

INTERACTIONS OF PARAQUAT AND NITRODIPHENYLETHER HERBICIDES WITH THE
CHLOROPLAST PHOTOSYNTHETIC ELECTRON TRANSPORT IN THE ACTIVATION OF TOXIC
OXYGEN SPECIES

by

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Dissertation submitted to the Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in

Plant Physiology

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(ABSTRACT)

The interactions of paraquat (methylviologen) and diphenylether herbicides with the Mehler reaction as investigated. Sera from two different rabbits (RS1 & RS2) were examined for their patterns of inhibition of the photosynthetic electron transport (PET) system. Serum from RS2 was greatly hemolyzed. Fifty μ l of RS1 serum were required for 100% inhibition of a $H_2O \rightarrow$ methylviologen(MV)/ O_2 reaction, whereas only 10 μ l of a 1:10 dilution of RS2 were needed for 100% inhibition. The γ -globulin fraction from purified rabbit serum (RS1) did not inhibit PET, indicating that the antibody fraction of the rabbit serum does not contain the inhibitor. It appears that the inhibitor is from the hemolyzed red blood cells. Rabbit sera, added to chloroplast preparations prior illumination, caused no inhibition of a $H_2O \rightarrow$ MV/ O_2 reaction while addition of rabbit sera during illumination inhibited the $H_2O \rightarrow$ MV/ O_2 reaction within 1-3 s. Various Hill reactions were used to determine the site of inhibition. Rabbit sera inhibited photosystem I (PSI) Hill reactions, but did not inhibit a photosystem II (PSI II) Hill reaction indicating that inhibition is on the reducing side of PSI. It would be

expected that a $\text{H}_2\text{O} \rightarrow \text{Ferredoxin (Fd)/NADP}$ Hill reaction should also be blocked. Surprisingly, rabbit sera did not inhibit this reaction. These results were interpreted as supportive evidence for parallel (branched) electron transport on the reducing side of PSI.

Six pyridyl derivatives {benzylviologen, 2-anilinopyridine, 1,2-bis(4-pyridyl)ethane, 1,2-bis(4-pyridyl)ethylene, 2-benzoylpyridine, and 2-benzylaminopyridine} and five heme-iron derivatives {hemoglobin, hemin, hematin, ferritin, and ferrocene} were screened for their potential to counteract paraquat toxicity on pea (Pisum sativum L. cv. Little Marvel) isolated chloroplasts. $\text{H}_2\text{O} \rightarrow \text{MV/O}_2$ and $\text{H}_2\text{O} \rightarrow \text{Fd/NADP}^+$ were the two Hill reactions assayed with these compounds. Antagonists of paraquat toxicity should inhibit the first Hill reaction but not the latter. None of the pyridyl derivatives examined inhibited the reaction $\text{H}_2\text{O} \rightarrow \text{MV/O}_2$. Ferritin and ferrocene were also ineffective as inhibitors of this reaction. Hemoglobin inhibited the reaction $\text{H}_2\text{O} \rightarrow \text{MV/O}_2$ without inhibiting the reaction $\text{H}_2\text{O} \rightarrow \text{Fd/NADP}^+$, providing protection to pea chloroplasts against paraquat. Hemin and hematin inhibited both Hill reactions examined. Hemin and hematin also inhibited $\text{H}_2\text{O} \rightarrow$ diaminodurene (ox) and durohydroquinone $\rightarrow \text{MV/O}_2$ Hill reactions but not the dichlorophenylindolphenol(red) $\rightarrow \text{MV/O}_2$ and diaminodurene(red) $\rightarrow \text{MV/O}_2$ Hill reactions. These results indicate that hemin and hematin are inhibiting photosynthetic electron transport in the plastoquinone pool region.

Potential involvement of hydroxyl and alkoxy radicals in the

peroxidative action of the *p*-nitro diphenyl ether herbicides acifluorfen was evaluated under laboratory conditions. Methional was added to illuminated pea thylakoids and its oxidation to ethylene was used as an indicator of hydroxyl and alkoxy radical synthesis. Oxyfluorfen-stimulation of the rate of methional oxidation was dependent on light, photosynthetic electron transport and hydrogen peroxide since it was not observed under dark conditions or in the presence of DCMU and catalase. Addition of FeEDTA, a catalyst of the Fenton reaction, stimulated the oxyfluorfen-induced enhancement of methional oxidation six-fold suggesting that hydroxyl radicals are synthesized through a Fenton reaction. Acifluorfen, nitrofen and nitrofluorfen inhibited the rate of methional oxidation whereas, acifluorfen-methyl had no effect on the rate of methional oxidation even at high concentrations (1 mM). Nitrofluorfen at 1 mM was the only *p*-nitro diphenyl ether herbicide tested which inhibited photosynthetic electron transport of pea thylakoids. In experiments with pea leaf discs, acifluorfen at low concentrations stimulated the rate of methional oxidation, while acifluorfen-methyl, nitrofen and nitrofluorfen had no effect. These data indicate that hydroxyl and alkoxy radicals could be involved in the mechanism of cellular damage caused by oxyfluorfen, but they are not important for the activity of the diphenyl ether herbicides acifluorfen, acifluorfen-methyl, nitrofen, and nitrofluorfen.

Diethyldithiocarbamate (DEDTC) does not accept electrons from the photosynthetic electron transport (PET), but can donate electrons to a photosystem I (PSI) Mehler reaction in the presence of the following PET

inhibitors: diuron, dibromothymoquinone, and bathophenanthroline. It cannot photoreduce PSI in the presence of cyanide, a PET inhibitor. These data indicate that the site of electron donation is after the plastoquinone pool. Ascorbate is not required for the ability of DEDTC to donate electrons to PSI. There is no photoreductant activity by DEDTC in a ferredoxin/NADP Hill reaction. Superoxide dismutase inhibits DEDTC/diuron or bathophenanthroline \rightarrow MV/O₂ Mehler reaction. Catalase does not restore the consumed O₂ from a DEDTC/diuron \rightarrow MV/O₂ Mehler reaction, indicating O₂⁻ has not been dismutating into H₂O₂. These results indicate that superoxide is required for DEDTC ability to donate electrons. therefore DEDTC is limited only to Mehler-type reactions.

ACKNOWLEDGEMENTS

I express sincere thanks to my major advisor, Dr. Kriton K. Hatzios for his instructional guidance in conducting, financing and publishing of this research project. I would like to express an even deeper appreciation for Dr. Hatzios' genuine concern in my overall professional development and his efforts in bringing recognition to my graduate achievements through nominations for various awards and fellowships. His kind and generous efforts will always be remembered. I would also like to express my gratitude to members of my committee, Drs. Joyce G. Foster, Laurence D. Moore, David M. Orcutt, John L. Hess and William E. Winner who provided needed advice and assistance throughout my graduate program. Additional thanks are expressed to Drs. Foster and Moore for their time and effort reviewing various manuscripts of this project submitted for publication. I also thank Dr. Susan A. Tolin for her generosity in the use of her centrifuge and spectrophotometer. An appreciation is also extended to my friends, fellow graduate students and department staff members for their support and help during my graduate studies. Thanks are extended to the financial support of this research project by the Thomas F. Jeffress and Kate Miller Jeffress Memorial Trust, Richmond, Virginia, USA. Finally, my deepest thanks go to my wife, Jenny E. Upham, for her extensive efforts in typing this dissertation and for her patience, sacrifices, understanding and love.

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LIST OF ABBREVIATIONS

RS1 and RS2, rabbit serum 1 and 2; MV, methylviologen; DCMU, 3,4-dichlorophenyl-N,N'-dimethylurea (diuron); $K_3Fe(CN)_6$, potassium ferricyanide; DCIP, dichlorophenolindolphenol; DAD, 2,3,5,6-tetramethyl-p-phenylenediamine (diaminodurene); DHQ, tetramethyl-p-hydroquinone (durohydroquinone); MES, [2-(N-morpholino)-ethanesulfonic acid]; HEPES, [N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid]; DBMIB, dibromothymoquinone; HCN, cyanic acid; BPT, bathophenanthroline; PET, photosynthetic electron transport; PSI and PSII, photosystem I and II; Fd, ferredoxin; PCN, plastocyanin; cyt f, cytochrome f; Fe-S, iron sulfur; PQ, plastoquinone; FRS, ferredoxin reducing substance; FNR, ferredoxin NADP⁺ oxidoreductase; PPO, polyphenol oxidase; Chl, chlorophyll; Asc, ascorbate; SOD, superoxide dismutase; hv, light.

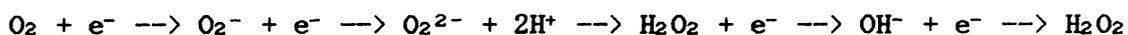
CHAPTER I

INTRODUCTION

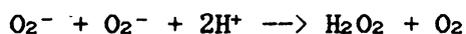
Oxygen is essential for most living organisms; however, it is also inherently toxic [1,2]. Toxicity of oxygen is linked, in part, to activated forms of oxygen such as the superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH\cdot$) and singlet oxygen (1O_2) [1,2]. Considerable insight into mechanisms of oxygen toxicity can be obtained from understanding the chemistry of diatomic oxygen. Diatomic oxygen is a paramagnetic molecule containing two unpaired electrons [2]. Each electron occupies a different molecular orbital and possesses the same value for the spin quantum number. The electrons in the outer orbital of most molecules have opposite spin quantum numbers. This creates a spin restriction between the diatomic oxygen radical with most other molecules making oxygen fairly unreactive which is unlike most free radicals [2]. If a spin transition occurs for the two unpaired electrons in the outer orbitals in which opposite values for the spin quantum number are attained, the spin restriction is removed [2]. The diatomic oxygen is now in a singlet state and is called singlet oxygen (1O_2). Removal of this spin restriction makes 1O_2 a very reactive and toxic molecule [2].

The spin restriction can also be removed by univalent reduction of diatomic oxygen. Although diatomic oxygen can accept four electrons and protons to form two molecules of water, the spin restriction of diatomic oxygen allows for only univalent reduction. In other words, oxygen can

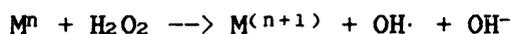
be reduced by one electron (e^-) at a time which forms reactive species of oxygen before it is reduced to water. The reduction of oxygen is expressed as:



The initial reduction of oxygen forms the superoxide radical. This reduction removes the spin restriction of O_2 making it more reactive. However, O_2^- is generally not very reactive towards biological molecules [4]. Its biological toxicity is believed to be related to superoxide's reactivity towards other forms of activated oxygen products [1,2]. Superoxide readily dismutates, in which one molecule of O_2^- is oxidized and the other is reduced. This dismutation is expressed as:



The product of this reaction, H_2O_2 , can further react with reduced transition metals producing the very toxic hydroxyl radical. This is commonly referred to as the Fenton reaction [4,5]. This reaction is expressed as:

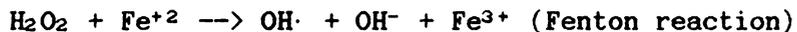


Superoxide and H_2O_2 can also react with each other to form hydroxyl radicals. This reaction is referred to as the Haber-Weiss reaction and

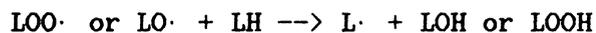
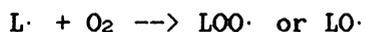
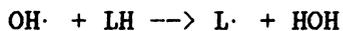
is expressed as :



The rate of the Haber-Weiss reaction is very slow [6,7] and considered biologically insignificant. However, an iron catalyzed Haber-Weiss cycle does occur at significant rates [8,9] and is represented as:



The hydroxyl radical, which is an extremely potent oxidant, is probably the most toxic oxygen reduction product [1,2]. Hydroxyl radicals readily react with unsaturated lipids, DNA, proteins and polysaccharides [1]. The most damaging aspect of hydroxyl radicals is believed to be its reactivity towards lipid molecules [1]. The hydroxyl radical is capable of abstracting a hydrogen atom, from an unsaturated fatty acid initiating a chain reaction of lipid peroxidation which disrupts membranes. This process is represented as:



LH = Lipid

LOOH = lipid peroxide

L· = Lipid radical

LOH = lipid alkoxide

LOO· = lipid peroxy radical

LO· = lipid alkoxy radical

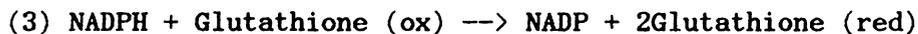
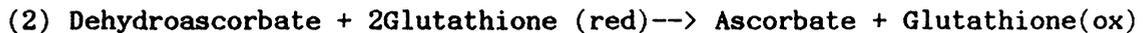
Disruption of membranes through lipid peroxidation causes a loss of membrane functions leading to impaired cell metabolism and even cell death. Similar results are seen by singlet oxygen.

The mechanism of biological reduction of oxygen varies with different organisms, tissues, cell types and cell organelles. The focus of this dissertation is the reduction of oxygen in higher plant chloroplasts. Chloroplasts undergoing photosynthesis will oxidize water to oxygen which creates a high internal oxygen concentration. The solubility of oxygen in organic solvents is several times greater than in water and O_2 will partition into the hydrophobic portions of the thylakoid membranes [1]. Therefore, thylakoid membranes probably experience a greater degree of oxygen stress than most organelles.

In addition to increased oxygen tensions, thylakoids contain an electron transport system with redox potentials favorable for the reduction of oxygen. The reduction of oxygen by isolated thylakoids was first reported by Mehler [10]. This reaction has since been termed the "Mehler reaction". The initial reduction of oxygen to superoxide occurs on the reducing side of photosystem I (PSI) [1]. The primary electron acceptor of PSI can photoreduce oxygen to O_2^- and H_2O_2 in the presence

or absence of ferredoxin (Fd) [11]. The Fd-mediated Mehler reaction has greater rate than a Mehler reaction in the absence of Fd [1,11]. Significant rate of the Mehler reaction have been monitored in isolated intact chloroplasts and cells [12,13,14].

Chloroplasts are have mechanisms to reduce the toxic effects of reduced oxygen species. Superoxide is removed through enzymatic dismutation by superoxide dismutase (SOD), a copper-zinc enzyme [1]. SOD is found in the chloroplast stroma and thylakoids [15,16]. Hydrogen peroxide produce is harmlessly reduced to H₂O through the Asada-Halliwell cycle [17]. This cycle utilizes several metabolites and enzymes [17] and is expressed as:



The first reaction is catalyzed by ascorbate peroxidase [EC.1.11.1.7] the second by dehydroascorbate reductase [EC.1.8.5.1] and the third by glutathione reductase [EC.1.6.4.2]. The second step can also proceed non-enzymatically at pH 8.0 or via NAD(P)H monodehydroascorbate reductase [17]. Ascorbate and α -tocopherol protect chloroplasts from the toxic effects of hydroxyl radicals in the stroma and thylakoids, respectively [18,19].

These protective mechanisms of the chloroplast prevent significant

injury from the toxic effects of reduced oxygen during normal photosynthesis. If these protective mechanisms are interfered with or overtaxed by a stress factor, the chloroplasts are subject to oxygen injury. Several herbicides can act as a stress factor that kills plants by elevating levels of toxic oxygen species.

The best understood class of herbicides which increase levels of oxygen toxicity are the bipyridinium compounds. Paraquat is a herbicidal member of this class. Paraquat is known to accept electrons from the reducing side of PSI, diverting electrons to oxygen instead of NADP [20]. The consequence is an accelerated rate of the Mehler reaction producing O_2^- , H_2O_2 , and $OH\cdot$ at levels that overcome the protective mechanisms of the chloroplasts. The result is lipid peroxidation of membranes leading to cell death. Originally, the primary electron acceptor of PSI, referred to as ferredoxin reducing substance, was thought to be responsible for paraquat reduction [21]. However, recent developments of the photochemistry of the reducing side of PSI indicate that several electron acceptors are involved in the reduction of Fd or paraquat [22]. These electron acceptors appear to be a monomeric chlorophyll anion, semiquinone and three iron-sulfur centers. The experimental evidence suggests that electron transfer to the iron-sulfur centers are in parallel under physiological conditions [22]. The physiological function of parallel electron flow has not been determined but it could possibly be involved in a differential reduction of NADP and O_2 . This introduces the concept of a separate pathway for the Mehler reaction which could be a potential site for inhibiting electron flow to

paraquat, rendering it non-toxic.

The p-nitro substituted diphenylether (NDPE) herbicides are also known to peroxidize lipid membranes. Light is required for this NDPE herbicide dependent lipid peroxidation. The mechanism of action for NDPE herbicides is not known but oxygen free radicals have been implicated since oxygen is also needed for NDPE-dependent lipid peroxidation [23]. It is not known how NDPE herbicides are activated by light. Experimental work with the NDPE herbicide, oxyfluorfen, has indicated that the photosynthetic electron transport (PET) is required [24,25,26], while the NDPE herbicide, acifluorfen, requires carotenoids and not PET [23,27,28]. At present, it is not known which oxygen free radicals are involved in lipid peroxidation induced by NDPE herbicides.

In studying the mode of action of the above herbicides, several scavengers can be used to identify which activated oxygen species are involved. Examples of these scavengers are SOD for O_2^- , catalase for H_2O_2 , 1,4-diazabicyclo (2,2,2 octane) (DABCO) for 1O_2 and $OH\cdot$, diethyldithiocarbamate (DEDTC) or formate for alkoxy and $OH\cdot$ radicals. However, concern must be given to compounds that scavenge oxidation/reduction products for their potential to interact with redox components of the photosynthetic electron transport. Enzymes are normally very specific, therefore SOD and catalase probably do not interact with the thylakoid electron transport system. However, it is possible that DABCO, formate, and DEDTC can interact with electron transport components. Preliminary work has indicated this to be true for DEDTC which

can reduce PSI [29].

The objectives of this research are to study the interactions of the bipyridinium and diphenylether herbicides with chloroplast components to produce oxygen toxicity. The particular objectives of this research were the following.

- 1) Find an inhibitor of parallel electron transport on the reducing side of PSI and characterize it.
- 2) Use this inhibitor to determine if parallel electron flow differentially reduces NADP and O_2 .
- 3) Screen compounds that could potentially counteract the toxicity of paraquat at the chloroplast level.
- 4) Determine if hydroxyl and alkoxy radicals are involved in the mode of action of several diphenyl ethers.
- 5) Determine if O_2^- and H_2O_2 are direct precursors to potential alkoxy and hydroxyl radical synthesis by NDPE herbicides.
- 6) Determine if PET activates NDPE herbicides that may potentially produce alkoxy and hydroxyl radicals.
- 7) Characterize in detail the PSI electron donating activity of the hydroxyl and alkoxy radical scavenger, DEDTC.

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CHAPTER II

INHIBITION OF THE PHOTOSYNTHETIC ELECTRON TRANSPORT OF ISOLATED CHLOROPLASTS BY HEMOLYZED RABBIT SERA. EVIDENCE FOR PARALLEL ELECTRON TRANSPORT ON THE REDUCING SIDE OF PHOTOSYSTEM I

Abstract

The inhibition patterns of rabbit sera from two different rabbits (RS1 & RS2) on the photosynthetic electron transport (PET) were studied. Serum from RS2 was greatly hemolyzed. Fifty μ l of RS1 were required for 100% inhibition of a $H_2O \rightarrow MV/O_2$ reaction, while only 10 μ l of a 1:10 dilution of RS2 were needed for 100% inhibition. The γ -globulin fraction from purified rabbit serum (RS1) did not inhibit PET, indicating that the antibody fraction of the rabbit serum does not contain the inhibitor. It appears that the inhibitor is from the hemolyzed red blood cells. Rabbit sera added prior to chloroplast illumination caused no inhibition, while addition of rabbit sera during illumination inhibited a $H_2O \rightarrow MV/O_2$ reaction within 1-3 s. Aminotriazole, a catalase inhibitor, did not affect the efficacy of the rabbit sera indicating that the unknown rabbit serum inhibitor is not catalase. Various Hill reactions were employed to determine the site of inhibition. Rabbit sera inhibited the following reactions: $DHQ/DCMU \rightarrow MV/O_2$, $DAD/Asc/DBMIB \rightarrow MV/O_2$, and $DCIP/Asc/DBMIB \rightarrow MV/O_2$. Rabbit sera did not inhibit a $H_2O \rightarrow DAD_{ox}$ reaction indicating that inhibition is on the reducing side of PSI. It would be expected that a $H_2O \rightarrow Fd/NADP$ reaction should also be blocked. Surprisingly, rabbit sera did not inhibit this reaction. These results were interpreted as supportive evidence

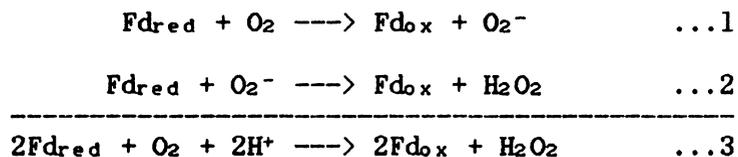
for parallel (branched) electron transport on the reducing side of
PSI.

INTRODUCTION

It has been known for some time that isolated, illuminated chloroplasts are able to use oxygen as a final electron acceptor in the light reaction. The photosynthetic reduction of oxygen, which leads to the formation of hydrogen peroxide (H_2O_2) and a consequent net consumption of oxygen (O_2) was first discovered by Mehler [18]. This reaction has since been termed the "Mehler reaction." The Mehler reaction can also be mediated by low redox potential acceptors, such as methylviologen (MV) and flavin mononucleotide (FMN) and therefore can be used to measure the rates of electron transport through the uptake of oxygen [28]. The rate of photoreduction of cytochrome c , a known superoxide scavenger, has been shown to be equivalent to the rate of the formation of H_2O_2 . Cytochrome c photoreduction is inhibited by superoxide dismutase (SOD) but H_2O_2 production is not [4]. This demonstrates that the Mehler reaction proceeds by a one-step reduction of O_2 . In other words, the reduced MV is involved only in the first O_2 reduction step ($O_2 \rightarrow O_2^-$), and not the second reduction step ($O_2^- \rightarrow H_2O_2$). This further suggests that reduced MV is directly involved only in O_2^- formation and not in H_2O_2 production.

Ferredoxin (Fd) is also an autooxidizable electron acceptor [23]. It is more likely to be of importance in vivo since Fd is a necessary electron transfer protein in photosynthetic membranes. Ferredoxin was found to produce O_2^- and H_2O_2 [12]. However, Fd-mediated oxygen uptake does not proceed in a one-step reduction. The enzyme SOD was found to

partially inhibit the rate of Fd-mediated oxygen uptake [1, 11]. This has been explained as a two-step reduction of oxygen:



In my opinion, the second reduction step by Fd does not occur in vivo. Physiologically significant amounts of SOD are found in chloroplasts [5], and localized predominantly in the stroma and to a lesser extent bound to the thylakoids [16]. It therefore seems more likely that O_2^- is dismutated by SOD, rather than reduced by Fd to H_2O_2 .

It has been accepted that MV and Fd accept electrons from the primary electron acceptor of PSI (X), referred to as ferredoxin reducing substance (FRS), before transferring an electron to oxygen or NADP^+ [29]. In light of recent developments on the primary electron acceptor of PSI, it has become clear that a series of electron acceptors is involved before the reduction of Fd [22]. The primary electron acceptor from P_{700} may be a monomeric chlorophyll anion designated A_0^- . Electron transfer probably proceeds to a semiquinone (A_1^-). The electron acceptors following A_1^- are three iron-sulfur (Fe-S) centers denoted as F_x , F_a and F_b . It is still unresolved whether these Fe-S centers act in series or in parallel. The experimental evidence tends to favor the concept that electron transfer to the Fe-S centers, particularly F_a and F_b , are in parallel under physiological conditions [22]. It is not known

which one of these electron acceptors (ie. F_x , F_b , F_a) interacts with F_d and MV in the reduction of O_2 . It may be possible that parallel electron flow has a significant role in the differential reduction of $NADP^+$ and O_2 by F_d . In this communication, we report on the patterns of inhibition of photosynthetic electron transport by an unknown compound from hemolyzed rabbit sera that supports the concept of parallel electron flow. A tentative model is proposed to explain the interaction of the Mehler reaction with PSI.

MATERIALS AND METHODS

Spinach (Spinacea oleracea L. cv Bloomsdale) was grown in a soilless mixture of peat:vermiculite:weblite (1:2:2), plus a slow release fertilizer (Osmocote®). Plants were kept in a growth chamber with a 12 hr light/dark cycle at temperatures of 20°C day/17°C night. The light intensity was varied during the day cycle, starting with 40 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and gradually increasing for 3 h to a maximum intensity of 800 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, which was held constant for 1 h. Then this process was reversed. After 4 weeks of growth in this environment, spinach plants were used for chloroplast isolation.

Chloroplast thylakoids were isolated as follows: 5-7 spinach leaves were gathered and macerated for 5 s in a partially frozen extraction medium (75 ml) containing 330 mM sorbitol, 5 mM MgCl_2 , 20 mM MES-NaOH/pH 6.5. The homogenate was filtered through one layer of Miracloth and centrifuged for 1 min at 2,000 x g at 0°C. The pellet was resuspended in a 1:20 dilution of the extraction medium (10 ml) then centrifuged for 1 min at 4,000 x g at 0 C. The pellet was resuspended in an assay medium containing 330 mM sorbitol, 50 mM HEPES-NaOH/pH 7.6, 2 mM MgCl_2 , 1 mM NH_4Cl , and 2 mM EDTA. Intact chloroplasts were isolated for the CO_2 -dependent O_2 evolution experiment after the method of Walker [26].

Hill reaction rates were determined by monitoring changes in O_2 concentration as a function of time using a Gilson-Oxygraph Clark-type oxygen electrode. Assay volumes, light intensities at the surface of

the reaction vessel and the assay temperature were 1.5 ml, 2000 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, and 20°C, respectively. All chemicals were from Sigma Chemical Company (St. Louis, MO), except for sorbitol and MES which were from Calbiochem (San Diego, CA). DBMIB was a gift from Dr. Draber, Bayer AG, Wuppertal, West Germany. Chlorophyll concentrations were determined according to the method of Arnon [3] and usually ranged from 35-50 μg Chl per assay.

Isolation of γ -globulins from serum involved repeated $(\text{NH}_4)_2\text{SO}_4$ precipitation and chromatography on DEAE cellulose according to the method of Campbell et al. [8]. RS1 and RS2 denote sera obtained from New Zealand White rabbits inoculated and not inoculated with spinach thylakoids, respectively. RS1 serum was obtained at the University of New Hampshire while RS2 was obtained at Virginia Polytechnic Institute and State University.

RESULTS AND DISCUSSION

The potential usefulness of antibodies as selective inhibitors of the Mehler reaction in broken chloroplasts was investigated. The initial approach was to use thylakoids as antigens. Several workers have produced antibodies against thylakoids that could inhibit Hill reactions, including the Mehler reaction [7, 21]. Rabbit antisera inhibited a MV-mediated Mehler reaction (Figure 1). Blood serum contains many different substances in addition to antibodies. This inhibition could be due to the non-antibody fraction. Subsequent experiments showed that this indeed was the case. Three lines of evidence suggested that this unknown inhibitor was not an antibody.

(1) Control serum as well as serum from rabbits inoculated with thylakoids caused total inhibition of a MV-mediated Mehler reaction when added during the light reaction.

(2) RSl was preincubated with thylakoids in the dark for 1.5, 5.0, and 8.5 minutes. No inhibitions of oxygen uptake at all time periods of dark preincubation were observed (Figure 1). Normally, 2 to 8 minutes incubation of antiserum with thylakoid preparations was sufficient time for antigen-antibody binding [7]. If the serum was added during the light reaction (Figure 1), then complete inhibition resulted. This pattern of inhibition was not typical of antibody inhibition of Hill reactions [6, 19, 24, 30]. It was found that any dark preincubation of antiserum with thylakoids did not cause a noticeable inhibition. In our

results, it is possible that inhibition required a particular protein to be in a specific conformational configuration which is induced in the light or the light made accessible the binding site to the inhibitor. The lack of inhibition by rabbit serum when added in the dark could be an unspecific binding of the inhibitor to the thylakoids which prevents binding of the inhibitor to the light activated site.

(3) The γ -globulin fraction was purified from RSl and no inhibition was detected from this fraction. The γ -globulin fraction of the serum is the fraction that contains the immunoglobulins (antibodies) formed in response to an antigen. This is the most persuasive evidence that the existing serum inhibitor is not an antibody. Taken collectively, the three lines of evidence strongly suggest no involvement of antibodies in the inhibition of the Mehler reaction.

The unknown inhibitor does not appear to be an H_2O_2 scavenger, such as catalase. The effects of three catalase inhibitors; cyanide (90 μ M), azide (90 μ M), and aminotriazole (50mM) on the ability of RSl to inhibit a MV-mediated Mehler reaction were examined. RSl inhibited the Mehler reaction 100% in the presence of aminotriazole and only 26% in the presence of cyanide and azide. Since aminotriazole is a catalase inhibitor that does not greatly affect photosynthesis or other biochemical reactions [2], it seems improbable that catalase is the unknown inhibitor from rabbit sera. Cyanide and azide did interfere with the effects of rabbit sera but they are known to affect many other biochemical reactions such as photosynthesis [2] and do not alter the above con-

clusion. Further, the inability of RS1 to inhibit the Mehler reaction when added in the dark suggests that the RS1 inhibitor is not scavenging products of the Mehler reaction.

We did not identify the unknown inhibitor or inhibitors in the rabbit sera. However, significant differences in serum volume required for maximum inhibition of a MV-mediated Mehler reaction were observed. Chloroplast preparations with approximately 25 μg chlorophyll required 50 μL of RS1 and 10 μL of a 1:10 dilution of RS2 for maximum inhibition of a MV-mediated Mehler reaction. The difference in the potency of these two rabbit sera can possibly be used in determining what the unknown inhibitor(s) is(are) in the serum. A difference between these sera (RS1 & RS2) could be visually observed. RS2 is a much deeper red than RS1 which is due to greater hemolysis of RS2 sera. It appears that the inhibitor is a product of hemolysis. It is not known whether more than one inhibitor from the hemolyzed portion of the sera is responsible for inhibition. In the remainder of the paper, the inhibitor will be referred to in the singular form. However, we are not ruling out the possibility that more than one inhibitor could be involved.

To localize the site of inhibition by rabbit sera on the electron transport chain various electron donors and acceptors were used. When water was used as a donor and MV as an acceptor of PET electrons, there was complete inhibition of this reaction by rabbit sera (Table 1). MV is reduced by an electron acceptor of PSI [29]. A PSII Hill reaction, $\text{H}_2\text{O} \rightarrow \text{DAD}$, was not inhibited (Table 1). Potassium ferricyanide was

added to keep the DAD in an oxidized state and DBMIB inhibited electron flow to KFeCN. Electron donors to PSI allow us to bypass PSII electron transport. When the electron donors DCIP/Ascorbate were used with MV as the acceptor and DBMIB as the PSII inhibitor, total or near total inhibition by the sera was observed (Table 1). Electrons are donated by DCIP/Ascorbate to P700 [15,20]. Similarly, if DAD/Ascorbate is used as the donor, total or near total inhibition is also observed (Table 1). DAD/Ascorbate is known to donate electrons to PSI at the cytochrome b-Rieske Fe-S-cytochrome f complex or plastocyanin [17]. Considerable inhibition by RS2 on a DHQ \rightarrow MV/O₂ reaction was also observed. DCMU was used as the inhibitor of electron flow from PSII. Durohydroquinone donates electrons to the plastoquinone pool [27]. From the above assumptions and data, the site of inhibition can be considered to be on the reducing side of PSI. It is generally assumed that MV is reduced by the primary electron acceptor (X) of PSI [6]. Since X also reduces NADP⁺ via Fd and Fd-NADP⁺ oxidoreductase [EC.1.18.1.2], one would predict that the electron flow from H₂O to Fd/NADP⁺ or H₂O to FeCN should be blocked by the rabbit sera. Surprisingly, rabbit sera did not block NADP⁺- or Fe(CN)₆⁻³-dependent O₂ evolution (Table 1). If MV is replaced by another autooxidizable substance such as Fd, we again observed complete inhibition of the Mehler reaction by the rabbit serum (Table 1). In contrast to assumptions made in the literature, these observations appear to be inconsistent with sequential linear electron flow between PSI to NADP⁺ or PSI to O₂ via Fd or MV. Tentative explanations may be put forward.

One possibility is the inhibitor binds to an activated form of MV (reduced). This would prevent the subsequent reduction of O_2 and not affect $NADP^+$ reduction. This hypothesis is difficult to reconcile with the data that RS1 serum preincubated with chloroplasts in the dark did not inhibit MV-mediated O_2 uptake. Instead, the lack of serum inhibition in the dark-pretreated chloroplast thylakoids might be explained as a slow binding reaction between the inhibitor and some other molecule in the system.

Another possibility is the presence of an intermediate molecule or subunit that is independent of the main chain (electron flow to $NADP^+$) and would transfer electrons from a PSI electron acceptor to O_2 . If this explanation is true, then inhibition at the site of this intermediate molecule or subunit would result in the inhibition of O_2 reduction while $NADP^+$ as well as $Fe(CN)_6^{-3}$ reduction would remain undisturbed. The experiments investigating inhibitions of Mehler and $NADP^+$ type Hill reactions were repeated using sera from six other New Zealand White rabbits (3 females and 3 males) which were obtained from the University of New Hampshire. Each of the six sera inhibited a $H_2O \rightarrow MV/O_2$ and not $H_2O \rightarrow Fd/NADP^+$ Hill reactions as was observed with RS1 and RS2.

An oxygen-reducing factor (ORF) has been reported [12]. This ORF is found to photoreduce O_2 to H_2O_2 . ORF was obtained by heat treatment of washed chloroplast lamellae from sugar beet leaves and spinach chloroplast lamellae and identified as 3-hydroxy-tyramine [10] and lumiflavin [13], respectively. The ORF was found to require phenoloxidase and PET

[10]. The ORF was considered an artifact of the chloroplast isolation procedure and not of physiological significance [13].

More recently, polyphenoloxidase (PPO) [EC. 1.10.3.1] has been implicated as a physiological modulator of the Mehler reaction [25]. To determine if PPO was involved in our system we examined whether diethyl-dithiocarbamate (DEDTC), a PPO inhibitor, exhibited the same inhibitory effects on the Mehler reaction as our rabbit sera inhibitors. The results were that DEDTC did not inhibit a MV-mediated Mehler reaction indicating that PPO was not the site of inhibition by the rabbit sera.

Although it is difficult to explain the inhibition patterns of our rabbit sera, it appears that a PET component is involved in the reduction of O_2 , mediated by Fd or MV, and not involved in the reduction of $NADP^+$. Since Fd is also involved in $NADP^+$ reduction via ferredoxin NADP reductase (FNR), the above hypothesis implicates a second binding site for Fd. Carrillo and Vellejos [9] proposed a model based on eosin binding experiments in which Fd binds to FNR and PSI, connecting the enzyme to PSI. This would give only one apparent binding site for Fd. Forti & Grubas [14] did not agree with this model because their experimental evidence indicated that the interaction of Fd with thylakoids occurs at two distinct sites. They proposed that FNR was not in close proximity to PSI and Fd was reduced at the reducing end of PSI and shuttled through the thylakoid membrane to FNR, the second binding site. It is possible that the second Fd binding site is independent of FNR and offers a site for O_2 reduction via Fd or MV, which could explain the

inhibition pattern observed in Table 1. The second binding site may or may not be one of the Fe-S centers F_a or F_b . This would also be consistent with parallel electron flow through these centers [22]. The second binding site may also be an unidentified redox molecule accepting electrons from one of the Fe-S centers F_a , F_b or even F_x . Such a model is illustrated in Figure 2.

This model agrees with our experimental results as well as with those presented by [14] and [9]. The author would like to emphasize that the model presented in Figure 2 is based upon indirect experimental evidence and that a need for direct experimental evidence is essential for confirming such a model. There are also practical implications of the results presented in this paper. Methylviologen, also known as paraquat, is a potent herbicide that destroys plant tissue by transferring electrons from PET to O_2 thereby creating oxy-radicals which peroxidize lipid membranes. Our data indicate that electron flow to O_2 , mediated by methylviologen, can be blocked without inhibiting electron flow to $NADP^+$. This biochemical information can be used to develop safeners that could protect plants from paraquat (methylviologen) toxicity.

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Table 1. The effects of rabbit sera, RS1 and RS2, on various Hill reactions.

Hill Reaction	% Inhibition by rabbit sera	
	RS1	RS2
H ₂ O --> MV/O ₂	100 ± 0	100 ± 0
H ₂ O --> DAD/DBMIB/KFeCN	ND	0 ± 0
DHQ/DCMU --> MV/O ₂	ND	72 ± 2
DAD/Asc/DBMIB --> MV/O ₂ /SOD	91 ± 7	77 ± 5
DCIP/Asc/DBMIB --> MV/O ₂ /SOD	93 ± 11	79 ± 8
H ₂ O --> Asc/MV/O ₂	78 ± 9	ND
H ₂ O --> Fd	100 ± 0	100 ± 0
H ₂ O --> K ₃ (FeCN) ₆	0 ± 0	0 ± 0
H ₂ O --> Fd/NADP ⁺	0 ± 0	0 ± 0
H ₂ O --> CO ₂	0 ± 0	ND

The sera were added during the light reaction. Assay medium was as described in Methods and Materials except for the CO₂ Hill reaction which had no NH₄Cl and the addition of Na₄P₂O₇ (5 mM) and K₂HPO₄ (0.25 mM). The concentrations of MV, DHQ, DAD, DCIP, Asc, DBMIB, DCMU, FeCN, NADP⁺, HCO₃⁻, Fd and SOD were 100 μM, 1 mM, 2 mM, 0.2 mM, 1 mM, 15 μM, 15 μM, 1 mM, 1 mM, 20 mM, 100 μg and 200 units, respectively. Serum volume required for maximum inhibition by RS1 and RS2 was 100 μL and 10 μL, respectively, for all reactions except those requiring ascorbate in which 300 μL and 20 μL was required for RS1 and RS2, respectively. Each value ± standard deviation represents an average of 3 replicates. ND = no data. All rates were greater than 2.00 μmoles O₂ · min⁻¹ · mg Chl⁻¹.

FIGURE LEGENDS

Figure 1. Oxygen electrode trace of a methylviologen-mediated Mehler reaction. Assay medium was as described in Experimental. 50 μ L of rabbit serum (RS1) was added each time.

Figure 2. A model indicating the potential interaction of PSI with ferredoxin- or methylviologen-mediated oxygen reduction. PCN, plastocyanin; Fd, ferredoxin; MV, methylviologen; FNR, ferredoxin NADP⁺ oxidoreductase; ORM, oxygen reduction molecule.

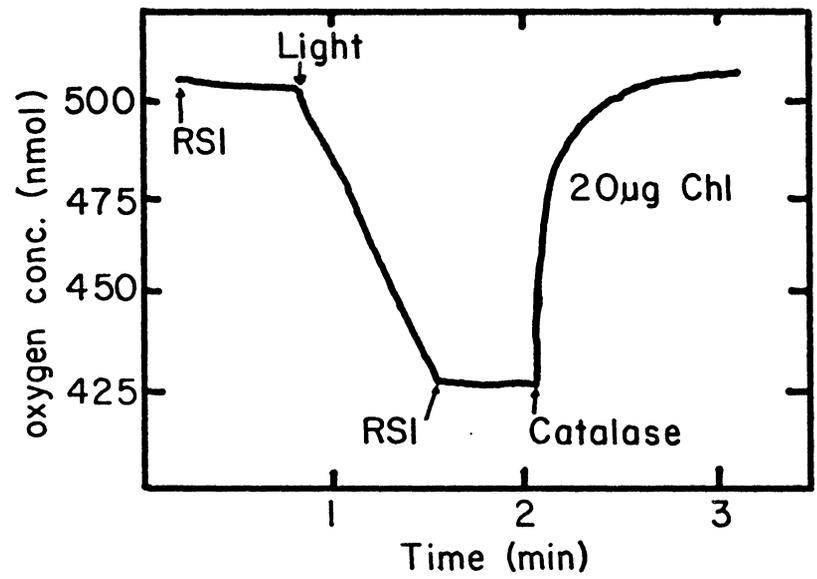


Figure 1. Oxygen electrode trace of a methylviologen-mediated Mehler reaction.

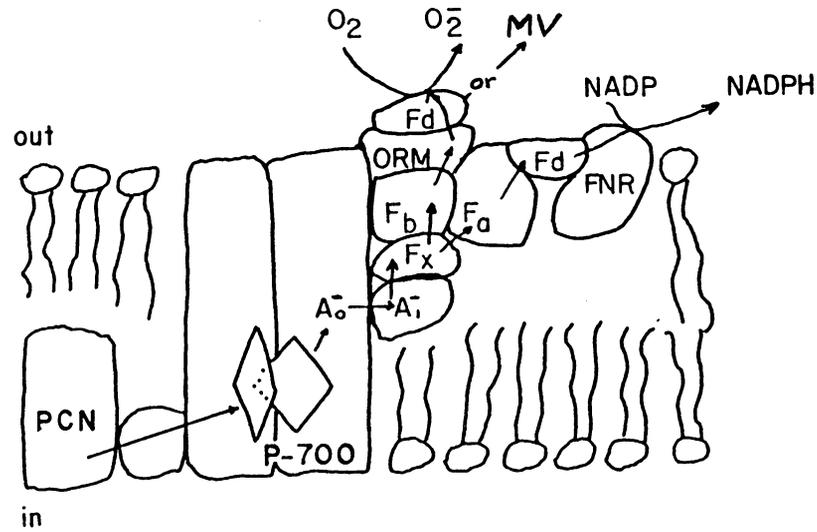


Figure 2. A model indicating the potential interaction of PSI with ferredoxin- or methylviologen-mediated oxygen reduction.

CHAPTER III

COUNTERACTION OF PARAQUAT TOXICITY AT THE CHLOROPLAST LEVEL

Abstract

Six pyridyl derivatives [benzylviologen, 2-anilinopyridine, 1,2-bis(4-pyridyl)ethane, 1,2-bis(4-pyridyl)ethylene, 2-benzoylpyridine, and 2-benzylaminopyridine] and five heme-iron derivatives [hemoglobin, hemin, hematin, ferritin, and ferrocene] were screened for their potential to counteract paraquat (1,1'-dimethyl-4,4'-bipyridinium ion) toxicity on pea (Pisum sativum L.) isolated chloroplasts. The $H_2O \rightarrow$ methylviologen(MV) / O_2 and $H_2O \rightarrow$ ferredoxin(Fd)/ $NADP^+$ were two Hill reactions assayed with these compounds. Antagonists of paraquat toxicity should inhibit the first Hill reaction but not the latter. The pyridyl derivatives examined did not inhibit the reaction $H_2O \rightarrow$ MV/ O_2 . Ferritin and ferrocene were also ineffective as inhibitors of this reaction. Hemoglobin inhibited the reaction $H_2O \rightarrow$ MV/ O_2 without inhibiting the reaction $H_2O \rightarrow$ Fd/ $NADP^+$, providing protection to pea chloroplasts against paraquat. Hemin and hematin inhibited both Hill reactions examined. They also inhibited $H_2O \rightarrow$ diaminodurene(DAD) $_{ox}$ and durohydroquinone \rightarrow MV/ O_2 Hill reactions but not the dichlorophenol $_{red} \rightarrow$ MV/ O_2 and DAD $_{red} \rightarrow$ MV/ O_2 Hill reactions. These results suggest that hemin and hematin inhibit photosynthetic electron transport in the plastoquinone-pool region.

INTRODUCTION

Chemical and genetic manipulation of crop tolerance to herbicides has challenged herbicide technologists for many years [1, 2, 3]. Recent advances in agricultural biotechnology offer new options and alternative approaches to meet this challenge. Plant tolerance to nonselective herbicides can be a result of one or more of the following mechanisms [1, 4]: a) altered uptake and translocation or compartmentation of the herbicide in tolerant plants; b) extensive metabolic detoxification of the herbicide in tolerant plants; c) modification of the target site of the herbicide in tolerant plants; d) increased synthesis of a target enzyme in tolerant plants; and e) oversynthesis of substrates able to reverse the herbicide-induced inhibition of growth in tolerant plants. Specific examples for the involvement of these mechanisms in the development of plant tolerance to herbicides have been reported [1,3]. Many of these mechanisms may be manipulated by genetic or chemical means.

Paraquat is a nonselective herbicide used extensively for total weed control in no-till crop production and as a harvest aid [5]. The active ingredient of paraquat is methylviologen (MV) which is routinely used in studying selected photochemical reactions mediated by plant chloroplasts. Paraquat exerts its phytotoxicity by accepting photosynthetic electrons from PSI and transferring these electrons to oxygen producing toxic oxygen species which cause lipid peroxidation and membrane breakdown [6]. During this process, paraquat is reduced to a cationic radical which is quickly reoxidized by molecular oxygen. Superoxide radical

(O_2^-) is the product of this reaction [7]. Disproportionation of superoxide radicals to H_2O_2 is catalyzed enzymatically by superoxide dismutase (SOD) (EC 1.15.1.1), which is located in plant chloroplasts [8]. Paraquat-mediated peroxide production is cytochemically located along the stroma lamellae and on the ends of the grana stack [9]. Hydrogen peroxide readily accepts electrons from reduced transition metals [10, 11, 12] or from reduced paraquat [13] to form hydroxyl radicals ($OH\cdot$). Lipid peroxidation is most likely initiated by $OH\cdot$ [10, 14, 15]. The superoxide radical is neither a strong oxidant nor reductant [16] and is probably not directly involved in lipid peroxidation [17]. However, superoxide is an efficient reductant of transition metals [11, 14, 18] and can play an indirect role in oxygen toxicity by maintaining transition metals in a reduced state [10, 12, 19]. The plant is normally protected from the harmful effects of O_2^- , H_2O_2 , and $OH\cdot$ through the action of SOD, ascorbate peroxidase (EC 1.11.1.7)/catalase (EC 1.11.1.6), and α -tocopherol, respectively [8, 20]. Treatment of plants with paraquat enhances the production of toxic oxygen species to levels far exceeding those that can be protected by the natural defense systems of plants.

Interest in manipulating crop tolerance to paraquat has been renewed recently, following the discovery of several weed biotypes that are tolerant to this herbicide. Biotypes of horseweed (*Conyza linefolia* L.) Cronq.) survive treatments with paraquat by excluding the herbicide from its site of action. Autoradiography and cytochemical, and biochemical studies showed that translocation of paraquat in tolerant *Conyza* biot-

ypes was limited to the major leaf veins and insufficient amounts of the herbicide reached the mesophyll cells containing the target site (chloroplast) of this herbicide [21, 22]. The tolerance of perennial ryegrass (Lolium perenne L.) biotypes to paraquat, however, is not due to differential uptake, translocation, or metabolism of paraquat [23]. It is the result of increased activity of the endogenous protectants of toxic oxygen species such as the enzymes catalase, SOD, and peroxidase [24]. Tobacco (Nicotiana tabacum L.) cell lines, tolerant to the herbicide paraquat, have been selected through tissue culture procedures [25]. Increased levels of peroxidase and catalase activities in these cell lines were identified as the causes for the tolerance of these lines to paraquat [26, 27].

Attempts to chemically regulate plant tolerance to paraquat have also been reported. D-penicillamine, a copper chelate with superoxide dismutating activity, offered partial protection to flax (Linum ussitatissimum L.) cotyledons against paraquat [28]. Preconditioning of plants to sublethal doses of stresses which increase oxygen toxicity, such as ferrous sulfate [29] and sulfite [30] tends to raise the levels of protective mechanisms toward toxic oxygen species and protects plants from paraquat injury. Ferrous sulfate applied as a spray with paraquat could protect wheat and oats from paraquat injury but the antidote to herbicide ratio was very uneconomical (100:1) [29].

An alternative approach for the chemical manipulation of plant tolerance to paraquat is the counteraction of its activity at the chloro-

plast level. Until recently, this approach was not apparently feasible since it is traditionally accepted that paraquat is reduced by the primary electron acceptor of PSI which is also responsible for the reduction of NADP^+ [6]. Current work on the primary electron acceptor of PSI, however, shows that a series of electron acceptors are involved before the reduction of ferredoxin (Fd) [31]. The primary electron acceptor from P700 may be a monomeric chlorophyll anion designated A_0^- . Electron transfer probably proceeds to a semiquinone (A_1^-) followed by three iron-sulfur (Fe-S) centers denoted as Fx, Fa, and Fb. It is still unresolved whether these Fe-S centers act in series or in parallel. The experimental evidence tends to favor the concept that electron transfer to the Fe-S centers, particularly Fa and Fb, is in parallel under physiological conditions [31]. It is not known which one of these electron acceptors (Fx, Fa, and Fb) interacts with Fd and paraquat in the reduction of O_2 . An unidentified inhibitor from hemolyzed rabbit sera is capable of inhibiting paraquat- or Fd-mediated O_2 uptake without inhibiting NADP^+ -dependent O_2 evolution [32]. The physiological implication of this finding is that electron flow after PSI is branched (parallel). One branch is involved in NADP^+ reduction whereas another branch is involved in the paraquat- or Fd-mediated O_2 reduction. The branch for reduction of O_2 offers a potential target site for regulating paraquat toxicity since electron flow to paraquat could be inhibited without interfering with NADP^+ -reduction.

This study is a report on the results to counteract paraquat toxicity at the chloroplast level with several chemical compounds that could

potentially inhibit electron flow to paraquat but not to NADP⁺. Two major groups of compounds, nonherbicidal pyridyl analogues of paraquat and heme/iron derivatives were screened.

MATERIAL AND METHODS

Peas (Pisum sativum cv. Little Marvel) were grown in a soilless mixture of 1:2:2 of peat:vermiculite:weblite, plus a slow release fertilizer (Osmocote®). Plants were maintained in a growth chamber with a 12 hr light/dark cycle at temperatures of 20°C day/17°C night. The light intensity was varied during the day cycle, starting with 40 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$ and gradually increasing for 3 hours to a maximum intensity of 800 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$, which was held constant for 1 hour, after which the process was reversed. After 4 weeks of growth in this environment, pea plants were used for chloroplast isolation.

Chloroplasts were isolated as follows: 6-7 leaves were gathered from 5-7 plants and macerated for 5 sec in a partially frozen extraction medium (75 ml) containing 330 mM sorbitol, 5 mM MgCl_2 , 20 mM MES-NAOH/pH 6.5. The homogenate was filtered through a single layer of Miracloth and centrifuged for 1 min at 2,000 x g at 0°C. The pellet was resuspended in a 1:20 dilution of the extraction medium (10 ml) then centrifuged for 1 min at 4,000 x g at 0°C. The pellet was resuspended in an assay medium containing 330 mM sorbitol, 50 mM HEPES-NAOH/pH 7.6, 2 mM MgCl_2 , 1 mM NH_4Cl , and 2 mM EDTA.

Hill reaction rates were determined by monitoring changes in O_2 concentration as a function of time using a Gilson-Oxygraph Clark-type oxygen electrode. Assay volumes, light intensities at the surface of the reaction vessel, and the assay temperature were 1.5 ml, 2000

$\mu\text{E}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$, and 20°C , respectively. All chemicals were from Sigma Chemical Company, except for sorbitol and MES which were from Calbiochem. Dibromothymoquinone (DBMIB) was a gift from Dr. Draber, Bayer AG, Wuppertal, West Germany. Chlorophyll concentrations (3 replicates) were determined according to the methods of Arnon [33] and usually ranged from 35–50 μg Chl per assay.

RESULTS AND DISCUSSION

Several compounds were screened for their ability to counteract paraquat toxicity. Each compound was added to a paraquat Hill reaction. The compounds that inhibited this reaction were then added to a NADP Hill reaction. Successful compounds should not inhibit the latter reaction. It has been established that the only pyridyl compounds exhibiting herbicidal action are those which contain coplanar rings [34]. Examples of such compounds are the 4,4'-and 2,2'-bipyridine series, such as paraquat (methylviologen), diquat and triquat. Other pyridyl compounds do not exhibit herbicidal activity. It is possible that pyridyl compounds which do not exhibit herbicidal activity could act as paraquat antagonists inhibiting the reduction of oxygen by binding to the active site of paraquat reduction. The pyridyl compounds tested were: benzylviologen; 2-anilinopyridine; 1,2-bis(4-pyridyl)ethane; 1,2-bis(4-pyridyl)ethylene; 2-benzoylpyridine and 2-benzylaminopyridine (Table 1). Unfortunately, none of these compounds inhibited paraquat-mediated O_2 uptake and most likely they do not act as paraquat antagonists. These results are in agreement with Lewinsohn and Gressel [35]. They found that benzylviologen protected Spirodela oligorrhiza (Kurz) Hegelm. colonies from damaging levels of diquat. However the protection was not found to be a direct interaction with thylakoids but was explained in part as an increase in SOD levels. Other paraquat analogues were also tested but these compounds did not offer any protection to S. oligorrhiza colonies [35].

In recent studies, Upham *et al.* [32] showed that an unidentified compound from rabbit sera inhibits O_2 reduction but not $NADP^+$ reduction in thylakoid preparations. This inhibitor is probably a product of red blood cell hemolysis [32]. Therefore, compounds that could be a product or resemble products of hemolysis were screened as potential paraquat antagonists. One such compound was hemoglobin which inhibited paraquat mediated O_2 uptake by 66% and had no effect on $NADP^+$ -dependent O_2 evolution (Table 1). However, to obtain reproducible results, hemoglobin had to be added three times with approximately 4 seconds or greater time between each addition. The final concentrations after each addition were 10, 20, and 30 μM . No inhibition was observed when hemoglobin was added once with a final concentration of 30 μM . No explanation could be offered for these results which appear to be concentration independent. Further, it should be noted that hemoglobin cannot be used on a live plant since it cannot traverse cell walls and membranes.

Another iron-containing protein used was ferritin. This compound did not inhibit O_2 uptake (Table 1). Other compounds chosen were those that may resemble breakdown products of hemoglobin. Benzylimidazole was chosen for its pyrrole nitrogen, which exists in the tetrapyrrole ring of heme-structures. This compound also did not inhibit O_2 uptake (Table 1). Hemin and hematin, two heme-structures containing reduced and oxidized iron, respectively did inhibit O_2 uptake (Table 1) but also inhibited $NADP$ reduction. These data show hemin and hematin are unsuitable for paraquat safening of isolated plant chloroplasts.

Ferrocene was also tested. This compound contains an iron molecule coordinated with two aromatic benzene rings in a non-planar configuration. This is in contrast to heme-structures in which iron is coordinated to aromatic rings in a planar configuration. Ferrocene did not inhibit paraquat-mediated oxygen uptake (Table 1).

The inhibitory effects of hemin and hematin on PET were further characterized to determine their approximate site of inhibition on PET. The electron donors: DCIP/ascorbate and DAD/ascorbate, donate electrons to P700 [36, 37] and the cytochrome b-Rieske(Fe-S)-cytochrome f/plastocyanin region [38], respectively. Hemin and hematin did not inhibit the above Hill reactions (Table 2). This indicates that inhibition by hemin and hematin is not on the reducing side of PSI. The inhibitor DCMU was used to block electron flow from PSII. Durohydroquinone is known to donate electrons to the plastoquinone region [39]. The electron acceptor DAD_{ox} is probably reduced in the same region [40]. Hemin and hematin inhibited these two Hill reactions (Table 2). Thus, it appears hemin and hematin are inhibiting PET in the region of the plastoquinone pool. The inhibitor DCMU was used to block electron flow from PSII for the DHQ reaction. Ferricyanide was added to DAD to keep it oxidized and DBMIB was used to inhibit electron flow to FeCN.

The results of our research on the chemical manipulation of plant tolerance to paraquat are not complete. The use of rabbit sera [32] demonstrates the potential for counteracting paraquat toxicity at the chloroplast level. The pyridyl and heme/iron compounds tested in this

study were not suitable for counteracting paraquat toxicity. However, hemin and hematin were effective inhibitors of PET at or near the plastoquinone pool. These heme compounds could be useful in studying the photosynthetic electron transport. Further screening of additional compounds may be successful. Also, continued research in the characterization of the O_2 reduction pathway may provide useful information for the development of a paraquat safener.

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Table 1. Efficacy of various compounds in counteracting paraquat toxicity in isolated pea thylakoids.

Experimental Compounds	% Inhibition by Experimental Compounds.	
	H ₂ O --> PQ/O ₂	H ₂ O --> Fd/NADP
<u>pyridyl compounds</u>		
benzylviologen	0 ± 0	ND
2-anilinopyridine	0 ± 0	ND
1,2-bis(4-pyridyl)ethane	0 ± 0	ND
1,2-bis(4-pyridyl)ethylene	0 ± 0	ND
2-benzoylpyridine	0 ± 0	ND
2-benzylaminopyridine	0 ± 0	ND
1-benzylimidazole	0 ± 0	ND
<u>heme/iron compounds</u>		
rabbit sera	100 ± 0	0 ± 0
hemoglobin	66 ± 3	0 ± 0
ferritin	0 ± 0	ND
hemin	100 ± 0	100 ± 0
hematin	100 ± 0	100 ± 0
ferrocene	0 ± 0	ND

Assay medium was as described in Experimental. The concentrations of PQ, Fd, NADP, hemoglobin, ferritin and the remaining experimental compounds were 10 µM, 100 µg/ml, 10 µM, 10 µM, and 1 mM, respectively. Each value ± standard deviation represents an average of 3 replicates. ND = not determined. All rates were greater than 2.00 µmol O₂ · min⁻¹ · mg Chl⁻¹.

Table 2. Localization of the PET site of inhibition by hemin and hematin.

Hill Reaction	% Inhibition by	
	hemin	hematin
H ₂ O --> PQ/O ₂	100	100
DCIP/Asc/DCMU/SOD --> PQ/O ₂	0	0
DAD/Asc/DCMU/SOD --> PQ/O ₂	0	0
DHQ/SOD --> PQ/O ₂	100	100
H ₂ O --> DAD/FeCN/DBMIB	100	100

Assay medium was as described in Experimental. The concentrations of hemin, hematin, PQ, DCIP, DAD, Asc, DCMU, DHQ, FeCN, DBMIB, and SOD were 100 μ M, 100 μ M, 10 μ M, 1 mM, 1 mM, 5 μ M, 1 mM, 1 mM, 10 μ M, and 150 units, respectively. Each value represents an average of 3 replicates with standard deviation of 0. All rates were greater than 2.00 μ mol O₂ \cdot min⁻¹ \cdot mg Chl⁻¹.

CHAPTER IV

POTENTIAL INVOLVEMENT OF ALKOXYL AND HYDROXYL RADICALS IN THE PEROXIDATIVE ACTION OF SELECTED p-NITRO DIPHENYL ETHER HERBICIDES

Abstract

The potential involvement of hydroxyl and alkoxy radicals in the peroxidative action of the p-nitro diphenyl ether herbicides acifluorfen {5-[2-chloro-4-(trifluoro-methyl)phenoxy]-2-nitrobenzoic acid}, acifluorfen-methyl (methyl ester of acifluorfen), nitrofen [2,4-dichloro-1-(4-nitrophenoxy)benzene], nitrofluorfen [2-chloro-1-(4-nitrophenoxy)-4-(trifluoromethyl)-benzene], and oxyfluorfen [2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(trifluoromethyl)benzene] was evaluated under laboratory conditions. Methional was added to illuminated thylakoids from peas (*Pisum sativum* L., cv Little Marvel) and its oxidation to ethylene was used as an indicator of hydroxyl and alkoxy radical production. Oxyfluorfen stimulated the rate of methional oxidation by 138% at 10 μ M and 175% at 1 mM. This oxyfluorfen-induced stimulation of the rate of methional oxidation was dependent on light, photosynthetic electron transport and hydrogen peroxide since it was not observed under dark conditions or in the presence of DCMU and catalase. Addition of FeEDTA, a catalyst of the Fenton reaction, stimulated the oxyfluorfen-induced enhancement of methional oxidation six-fold suggesting that hydroxyl radicals are synthesized through a Fenton reaction. Acifluorfen, nitrofen and nitrofluorfen inhibited the rate of methional oxidation, whereas acifluorfen-methyl had no effect on the rate of methional oxidation, even at high concentrations (1 mM). Nitrofluorfen at 1

mM was the only p-nitro diphenyl ether herbicide tested to inhibit photosynthetic electron transport of pea thylakoids. In experiments with pea leaf disks, acifluorfen at low concentrations stimulated the rate of methional oxidation, whereas acifluorfen-methyl, nitrofen and nitrofluorfen had no effect. These data indicate that hydroxyl and alkoxy radicals could be involved in the mechanism of cellular damage caused by oxyfluorfen but they are not important for the activity of the diphenyl ether herbicides acifluorfen, acifluorfen-methyl, nitrofen and nitrofluorfen.

INTRODUCTION

The p-nitro substituted diphenyl ether (NDPE) herbicides are known to disrupt membranes (1,2,3,4) and cause cell leakage. Membrane leakage induced by NDPE herbicides is a result of lipid peroxidation (5,6,7,8) and this process requires light (2,4,9). Yet, the mechanism of light activation of NDPE herbicides is not clearly understood. Matsunaka (10) demonstrated that yellow rice mutants were susceptible to nitrofen, an NDPE herbicide, whereas white mutants were tolerant to the herbicide. Similar results were observed with soybean and corn mutants (9). Corn seedlings and cucumber cotyledons treated with norflurazon, a carotenoid biosynthesis inhibitor, became insensitive to acifluorfen (11), and partially tolerant to oxyfluorfen (12). Therefore, it appears that carotenoids are involved in the herbicidal activity of NDPE herbicides. However, an action spectrum of the toxicity of acifluorfen-methyl on Chlamydomonas cells revealed absorbance maxima at 450 nm and 650-670 nm which correspond to carotenoids and chlorophylls (13).

The involvement of photosynthetic electron transport (PET) in the light activation of NDPE herbicides is also controversial. Considerable evidence has been gathered suggesting that PET is not required for NDPE herbicide toxicity. Addition of various PET inhibitors such as DCMU, atrazine, or bentazon to greened cucumber cotyledons treated with acifluorfen-methyl (4) and acifluorfen (12, 14) did not prevent membrane damage induced by these NDPE herbicides. DCMU also could not prevent Chlamydomonas eugametos cells from acifluorfen and oxyfluorfen toxicity (15). Photosynthetically inactive cucumber cotyledons grown under far

red light were hypersensitive to acifluorfen compared to photosynthetically competent cotyledons grown under white light (14).

In contrast to the above findings there is also good evidence that PET may be involved in the herbicidal activity of selected NDPE herbicides. DCMU inhibited the light-dependent peroxidative activity of oxyfluorfen (7, 8). Cells treated with difunon, a carotenoid biosynthesis inhibitor, exhibited the same phytotoxicity to oxyfluorfen as untreated cells (8). This indicates that carotenoids in Scenedesmus cells are not essential for the mode of action of oxyfluorfen. Trapped ESR-signals of radicals were detected in oxyfluorfen-treated spinach thylakoids (16). These signals were eliminated by treatments with DCMU. At present it is not clear how light activates NDPE herbicides and whether PET, along with carotenoids are involved in this light-activation process.

Toxic free radicals have been implicated as the cause of lipid peroxidation induced by light-activated NDPE herbicides. In addition to light, oxygen is also needed for lipid peroxidation (4). However, it is not clearly understood how light-activated NDPE herbicides peroxidize cell membranes. The lipophylic antioxidants, α -tocopherol and ethoxyquin, two well known scavengers of singlet oxygen and hydroxyl radicals (OH \cdot), protected higher plants and algae from peroxidative action of NDPE herbicides (4, 17, 18). Copper penicillamine, a scavenger of superoxide radicals (19) offered marginal or no protection to cucumber cotyledons from the effects of acifluorfen (12, 15). This indicates that the superoxide radical is not involved in acifluorfen action. Duke et al [11]

detected superoxide (O_2^-) and hydrogen peroxide (H_2O_2) synthesis in the mitochondria of acifluorfen-treated cucumber cotyledons using cytochemical techniques. Acifluorfen did not stimulate H_2O_2 synthesis in intact chloroplasts (22). In vitro enhancement of O_2^- production by nitrofen has been observed (20). Normally H_2O_2 and O_2^- cannot peroxidize lipids directly, but in the presence of reduced transition metals, highly reactive hydroxyl radicals ($OH\cdot$) are normally synthesized (21). The $OH\cdot$ radical reacts with unsaturated fatty acids forming additional radicals such as alkyl, alkoxy and peroxy radicals which react further with other lipids, causing a breakdown of lipids (21).

The exact kinds of free radicals which are promoted by NDPE herbicides have not been determined. Orr and Hess (4) proposed that carotenoid-sensitized acifluorfen can directly form a lipid radical which causes lipid peroxidation. The potential involvement of hydroxyl and alkoxy radicals in the peroxidation action of NDPE herbicides has not been examined. In this communication, a sensitive technique for measuring hydroxyl and alkoxy radicals was used to determine if various NDPE herbicides produce these toxic oxygen species and whether PET is involved in the synthesis of these free radicals.

MATERIALS AND METHODS

Plant material

Peas (Pisum sativum cv 'Little Marvel') were grown in a soilless mixture of 1:2:2 of peat:vermiculite:weblite, plus a slow release fertilizer (Osmocote®). Plants were kept in a growth chamber with a 12 hr light/dark cycle at temperatures of 20°C day/17°C night. The light intensity was varied during the day cycle, starting with 40 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and gradually increasing for 3 hours to a maximum intensity of 800 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, which was held constant for 1 hr. Then this process was reversed. After 4 weeks of growth in this environment, pea plants were used for chloroplast isolation.

Chloroplast isolation and photochemical reactions

Chloroplasts were isolated as follows: leaves were gathered from 5-7 plants and macerated for 5 s in a partially frozen extraction medium (75 ml) containing 330 mM sorbitol, 5 mM MgCl_2 , 20 mM MES-NaOH/pH 6.5. The homogenate was filtered through one layer of Miracloth and centrifuged for 1 min at 2,000 x g at 0°C. The pellet was resuspended in a 1:20 dilution of the extraction medium (10 ml) then centrifuged for 1 min at 4,000 x g at 0°C. The pellet was resuspended in an assay medium containing 330 mM sorbitol, 50 mM HEPES-NaOH/pH 7.6, 2 mM MgCl_2 , 1 mM NH_4Cl , and 2 mM EDTA. Hill reaction rates ($\text{H}_2\text{O} \rightarrow \text{MV}/\text{O}_2$) were determined by monitoring changes in O_2 concentration as a function of time using a Gilson-Oxygraph Clark-type oxygen electrode. Assay volumes, light intensities at the surface of the reaction vessel and the

assay temperature were 1.5ml, 2000 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$, and 20 C, respectively. All chemicals were from Sigma Chemical Company, except for sorbitol and MES which were from Calbiochem. Chlorophyll concentrations were determined according to the methods of Arnon (23) and usually ranged from 35-50 μg Chl per assay.

Preparation of leaf disks

Leaf disks were prepared by cutting 2 mm diameter sections with a cork borer from fully expanded pea leaves. Six leaf disks were used for each replicate of each experiment. The leaf disks were placed in a solution of 20 mM NaHCO_3 and 50 mM phosphate buffer, pH 7.6/KOH-HCl. The leaf disks were incubated with the designated herbicide (see Table 3) and methional for 15 min in the dark prior to illumination in gas-tight vials. Leaf disk area was calculated for both sides of the leaf disk.

Methional assay

The method for ethylene determination from methional was based after the method of Beauchamp and Fridovich (24). Methional (2mM) was added to the assay mixture which consisted of the assay buffer described above, depending upon the plant material used (chloroplasts or leaf disks). Methional oxidation to ethylene was carried out in a total volume of 1 ml in 5-ml gas tight vials. Leaf disks or chloroplasts were exposed to saturating light intensities between duplicate sets of two Sylvania 20 W fluorescent lamps (cool white). Reactions usually ran for 8 minutes at 25 C; lights were turned off to terminate the reaction.

Gas-tight syringes were used to take 1 ml gas samples from the head space of each vial. These samples were analyzed for ethylene with a Bendix 2600 and a Varian 3700 gas chromatograph equipped with 1.61 m x 2.0 mm stainless steel columns of 80/100 mesh Poropak N (Supelco, Inc.) and flame ionization detectors. The carrier was pre-purified nitrogen (Airco, Inc.) at a flow of 40 ml·min⁻¹. Detector gas was air and ultra-high purity hydrogen (Airco, Inc.). Operating temperatures for the injection block and detector were 60 and 150 C, respectively. Signals were recorded on a Bendix 1200 recorder. Minimum sensitivity was to 50 pmole of ethylene. An external standard of 30 nmole·ml⁻¹ ethylene in nitrogen was prepared by diluting pure ethylene (Airco, Inc.) in nitrogen (Airco, Inc.) using gas impermeable plastic bags which were obtained from Dr. J. L. Neal, Dept. Biology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

All data presented in Figures and Tables are averages of four replicates in methional assays and three replicates for Hill reaction rate determinations. A standard deviation (SD) for each datum is represented as an error bar in the figures and \pm SD in the tables.

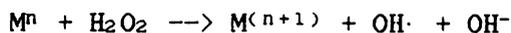
RESULTS AND DISCUSSION

A sensitive technique for detecting oxygen radicals was used to study the involvement of NDPE herbicides in oxygen radical synthesis. This procedure involved gas chromatographic determination of ethylene from the oxy radical-dependent oxidation of methional. The oxy radicals most likely oxidizing methional to ethylene are hydroxyl and alkoxy radicals. Beauchamp and Fridovich (24) were able to oxidize methional with hydroxyl radicals generated from an in vitro Fenton reaction ($\text{H}_2\text{O}_2 + \text{Fe}^{+2} \rightarrow \text{OH}\cdot + \text{OH}^- + \text{Fe}^{+3}$). They also determined that neither H_2O_2 nor O_2^- could oxidize methional. In addition to hydroxyl radicals, alkoxy radicals were found to efficiently oxidize methional while peroxy and alkyl radicals did not (25). It is not clear if singlet oxygen ($^1\text{O}_2$) can oxidize methional to ethylene. Evidence for the oxidation of methional by $^1\text{O}_2$ was presented by Klebanoff and Rosen (26). However, in vitro experiments with $^1\text{O}_2$ generated by Rose Bengal (4,5,6,7-tetrachloro-2',4',5',7'-tetraiodofluorescein) indicated that methional is not oxidized by $^1\text{O}_2$ (27).

Isolated pea thylakoids oxidized methional to ethylene in the absence of NDPE herbicides and the addition of oxyfluorfen increased the rate of methional oxidation (Fig. 1). Significant oxygen radical synthesis was detected at 10 μM oxyfluorfen and increased considerably when oxyfluorfen was present at 1 mM (Fig. 1). Oxyfluorfen concentrations of 10 μM are considered to be herbicidal while 1 mM is a very high concentration. Inhibition of PET by high oxyfluorfen concentrations (100-1000

μM) has been reported (28). However, oxyfluorfen at 1 mM did not inhibit PET in isolated pea thylakoid preparations under the conditions of the present study (Table 1).

These findings show that oxyfluorfen can promote hydroxyl and/or alkoxy radical synthesis in pea thylakoid membranes. Further experiments comparing the oxidation of methional to ethylene by pea thylakoids in the presence or absence of oxyfluorfen were conducted. Data in Table 2 include two controls, one corresponding to thylakoids with no oxyfluorfen and the other corresponding to thylakoids with oxyfluorfen. Each column was compared, on a percent basis, to the appropriate control rate. Chloroplasts, incubated with and without oxyfluorfen in the dark, did not oxidize methional to ethylene (Table 2), indicating that oxyfluorfen induced-stimulation of hydroxyl and/or alkoxy radical synthesis requires light. The PET inhibitor DCMU prevented ethylene formation in irradiated chloroplasts with and without oxyfluorfen (Table 2). This is good evidence that oxyfluorfen-induced stimulation of hydroxyl and/or alkoxy radical synthesis is dependent upon photosynthetic electron transport. Addition of catalase inhibited the rate of methional oxidation by approximately 40% in each set of experiments (Table 2), which strongly suggests that H_2O_2 is a direct precursor for free radical synthesis. In the presence of certain reduced transition metals H_2O_2 , can form hydroxyl radicals (29).



M^n represents a transition metal in a redox state n . When ferrous ions are used, this reaction is known as the Fenton reaction.

The addition of a Fenton catalyst should stimulate the rate of methional oxidation by irradiated chloroplasts with and without oxyfluorfen, since H_2O_2 appears to be a component of methional oxidation. Addition of Fe-EDTA to pea thylakoids in the presence and absence of oxyfluorfen caused approximately a 6-fold increase in the rate of methional oxidation (Table 2). This suggests that a Fenton-type reaction is probably generating the hydroxyl radicals detected by the methional assay. The EDTA chelator is not an inhibitor of Fenton reactions. To the contrary, iron chelated to EDTA is a more effective Fenton catalyst than free iron (30). Sugar alcohols, particularly mannitol, can scavenge hydroxyl radicals (30). However, when sorbitol was replaced with a phosphate/NaCl buffer an increase of only 110% was observed in the rate of methional oxidation (Data not shown). Similarly, Upham and Jahnke (27) observed only a 30% inhibition by mannitol on the rate of methional oxidation by irradiated chloroplasts. They proposed site-specific radical formation within lipid membranes and/or the involvement of alkoxy radicals as possible explanations for the above observation. Site-specific radical formation in lipid membranes also serves as a good explanation of why catalase did not totally inhibit the rate of methional oxidation (Table 2). Because of these results all experiments were conducted with sorbitol as an osmoticum to maintain higher quality chloroplasts.

Superoxide dismutase (SOD) is a specific enzymatic scavenger of the superoxide radical and dismutates O_2^- to H_2O_2 . The addition of SOD to irradiated thylakoids showed no significant differences in the rate of methional oxidation as compared to the control (Table 2). The lack of inhibition by SOD indicates that O_2^- is not directly involved in hydroxyl or alkoxy radical synthesis. Similar results were observed with SOD plus oxyfluorfen (Table 2). From the above results, many similarities between thylakoids in the presence and absence of oxyfluorfen were observed. In both cases H_2O_2 , PET and Fenton catalysts seem to be involved. Unlike paraquat, oxyfluorfen does not reduce O_2 to O_2^- and H_2O_2 [7]. Therefore, oxyfluorfen is probably stimulating hydroxyl and/or alkoxy radical synthesis after the thylakoids initially reduce oxygen.

Oxyfluorfen was also compared to the herbicide methylviologen (paraquat). Methylviologen (MV) is known to produce O_2^- , H_2O_2 , $OH\cdot$ and $RO\cdot$ (19,21,27). Data in Table 2 show that oxyfluorfen can cause oxidation of methional as effectively as MV. SOD has been found to be an effective inhibitor of MV mediated methional oxidation (27). This inhibition was not observed with oxyfluorfen-induced stimulation of free radical synthesis (Table 1). Therefore, it appears that oxyfluorfen is not identical to MV in mediating the oxidation of methional by isolated illuminated thylakoids.

In addition to oxyfluorfen, the NDPE herbicides nitrofen, nitrofluorfen, acifluorfen, and acifluorfen-methyl were tested. Nitrofen,

nitrofluorfen, and acifluorfen decreased the rate of methional oxidation by 50%, 50% and 80%, respectively (Figs. 2 and 3). The methyl ester of acifluorfen did not alter oxygen radical synthesis (Fig. 2a). High concentrations of NDPE herbicides are known to inhibit PET (28,31,32). Therefore, it is possible that at high concentrations of NDPE herbicides, an inhibition of methional oxidation would be expected since the control rate depends upon PET. However, under the conditions of the present study, nitrofen and acifluorfen at 1 mM did not inhibit PET (Table 1). Therefore, the decline in the rate of methional oxidation by these NDPE herbicides can not be attributed to inhibition of PET. Nitrofluorfen did inhibit PET at 1mM (Table 1) and this could explain in part why methional oxidation was inhibited. Overall, no good explanation for the inhibition pattern of the above NDPE herbicides could be found. The only conclusion that could be made from the above observations is that these NDPE herbicides do not produce oxy radicals via the photosynthetic electron transport or by reacting with thylakoid components.

The above experiments were repeated with pea leaf disks to see whether oxygen-radical production could occur by other components in the cell. The results are presented in Table 3. Acifluorfen was the only herbicide to increase methional oxidation at concentrations that were probably not herbicidally significant. Acifluorfen may interfere with the enzymatic mechanisms of plant cells that remove H_2O_2 (33). Since these enzymes are absent in thylakoids and present in leaf disks could explain why an increase in methional oxidation was observed in leaf disks and not thylakoids.

It is not clear how NDPE herbicides are activated by light and how light-activated NDPE herbicides peroxidize membranes. Most studies suggesting that photosynthesis is involved in NDPE herbicide dependent lipid peroxidation were conducted with oxyfluorfen, while results to the contrary were obtained with acifluorfen. An exception to the above generalization is the lack of protection in oxyfluorfen-treated Chlamydomonas cells by PET inhibitors (15). These observations could indicate that species differences may also be an important factor influencing NDPE herbicides. Until experiments are duplicated using more than one NDPE herbicide and plant species, the above experimental discrepancies could remain unexplained. Our results demonstrate that oxyfluorfen interacts with PET of isolated thylakoids to produce hydroxyl and/or alkoxy radicals. It is not clear how oxyfluorfen stimulates hydroxyl and/or alkoxy radical synthesis but a Fenton-type reaction probably plays an important role. Other NDPE herbicides such as acifluorfen, acifluorfen-methyl, nitrofen, and nitrofluorfen do not interact with the thylakoids to produce oxygen radicals. Our data is in agreement with general observations reported in literature that the herbicide activity of oxyfluorfen is dependent on the photosynthetic electron transport while other NDPE herbicides are not.

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Table 1. Inhibitory effects of NDPE herbicides on the photosynthetic electron transport.

NDPE Herbicides	H ₂ O → MV/O ₂ Hill Reaction		% Inhibition
	NDPE Herbicide (mM)		
	0	1	
	(μmol O ₂ · min ⁻¹ · mg Chl ⁻¹)		(%)
Oxyfluorfen	4.21 ± 0.47	4.21 ± 0.47	0
Nitrofen	2.46 ± 0.24	2.46 ± 0.24	0
Nitrofluorfen	3.07 ± 0.36	0.39 ± 0.09	87
Acifluorfen	3.15 ± 0.41	3.15 ± 0.41	0
Acifluorfen-methyl	2.05 ± 0.15	2.05 ± 0.15	0

Experimental conditions as described in Materials and Methods. Each datum represents an average of three replicates ± standard deviation.

Chlorophyll concentration = 43 ug.

Table 2. Methional oxidation by isolated, illuminated pea thylakoids in the absence and presence of oxyfluorfen.

Treatment	Methional Oxidation	
	Oxyfluorfen (uM)	
	0	100
	Percent (%) of Control	
Control	100 ± 7	100 ± 4
No methional	0 ± 0	0 ± 0
No light	0 ± 0	0 ± 0
DCMU (10 uM)	3 ± 1	2 ± 1
Catalase (100 units)	40 ± 12	41 ± 7
FeEDTA _(red) (50 uM)	658 ± 44	521 ± 38
SOD (100 units)	121 ± 23	80 ± 42
Methylviologen (100 uM)	160 ± 7	ND
Oxyfluorfen (100 uM)	175 ± 13	ND

Experimental conditions as described in Materials and Methods. Each datum represents an average of four replicates ± standard deviation. Control rate = 10.9 nmol C₂H₄ · min⁻¹ · mg Chl⁻¹ in the absence of oxyfluorfen. Chlorophyll concentration = 30 ug. ND = no data.

Table 3. The effects of various NDPE herbicides on the rate of methional oxidation in pea leaf disks.

Herbicide Conc. (μM)	Acifluorfen	Acifluorfen-methyl	Nitrofen	Nitrofluorfen
	(pmol C_2H_2 evolution $\cdot \text{min}^{-1} \cdot \text{mm}^{-2}$ leaf disk area)			
0	0.22 \pm 0.04	0.22 \pm 0.04	0.22 \pm 0.04	0.22 \pm 0.04
10	0.25 \pm 0.02	0.22 \pm 0.02	0.21 \pm 0.00	0.30 \pm 0.02
100	0.25 \pm 0.03	0.23 \pm 0.03	0.29 \pm 0.03	0.27 \pm 0.04
1000	0.48 \pm 0.06	0.23 \pm 0.03	0.27 \pm 0.05	0.24 \pm 0.04

Experimental conditions as described in Materials and Methods. Each datum represents an average of three replicates \pm standard deviation.

Chlorophyll concentration of 6 pea leaf disks = 202 μg .

FIGURE LEGENDS

- Fig. 1 The influence of oxyfluorfen on the rate of methional oxidation in isolated pea thylakoids. Control rate = 18.4 nmol C₂H₄ min⁻¹ · mg Chl⁻¹. Chlorophyll concentration = 57 µg.
- Fig. 2a The influence of acifluorfen-methyl on the rate of methional oxidation in isolated pea thylakoids. Control rate = 6.9 nmol C₂H₄ · min⁻¹ · mg Chl⁻¹. Chlorophyll concentration = 15 µg.
- b The influence of acifluorfen on the rate of methional oxidation in isolated pea thylakoids. Control rate = 17.7 nmol C₂H₄ min⁻¹ · mg Chl⁻¹. Chlorophyll concentration = 24 µg.
- Fig. 3 The influence of nitrofen and nitrofluorfen on the rate of methional oxidation in isolated pea thylakoids. Control rate = 9.9 nmol C₂H₄ · min⁻¹ · mg Chl⁻¹. Chlorophyll concentration = 18 µg.

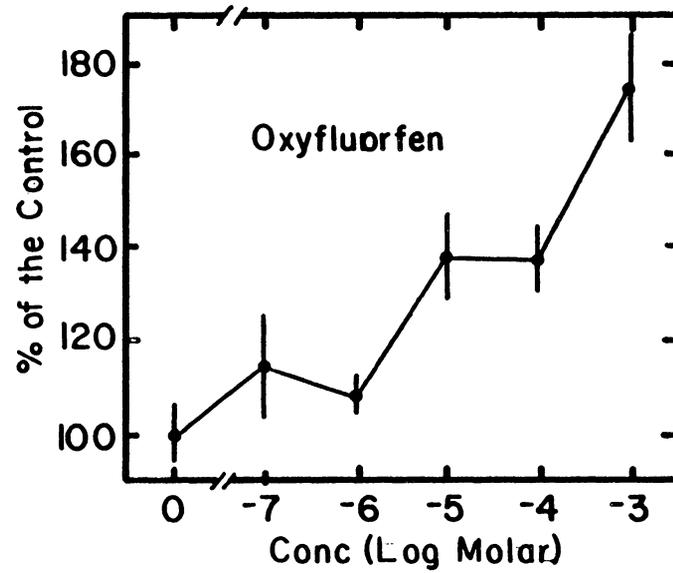


Figure 1. The influence of oxyfluorfen on the rate of methional oxidation in isolated pea thylakoids.

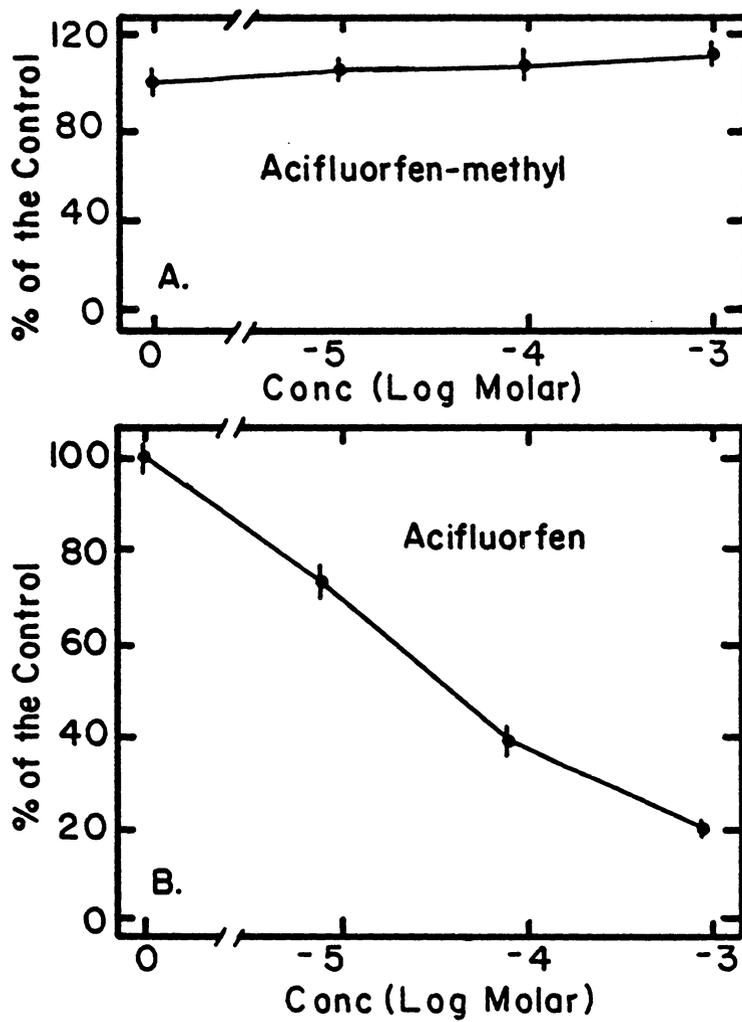


Figure 2a. The influence of acifluorfen-methyl on the rate of methional oxidation in isolated pea thylakoids.
2b. The influence of acifluorfen on the rate of methional oxidation in isolated pea thylakoids.

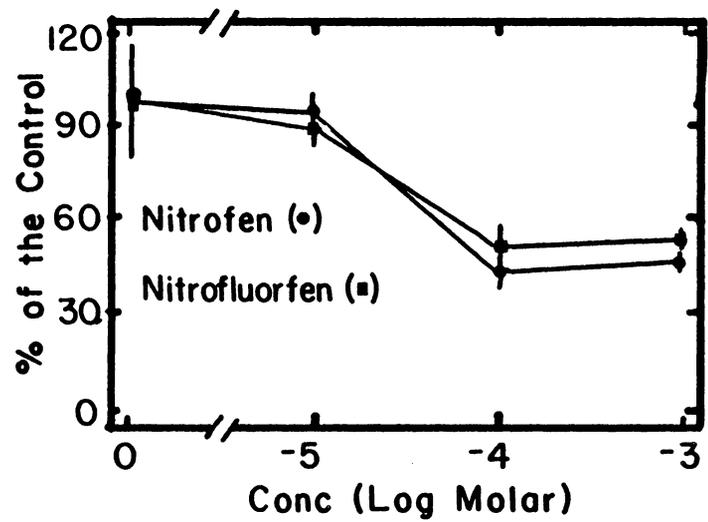


Figure 3. The influence of nitrofen and nitrofluorfen on the rate of methional oxidation in isolated pea thylakoids.

CHAPTER V

DIETHYLDITHIOCARBAMATE, A NEW PHOTOSYSTEM I ELECTRON DONOR OF MEHLER-TYPE HILL REACTIONS.*

Abstract

Diethyldithiocarbamate (DEDTC) does not accept electrons from the photosynthetic electron transport (PET) but can donate electrons to a photosystem I (PSI) Mehler reaction in the presence of the following PET inhibitors: DCMU, DBMIB, and bathophenanthroline. It cannot photoreduce PSI in the presence of cyanide, a PET inhibitor. These data indicate that the site of electron donation is after the plastoquinone pool. Ascorbate is not required for the ability of DEDTC to donate electrons to PSI. There is no photoreductant activity by DEDTC in ferredoxin/NADP Hill reactions. Superoxide dismutase inhibits DEDTC/DCMU or bathophenanthroline \rightarrow methylviologen/ O_2 Mehler reaction. Catalase does not recover the consumed O_2 from a DEDTC/DCMU \rightarrow methylviologen/ O_2 Mehler reaction, indicating O_2^- has not been dismutating into H_2O_2 . These results indicate that superoxide is required for DEDTC ability to donate electrons, therefore DEDTC is limited only to Mehler type reactions.

INTRODUCTION

The use of artificial electron donors and acceptors has been very useful in the study of the chloroplast electron transport system [1, 2]. They have been used in elucidating the sequence of electron transport components, the energy conserving steps, and the topography of the chloroplast membrane. These compounds are also used to locate sites of inhibition by xenobiotics and to develop assays for isolated chloroplasts components [1, 2].

The first artificial electron donor introduced was dichlorophenoldimethylphenol (DCPIP) [3], which donates electrons to photosystem I. Other suitable PSI electron donors include the substituted phenylenediamines [4-6], indamines [7], diaminobenzidine [8, 9], and durohydroquinone [10, 11]. With the exception of durohydroquinone, the above photoreductants require ascorbate as an electron reservoir, which is advantageous in that only catalytic amounts of the donors are required. However, ascorbate will enhance Mehler reactions through superoxide radical (O_2^-)-dependent oxidations of ascorbate [12-14]. Normally $2O_2^-$ will dismutate to $H_2O_2 + O_2$, but in the presence of ascorbate, $2O_2^-$ are reduced to $2H_2O_2$, therefore stimulating Mehler reaction rates. In addition, ascorbate will reduce catalytic amounts of transition metals (ie. Fe^{3+}), and increase the rate of O_2 uptake through a Fenton reaction: $Fe^{3+} + Asc \rightarrow Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH\cdot$ [15]. The addition of superoxide dismutase (SOD) eliminates the side reactions of O_2^- with ascorbate. However, SOD does not eliminate the stimulatory

effects of transition metals, reduced by ascorbate, on Mehler type reactions. Therefore, it would be advantageous to use electron donors that do not require ascorbate when studying Mehler type reactions (reduction of $O_2 \rightarrow O_2^- \rightarrow H_2O_2$). Durohydroquinone is such a donor, but it is susceptible to air oxidation and is only stable for short term experiments (3-4 min.) [10, 11]. It is also sensitive to oxidations by the O_2^- radical making the addition of SOD necessary [10].

It would be of interest to find a PSI electron donor for Mehler type reactions that does not require ascorbate and would not be impaired by O_2^- . A substituted carbamate compound (p-nitroacetophenoxime n-methylcarbamate) was recently reported to function as a PSI electron acceptor [17]. Electron acceptors can often be electron donors if they are in a reduced state [1]. The potential activity of the diethyldithiocarbamate (DEDTC) as an electron donor or acceptor in photosynthesis has not been studied. Diethyldithiocarbamic acid (DEDTC), a copper chelator, is often used for spectrophotometric analysis and separations of trace metals [16] and inhibition of copper containing-enzymes such as polyphenol oxidase [19]. The objectives of this study were to determine (a) whether DEDTC may serve as an electron donor to PSI, (b) whether mediation of electron transport at PSI by DEDTC requires the presence of ascorbate and (c) if O_2^- is detrimental to DEDTC activity, as an electron donor.

MATERIALS AND METHODS

Peas (*Pisum sativum* cv 'Little Marvel') were grown in a soilless mixture of 1:2:2 of peat:vermiculite:weblite, plus a slow release fertilizer (Osmocote®). Plants were kept in a growth chamber with a 12 hr light/dark cycle at temperatures of 20°C day/17°C night. The light intensity was varied during the day cycle, starting with 40 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$ and gradually increasing for 3 hours to a maximum intensity of 800 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$, which was held constant for 1 hour. Then this process was reversed. After 4 weeks of growth in this environment, pea plants were used for chloroplast isolation.

Chloroplasts were isolated as follows: leaves were gathered from 5-7 plants and macerated for 5 sec in a partially frozen extraction medium (75 ml) containing 330 mM sorbitol, 5 mM MgCl_2 , 20 mM MES-NaOH/pH 6.5. The homogenate was filtered through one layer of Miracloth and centrifuged for 1 min at 2,000 x g at 0°C. The pellet was resuspended in a 1:20 dilution of the extraction medium (10 ml) then centrifuged for 1 min at 4,000 x g at 0°C. The pellet was resuspended in an assay medium containing 330 mM sorbitol, 50 mM HEPES-NaOH/pH 7.6, 2 mM MgCl_2 , 1 mM NH_4Cl , and 2 mM EDTA.

Hill reaction rates were determined by monitoring changes in O_2 concentration as a function of time using a Gilson-Oxygraph Clark-type oxygen electrode. Assay volumes, light intensities at the surface of the reaction vessel and the assay temperature were 1.5ml, 2000

$\mu\text{E}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$, and 20°C , respectively. All chemicals were from Sigma Chemical Company, except for sorbitol and MES which were from Calbiochem. Chlorophyll concentrations were determined according to the methods of Arnon [18] and usually ranged from 35-50 μg Chl per assay.

RESULTS AND DISCUSSION

Diethyldithiocarbamate (DEDTC) was found to be unable to accept electrons in a chloroplast preparation containing no exogenous electron acceptors. DEDTC reverses the inhibition of a methylviologen (MV)-mediated Mehler reaction caused by DCMU (Fig.1, Table 1), DBMIB (Table 1), and BPT (Table 1). The inhibition of a MV-mediated Mehler reaction by HCN is not alleviated by DEDTC (Table 1). The photosynthetic electron transport (PET) inhibitor, DCMU, blocks electron flow at the Q_B binding site of PSII [20]. DBMIB inhibits PET at the oxidizing side of the plastoquinone pool [21, 22], while bathophenanthroline (BPT) inhibits electron flow at Cyt f or the "Rieske" Fe-S center [23, 24]. HCN blocks electron flow at plastocyanin (PCN) [25, 26]. The reversal of inhibitions at the Q_B binding site, Cyt f, and oxidizing side of PQ but not PCN with the addition of DEDTC, indicates that DEDTC donates electrons after the PQ pool. The rate of a DEDTC/DBMIB \rightarrow MV/ O_2 Mehler reaction is less than reactions containing DCMU or BPT (Table 1). Thio-compounds are known to interfere with DBMIB inhibition of PET [27], which means DBMIB could interfere with the activity of thio-compounds such as DEDTC. Ascorbate was not required and DEDTC was stable for many days. This makes DEDTC valuable when electron transport studies of a Mehler reaction must be done in the absence of ascorbate.

The apparent PET rate should double when H_2O is replaced with DEDTC since two electrons from H_2O will cause the consumption of $1/2 O_2$ into H_2O_2 [13], while two electrons from DEDTC will have a net consumption of

1 O_2 into H_2O_2 . However, when DEDTC replaces H_2O as an electron donor, the rate does not double at 100mM DEDTC (Table 1). The concentration of DEDTC in Table 1 has been found to be saturating for these experiments. An exponential increase in the rate of the Mehler reaction as a function of the DEDTC concentrations was observed (Fig. 2). This biphasic response suggests that a second factor may be involved in the photoreduction of PSI by DEDTC, particularly at lower concentrations.

One such factor may be O_2^- . Durohydroquinone, a PSI photoreductant is an example of a donor which requires no ascorbate but is sensitive to the O_2^- radical [10]. Superoxide inhibits the overall durohydroquinone $\rightarrow MV/O_2$ reaction, and SOD reverses the inhibition. When SOD is added to a DEDTC/DCMU $\rightarrow MV/O_2$ or DEDTC/BPT $\rightarrow MV/O_2$ reaction, the reaction was unexpectedly inhibited (Table 2). This shows that O_2^- was not inhibiting these reactions, and does not explain the reduction in the predicted rate. In contrast, it appears that O_2^- is essential for DEDTC caused photoreduction. The possibility that DEDTC chelates with the copper from SOD and the Cu-DEDTC chelate becomes ineffective was considered. Data in Table 2 show that additions of copper salts ($CuCl_2$ & $CuSO_4$) did not affect DEDTC's efficacy to donate electrons to PSI, ruling out the above hypothesis.

If O_2^- is essential for the efficacy of DEDTC to donate electrons to PSI, one can predict that DEDTC will not donate electrons to a $H_2O \rightarrow Fd/NADP$ reaction, since no superoxide is produced in this reaction.

DEDTC did not reverse the inhibitory effects of DCMU on a $H_2O \rightarrow$ Fd/NADP reaction (Fig.3). This supports further the hypothesis that O_2^- is essential for DEDTC's ability to donate electrons. The uptake of oxygen, exhibited in Fig.3 after the addition of DEDTC is due to the ability of the chloroplasts to reduce oxygen in the absence of an exogenous electron acceptor (Fig.4) and DEDTC can reverse the inhibition of this reaction by DCMU. The assay chamber was washed several times with 10% Micro-cleaner (International Products Corp.), to assure that MV contamination was not a reason for the results observed in Fig. 4.

If O_2^- is required for the reduction of DEDTC, very little dismutation of O_2^- to H_2O can take place. The use of catalase can determine how much H_2O_2 is present. In a $H_2O \rightarrow MV/O_2$ Mehler reaction, catalase liberated 100% of the O_2 incorporated into H_2O_2 . While in a DEDTC $\rightarrow MV/O_2$ Mehler reaction, only $14 \pm 7\%$ of the O_2 was recovered. This indicates that H_2O_2 is not being formed and is probably the result of O_2^- donating electrons to DEDTC as expressed in eq. 1 and 2.



At lower concentrations of DEDTC, it becomes essential that O_2^- reduces the oxidized DEDTC, while at higher concentrations an excess of reduced DEDTC minimizes the need for O_2^- . This allows O_2^- to undergo normal dismutation which gives a greater apparent electron transport rate as observed in Fig.2. The implications of eq 1 and 2 suggest that

the redox state of DEDTC is dependent on the presence or absence of O_2^- .

Absorption spectra of reduced and oxidized DEDTC are given in Fig.5. At a wavelength of 235 nm, a large change in absorbance is observed between the oxidized and reduced states of DEDTC. The assay medium and the assay medium plus $K_3(FeCN)_3$ were used as blanks for reduced and oxidized DEDTC, respectively. The absorbance of the assay medium and assay medium plus DEDTC and $K_3(FeCN)_3$ at 235 nm when blanked against water was 1.1 and 2.3, respectively. $K_3(FeCN)_3$ did not absorb light at 235 nm when water was used as a blank. Therefore, the effects of O_2^- on the redox state of DEDTC can be determined by monitoring absorbance changes at 235 nm. In a DEDTC/DCMU \rightarrow MV/ O_2 reaction, O_2^- is produced and DEDTC, according to eq.2, will predominantly be in the reduced state. The addition of SOD to this reaction will remove the O_2^- and the oxidized DEDTC will remain in this redox state (eq.1).

A DEDTC/DCMU \rightarrow MV/ O_2 reaction was conducted in the absence and presence of SOD for seven minutes. Following the reaction, the chloroplasts were centrifuged out of the assay medium. An aliquot of the supernatant was taken for an absorbance measurement at 235 nm. In the O_2^- (presence of SOD) indicates the oxidation of DEDTC as predicted in eq.1. The absorbance of the supernatant of the control (DEDTC added to chloroplasts in the dark) was 0.063. Therefore, the absorbance reading of 0.036 which was from chloroplasts reacted in the presence of O_2^- (absence of SOD) indicated that the DEDTC was in the reduced state as predicted in eq.2. The above experiments were repeated and absorbance

readings were taken at 255 nm. At 255 nm there should be no change in absorbance between the oxidized and reduced states of DEDTC (Fig.5). The resulting absorbance measurements at 255 nm were 0.079, 0.080, 0.079 for the control, -SOD, +SOD, respectively. The DEDTC purchased from Sigma Chemical Co. was 99% pure. The 1% impurities were mainly inorganic compounds. This makes it highly unlikely that an impurity is responsible for the observed responses in Table 1 and Fig. 1 and 2.

DEDTC will not replace or become more important than existing PSI reductants but it should be of use in many photochemical studies with chloroplasts particularly in experiments that require the absence of ascorbate. Furthermore, DEDTC is a stable molecule and could facilitate chloroplast experiments which require a more extensive time period than can be accomplished with durohydroquinone.

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Table 1. The effects of DEDTC on Mehler reaction rates in the presence of various PET inhibitors.

Control Rates		Experimental Rates	
H ₂ O → MV/O ₂		DEDTC + Inhibitor → MV/O ₂	
(-μmol O ₂ ·min ⁻¹ ·mg Chl ⁻¹)	Inhibitor	(–μmol O ₂ ·min ⁻¹ ·mg Chl ⁻¹)	
4.54 ± 0.26	DCMU	3.82 ± 0.36	
2.42 ± 0.51	HCN	0.29 ± 0.46	
2.87 ± 0.13	DBMIB	1.11 ± 0.31	
3.43 ± 0.14	BPT	3.68 ± 0.20	

Experimental conditions were as described in Materials and Methods. Mehler reaction rates in the presence of the respective inhibitor alone (-DEDTC) were zero. Concentrations of DEDTC, MV, DCMU, HCN, DBMIB, and BPT were 10 mM, 100 μM, 10 μM, 50 mM, 10 μM, and 50 μM, respectively. Each datum represents an average of at least 3 replicates ± standard deviation.

Table 2. The effects of SOD and copper on DEDTC + DCMU \rightarrow MV/O₂ Mehler reactions.

DEDTC Conc.(mM)	Rate ($-\mu\text{mol O}_2 \cdot \text{min}^{-1} \cdot \text{mg Chl}^{-1}$)	
	-SOD	+SOD
0	4.30 \pm 0.64	4.30 \pm 0.64
10	0.36 \pm 0.04	00.0 \pm 0.00
100	2.93 \pm 0.21	1.52 \pm 0.18
	-CuCl ₂	+CuCl ₂
10	0.84 \pm 0.10	1.16 \pm 0.19
	-CuSO ₄	+CuSO ₄
10	0.91 \pm 0.24	0.80 \pm 0.22

Experimental conditions were as described in Materials and Methods. Concentrations of SOD, MV, DCMU, CuCl₂ and CuSO₄ were 500 units, 100 μM , 10 μM , 100 μM and 100 μM , respectively. Each datum represents the average of 3 replicates \pm standard deviation.

FIGURE LEGENDS

- Fig.1 Oxygen electrode trace of a DEDTC/DCMU \rightarrow MV/O₂ Mehler reaction. Experimental conditions were as described in Materials and Methods. Concentration of MV was 100 μ M. This experiment was repeated at least three times, giving the same results.
- Fig.2 The effects of various DEDTC concentrations on DEDTC/DCMU \rightarrow MV/O₂ Mehler reaction rates. Experimental conditions were as described in Materials and Methods. Each datum represents an average of three replicates. The error bars are the standard deviation of the three replicates. Concentrations of DEDTC, MV, and DCMU were 10 mM, 100 μ M, and 15 μ M, respectively.
- Fig.3 Oxygen electrode trace of a DEDTC/DCMU \rightarrow Fd/NADP Hill reaction. Experimental conditions were as described in Materials and Methods. Concentrations of Fd and NADP were 50 μ M and 1 mM, respectively. This experiment was repeated three times, giving the same results.
- Fig.4 Oxygen electrode trace of a chloroplast preparation that contained no exogenous electron acceptors. Experimental conditions were as described in Materials and Methods. This experiment was repeated three times, giving the same results.

Fig.5 Absorption spectra of reduced and oxidized DEDTC. The reduced DEDTC was gased with nitrogen for 20 minutes and the oxidized state of DEDTC was maintained with excess KFeCN. A Beckman Du-6 spectrophotometer was used. Blanks contained the assay medium (+ KFeCN for oxidized DEDTC curve). Concentrations of DEDTC and KFeCN were 100 μM and 150 μM , respectively. This experiment was repeated three times, giving the same results.

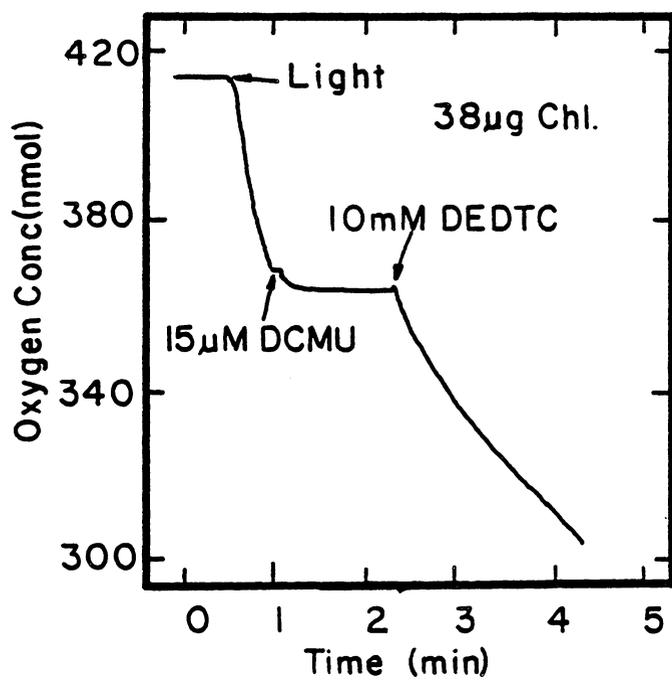


Figure 1. Oxygen electrode trace of a DEDTC/DCMU
-- MV/O₂ Mehler reaction.

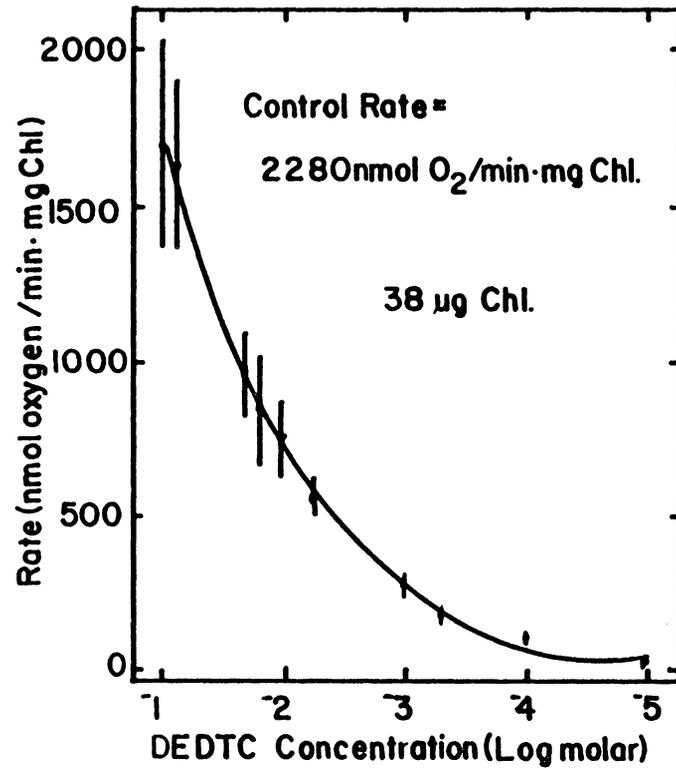


Figure 2. The effects of various DEDTC concentrations on DEDTC/DCMU -- MV/O₂ Mehler reaction rates.

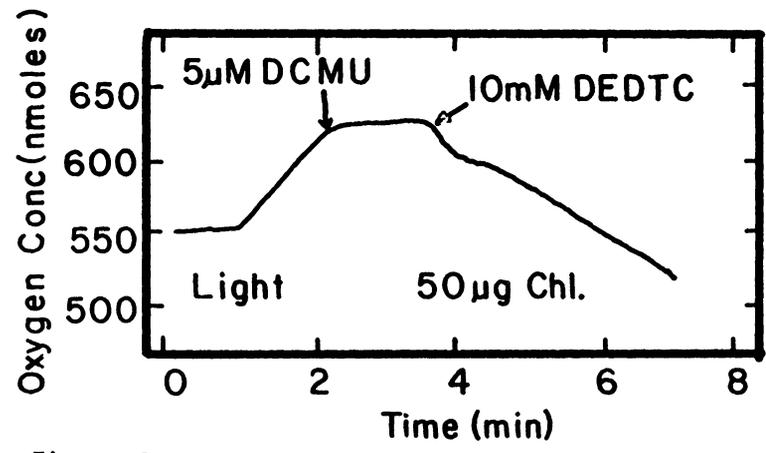


Figure 3. Oxygen electrode trace of a DEDTC/DCMU -- Fd/NADP Hill reaction.

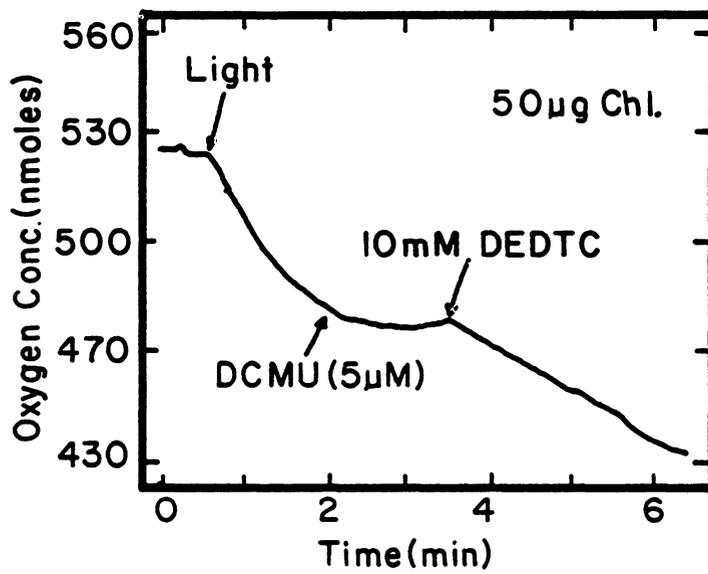


Figure 4. Oxygen electrode trace of a chloroplast preparation that contained no exogenous electron acceptors.

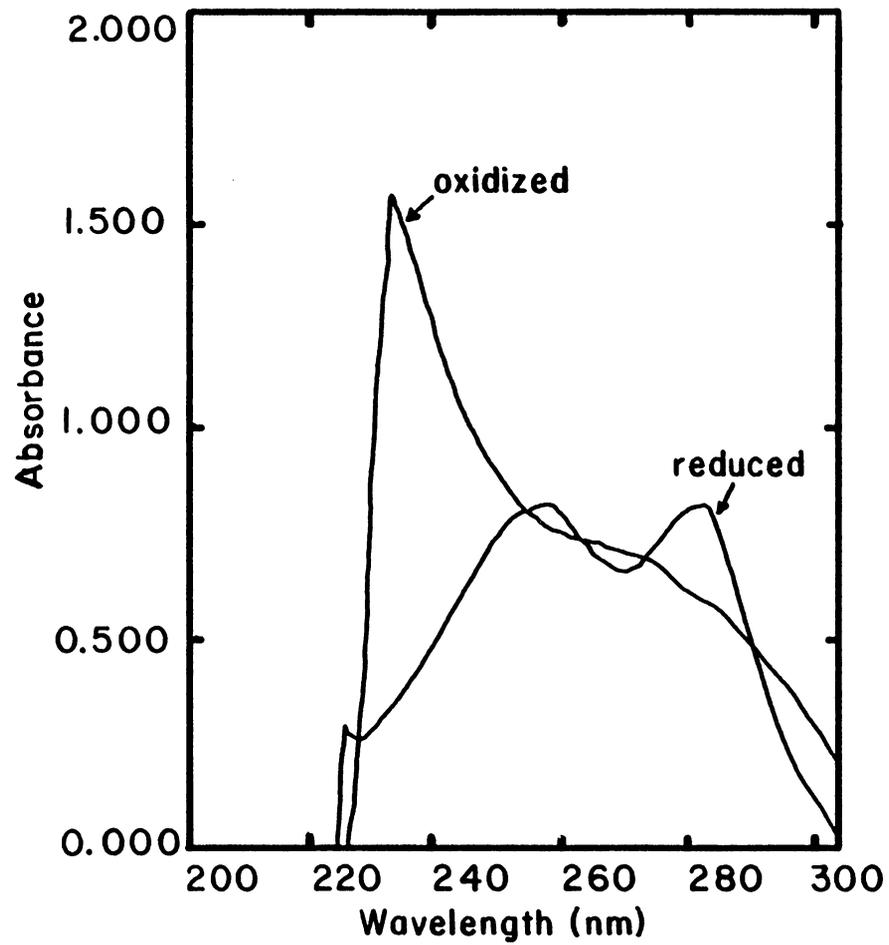


Figure 5. Absorption spectra of reduced and oxidized DEDTC.

CHAPTER VI
SUMMARY AND CONCLUSIONS

Photosystem I (PSI) is known to photoreduce oxygen to a superoxide ion and hydrogen peroxide. This reaction was first discovered by Mehler [1] and has since been termed the "Mehler reaction". The herbicide paraquat (methylviologen) stimulates the Mehler reaction. The result is an increase in activated oxygen with consequent lipid peroxidation. Paraquat is reduced by PSI and oxygen oxidizes the reduced paraquat inducing the formation of superoxide. It is not known which PSI components reduce paraquat. Another class of herbicides peroxidizes lipids in the presence of oxygen and light. These are the p-nitrodiphenyl ether (NDPE) herbicides. The mechanism of lipid peroxidation induced by NDPE herbicides is not known. The results of this dissertation give some insight into the unknown areas discussed above.

An unidentified compound from rabbit sera was effective in inhibiting the photosynthetic electron transport (PET) of spinach chloroplasts. This inhibitor blocked electron flow of PSI in Mehler type reactions (Table 1, Chap. 2). It did not inhibit a PSII Hill reaction or the photoreduction of PSI electron acceptors that do not promote Mehler reactions (Table 1, Chap. 2). These results indicate the existence of a branch in electron flow on the reducing side of PSI. It is not known where this branch occurs. However, branched (parallel) electron flow through the iron-sulfur centers has been reported [2]. It can be specu-

explain the results of Table 1, Chap. 2. This branch of electron flow that reduces oxygen independently of NADP reduction is termed in this report as the Mehler reaction pathway.

An inhibitor of the Mehler reaction pathway could offer practical applications if it could traverse leaf cuticles, cell walls and cell membranes. This inhibitor could possibly counteract paraquat toxicity. Such a compound would be termed an herbicide safener. Herbicide safeners are currently used to introduce or increase crop selectivity in herbicide tolerance. A good example of this is the treatment of crop seeds with a chemical safener which protects crop seedlings in the field from herbicide injury. An inhibitor of the Mehler reaction pathway could also be used to answer basic questions such as: is the Mehler reaction pathway essential for the plant at various growth stages or can plants be protected from stresses such as herbicides through partial inhibition?

Two major groups of compounds were screened for possible counteraction of paraquat toxicity at the chloroplast level. These were non-herbicidal pyridyl analogues of paraquat and heme/iron derivatives. It was postulated that pyridyl compounds could act as paraquat antagonists by inhibiting the Mehler reaction by binding to the active site of paraquat reduction. The compounds tested were: benzylviologen, 2-anilinopyridine, 1,2-bis(4-pyridyl)ethane, 1,2-bis-(4-pyridyl)ethylene, 2-benzoylpyridine and 2-benzylaminopyridine. Unfortunately, none of these compounds inhibited the Mehler reaction (Table, Chap. 3). Heme/iron

derivatives were chosen based on results from Chapter 1, which indicated that the inhibitor of the Mehler reaction was from the hemolyzed portion of rabbit sera. The compounds tested were ferrocene, ferritin, hemoglobin, hemin and hematin. Ferrocene and ferritin did not inhibit a methylviologen-mediated Mehler reaction (Table 1, Chap. 3), but hemoglobin, hemin and hematin did (Table 1, Chap. 3). However, heme and hematin also inhibited NADP reduction (Table 1, Chap. 3), indicating that these compounds are not inhibiting the Mehler reaction pathway. Hemoglobin did not inhibit NADP reduction (Table 1, Chap. 3) and probably blocks electron flow at the Mehler reaction pathway. Although hemoglobin can partially counteract paraquat toxicity, it cannot be used on a live plant since it cannot traverse cell walls or membranes. Heme and hematin inhibition of PET was further characterized. The results of Table 2, Chap. 3 suggest that inhibition is occurring at or near the plastoquinone pool.

Free radicals have been implicated as the cause of NDPE herbicide-induced lipid peroxidation [3,4,5,6] but the exact kinds of free radicals involved have not been determined. The methional assay was used to determine if alkoxyl and hydroxyl radicals are induced by NDPE herbicides. Methional is oxidized to ethylene by hydroxyl and alkoxyl radicals [7] and the ethylene is detected by gas chromatography. This assay revealed that the following NDPE herbicides do not promote hydroxyl and alkoxyl radicals in isolated illuminated thylakoids: acifluorfen and acifluorfen-methyl (Fig. 2a & b, Chap. 4), nitrofen and nitrofluorfen (Fig. 3, Chap. 4). However, the NDPE herbicide oxyfluorfen does promote

synthesis of hydroxyl and/or alkoxy radicals in isolated illuminated thylakoids (Fig. 1, Chap. 4). Catalase inhibited and a Fenton-catalyst stimulated the rate of methional oxidation (Table 1, Chap. 4). The photosynthetic electron transport was also essential for oxyfluorfen induced methional oxidation (Table 1, Chap. 4). Unlike paraquat, oxyfluorfen does not reduce oxygen to superoxide and hydrogen peroxide [8]. Therefore, oxyfluorfen is probably interacting with oxygen species after the reduction of oxygen to superoxide. Isolated, illuminated thylakoids can reduce oxygen in the absence of artificial electron acceptors (Fig. 4, Chap. 5) thus, supplying the reduced oxygen interacting with oxyfluorfen. The exact mechanism of oxyfluorfen interaction with thylakoid-derived oxygen reduction products cannot be determined from the above data and/or from results reported in literature.

Diethyldithiocarbamate (DEDTC) is commonly used as an hydroxyl and alkoxy radical scavenger [7]. In addition to its ability to scavenge these free radicals, DEDTC is reported here for the first time as capable of donating electrons to PSI (Fig. 1 and 2, Chap. 5). Inhibitors such as diuron (DCMU), dibromothymoquinone (DBMIB) and bathophenanthroline (BPT) did not block a DEDTC \rightarrow MV/O₂ Mehler reaction (Table 1, Chap. 5). Cyanide did effectively block this Mehler reaction. These data indicate that DEDTC is donating electrons to PSI after the plastoquinone pool. Superoxide dismutase (SOD) also inhibited a DEDTC \rightarrow MV/O₂ Mehler reaction (Table 2, Chap. 5) suggesting that superoxide is required for this reaction. DEDTC did not donate electrons to a NADP Hill reaction which does not generate superoxide. Oxidized DEDTC exhi-

bits greater absorbance at 235 nm than reduced DEDTC (Fig. 5, Chap. 5). DEDTC plus illuminated chloroplasts, in the absence of superoxide (presence of SOD), had a greater absorbance value than DEDTC in the presence of superoxide (absence of SOD). These data suggest that DEDTC donates electrons to PSI of Mehler-type reactions and the oxidized DEDTC is reduced by superoxide. This cyclic system for Mehler reactions may be an important tool in studying various aspects of PSI electron transport components related to Mehler reactions.

Many of the above results are illustrated schematically in the diagram of Fig. 1, Chap. 6. Future research is necessary for verifying and describing the Mehler reaction pathway in more detail. Monoclonal antibodies that have been chosen for their ability to selectively inhibit the Mehler reaction pathway would be very useful in studying this pathway. Monoclonal antibodies could be used to isolate the electron transport components of the Mehler reaction pathway which would make possible the characterization of these components. Diethyldithiocarbamate may be a useful compound in studying the Mehler reaction pathway of isolated thylakoids or isolated PSI particles. Future studies on the NDPE herbicide oxyfluorfen are necessary to determine how it interacts with the Mehler reaction. The use of other free radical scavengers and in vitro experiments generating superoxide radicals, hydrogen peroxide, Fenton reactions, and Haber-Weiss reactions could give considerable insight to oxyfluorfen's mechanism of action. It appears from the above results that the Mehler reaction is not involved in the mode of action of other diphenylether herbicides. Carotenoids and the mitochondria

seem to be involved in the mode of action of acifluorfen [9]. Therefore, future research of acifluorfen and other NDPE herbicides should focus on the mitochondria and carotenoids.

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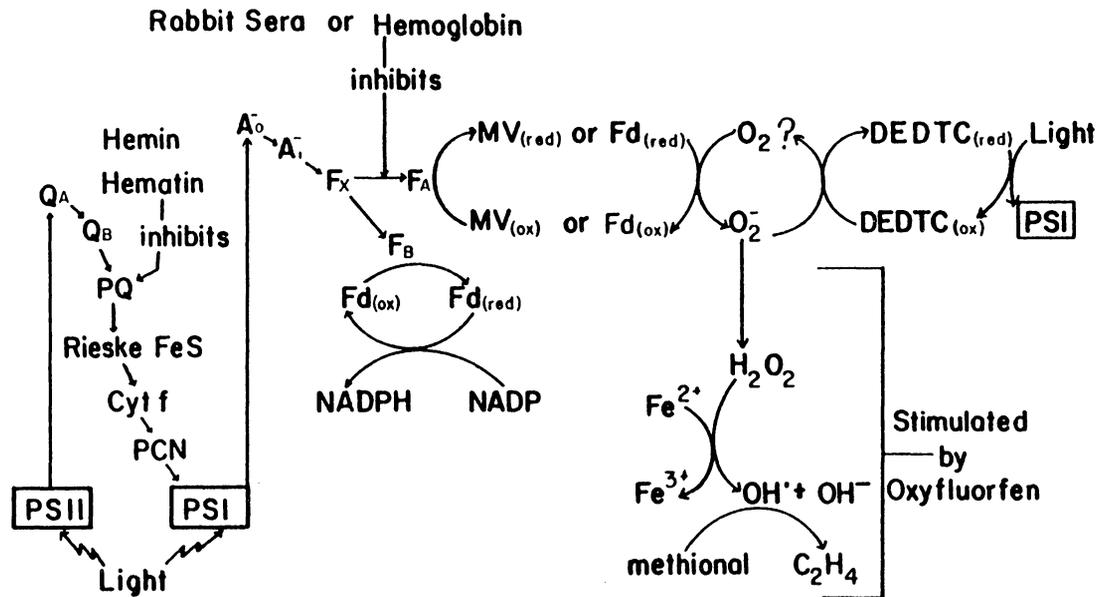


Figure 1. Schematic summary of results for the interaction of methylviologen, oxyfluorfen, DEDTC, hemin and hematin with the photosynthetic electron transport.

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