Inhibition of Chloroplast Reactions with Phenylmercuric Acetate$^{1,2}$

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ABSTRACT
Phenylmercuric acetate is a selective inhibitor of the photosynthetic activities of isolated spinach (Spinacia oleracea) chloroplasts. At 5 $\mu$M concentration of phenylmercuric acetate, photophosphorylation is inhibited. At 33 $\mu$M phenylmercuric acetate, ferredoxin is inactivated. Ferredoxin-NADP oxireductase is 50% inhibited at 100 $\mu$M phenylmercuric acetate. Photosystem II reactions are 50% inhibited at 100 $\mu$M phenylmercuric acetate and very much higher concentrations—500 $\mu$M—are needed to approach complete inhibition. Phenylmercuric acetate inhibition of photosystem II appears to be selective, blocking a site between the 3-(3,4-dichlorophenyl)-1,1-dimethyl urea sensitive site and the site inactivated by high concentrations of tris buffer.

Mercurials have long been known to inhibit photosynthetic electron transport. Siegenthaler and Packer (13) reported that phenylmercuric acetate inhibits both Hill activity and photophosphorylation. Davenport and Hill (7) determined that PMA$^1$ inhibited methemoglobin reduction supported by ferredoxin. Hiyama et al. (9) found that PMA inhibits the dark reduction of cytochrome $c_6$ in whole cells of Chlamydomonas reinhardtii and inhibits either ferredoxin or ferredoxin-NADP oxireductase. The work presented in this paper shows that 33 $\mu$M PMA inhibits ferredoxin specifically, thereby blocking the photoreduction of NADP. The diaphorase activity associated with ferredoxin-NADP oxireductase in inhibited at higher concentrations of PMA than inhibit the ferredoxin-dependent reactions of photosystem II.

Recently many workers have directed their attention to the dark reactions of photosystem II associated with oxidation of water and oxygen evolution. From these studies several different sites of inhibition of photosystem II have been uncovered. For example, photosystem II is inhibited by heat (10), UV light (17), and high concentrations of tris buffer (18). Epel and Levine (8) have found mutants of C. reinhardtii which have lost partial function in photosystem II. These blocks in photosystem II occur on the oxidizing side of the photoact, and many of the manifestations of this photosystem can be restored with the appropriate electron donor system. This paper presents evidence that PMA inhibits photosystem II at a site between the DCMU and the photoact. Alternate oxidation sites for artificial donors such as hydroxylamine, hydroquinone, and diphenyl carbazide have been discerned through the use of PMA to study the complex series of reactions associated with the evolution of oxygen.

MATERIALS AND METHODS
The method for the spectrophotometric measurement of the photoreduction of NADP has been described previously (15). A typical 3-ml reaction mixture contained the following components in $\mu$moles: sodium phosphate buffer, pH 7.8, 50: NADP, 0.5; ADP, 1: MgCl$_2$, 10; and a saturating amount of spinach ferredoxin. If TCIP-ascorbate was to be used as a donor, 5 $\mu$moles of DCMU, 20 $\mu$moles of sodium ascorbate, and 0.3 $\mu$moles of TCIP were added. For metmyoglobin reduction NADP was replaced by 0.4 $\mu$ mole of metmyoglobin, and the light-induced absorbance change at 580 nm was measured. When ferriyanide or TCIP was used as acceptor, NADP and ferredoxin were replaced by 1.0 $\mu$ mole of potassium ferricyanide or 0.1 $\mu$ mole of TCIP, and the light-induced change at 410 or 620 nm respectively was followed. The luminous flux for such experiments was $5 \times 10^3$ erg $\cdot$ cm$^{-2}$ $\cdot$ sec$^{-1}$. Methyl viologen reduction was measured by taking advantage of its autoxidizability. Oxygen uptake was measured using a Yellow Springs Instrument Company oxygen electrode. A representative 3-ml reaction mixture usually contained the following in $\mu$moles: Tricine buffer, pH 7.8, 45; methyl viologen, 2; sodium azide, 1; and NaCl, 6. When TCIP was the electron donor, 5 $\mu$ moles of DCMU, 30 $\mu$ moles of TCIP, and 15 $\mu$ moles of sodium ascorbate were used. The light intensity used for methyl viologen reduction was the same as noted above. Photophosphorylation was assayed according to the procedure of Krogmann and Olivero (12).

The diaphorase activity of ferredoxin-NADP oxireductase was measured with a modification of the method of Avron and Jagendorf (2). A 3-ml reaction mixture containing 50 $\mu$moles of TCIP, 50 $\mu$moles of sodium phosphate buffer, pH 7.0, and 0.04 ml of partially purified spinach flavoprotein (approximately 3 mg protein/ml) was placed in a cuvette and the absorbance measured at 620 nm. The reaction was initiated by adding 0.3 $\mu$ mole NADPH. The rate of decrease in absorbance at 620 nm is a measure of diaphorase activity.

Mn$^{2+}$ photoxidation was measured according to Ben-Hayyim and Avron (4). A standard reaction mixture contained exactly one-half of the amounts of components described by

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$^1$Publication No. 4513 of the Purdue Agriculture Experiment Station.

$^2$This work was supported by National Science Foundation Grant GB-27466.

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$^4$Abbreviations: PMA: phenylmercuric acetate; TCIP: 2,3',6-trichlorophenol indophenol; TCIPH$: reduced TCIP; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; DPC: diphenyl carbazide; Fd: ferredoxin.

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the above authors except that 3 μmoles of methyl viologen were used, and the final reaction volume was 3 ml. The reaction mixture was placed in a 50-ml Erlenmeyer flask and illuminated in a Warburg bath for 3 min with red light at an intensity of $8 \times 10^3$ ergs cm$^{-2}$ sec$^{-1}$. Aliquots were then taken for Mn$^{2+}$ determination.

Chloroplasts from Spinacia oleracea were prepared by the procedure of Avron et al. (3). Tris treated chloroplasts were prepared according to the procedure of Yamashita and Butler (17). The chloroplasts were incubated in 0.4 m tris buffer, pH 7.8, for 30 min, then washed twice with 0.4 m sucrose-50 mm NaCl solution. PMA-treated chloroplasts were prepared by suspending fresh chloroplasts in 10 mm PMA-0.4 m sucrose, and then washed twice with 20 ml of 0.4 m sucrose-50 mm NaCl solution.

**Chemicals.** PMA and myoglobin were purchased from Sigma Chemical Company. Metmyoglobin was prepared from myoglobin using the procedure of Smith (14). DPC was purchased from Aldrich Chemical Company, and hydroxylamine was obtained from Matheson, Coleman and Bell. Ferredoxin was prepared by the method of Bürger et al. (5). Chlorophyll concentration was estimated by the method of Arnon (1).

**RESULTS**

While studying various manifestations of Hill activity in spinach chloroplasts, a variety of effects of PMA were observed. Figure 1 is representative of such experiments. The top curve shows the effects of PMA on the reduction of methyl viologen using TCIPH$_2$ as an electron donor. Photosystem I appears to be insensitive to PMA up to 500 μM. The closed and open triangles show the effect of PMA on photosystem II reactions, i.e., ferricyanide reduction and TCIP reduction respectively. There is a gradual inhibition of photosystem II with incomplete inhibition above 500 μM PMA. The open circles and closed circles respectively represent ferredoxin requiring NADP and metmyoglobin photoreduction using electrons from water. PMA appears to inhibit these activities at much lower concentrations (50 μM). Identical results were obtained when TCIF$_2$ was used to replace water as the electron donor in the NADP reducing reaction. Pyocyanin-mediated cyclic photosphorylation (closed rectangles) is inhibited at much lower PMA concentrations than inhibit photosynthetic electron transport. We found that noncyclic photosphorylation is inhibited by PMA at the same concentration that inhibits cyclic photosphorylation. Although the data are not shown, methyl viologen reduction using electrons from water behaves toward PMA as does TCIP photoreduction. Thus PMA inhibits photosynthetic electron transport in at least 2 sites, a sensitive site in the ferredoxin-dependent reactions and a less sensitive site in photosystem II.

We attempted to determine whether the flavoprotein ferredoxin-NADP oxidoreductase or ferredoxin itself was being inhibited by low PMA. Hiyama et al. (9) have suggested either protein might be the site of PMA inhibition. Table I shows that PMA specifically inhibits the ability of exogenous ferredoxin to support NADP photoreduction using electrons from TCIPH$_2$. Adding excess ferredoxin overcomes the PMA effect. Experiment 2 shows that PMA does irreversible damage to ferredoxin. Dialysis of PMA-treated ferredoxin does not relieve the inhibitory effect of PMA. Keister and San Pietro (11) have shown that the mercurial p-chloromercury phenyl sulfonic acid bleached the visible spectrum of ferredoxin. We have found that PMA causes a similar bleaching of ferredoxin.

The effect of PMA on the diaphorase activity of isolated ferredoxin-NADP oxidoreductase is represented in Figure 2. PMA inhibits soluble or bound diaphorase at much higher concentrations than those needed to inhibit ferredoxin. At 33 μM PMA, where NADP photoreduction is inhibited 90%, the chloroplast diaphorase activity is inhibited only 30%. One must conclude that the inhibition of NADP reduction caused by 33 μM PMA is due to inhibition of ferredoxin.

Figure 1 presented evidence that high concentrations of PMA inhibited the photoreduction of TCIP or ferricyanide using water as an electron donor. An attempt was made to relate PMA inhibition of photosystem II to tri inhibition and other known lesions occurring in this part of the electron transport sequence. Figure 3 presents evidence to show that PMA

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**Table I. PMA Inhibition of Soluble Ferredoxin**

<table>
<thead>
<tr>
<th>Additions to Fresh Chloroplasts</th>
<th>Rate of TCIPH$_2$ → NADP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% control</td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
</tr>
<tr>
<td>200 μg Fd</td>
<td>100</td>
</tr>
<tr>
<td>200 μg Fd + PMA</td>
<td>0</td>
</tr>
<tr>
<td>400 μg Fd + PMA</td>
<td>71</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
</tr>
<tr>
<td>200 μg Fd before dialysis</td>
<td>100</td>
</tr>
<tr>
<td>200 μg Fd after dialysis</td>
<td>100</td>
</tr>
<tr>
<td>(200 μg Fd + PMA) before dialysis</td>
<td>0</td>
</tr>
<tr>
<td>(200 μg Fd + PMA) after dialysis</td>
<td>0</td>
</tr>
<tr>
<td>(200 μg Fd + PMA) after dialysis + 200 μg excess Fd</td>
<td>100</td>
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</tbody>
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**FIG. 1.** Differential inhibition of photosynthetic electron transport by PMA. The reaction conditions for all measurements are given in "Materials and Methods." Numbers within brackets below indicate the control (100%) rate in terms of μmoles electrons per mg chl per hour. The open blocks represent methyl viologen reduction by electrons from TCIP-ascorbate [170]. The closed blocks represent pyocyanine-mediated cyclic photosphorylation [360 μmoles ATP/mg chl hr = 100%]. The open triangles represent TCIP photoreduction by electrons from water [320], while the closed triangles represent ferricyanide photoreduction by electrons from water [77]. The open circles represent photoreduction of NADP by electrons from water [315], while the closed circles represent photoreduction of metmyoglobin by electrons from water [50]. Chloroplasts representing 40 μg of chlorophyll were used in this experiment.
photooxidation in photosystem II concentrations, the plastids. The inhibition of photosystem II shows a saturating light at high concentrations since it inhibits the flow of electrons from water to methyl viologen. DPC and hydroxylamine reverse the inhibition in a DCMU-sensitive fashion. However, hydroxylamine but not DPC reverses the PMA block. The reversal by hydroxylamine is DCMU sensitive, indicating PMA inhibits on the

inhibits a dark reaction associated with photosystem II. The top curve shows the effect of varying light intensity on TCIP photoreduction in the absence of PMA. The bottom curve shows the same experiment with PMA present (165 μM). The inhibitory effect of PMA appears to be on a dark reaction since saturating light intensities do not overcome the inhibition.

Figure 4 presents some observations of PMA effects on photosystem II. PMA at low concentrations stimulates the photooxidation of Mn2+ by whole chloroplasts. At higher concentrations, PMA inhibits this same reaction. As the concentration of PMA approaches 150 μM, TCIP photoreduction by photosystem II is inhibited in the same concentration range as is Mn2+ photooxidation. Tris-treated chloroplasts which have photosystem II activity supported by DPC behave toward PMA as do the control chloroplasts. Table II indicates that PMA inhibition of photosystem II is of a specific nature. Both PMA and tris inhibit the flow of electrons from water to methyl viologen. DPC and hydroxylamine reverse the tris inhibition in a DCMU-sensitive fashion. However, hydroxylamine but not DPC reverses the PMA block. The reversal by hydroxylamine is DCMU sensitive, indicating PMA inhibits on the
oxidizing side of photosystem II. These data also show that the PMA block is closer to the photoact than is the tris inhibition site. PMA pretreatment of chloroplasts followed by washing with sucrose-sodium chloride solution does not relieve the inhibition of photosystem II and causes a gradual loss in the ability of the chloroplasts to use hydroxylamine as an electron donor. A ratio of 1 μmole of PMA to 300 μg of chlorophyll is optimal for inhibition of oxygen evolution when attempting to substitute another donor for water. Table III presents the effect of donor systems on tris- and PMA-treated chloroplasts. DPC reverses inhibition of methyl viologen reduction caused by tris. This reversal is DCMU sensitive. DPC will not reverse the PMA inhibitory effect. Semicarbazide is a poor substitute for DPC in tris-washed chloroplasts. Hydroxylamine now acts like DPC when used for reversal of inhibition of PMA-pre-treated plastid. There appears to be a time-dependent PMA inhibition phenomenon associated with hydroxylamine oxidation resulting in a much poorer reversal of PMA inhibition by hydroxylamine. Benzidine and MnCl₂ are poor donors to both tris- and PMA-treated chloroplasts. The reduced aromatic donors such as hydroquinone or phenylenediamine appear to reverse both types of inhibition in a DCMU-sensitive fashion. Ascorbate alone is a poor donor to either tris- or PMA-treated chloroplasts.

**DISCUSSION**

Siegenthaler and Packer (13) reported that 10 μM PMA inhibits photophosphorylation as well as NADP and 2,6-dichlorophenol indophenol reduction. Our findings indicate that while 10 μM PMA inhibits photophosphorylation, it takes much higher concentrations of PMA (50 μM) to inhibit NADP reduction. Even higher concentrations (200 μM) are required to inhibit photosystem II-mediated TCIP reduction. In 1960 Davenport and Hill (7) showed that 150 μM PMA gave 50% inhibition of methemoglobin photoreduction supported by ferredoxin. We find that metmyoglobin reduction is inhibited 50% at lower concentrations of PMA (25 μM). The discrepancies between our data and Davenport and Hill’s data probably arise from the use of different amounts of ferredoxin. Tagawa and Arnon (16) have shown that mercurials react stoichiometrically with pure spinach ferredoxin.

We have further established that PMA inhibits at least three sites of photosynthetic electron transport. PMA at 33 μM inhibits ferredoxin specifically and thereby inhibits NADP photoreduction. PMA at 33 μM also inhibits metmyoglobin photoreduction, a reaction which requires only ferredoxin and not the flavoprotein (6). Inhibition of diaphorase activity occurs at higher concentrations of PMA. Thus ferredoxin-NADP oxidoreductase is eliminated as a point of inhibition when using 33 μM PMA to inhibit NADP photoreduction. Hiyama et al. (9) established that both ferredoxin or ferredoxin-NADP oxidoreductase of *C. reinhardtii* could be inhibited by 100 μM PMA. We have confirmed their observations for spinach chloroplasts by measuring the effect of 100 μM PMA on cytochrome c₅₅₅ turnover using dual wavelength spectrophotometry. The third site of inhibition by PMA is on the oxidizing side of photosystem II and occurs around 200 μM. PMA inhibits TCIP and ferricyanide photoreduction and also inhibits Mn⁺ photooxidation. Yamashita and Butler (17) have established that high concentrations of tris buffer inhibit on the oxidizing side of photosystem II and that various donors will re-establish electron flow by donating electrons past the tris block at a point closer to the photoact (see scheme below). Our data indicate that PMA blocks photosystem II at a point between the tris block and the photoact. DPC will reverse the tris block but not PMA inhibition. Thus DPC must be oxidized at a point between the two inhibitory sites. Hydroquinone-ascorbate, phenylene diamine-ascorbate, and hydroxylamine (under the proper conditions) will reverse both PMA and tris inhibition. It appears that hydroquinone-ascorbate and other related donors are oxidized at a site closer to the photoact than are donors such as DPC or semicarbazide. It also appears that Mn⁺ donates electrons at a site further from the photoact than the tris or PMA block since tris or PMA inhibit Mn⁺ photooxidation. The scheme (Fig. 5) represents our interpretation of the above data. PMA inhibition suggests the sequential relations of two inhibitor sites and three donor sites on the oxidizing side of photosystem II.
Fig. 5. Inhibition of photosynthetic electron transport. Tris buffer prevents the use of electrons from manganous ions (Mn) and this block is circumvented by diphenyl carbazide (DPC). High concentrations of phenyl mercuric acetate (PMA) will block electron flow from both Mn and DPC but can be bypassed by using either hydroquinone and ascorbate (HQ/Asc) or hydroxylamine (NH₄OH) as electron donors to the photoact (Z → Q). All of these reactions are inhibited by DCMU. Low concentrations of PMA block the activity of ferredoxin (Fd) and the ferredoxin-NADP oxidoreductase (FPR) which prevents the flow of electrons from cytochrome c₅₅₃ (Cyt. c₅₅₃) and other carriers in the intermediary dark electron transport chain through F₉₀₀, photosystem I and the ferredoxin reducing substance (FRS) to NADP.

**LITERATURE CITED**


