPIPH_2$/MV) in which electron transfers from donor to $P700^+$ occur at rates an order of magnitude greater than those given in (*a*) show lead induced changes only in the rate constants (last two columns in Tables 4 and 5), the reactions remain first-order (Fig.4). In the $DCPIPH_2$ to methyl viologen system, the "lead effect" is an increased rate of electron donation from $DCPIPH_2$ to $P700^+$ at pH 6.5 (see Table 5).

The mechanism outlined above provides a unified explanation for the following observations: (*1*) the decrease in the amount of active $P700$ in the presence of lead ions, as detected by the steady-state absorbance change at 703 nm, and by ESR signal I; (*2*) the change from first- to second-order decay of $P700^+$ in normal and DCMU treated chloroplast samples upon the addition of lead ions; (*3*) the increased first-order rate constant of electron donation to $P700^+$ by $PMSH_2$ and $DCPIPH_2$ at pH 6.5; (*4*) the decreased rate of methyl viologen reduction in samples incubated with lead ions for 30 min (data of MILES *et al.* 1972); and (*5*) the lead inhibited PMS-mediated light-induced proton translocation reported by MILES *et al.* (1972).

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