Inhibition of Photosystem II in Isolated Chloroplasts by Lead[1]

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ABSTRACT

Inhibition of photosynthetic electron transport in isolated chloroplasts by lead salts has been demonstrated. Photosystem I activity, as measured by electron transfer from dichlorophenol indophenol to methylviologen, was not reduced by such treatment. However, photosystem II was inhibited by lead salts when electron flow was measured from water to methylviologen and Hill reaction or by chlorophyll fluorescence. Fluorescence induction curves indicated the primary site of inhibition was on the oxidizing side of photosystem II. That this site was between the primary electron donor of photosystem II and the site of water oxidation could be demonstrated by hydroxylamine restoration of normal fluorescence following lead inhibition.

A great deal of interest has been generated recently by the effect of lead on plants and animals (7). The large concentration of airborne lead accumulates in soils (9, 33). Lead pollutants are incorporated into animal tissues (4, 8) and have been shown to inhibit various enzyme systems (12, 29, 31, 34). Despite these effects on animal systems, little is known about the effect of lead on plant enzymes. We know that lead is taken up and transported in plants (3, 4, 24, 32, 33) and can decrease cell division at very low concentration (13, 14). Koeppe and Miller (23) examined the effect of lead salts on corn mitochondria and found an inhibition of electron transport, especially in the absence of phosphate.

Because of the increasing concentration of lead in our environment, the toxigenic effects of lead on animal enzyme systems, the increasing reliance we have upon photosynthesis to sustain our environment, and the similarity of the electron transport chain of mitochondria to that of photosynthetic electron transport, we feel a preliminary investigation of the effects of lead upon the light reactions of photosynthesis seems to be warranted.

MATERIALS AND METHODS

Plant Material. Fresh spinach (*Spinacia oleracea* L.) leaves were obtained from local markets. Young leaves were collected from tomato (*Lycopersicon esculentum* Mill., var. Rutgers) grown in a controlled environment of 16 hr light (28 C) and 8 hr dark (19 C).

Isolation of Chloroplasts. The procedure used was a modification of the technique of Jagendorf and Avron (19). Fresh leaves (20 g) were deveined and washed twice with distilled water. They were cut into small pieces and macerated in a cold blender (Eberbach No. 8475) operated at full speed for 10 sec with 60 ml of cold grinding medium which consisted of 0.8 M sucrose, 20 mM Tricine, and 10 mM NaCl adjusted to a final pH of 7.8. Defatted bovine serum albumin (50 mg) was included in the grinding medium during each extraction. The resulting homogenate was strained through Miracloth (Chicopee Mills, Inc., N.Y.) into chilled centrifuge tubes which were then placed into a refrigerated centrifuge and accelerated to 1,500g and immediately turned off. The supernatant obtained was centrifuged at 1,500g for 8 min to sediment chloroplasts which were resuspended in fresh grinding medium and diluted to a concentration of 1 mg of chlorophyll per ml as determined by the method of Arnon (2).

Hypotonic chloroplast fragments were prepared as described by Jagendorf and Uribe (22) by resuspension of chloroplasts in 25 ml of cold 10 mM NaCl for 20 min and collected by centrifugation at 10,000g for 10 min. The resulting swollen thylakoid fragments were suspended in cold 10 mM NaCl to a concentration of 1 mg chlorophyll per ml and stored until use.

Electron Transport. Electron transport was measured by a modified Mehler reaction (26) as described previously (33). In this assay, methylviologen accepts electrons from the reducing side of the photosystem I reaction and is itself reoxidized from water with an H_2O_2 intermediate. The progress of the reaction is followed by monitoring oxygen concentration in the reaction mixture. The reaction medium contained, in millimolar concentrations, Tricine, 50; NaCl, 30; $MgCl_2$, 2.5; NaN_3, 0.5; methylviologen, 0.1; and CCCP[3] to a final concentration of 1 μM. The pH was adjusted to 7.8. Chloroplasts were added to a concentration of 200 μg chlorophyll. The reaction was performed in a 5-ml chamber with a tight fitting stopper. Temperature of the reaction mixture was maintained at 24 C by circulation of water through jackets surrounding the chamber. Light was provided by a GE-DDB, 750-watt projection lamp focused through an 11-cm diameter planoconvex lens, 4 cm of water, a Corning 2408 red cut-off filter passing light at wavelengths longer than 610 nm, and the plastic window (3 mm) of the chamber. Light intensity was 8×10^5 ergs/cm^2·sec as measured with a Yellow Springs Kettering Radiometer, Model 65. The uptake of oxygen from the reaction mixture was measured with a Clark electrode (Yellow Springs 4044) polarized at -0.7 v. The signal from the electrode was amplified by a Keithley 414S picoammeter and recorded on a Mosley Autograph 680 strip chart recorder. Where indicated, 0.5 μmole of ascorbate-reduced DCIP was included in the reaction mixture.

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[3] Abbreviations: CCCP: carbonyl cyanide *m*-chlorophenylhydrazone; DCIP: 2,6-dichlorophenol indophenol; PMS: phenazine methosulfate; FMN: flavin mononucleotide.

Hill Reaction. Oxygen evolution was measured with the oxygen electrode apparatus described above. For Hill reaction, the mixture contained, in millimolar concentrations, Tricine, 50; NaCl, 30; $K_3(Fe)CN_6$, 1; and 200 μg chlorophyll of chloroplasts at a pH of 7.8.

Proton Transport. Light-induced pH rise was measured with a Thomas 4858-L15 combination pH electrode and an Instrumentation Laboratory 245 meter and recorded on a Houston Omnigraphic 2000 recorder. The 7.5-ml reaction mixture in a water jacket-cooled reaction vessel contained, in micromoles, NaCl, 70; PMS, 0.5; and 300 μg of chloroplasts. The initial pH of the reaction mixture was adjusted from 6.2 to 6.3. The vessel was illuminated with a ribbon filament DC microscope illuminator (GE-EDT) at a light intensity of 3×10^5 ergs/$cm^2 \cdot$ sec. The extent of the pH change was taken as the largest change observed in light which came 30 to 60 sec after initial illumination.

Fluorescence. Chloroplasts were suspended in a four-sided transparent cuvette 1×1 cm, and fluorescence was activated by a broad blue light of 350 to 550 nm with a peak intensity at 465 nm, generated by a blue Rohm and Haas acrylic filter. A light intensity of 1×10^5 ergs/$cm^2 \cdot$sec was supplied by a Bausch and Lomb, Nicholas microscope illuminator. Light emission from the chloroplasts was measured at a 90° angle with an EMI 9558B photomultiplier tube (S-20 cathode) which was blocked by a red cut-off filter, Corning 2030, transmitting only wavelengths above 640 nm. The anode current of the tube was amplified by a Keithley 414S picoammeter and recorded on a Houston Omnigraphic 2000 recorder. The reaction cuvette contained 80 mM sucrose, 2 mM Tricine, 1 mM NaCl, and 50 μg of chlorophyll at pH 7.6. Fluorescence was measured at 24 C. A fresh sample of dark-adapted chloroplasts was used for each reaction. Where indicated, 1 μM DCMU, 1 mM hydroxylamine, or 670 μM $MnCl_2$ were added.

Lead salts were ACS certified from Fisher Scientific, and

FIG. 2. Effect of lead on photosynthetic electron transport in the presence (upper curve) or absence of the electron donor, ascorbate-reduced DCIP. Spinach chloroplasts were preincubated for 10 min with lead, and the reaction was performed as indicated in "Materials and Methods."

DCMU and CCCP were the gift of P. G. Heytler, E. I. DuPont De Nemours and Co.

RESULTS

Electron Transport. Using a modified Mehler reaction (26), photosynthetic electron transport was measured in isolated spinach chloroplasts with saturating red light. Figure 1 shows the effect of two different lead chloride concentrations on CCCP uncoupled electron transport as a function of time. Extensive inhibition of electron transport was noted after a few minutes at the highest concentration of lead, 2.4 mM, if water served as the electron donor. When a lower concentration of lead was used (1.6 mM) the inhibition was less; if still lower concentrations were used (data not shown), inhibition did not occur until proportionally longer times. Little, if any, inhibition occurred when photosystem II was by-passed and ascorbate-reduced DCIP was added as the electron donor (upper curves, Fig. 1). The small amount of inhibition indicated in this case could not be correlated with lead concentration and was not significantly lower than a similar concentration of KCl.

The effects of various lead salt concentrations, all uniformly incubated with the chloroplast for 10 min at 0 C, is illustrated in Figure 2. These data show more dramatically the inhibition of photosynthetic electron transport by lead when water was the electron donor (lower curve) and the lack of lead inhibition when reduced DCIP acted as the electron donor (upper curve). Nearly identical results were obtained using the nitrate anion, $Pb(NO_3)_2$, to those shown in Figures 1 and 2. No inhibitory effect of KCl at similar concentration was noted; therefore, inhibition was limited to the Pb^{2+} ion.

Oxygen Evolution. Since the preceding experiments suggested a site for lead inhibition more closely associated with photosystem II than photosystem I, we examined the effects of lead salts on the Hill reaction, a photosystem II process. Chloroplasts were preincubated for 10 min with lead salts, but
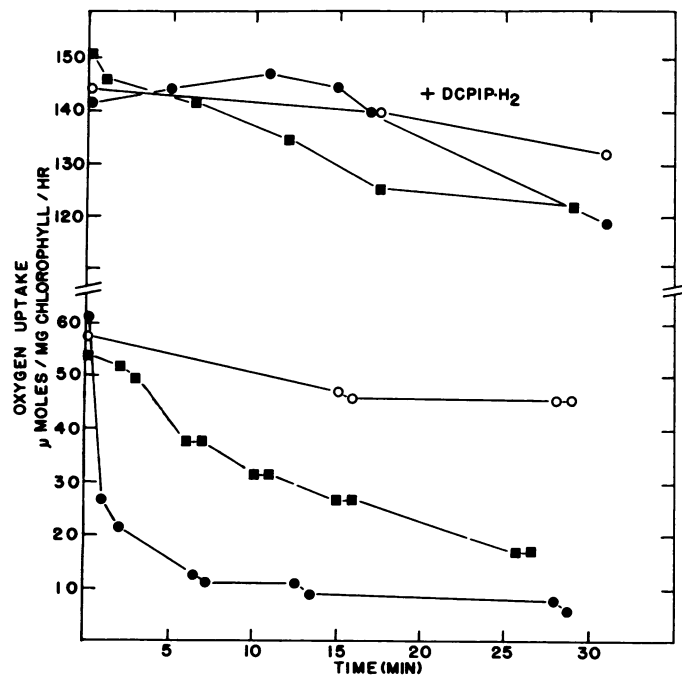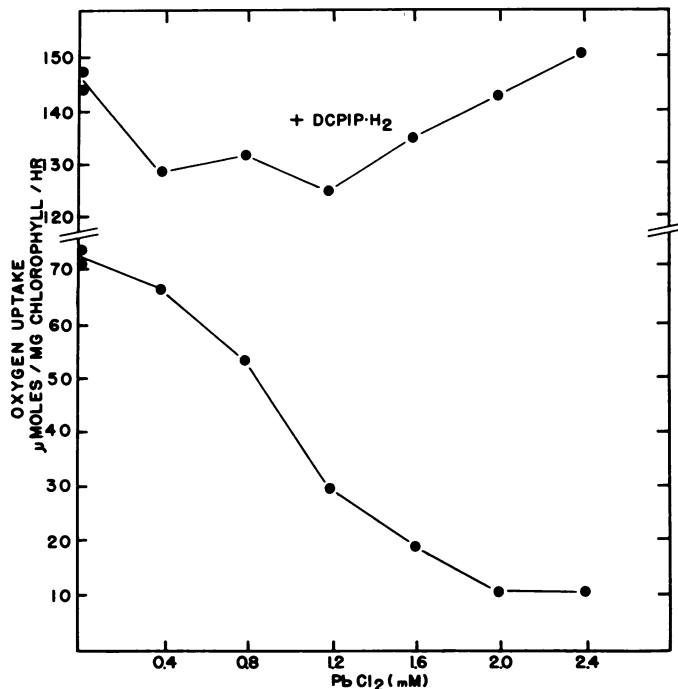

FIG. 1. Time course for lead inhibition of photosynthetic electron transport in spinach chloroplasts as measured by methylviologen reduction and reoxidation from water. Upper curves represent experiments with ascorbate reduced DCIP added. Symbols equal in mM; ●: $PbCl_2$, 2.4; ■: $PbCl_2$, 1.6; ○: KCl, 2.4. Reaction protocol as indicated in "Materials and Methods."
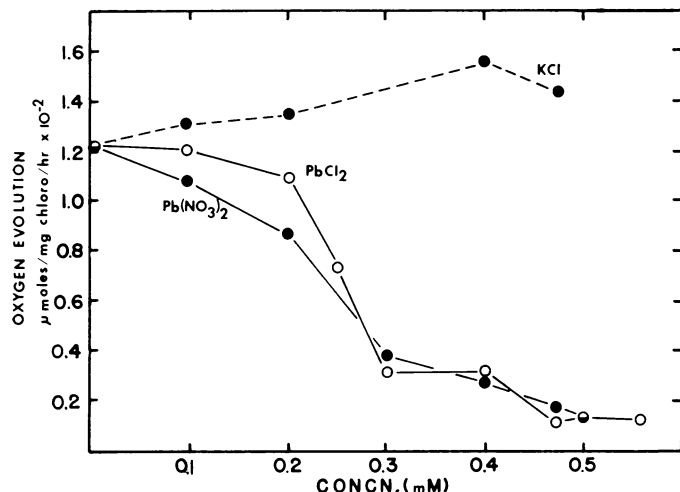
FIG. 3. Effects of lead salts on oxygen evolution of spinach chloroplasts with $K_3(Fe)CN_6$ as the electron acceptor. Preincubation was for 10 min in the absence of reaction mixture. Dashed line indicates the effect of similar concentrations of KCl.
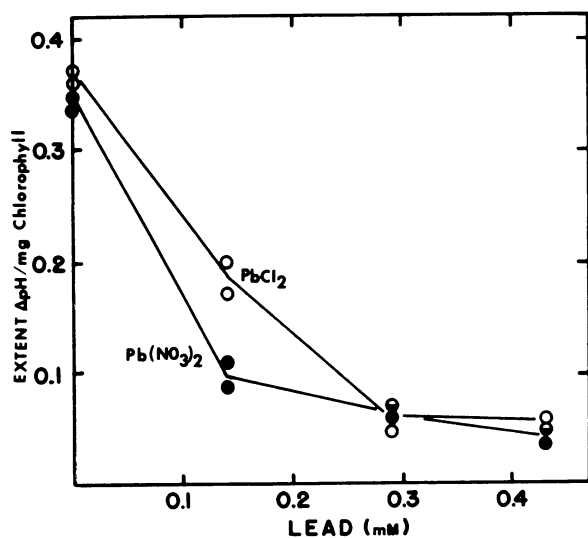

FIG. 4. Inhibition of proton transport measured as the extent of the light-induced pH gradient formed by spinach chloroplast fragments. Preincubation in lead was for 10 min without PMS. The extent was measured at its highest point during illumination. See "Materials and Methods" for complete procedure.

without ferricyanide, and oxygen evolution was measured polarographically in the presence of ferricyanide. The effective inhibitory concentration (Fig. 3) of lead was lower for the Hill reaction with a half-maximal concentration of about 0.25 mM as compared to about 1 mM for methylviologen reduction. Note (Fig. 3) that nitrate and chloride salts of lead again have nearly identical effects, and KCl has no inhibitory effect. Another electron accepter (DCIP) was used as a Hill acceptor with similar results.

Proton Transport. Light-induced proton transport was measured by the increase in pH of the chloroplast medium. It was of interest to measure the rise in pH as a measure of electron transport and also because of its relation to ATP synthesis (21, 28). The change in pH driven by cyclic electron flow in the presence of PMS is indicated in Figure 4. Lead salts decreased the extent of the pH gradient which was formed in light. Lead salts had little effect on the initial burst of proton translocation

(up to 0.04 ΔpH), but slowed the rate of translocation after this small burst (kinetic data not shown). In this case the concentration of lead for a half-maximal inhibition (about 0.1 mM) was only slightly lower than that for inhibition of the Hill reaction, but an order of magnitude lower than for methylviologen reduction.

Another characteristic of Pb^{2+} inhibition which is rather surprising is that the inhibition can be reversed if chloroplasts are sedimented from the lead containing medium, washed once by centrifugation at 1,500g for 7 min, and resuspended in fresh, lead-free medium. Chloroplasts which had been inhibited 70% of the control value by $PbCl_2$ could be restored to 30% of that value following such a wash procedure. This observation may rule out irreversible conformational changes resulting from lead salt treatment.

Fluorescence. All previous data on lead inhibition indicated that the site of inhibition was more closely associated with photosystem II than photosystem I. Therefore, we decided to examine the function of photosystem II in the presence of lead salts more closely. The results reported are all for tomato chloroplasts; however, similar effects of lead were observed on spinach chloroplasts.

One measure of photosystem II activity is chloroplast fluorescence. The rise of chlorophyll fluorescence upon illumination reflects the photoreduction of the primary electron acceptor of photosystem II (10). The usual chloroplast fluorescence induction kinetics and the effect of $PbCl_2$ on them is shown in Figure 5A. The induction curve appeared normal as exemplified by a fast increase to F_o which was followed by a slower increase in fluorescence to F_∞. Curve B of Figure 5A shows the characteristic rapid increase in fluorescence yield to F_∞ when electron transport from Q to the pool of carriers between the photosystems is blocked by DCMU.

Lead salts at a concentration of 200 μM or more limited fluorescence to a level slightly above the initial level (curve D). At lower concentrations of 50 μM or less (curve C), inhibition was incomplete, allowing a slow increase in fluorescence above F_o. At all concentrations of lead tested, the level of fluorescence seemed to break or remain at the initial plateau level, F_{pl} (11). KCl at 200 μM had little effect on the kinetics of fluorescence yield (curve E, Fig. 5A), thereby eliminating a possible osmotic effect or anion effect of the lead salt. The reduction of fluorescence to this low level indicated an effect of Pb^{2+} on the oxidizing side of photosystem II. If inhibition were on the reducing side, the results should be similar to the effect of DCMU on fluorescence.

The concentration of lead salt required to reduce the variable fluorescence 50% can be estimated from Figure 6 to be 50 μM. In this figure, only the variable fluorescence ($F_\infty - F_o$) was plotted; note that fluorescence was never reduced to F_o. The inhibitory concentration represents a value slightly lower than that required for half-maximal inhibition of proton transport and much less than for half-maximal inhibition of the other electron transport reactions previously presented.

In order to test the location of lead inhibition further, we questioned if the reduction of fluorescence yield could be reversed by an agent which donates electrons to the oxidizing side of the photosystem. Hydroxylamine is such an electron donor (16), and its effect on restoring the high level of chloroplast fluorescence is shown in Figure 5B. Curves E and F show the effect of 1 mM hydroxylamine in restoration of the high level of fluorescence which Pb^{2+} had inhibited. At two different concentrations of lead the level of fluorescence was restored to the steady-state level after 1.3 sec, as compared to 0.9 sec for the control.

Inhibition of photosystem II by hydroxylamine as described
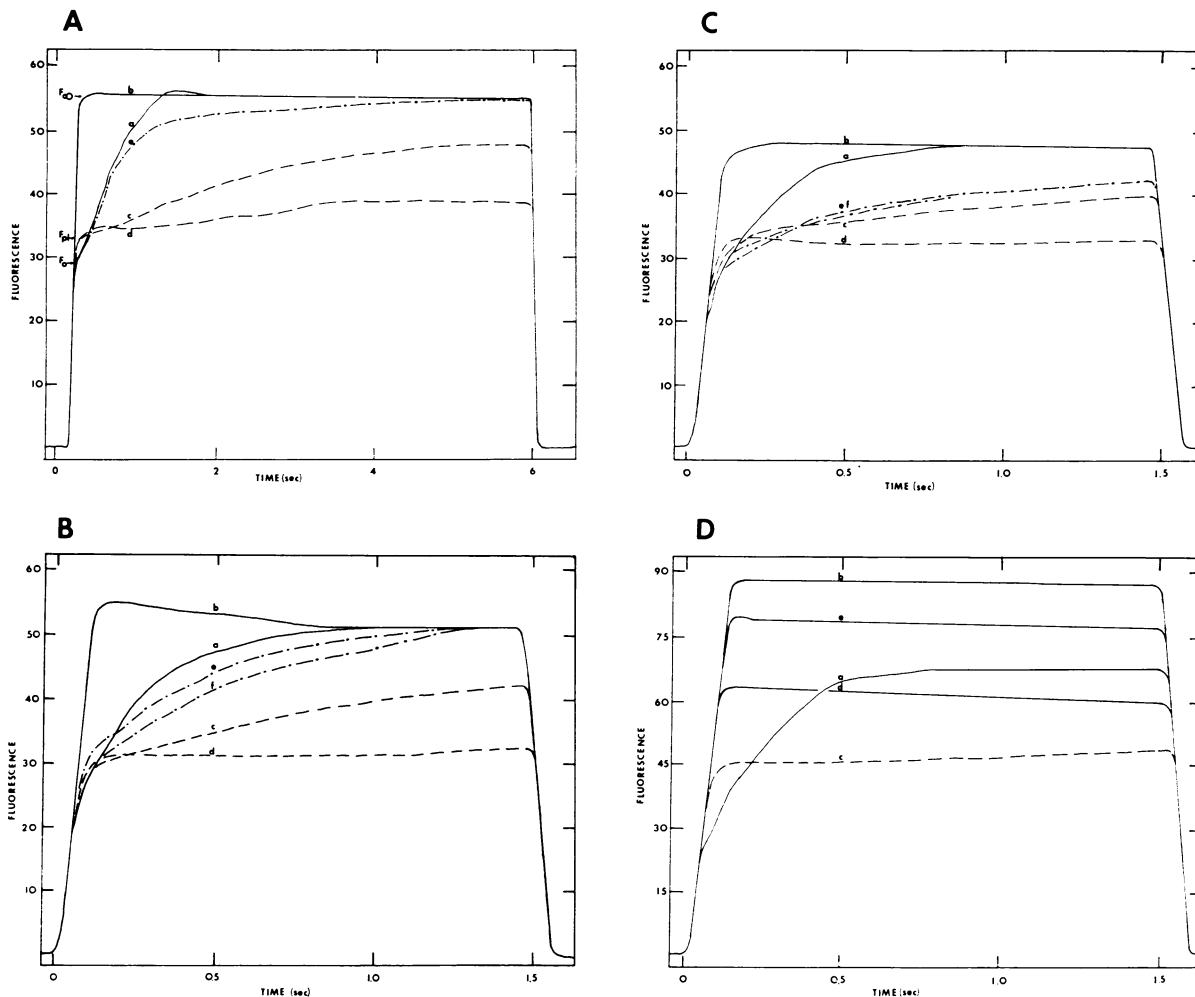
A

C

B

D

FIG. 5. Fluorescence rise following illumination of chloroplasts from tomato leaves. Procedure was as presented in "Materials and Methods." Fluorescence intensity is in arbitrary units. In all experiments, curve A represents untreated control chloroplasts, and curve B has 1 μM DCMU. A: Inhibition of fluorescence rise by lead. Curve C, 50 μM PbCl$_2$; curve D, 200 μM PbCl$_2$; curve E, 200 μM KCl. B: Restoration of fluorescence by hydroxylamine following lead inhibition. Curve C, 50 μM PbCl$_2$; curve D, 200 μM PbCl$_2$; curve E, 50 μM PbCl$_2$, plus 1 mM hydroxylamine; curve F, 200 μM PbCl$_2$, plus 1 mM hydroxylamine. C: Fluorescence with lead and Mn^{2+}, the same as B, except that 670 μM MnCl$_2$ was added in E and F instead of hydroxylamine. D: Inhibition and restoration of DCMU fluorescence rise. Curve C, 200 μM PbCl$_2$; curve D, 200 μM PbCl$_2$, 1 μM DCMU; curve E, 200 μM PbCl$_2$, 1 μM DCMU, 1 mM hydroxylamine.

by Cheniae and Martin (6) was not seen in our reactions, since the reaction mixture was maintained in near darkness for very short periods of time before fluorescence was measured.

Reduced fluorescence similar to Pb^{2+} inhibition (limited to F$_0$) was reported in manganese-depleted chloroplasts (17) or chloroplasts from which manganese has been extracted (1). Normal steady-state fluorescence can be restored to those chloroplasts when Mn^{2+} was added again (16). It is conceivable that lead salts could be depleting or interfering with manganese in the chloroplast. If this were true, the addition of Mn^{2+} ions should promote higher levels of fluorescence similar to those seen when hydroxylamine was added to the reaction. A restoration was not observed (Fig. 5C, curves E and F) when Mn^{2+} was used, although a slightly higher level of fluorescence was noted.

Additionally, we tested lead salts and hydroxylamine on the high level DCMU-induced fluorescence. During DCMU-"poisoned" electron transport, the quencher of fluorescence (Q) is not reoxidized by the large pool of intermediates between the photosystems, the A pool (25), and therefore photosystem I should have no effect on this measurement. Any inhibition of

fluorescence with DCMU present would have to be a photosystem II effect. Figure 5D, curve B, shows the typical DCMU fluorescence, while curve C shows fluorescence with lead chloride alone. When lead and DCMU were both present, there was a reduction of the high fluorescence yield, curve D. Therefore, the inhibition of fluorescence by lead was limited to photosystem II alone. If the electron donor hydroxylamine was added along with DCMU and lead chloride, the lowering of fluorescence caused by lead was more than half restored to the high DCMU level. However, if DCMU, lead chloride, and Mn^{2+} were all added, a tracing similar to curve D results; that is, there was no restoration of fluorescence. This is similar to our results observed earlier in the absence of DCMU.

Inhibition of electron flow by low concentrations of Pb^{2+} seems to be clearly limited to the oxidizing side of photosystem II; however, when the concentration of lead was increased, it appeared that the inhibitory effect might be extended to the reducing side of the photoact as well. This is suggested from the data shown in Figure 7. At the lowest concentrations of lead, F$_0$ was identical to the control, but F$_\infty$ was lowered. At intermediate levels, F$_0$ was slightly increased, and F$_\infty$ was
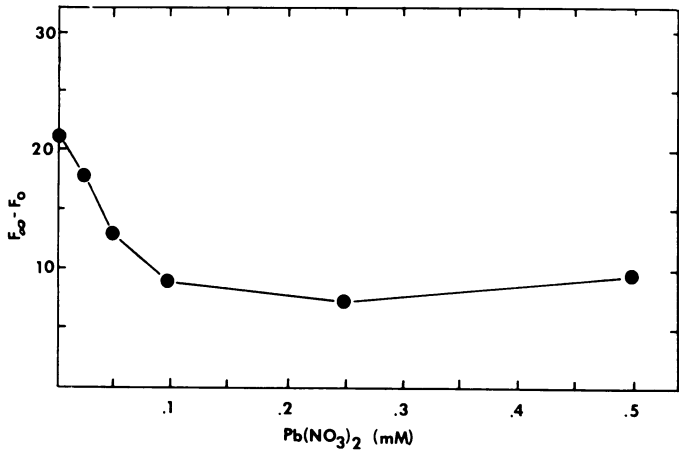
FIG. 6. Variable fluorescence of tomato chloroplasts (steady-state fluorescence, F_∞ minus initial fluorescence, F_0) compared to various added concentrations of lead nitrate. There was no period of preincubation of chloroplasts with lead.
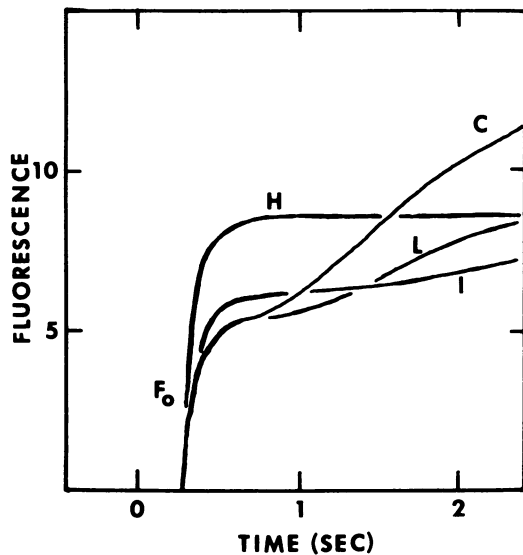

FIG. 7. The effects of lead on the kinetics of the variable fluorescence of tomato chloroplasts. Trace C is control chloroplasts minus lead; trace L is low concentration of $Pb(NO_3)_2$ (35 μM); trace I is intermediate concentration of $Pb(NO_3)_2$ (80 μM); and trace H is a high concentration of $Pb(NO_3)_2$ (220 μM).

reduced to near the F_0 level; at high concentrations of lead, F_0 was noticeably increased with no slow rise observed. This increase in the level of F_0 could be an indication that lead at high concentrations was inhibiting on the reducing side of the photosystem and preventing normal oxidation of Q in a manner similar to the DCMU effect.

If Pb^{2+} inhibits methylviologen reduction by its action on the oxidizing side of the photosystem II, then this inhibition could be relieved, just as electron transport to the quencher of photosystem II was relieved, by adding the electron donor, hydroxylamine. Restoration of electron transport could only be possible if the Pb^{2+} limiting site was in the chain between the point where electrons are donated to the chain by hydroxylamine, to Z (5) and the oxidation of water. Figure 8A indicates that lead chloride-inhibited methylviologen reduction (curve C) could be restored by 1 mM hydroxylamine (curve D). Further, if our suggestion of the site of inhibition is correct, then we should not observe a restoration of oxygen evolution

with hydroxylamine. Indeed, no restoration was noted (Fig. 8B).

A marked difference between the effect of lead on chloroplast fluorescence and on the other electron transport reaction measured was that in order to extensively inhibit fluorescence no preincubation with lead was required. Lead salts reduce fluorescence yield to the same extent whether the treatment was for 20 sec or for 10 min. This faster action may indicate that fluorescence pigments are very near the site of action of the lead salts.

DISCUSSION

These data are discussed within the framework of the "Z-scheme" for photosynthetic electron transport, which postulates two photochemical reactions, I and II, in series (15). The experimental results demonstrate the inhibitory effects of lead salts on chloroplast electron transport. Pb^{2+} inhibits electron flow from water through both photosystems to methylviologen or the Hill reaction electron flow from water through only photosystem II. However, if electron flow is only through photosystem I from reduced DCIP to methylviologen, then there is no effect of Pb^{2+}. These data indicate the inhibition is primarily with photosystem II.

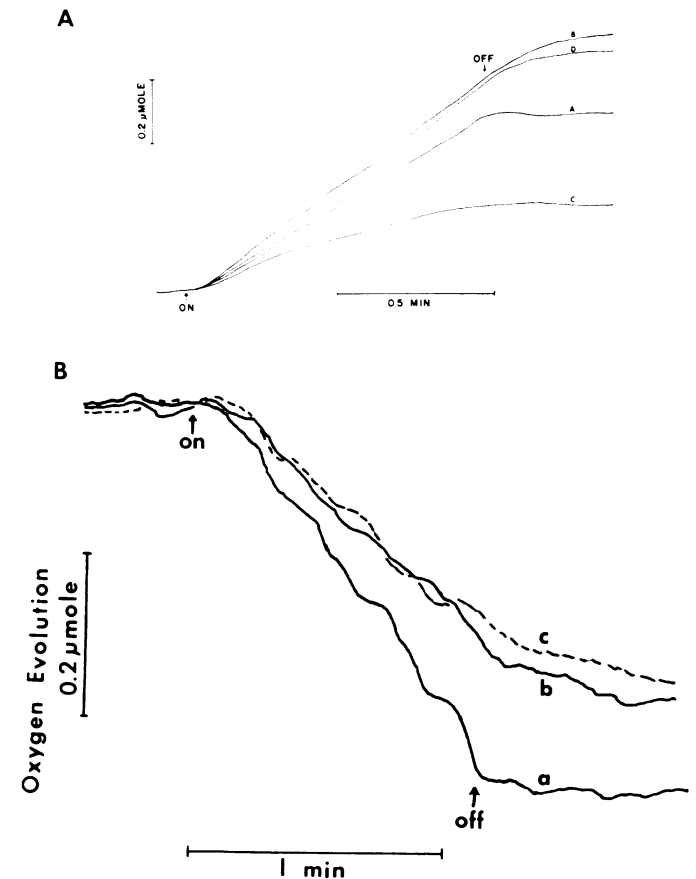When light-induced proton transport and pH gradient for-

FIG. 8. A: Oxygen uptake due to photosynthetic electron transport as measured by methylviologen reduction and reoxidation from water. Curve A: control rate; B: 1 mM hydroxylamine; C: 200 μM $PbCl_2$; D: both $PbCl_2$ and hydroxylamine. Arrows indicate illumination on and off. B: Oxygen evolution in the Hill reaction with potassium ferricyanide as the electron acceptor. Curve A: control rate; B: 200 μM $PbCl_2$; C: 200 μM $PbCl_2$, 1 mM hydroxylamine.

mation (21) was measured, the observed inhibition by lead did not seem to agree with our previous data. Lead salts exhibit a marked inhibition of FMN, methylviologen, or PMS-mediated light-driven proton translocation. Suppression of proton transport was more sensitive than the other electron-transport reactions with a half-maximal inhibitory concentration of 0.1 mM. If membrane proton transport is associated with photosystem I, as is indicated by the stimulating effects of cofactors which promote cyclic electron flow (18), then it does not fit with the observed lack of inhibition of electron flow from reduced DCIP to methylviologen. The observation may in fact lend evidence to the suggestion that the site (or at least one of the sites) for proton translocation is more closely associated with photosystem II (18).

A clear assignment of the Pb^{2+} effect to photosystem II comes from the demonstration of inhibition of chloroplast variable fluorescence. The major fluorescence at room temperature is emitted from photosystem II (10) and the portion of this associated with electron transport is inhibited by lead with a half-maximal concentration less than for inhibition of pH gradient formation, i.e., 50 μM. The only reasonable interpretation of the fluorescence inhibition is that the effect is on the oxidizing side of the photoact, since maximum fluorescence associated with reduction of the quencher of fluorescence (Q) is not reached. The effect of lead in reducing fluorescence yield is reminiscent of the effect of Hill oxidants or manganese deficiency (1). However, reduced fluorescence due to manganese deficiency can be restored with the addition of Mn^{2+} (16). We noted little, if any, restoration by Mn^{2+} following lead inhibition. We also believe it unlikely that the lead salt would be acting as a Hill oxidant, since, if it did, lead should support and increase the Hill reaction rather than inhibit it. Because lead-inhibited variable fluorescence and methylviologen reduction can be restored when hydroxylamine adds electrons to the primary electron donor (5), Z, of photosystem II, we must assign the primary inhibitory site to the electron transport chain between Z and the site of water oxidation. The inhibition by lead may not involve manganese, because there was no restoration of electron flow when manganese was added. It is possible that the site for lead inhibition is similar to the site where amines and ammonia inhibit electron flow (17).

Lead may be somewhat unique in its effect, since no divalent or trivalent metal ion tested by Murata et al. (30) had the effect of reducing variable fluorescence. Mg^+, Ca^{2+}, Sr^{2+}, Mn^{2+}, Al^{3+}, Zn^{2+}, and Cd^{2+} all either increased or had no effect on fluorescence yield of spinach chloroplasts at room temperature. We tested some of these divalent ions and found, while using our techniques and tomato chloroplasts, that there was an increase in fluorescence compared to a decrease in fluorescence following lead treatment (data not presented).

All of the fluorescence data presented used tomato chloroplasts. However, the most important reactions were also done with spinach chloroplast, to eliminate any species difference.

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