PHD

The role of oxygen in the herbicidal action of paraquat.

Youngman, Richard J.

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THE ROLE OF OXYGEN IN THE
HERBICIDAL ACTION OF PARAQUAT

Submitted by Richard J. Youngman
for the degree of Ph.D.
of the University of Bath
1980

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To my parents

"What is a weed? A plant whose virtues have not yet been discovered."

Ralph Waldo Emerson (1803-1882)
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Finally, I should like to thank Judy Harbutt and Jean Pitman for their efforts in typing this thesis.
ABSTRACT

The mode of action of paraquat has been studied with particular reference to the roles of superoxide and hydrogen peroxide. A variety of redox compounds was investigated for an ability to mediate chloroplastic oxygen reduction via photosynthetic electron transport. In spite of the relatively wide range of chemical types studied, most compounds were able to catalyse superoxide and hydrogen peroxide formation, but this was not in direct proportion to their one-electron redox potentials. The most active compounds were also shown to stimulate oxygen uptake in isolated asparagus mesophyll cells and in a whole plant screen, produced phytotoxic symptoms reminiscent of paraquat treatment.

The role of superoxide in the herbicidal action of paraquat in flax cotyledons was investigated using penicillamine-copper complex (PA-Cu) which possessed superoxide dismutase (SOD) activity. Paraquat-induced chlorophyll and carotenoid pigment breakdown were inhibited by PA-Cu. The evolution of ethane, as an indicator of herbicide induced membrane lipid peroxidation was inhibited by the complex and correlated with chloroplast fatty acid levels notably linolenic acid, isolated from treated cotyledons. Similarly, ultrastructural examination revealed that cellular damage was markedly reduced by PA-Cu. These results indicated that the paraquat stimulation of superoxide production was of major importance in the herbicidal action of the compound.

The simple hydrocarbons ethylene and ethane are produced during wounding and lipid peroxidative reactions respectively.
and active oxygen species are believed to be involved in both the biosynthetic pathways. A series of model reactions showed that ethylene production from methionine and pyridoxal phosphate was promoted by SOD, PA-Cu and ferredoxin, but was inhibited by catalase and paraquat. In contrast, ethane formation was stimulated by α-linolenic acid, paraquat and DCMU, but was inhibited by SOD, PA-Cu and the carotenoid, crocin. The results indicated that ethylene and ethane were formed via different biosynthetic pathways. The mechanism of production of ethylene appeared to involve hydrogen peroxide, whereas ethane formation required superoxide and/or singlet oxygen.

A study of the mechanism of resistance of a biotype of Conyza linefolia to paraquat showed that tolerance was not based on reduced uptake of the herbicide. A comparison of the SOD enzymes showed that the paraquat resistant biotype possessed almost three times the amount of SOD found in susceptible plants. This was shown to be due at least in part, to the additional presence of two cyanide-insensitive isozymes in the resistant plant. CO₂ fixation in the resistant biotype was stimulated on prolonged incubation with paraquat. A mechanism of herbicide resistance is proposed based on a decreased reduction of paraquat by the chloroplast which could account for the observed differences in CO₂ fixation and SOD levels between the biotypes.

In summary, superoxide would appear to occupy a central role in the herbicidal action of paraquat, but this does not preclude the possible involvement of other active oxygen species, notably singlet oxygen.
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<tr>
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<th>Full Form</th>
<th>Description</th>
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<td>ATP</td>
<td>adenosine triphosphate</td>
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</tr>
<tr>
<td>ATPase</td>
<td>adenosine triphosphatase</td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
<td></td>
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<tr>
<td>CF&lt;sub&gt;1&lt;/sub&gt;</td>
<td>coupling factor 1</td>
<td></td>
</tr>
<tr>
<td>CMU</td>
<td>3-(4-chlorophenyl)-1,1-dimethylurea</td>
<td></td>
</tr>
<tr>
<td>CPM</td>
<td>counts per minute</td>
<td></td>
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<tr>
<td>DABCO</td>
<td>1,4-diazabicyclo(2,2,2)octane</td>
<td></td>
</tr>
<tr>
<td>DAD</td>
<td>diaminodurene</td>
<td></td>
</tr>
<tr>
<td>DBMIB</td>
<td>dibromothymoquinone</td>
<td></td>
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<tr>
<td>DCMU</td>
<td>3-(3,4-dichlorophenyl)-1,1-dimethylurea</td>
<td></td>
</tr>
<tr>
<td>DCPIP</td>
<td>dichlorophenol indophenol</td>
<td></td>
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<tr>
<td>DEGS</td>
<td>diethylene glycol succinate</td>
<td></td>
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<tr>
<td>DIMEB</td>
<td>2,3-dimethyl-5,6-methylenedioxy-p-benzoquinone</td>
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<tr>
<td>DMAP</td>
<td>4-dimethylaminophenol</td>
<td></td>
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<tr>
<td>DPC</td>
<td>diphenylcarbazide</td>
<td></td>
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<tr>
<td>E&lt;sub&gt;1&lt;/sub&gt;</td>
<td>one-electron redox potential</td>
<td></td>
</tr>
<tr>
<td>EDAC</td>
<td>1-ethyl-3-(3'-dimethylaminopropyl)-carbodiimide</td>
<td></td>
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<tr>
<td>EDAX</td>
<td>electron dispersive analysis of X-rays</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
<td></td>
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<tr>
<td>esr</td>
<td>electron spin resonance</td>
<td></td>
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<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
<td></td>
</tr>
<tr>
<td>GDA</td>
<td>glutaraldehyde</td>
<td></td>
</tr>
<tr>
<td>GLC</td>
<td>gas-liquid chromatography</td>
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<tr>
<td>GSH</td>
<td>reduced glutathione</td>
<td></td>
</tr>
<tr>
<td>GSSG</td>
<td>oxidised glutathione</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid</td>
<td></td>
</tr>
<tr>
<td>IRGA</td>
<td>infra-red gas analyser</td>
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MES 2-(N-morpholino) ethane sulphonic acid
NADH reduced nicotinamide dinucleotide
NADP nicotinamide dinucleotide phosphate
NADPH reduced nicotinamide dinucleotide phosphate
NBT nitroblue tetrazolium
NMR nuclear magnetic resonance
P-430 primary electron acceptor of PSI
P-680 reaction centre trap of PSII
P-700 reaction centre trap of PSI
PA-Cu penicillamine-copper complex
3-PGA 3-phosphoglycerate
PNDA p-nitrosodimethylaniline
POPOP 1,4 di [2-(5-phenyloxazoyl)] benzene
PPO 2,5-diphenyloxazole
PQ paraquat, 1,1'-dimethyl-4,4'bipyridylium dichloride
PSI photosystem I
PSII photosystem II
PVP polyvinylpyrrolidone
Q primary acceptor of PSII
RUDP ribulose diphosphate
SDS sodium dodecyl sulphate
S\textsubscript{n} charge accumulating enzyme associated with PSII
SOD superoxide dismutase
STEM scanning transmission electron microscope
TEMED N,N,N'N'tetramethylethylene diamine
TLC thin layer chromatography
tricine N- (trishydroxymethyl) propane-1,3-diol
TMPD N,N,N'N'-tetramethyl-p-phenylenediamine
TRIS 2-amino-2-(hydroxymethyl) propane-1,3-diol
X primary electron acceptor of PSI

$^{3}\Sigma_{g}^{-}$ ground state dioxygen

$^{1}\Gamma_{g}^{+}$ high energy singlet excited oxygen

$^{1}\Delta_{g}$ low energy singlet excited oxygen
LIST OF PUBLICATIONS

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Oxygen activation in chloroplasts: models for in vivo observations.

Youngman, R.J. and Dodge, A.D. (1979)*
Mechanism of paraquat action: inhibition of the herbicidal effect by a copper chelate with superoxide dismutating activity.
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Active oxygen species in herbicide action.

* copy included at end of thesis.
INTRODUCTION
1. PHOTOSYNTHESIS

Photosynthesis is the mechanism by which solar energy is used to reduce carbon dioxide resulting in the formation of sugars and eventually a great range of biological molecules. The total amount of solar energy reaching the earth is $3 \times 10^{24}$ J per annum, compared with the world energy expenditure of $10^{20}$ J. Conventional methods of agriculture yield about 3% maximum conversion efficiency, thus in theory, 0.1% of the earth's surface could provide by photosynthesis, the total energy required by the world. It is clear that photosynthesis is a process of fundamental importance to all forms of life, which is reflected in the considerable interest that has been shown in the subject over the past few decades.

1.1 Discovery of Photosynthesis

The process of photosynthesis was demonstrated in a number of empirical studies, during the eighteenth and nineteenth centuries. However, it was not until the 1930's that Hill succeeded in isolating chloroplasts which were able to reduce artificial electron acceptors (Hill, 1937, 1939), thus laying the foundations for the present knowledge of photosynthesis. In the intervening years since Hill's initial observations, chloroplast isolation procedures have greatly improved and it is now known that the entire photosynthetic process occurs in this organelle. Photosynthesis is instigated by the photolysis of water which forms oxygen. The reducing power derived from this reaction is used to generate ATP and NADPH which are required as cofactors in
the reduction of CO₂. The absorption of light and all processes leading to the formation of NADPH occur in the thylakoid lamellae, whereas the subsequent reactions of CO₂ assimilation are confined to the chloroplast stroma.

1.2 Primary Processes and Electron Transport

Various models have been proposed to account for the relationship between light absorption, electron transport and the formation of ATP and NADPH. However, the most widely accepted scheme is that based on the concept of Hill and Bendall (1960), which requires two photosystems operating in series, separated by a sequence of redox compounds arranged in order of decreasing electronegativity (Trebst, 1974; Radmer and Kok, 1975). This model is generally referred to as the "Z-scheme" and is shown in Fig. 1.1. The two photosystems are each composed of photochemically active pigment protein complexes, which are membrane-bound and maintained in a particular orientation (Bolton, 1977; Williams, 1977). The light harvesting pigment protein complexes of PSII absorb incident light and a migration of excitation energy occurs through several hundred chlorophyll a molecules to a reaction centre P680, composed of a chl a dimer maintained in a specialised environment (Golbeck, Lien and San Pietro, 1977). The excitation energy causes a charge separation of the reaction centre pigment molecules, which leads to a reduction of the primary acceptor Q (E₀' = -0.1V) and the formation of an oxidant Z(E₀' = + 0.81V) (Govindjee and Govindjee, 1975; Golbeck et al., 1977).
Fig. 11 Z-Scheme (Govindjee 1975)
A similar process of light absorption resulting in pigment charge separation and reduction of a primary acceptor occurs at PSI. The reaction centre of PSI is known to be composed of a different type of chlorophyll a, P700, but the nature of X, the primary acceptor of PSI ($E'_0 = -0.55V$) is still a matter of controversy (see Golbeck et al., 1977). The reduction of oxidised P700 is accomplished by electrons derived from PSII photoreactions. The primary acceptor X, is oxidised by the non-haem iron protein, ferredoxin which then reduces NADP via the FAD flavoprotein ferredoxin-NADP-reductase.

1.3 Artificial Electron Donors, Acceptors and Inhibitors

The use of artificial electron donors, acceptors and inhibitors has been a major factor in the elucidation of photosynthetic electron transport. This biochemical approach has permitted the study of parts of the chain in isolation, in order to determine the nature and sequence of individual components. Fig. 1.2 summarises the sites of interaction of exogenous compounds with photosynthetic electron transport.

The majority of inhibitors are known to act at or near PSII (Izawa, 1977), either by preventing the oxidation of water e.g. hydroxylamine, Tris-washing (Bennoun and Joliot, 1969; Elstner, Heupel and Vaklinova, 1970a; Yamashita and Butler, 1968, 1969), or by blocking the passage of electrons from PSII eg. DCMU (Duysens and Sweers, 1963). During the last decade, antagonists of plastoquinone have gained prominence, notably DBMIB which prevents electron flow between the photosystems, but
Fig. 1.2 Sites of interaction with electron transport
allows reactions of each to be studied (Trebst, Harth and Draber, 1970; Trebst, 1972). Other electron transport inhibitors are known to act between plastoquinone and cytochrome f e.g. EDAC (Uribe, 1972; McCarty, 1974), at or near plastocyanin e.g. HgCl2, KCN (Kimimura and Katoh, 1972; Ouitrakul and Izawa, 1973; Izawa, Kraayenhof, Ruuge and De Vault, 1973) and in the region of ferredoxin (disalicylidene-diamines) (Trebst and Burba, 1967; Ben-Amotz and Avron, 1972).

Electron donation to PSII by artificial redox compounds may be complicated by the fact that some donors are also oxidised by PSI (Hauska, 1977). However, DPC and hydroquinone have been used to donate electrons to PSII in Tris-washed chloroplasts (Vernon and Shaw, 1969; Yamashita and Butler, 1969). Most investigations of electron donation to PSI have been made in the presence of DCMU. Ascorbate has often been used as the electron donor, but it is generally unable to traverse the thylakoid membrane and so mediators such as DCPIP (Vernon and Zaugg, 1960) o-substituted p-phenylenediamines are required (Trebst, 1974). The choice of catalyst is important as some systems, such as DAD/ascorbate, are coupled to phosphorylation, whereas others e.g. TMPD/ascorbate, do not result in ATP formation. The capacity of donors to cause phosphorylation is dependent on their ability to lose protons upon oxidation on the inner side of the thylakoid membrane (Hauska, Trebst and Draber, 1973; Hauska, Oettmeier, Reimer and Trebst, 1975).

Early studies showed that reactions of artificial acceptors such as ferricyanide involved only PSII (Rumberg et al., 1965;
Avron and Ben-Hayyim, 1969), but more recently PSI has been implicated (Bohme, Reimer and Trebst, 1971). In general, PSII acceptors are lipophilic, while those of PSI are hydrophilic. Many classes of compound are known to accept electrons from PSI. These include quinones, tetrazolium salts, bipyridyls and various dyes (see Hauska, 1977). Some compounds are able to accept and donate electrons, thereby causing a cyclic electron flow around PSI. Phenazine derivatives and naphthoquinones are examples of this type of compound, which also effect cyclic phosphorylation (Jagendorf and Avron, 1958; Hauska, 1977).

1.4 Photophosphorylation

Since the discovery of photophosphorylation in chloroplasts by Arnon, Whatley and Allen (1954), there has been a considerable controversy regarding the number and nature of phosphorylating sites. In linear electron transport from water to NADP, it is probable that two energy conserving sites exist; one coupled to the oxygen evolving side of PSII and the other situated between plastoquinone and cytochrome f (Hall, 1976; Jagendorf, 1977). The postulation of two sites of phosphorylation originated from stoichiometric studies of the ATP/2e ratio. This is now known to be very sensitive to the chloroplast isolation procedure and the value appears to lie between 1.3 and 2.0 (Winget, Izawa and Good, 1965; Reeves and Hall, 1973; Hall, 1976). Evidence for two sites has been derived from experiments with various artificial electron donors, acceptors and inhibitors, notably DBMIB. A cyclic photophosphorylation around PSI has been demon-
strated in isolated chloroplasts, in the presence of such compounds as PMS (see Arnon, 1977), but the physiological significance of this has not been conclusively established.

The mechanism of photophosphorylation has been the subject of considerable debate, although one model is generally regarded as being close to the in vivo situation. One hypothesis predicts the formation of a chemical intermediate derived from electron carriers and enzymes or substrates of the phosphorylation reaction (Slater, 1953; Chance and Williams, 1956). Oxidation of the complex is believed to produce a high energy intermediate which is used to form the anhydro bond of ATP. Another proposal (Boyer, 1965, 1974) explains energy transfer in terms of conformational changes of phosphorylation enzymes and membrane components. Neither of these hypotheses are supported by any convincing evidence, unlike the chemiosmotic theory advanced by Mitchell (1963, 1966, 1974), which is based on the establishment of a pH gradient across the thylakoid membrane. This presupposes an asymmetrical arrangement of electron carriers within the thylakoid membrane so that electron transport causes a vectorial proton movement, leading to a pH decrease of the intrathylakoid space. The coupling membrane is assumed to be relatively impermeable to protons except at certain sites. These are known as coupling factor 1 (CF1) and are essentially vectorially aligned ATPases. These permit controlled dissipation of the proton gradient and lead to ATP formation (Fig. 1.3). The stimulation of electron transport rates by uncouplers is due to membrane leakage resulting in uncontrolled dissipation of the proton gradient and therefore the inhibition of ATP formation.
Fig. 13 Master scheme for photosynthetic electron flow coupled to ATP formation. According to a chemiosmotic mechanism of coupling the electron flow system is separated in the membrane from the ATP synthase. Non-cyclic electron flow generates NADPH, 1/2 O₂, and 4 protons inside the thylakoid membrane. It includes two energy-conserving sites defined as an electrophotocycle + a proton-translocating electron neutral redox reaction across the membrane. 3 protons (n = 3 in the PMF) are assumed to drive the formation of 1 ATP in the coupling system consisting of a base piece and the coupling factor CF₁ with 5 different subunits. This yields a stoichiometry of 1.33 ATP per NADPH. In cyclic photophosphorylation in vivo ferredoxin reacts back with PQ. It includes one energy-conserving site: P₇₅₀, P₂₅₀: reaction centers of photosystem II and I; Q-acceptor; PQ: plastoquinone; P₇₅₀: plastohydroquinone; PCy: plastocyanin; Fd: ferredoxin; reduct: ferredoxin-NADP⁺ oxidoreductase. Artificial donors and acceptors: DPC: diphenylcarbazide; DCPPE: dichlorophenolindophenol; TMPD: N-tetramethyl-p-phenylenediamine; DAD: diaminoduroyl; PD: p-phenylenediamine. Flow inhibitors: DCMU: dichloromethylene; DBMIB: dibromomethyl-isopropyl-p-benzoquinone; DSPD: disalicyliden-propanediamine. Energy-transfer inhibitors: DCCD: dicyclohexylcarbodiimide, also closing the proton conducting channel.

Trebst & Avron 1977
1.5 Oxygen Evolution

Oxygen is evolved during photosynthesis according to the simple redox equation (Eqn 1)

\[
2\text{H}_2\text{O} \rightarrow 4\text{H}^+ + 4\text{e}^- + 0_2
\]  

However, little can be discerned from this equation regarding the mechanism of the photosynthetic oxidation of water. It is known that in addition to P680, a primary donor Z and a secondary donor S_n are involved (Joliot and Kok, 1975). S_n is a charge accumulating, oxygen evolving enzyme. The flow of electrons in PSII is shown in Fig. 1.4. S_n accumulates four oxidising equivalents which are then used to rapidly decompose two molecules of water. Manganese is essential for oxygen evolution and appears to be associated with a readily denatured protein (Joliot and Kok, 1975; Lawrence and Sawyer, 1978). However, manganese is not required for photooxidations of donors other than water. Chloride ions also play a role in oxygen evolution.

1.6 CO₂ Fixation

The pathway by which C₃ plants incorporate CO₂ and subsequently reduce it to carbohydrate was discovered by Calvin and co-workers at Berkeley during the period 1946-1953. Their success was due in part, to the newly available isotope ^14C, which permitted the pathways of incorporated CO₂ to be followed, and also to the recent advent of two dimensional paper chromatography.

CO₂ is incorporated by the chloroplast through the carboxylation
Fig. 1.4 Oxygen evolution in photosynthesis
Fig. 1.5 Calvin Cycle
of ribulose 1,5-diphosphate (RuDP) at the C-2 position. The enzyme responsible for this reaction, RuDP carboxylase is abundant in leaves, comprising about half the total soluble leaf protein (Akazawa, 1970) and can act both as a carboxylase and as an oxygenase. This latter activity may be the mechanism of phosphoglycolate for the process of photorespiration. Following the carboxylase activity of this enzyme, the C₆ intermediate is hydrolytically cleaved to form two molecules of phosphoglycerate (3-PGA). ATP and NADPH generated by electron transport are then utilised in the phosphorylation and reduction of 3-PGA to form triose phosphate. The condensation of these compounds followed by phosphatase action results in the formation of fructose 6-phosphate. The final sequence of reactions consists of isomerisations, condensations and rearrangements which eventually results in the regeneration of RuDP. The Calvin cycle is shown in Fig. 1.5.

Since the elucidation of the basic CO₂ reduction pathway, plants with variations have been discovered (in particular C₄ photosynthesis) and the knowledge concerning the regulation of these reactions has greatly increased (see Bassham, 1977).
2. BIPYRIDYL HERBICIDES

The herbicidal activity of the bipyridyl compounds was first discovered in 1955 by ICI (Calderbank, 1968), although they had previously been used as redox indicators. It was shown by Michaelis and Hill (1933) that the addition of one electron to these compounds produced highly coloured free radicals, which were stable in the absence of oxygen.

These compounds are used as contact herbicides and lead to a rapid loss of colour and desiccation of green plant tissue upon illumination (Dodge, 1971). Non-photosynthetic tissues is killed only slowly by the bipyridyl herbicides and light has no effect (Mees, 1960), indicating that although these compounds possess an activity not connected with photosynthesis, this is of very secondary importance compared to the main effect (Corbett, 1974).

2.1 Site of Action

Early studies showed that the site of action of the bipyridyls was quite distinct from that of the photosynthetic inhibitor herbicides, CMU and DCMU. These latter compounds were shown to inhibit photophosphorylation, NADP reduction and ferricyanide reduction in isolated chloroplasts, suggesting that their site of action was at or near the primary acceptor of PSII (see Fig. 1.2). By contrast, the bipyridyls do not inhibit photophosphorylation or ferricyanide reduction, but NADP reduction is decreased as a function of the herbicide concentration (Zweig, Shavit and Avron, 1965). This suggests that the bipyridyls compete for electrons emanating from the
primary acceptor of PSI (Van Rensen, 1969). The photoreduction of the bipyridyls has been demonstrated in illuminated chloroplasts (Zweig and Avron, 1965; Zweig et al., 1965; Kok, Rurainski and Owens, 1965; Black, 1966). PSI is believed to generate a reducing potential of -0.5V (Golbeck et al., 1977) and is thus the only photosystem capable of reducing the bipyridyls.

2.2 Mechanism of Action

The early investigations of Mees (1960) and Homer, Mees and Tomlinson (1960) provided a valuable insight to key features in the mode of action of these herbicides. Light and oxygen are both essential for the toxic effect and after the demonstration that reduced diquat was immediately reoxidised by molecular oxygen to form hydrogen peroxide (Davenport, 1963), it was proposed that this was the toxic species (Calderbank, 1964, 1968).

One of the first visual signs of paraquat treatment is a breakdown of membranes, particularly the tonoplast and plasmalemma (Harris and Dodge, 1972a), which is accompanied by the gradual formation of malondialdehyde (Baldwin, Harris and Dodge, 1968; Harris and Dodge, 1972b). This product is believed to arise from the breakdown of unsaturated fatty acid hydroperoxides, instigated by the abstraction of hydrogen, mediated by free radicals. Pulse radiolytic studies have shown that the paraquat radical decays rapidly in the presence of oxygen (k = 7.7 x 10^8 M^-1 s^-1) and is thus an unlikely candidate
as the agent directly responsible for causing lipid peroxidation (Farrington, Ebert, Land and Fletcher, 1973). It is more likely that free radicals of oxygen are the species which attack membrane lipids (Van Rensen, 1975). In this way, the paraquat ion is regenerated and can act catalytically in a series of reactions (Eqns 2-8) (Farrington et al., 1973).

\[
PQ^{2+} + e^- \rightarrow PQ^+ \quad (2)
\]

\[
PQ^+ + O_2 \rightarrow PQ^{2+} + O_2^- \quad (3)
\]

\[
PQ^+ + O_2^- \rightarrow PQ^{2+} + O_2^- \quad (4)
\]

\[
O_2^- + 2H^+ \rightarrow H_2O_2 \quad (5)
\]

\[
PQ^+ + H_2O_2 \rightarrow PQ^{2+} + OH^- + OH^- \quad (6)
\]

\[
PQ^+ + OH^- \rightarrow PQ^{2+} + OH^- \quad (7)
\]

\[
OH^- + H^+ \rightarrow H_2O \quad (8)
\]

Each of the oxygen radicals formed by these reactions has been proposed as the instigative species of lipid peroxidation reactions (Harris and Dodge, 1972b; Farrington et al., 1973; Dodge, 1975). The plant cell possesses a range of complex defence mechanisms to deal with active oxygen species, especially superoxide and hydrogen peroxide, but it has been proposed that these may only be sufficient under normal conditions (Calderbank, 1968; Dodge, 1977) and that the increased radical production due to herbicidal action may swamp these protective devices.

The initial damage to certain membranes after paraquat treatment is followed by a progressive breakdown of cell organelles (Harris and Dodge, 1972a). It is proposed that disruption of the tonoplast would result in the release of toxic waste products, especially those of an acidic nature which would drastically alter the pH and osmotic potential of the cytoplasm.
(Dodge, 1975). The loss of chlorophyll and other pigments is assumed to occur via photooxidative processes.

2.3 Structure-Activity Relationships of the Bipyridyls

It has been inferred in the preceding section that the herbicidal activity of the bipyridyls is dependent upon the cationic part of the compound. The basic feature of the bipyridyl herbicides is that they can be readily reduced to a water soluble free radical by the addition of one electron. These can then undergo autoxidation with dioxygen (molecular oxygen), reforming the cation and concomitantly reducing oxygen. The ease of reduction by PSI in chloroplasts is correlated with herbicidal activity and is confined to compounds with redox potentials of between -0.3v and -0.5v. It has been observed (Brian, 1965) that herbicidal activity is relatively independent of fine structure and stereochemistry. However, there is a requirement for the two pyridine rings to lie in the same plane, so that the odd electron of the cation radical can be delocalised over all positions of the ring system (Summers, 1979). These features imply that the bipyridyls do not bind to a specific site in the chloroplast, although their mode of action requires that they be close to the primary acceptor of PSI, in order to compete favourably with ferredoxin for electrons.
3. CHEMICAL AND BIOCHEMICAL CONSIDERATIONS OF OXYGEN

3.1 Properties of Dioxygen

Molecular oxygen or dioxygen exists in the ground state as a triplet \( ^3\Sigma_g^- \) and so the dioxygen molecule is correctly represented as \( \cdot O-O\cdot \) and not \( O=O \). Thus, the \( O-O \) bond may be visualised as one sigma bond and one pi bond. The two unpaired electrons of the diradical possess the same spin quantum number and so occupy different \( 2p_{\pi g^*} \) antibonding orbitals (Fig. 1.6). Ground state dioxygen is relatively unreactive towards biological molecules and this is due to a spin restriction encountered when triplet dioxygen reacts with a singlet state substrate to form singlet products (Taube, 1965; Hamilton, 1974). This spin restriction may be circumvented by a reaction mechanism involving free radicals (Uri, 1962), as follows:

\[
\begin{align*}
X' + RH & \longrightarrow R' + XH & (9) \\
R' + O_2 & \longrightarrow RO_2' & (10) \\
RO_2' + RH & \longrightarrow ROOH + R' & (11)
\end{align*}
\]

There are two other ways in which the two electrons can be placed in the \( 2p_{\pi g^*} \) orbitals (Fig. 1.6) and these account for the two excited states of dioxygen: \( ^1\Delta_g \) and \( ^1\Sigma_g^+ \), which are both singlets. There is no evidence that the \( ^1\Sigma_g^+ \) excited state is generated in biological systems (Foote, 1976; Krinsky, 1977). The direct decay of the two singlet excited states to the \( ^3\Sigma_g^- \) ground state is forbidden and thus accounts for the relative longevity of these excited states compared to
Fig. 16 Molecular orbitals of oxygen species

$2p_{\pi}^*$ $2p_{\pi u}$ $2p_{\sigma g}$ $2s_{\sigma}^*$ $2s_{\sigma}$

$O_2^-$

$\Sigma^+$

$\Delta_g$

$3\Sigma_g^-$
excited states of other molecules which can decay via allowed transitions.

3.2 Reduction of Oxygen

The mechanism of the reduction of dioxygen is determined by the occupancy of its molecular orbitals. Direct divalent reduction is forbidden as this would result in a molecular orbital being occupied by two electrons of parallel spin. This spin restriction may be overcome in three ways. In the first, the conversion of the $^3\Sigma^+$ state to either of the two excited singlet states would result in the spin inversion of an electron, which would then allow conventional reduction to occur. The second possibility involves complexing dioxygen to a transition metal with unpaired electrons (Hamilton, 1969). Under these circumstances, the complex can accept a pair of electrons and oxygen is reduced by an ionic mechanism. The third solution avoids the spin restriction by the formation of free radicals in the univalent reduction of oxygen. The complete reduction of dioxygen to water requires four electrons and thus the univalent mechanism inevitably involves intermediates (Fig. 1.7). All these partially reduced species are more reactive than either of the fully oxidised or reduced forms of oxygen and therefore present a potential hazard wherever univalent oxygen reduction occurs in biochemical systems. These intermediates will be considered individually with special reference to their occurrence in biological systems and the protective mechanisms that have been developed to deal with each potentially toxic oxygen species.
Fig 1.7 Univalent reduction of oxygen
3.3 Production of Superoxide

The univalent reduction of oxygen gives rise to the hydroperoxy radical, H$_2$O$_2^\cdot$ or its conjugate base, superoxide O$_2^\cdot^-$. Most of the known properties of this radical have been derived from studies which generated the species in high concentrations and allowed rapid investigation of the decay process. Such methods include flash photolysis (Baxendale, 1962; Hayon and McGarvey, 1967), pulse radiolysis (Behar, Czapski and Duchovny, 1970; Bielski and Saito, 1971; Czapski, 1971) and fast flow radiolysis techniques (Bielski and Gebicki, 1977; Bielski and Richter, 1977).

The redox potential for the O$_2$/O$_2^\cdot$ couple has been determined as -0.16V (Wood, 1974; Sawada, Iyanagi and Yamazaki, 1975; Meisel and Czapski, 1975; Ilan, Czapski and Meisel, 1976). This value has important consequences for biological systems where numerous compounds possess potentials more negative than -0.16V and thus theoretically could reduce oxygen to superoxide. The generation of superoxide has been demonstrated in a wide range of biological systems (for a review see, McCord, Crapo and Fridovich, 1977 and other papers in the same proceedings). Many enzymes are capable of producing the radical and these include xanthine oxidase (McCord and Fridovich, 1968, 1969; Fridovich, 1970; Fridovich and Handler, 1962), aldehyde oxidase (Rajagopalan, Fridovich and Handler, 1962; Rajagopalan and Handler, 1964) and flavoprotein dehydrogenases (Massey et al., 1969; Rich and Bonner, 1978). The essential processes of respiration and photosynthesis are known to be capable of generating superoxide. The electron carriers of respiration
are arranged so that only the terminal component is directly exposed to dioxygen. Nevertheless, univalent oxygen reduction still occurs both \textit{in vitro} (Flohe, Loschen, Azzi and Richter, 1977; Loschen, Flohe and Chance, 1972; Boveris and Cadenas, 1975; Boveris, Oschino and Chance, 1972) and \textit{in vivo} (Nohl and Hegner, 1978). It appears that the univalent reduction of oxygen by mitochondria is mediated by cytochrome $b_{566}$ (Erecinska; Veech and Wilson, 1974; Wilson, Koppelman, Erecinska and Dutton, 1971) and not cytochrome oxidase (Rich and Bonner, 1978).

Isolated chloroplast lamellae were shown to produce superoxide by Allen and Hall (1973), Asada and Kiso (1973a, b), Elstner and Kramer (1973) and Epel and Neumann (1973). It is now known that oxygen reduction occurs both \textit{in vitro} and \textit{in vivo} (Patterson and Myers, 1973; Radmer and Kok, 1976). Experiments with $^{18}$O$_2$ and intact algae have shown that PSI catalysed oxygen reduction occurs under physiological conditions and that superoxide is not an artefact of chloroplast isolation procedures (Glidewell and Raven, 1975; Radmer and Kok, 1976). The site of superoxide production has been established as the reducing side of PSI (Asada, Kiso and Yoshikawa, 1974; Miller and Macdowell, 1975) and it is believed to be formed during the autoxidation of low potential electron acceptors such as ferredoxin (Misra and Fridovich, 1971; Allen, 1975), the bound iron-sulphur protein P-430, which is thought to be the primary acceptor of PSI (Ke, 1973) and FMN (Misra and Fridovich, 1972). Oxygen reduction has been demonstrated to occur in isolated chloroplast lamellae when about 80% of the NADP pool is in the
reduced form (Elstner and Heupel, 1973; Elstner, Stoffer and Heupel, 1975). This is consistent with the observation that isolated chloroplasts take up oxygen in the absence of pseudocyclic acceptors, resulting in the formation of \( \text{H}_2\text{O}_2 \) (Mehler, 1951a,b; Mehler and Brown, 1952; Whitehouse, Ludwig and Walker, 1971). The rate of oxygen uptake is stimulated by the addition of various autoxidisable electron acceptors such as dyes, flavin derivatives and bipyridyls (Good and Hill, 1955). There is evidence that superoxide may also be produced during the decomposition of linoleic acid hydroperoxide (Yamashoji, Yoshida and Kajimoto, 1979). Thus, illuminated chloroplasts \textit{in vitro} and \textit{in vivo} are an important biochemical source of superoxide with perhaps up to 10% of electron transport being diverted to the reduction of oxygen (Asada, Takahashi, Tanaka and Nakano, 1977).

### 3.3.1 Reactivity of Superoxide

The radical nature of superoxide permits it to interact with a variety of compounds; the ease of reaction being determined by its anionic character and redox potential. The \( \text{pK}_a \) of the weak acid \( \text{H}_2\text{O}_2 \) is 4.8 (Rabani and Nielson, 1969; Behar, Czapski, Rabani, Dorfman and Schwartz, 1970) and therefore at physiological pH values, the radical will be present as the superoxide anion. Superoxide has been shown to react with numerous biological materials and thus its presence could have serious consequences for the cell. It can react with proteins (Koppenol, 1976), especially those containing sulphydryl groups (Fridovich, 1974a). This is consistent with the observation that
thiols could scavenge superoxide (Misra, 1974; Asada and Kanematsu, 1976). Ribonuclease has also been shown to be inhibited by superoxide (Målmmstromm, Andréasson and Reinhammer, 1975).

Particular interest has been focused on the role of superoxide in the peroxidation of lipids which eventually results in membrane disruption (Fee and Teitelbaum, 1972; Pederson and Aust, 1972, 1973; Zimmerman, Flohé, Weser and Hartmann, 1973). Superoxide is a relatively stable radical and does not possess sufficient energy to abstract allylic hydrogen atoms necessary for the initiation of lipid peroxidation (Pryor, 1978; Bors, Michel and Saran, 1979a). However, it would appear to be involved in lipid peroxidation, perhaps through an ability to generate more toxic species. The hydroxyl radical OH' has been invoked by McCay and coworkers as the oxidising species (King, Lai and McCay, 1975; Fong, McCay, Poyer, Keele and Misra, 1973), whereas other groups believe singlet oxygen, \(^1\text{O}_2\) to be the agent responsible (Kellogg and Fridovich, 1975; Pederson and Aust, 1973, 1975). The mechanism of generation of these more reactive species is still a matter of some controversy centred around the Haber-Weiss reaction (Haber and Weiss, 1934) and will be discussed further in the relevant sections.

Superoxide has also been implicated in hydroxylation reactions (Halliwell, 1977), ethylene production and in sulphite oxidation (see Fridovich, 1974b).
3.3.2 Detection of Superoxide

Direct and unequivocal detection of superoxide is limited to physical methods such as UV absorption (Czapski, 1971) and esr measurements (Knowles, Gibson, Pick and Bray, 1969), often coupled with spin trapping techniques (Harbour, Chew and Bolton, 1974; Harbour and Bolton, 1975). In general, these procedures are of limited value with biological material due to their lack of sensitivity (Fridovich, 1976) and the problems associated with interfering substances. Most biochemical methods trap superoxide in dependent reactions that can be followed optically, manometrically or polarographically. The concentration of the substrate is such that it competes favourably with the dismutation reaction so that all superoxide produced is detected. During the last decade, many biochemical assays of this type have been developed (see McCord, Crapo and Fridovich, 1977 for a review). Of these the most widely used are the autoxidation of epinephrine (Misra and Fridovich, 1972b; Asada and Kiso, 1973), the reduction of ferricytochrome c (McCord and Fridovich, 1968, 1969a), the reduction of nitroblue tetrazolium (Beauchamp and Fridovich, 1971) and the formation of nitrite from hydroxylamine (Elstner, Heupel and Vaklinova, 1970a; EJstner and Heupel, 1974, 1975, 1976a). The specificity of these reactions for superoxide is not absolute, but the involvement of this species is generally determined with the aid of superoxide dismutase (McCord and Fridovich, 1970).
3.3.3 Protective Mechanisms against Superoxide

It is apparent from the preceding sections that the presence of oxygen in biological systems coupled with the ability to generate a strong reductant provide the basic requisites for superoxide production. Superoxide has been implicated in a variety of deleterious reactions although it may not necessarily be the species directly responsible for causing damage. Therefore, any organism which requires aerobic conditions for all or part of its life cycle must possess mechanisms enabling it to deal with any superoxide formed. The rate of superoxide production may be controlled or its presence tolerated if it can be prevented from instigating reactions detrimental to the system. Most organisms have evolved complex defence mechanisms which make use of both these features. In both respiratory and photosynthetic electron transport, the carriers are specifically arranged so that only the terminal stages of the sequence are exposed to dioxygen. Of paramount importance in the defence against any superoxide produced are the superoxide dismutase (SOD) enzymes. These are metalloproteins which are virtually ubiquitous in aerobic organisms and catalyse the dismutation of superoxide (McCord and Fridovich, 1968, 1969a,b), according to Eq. 12

\[ O_2^- + O_2^- + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2 \] (12)

This reaction proceeds at a diffusion controlled rate with \( k = 1.9 \times 10^9 \text{ M}^{-1}\text{s}^{-1} \) (Klug, Rabani and Fridovich, 1972; Rotilio, Bray and Fielden, 1972). Although superoxide can undergo spontaneous dismutation the rate is dependent upon the pH and is much slower (Eqns 13-15).
\[ \text{HO}_2^+ + \text{HO}_2 \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad k = 7.6 \times 10^5 \text{ M}^{-1}\text{s}^{-1} \quad (13) \]

(Bielski and Saito, 1962; Czapski, 1971)

\[ \text{HO}_2^+ + \text{O}_2^- + \text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad k = 1.5 \times 10^7 \text{ M}^{-1}\text{s}^{-1} \quad (14) \]

(Bielski and Schwartz, 1968; Czapski, 1971)

\[ \text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2^- + \text{O}_2 \quad k < 6\text{M}^{-1}\text{s}^{-1} \quad (15) \]

(Divisek and Kastening, 1975)

The enzyme function of the protein now known as SOD was discovered by McCord and Fridovich (1969a,b), who found that it inhibited the reduction of ferricytochrome c by xanthine oxidase. The action of this latter enzyme was believed to be mediated by superoxide (Fridovich and Handler, 1958a,b; McCord and Fridovich, 1968). Over the past decade since its discovery, SOD has been isolated from a great variety of sources. In general, the cytoplasm of eucaryotes contains Cu, Zn-SOD (McCord and Fridovich, 1969a; Asada, Urano and Takahashi, 1973; Sawada, Ohyama and Yamazaki, 1972), which is a dimer composed of identical subunits. Procaryotes and eucaryotic mitochondria have been shown to contain Fe- and/or Mn-SOD but no Cu, Zn enzyme (Keele, McCord and Fridovich, 1970; Asada, Kanematsu and Uchida, 1977; Cseke et al., 1979). The manganic and ferric enzymes possess a dimeric or tetrameric structure with identical subunits (Kanematsu and Asada, 1979). In spite of differences in the apoenzyme and the prosthetic metal ions, this group of enzymes appears to be specific for the dismutation of superoxide. The variations may reflect differences in the evolution of the enzymes (Lumsden and Hall, 1975; Lumsden, Henry and Hall, 1977; Okada, Kanematsu and Asada, 1979).
The chloroplast is well protected against superoxide by possessing both soluble and thylakoid-bound SOD (Asada et al., 1973; Lumsden and Hall, 1974; Elstner and Heupel, 1975; Jackson et al., 1978). This protection is essential as many chloroplast components are able to react with superoxide as summarised in Fig. 1.8. Ascorbate is present in the chloroplast stroma at concentrations up to 50 mM (Walker, 1971; Halliwell, 1978) and reacts non-enzymatically with superoxide as shown in Eqn 16 (Elstner and Kramer, 1973; Epel and Neumann, 1973; Nishikimi and Yagi, 1977).

\[
\text{Ascorbate} + 2\text{O}_2 \rightarrow \text{H}_2\text{O}_2 + \text{dehydroascorbate}
\]  

(16)

3.4 Production of Hydrogen Peroxide

By comparison with superoxide, hydrogen peroxide is a stable intermediate of oxygen reduction. It is a product of both the enzymatic and non-enzymatic dismutation of superoxide and so would be expected to be generated in any system where the univalent reduction of oxygen occurs, and should be stimulated by similar cofactors. Dioxygen was first shown to act as the terminal oxidant of photosynthetic electron transport under certain conditions by Mehler, who demonstrated the accumulation of hydrogen peroxide (Mehler, 1951a, b; Mehler and Brown, 1952). Endogenous and artificial acceptors of PSI such as ferredoxin, flavins and methyl viologen were found to stimulate the Mehler reaction (Good and Hill, 1955; Hill and Walker, 1959; Ludwig et al., 1971; Telfer, Cammack, Evans, 1970). Under in vivo conditions, hydrogen peroxide is not generally accumulated although it is still rapidly produced (Patterson and Myers, 1973).
Table 1. Reactivity of Chloroplast Components with Superoxide (based on ASADA et al., 1977).

<table>
<thead>
<tr>
<th>Chloroplast Component</th>
<th>Rate Constant K (M⁻¹ s⁻¹)</th>
<th>Chloroplastic Concentration (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome f (Fe³⁺→Fe²⁺)</td>
<td>6.1 x 10⁶</td>
<td>6.2 x 10⁻⁵</td>
</tr>
<tr>
<td>Plastocyanin (Cu²⁺→Cu⁺)</td>
<td>1.1 x 10⁶</td>
<td>6.2 x 10⁻⁵</td>
</tr>
<tr>
<td>Ferredoxin (Fe³⁺→Fe²⁺)</td>
<td>&lt;1 x 10⁴</td>
<td>6.2 x 10⁻⁵</td>
</tr>
<tr>
<td>Mn²⁺→Mn³⁺</td>
<td>6.0 x 10⁶</td>
<td>4.0 x 10⁻⁴</td>
</tr>
<tr>
<td>Ascorbate→dehydroascorbate</td>
<td>2.7 x 10⁵</td>
<td>2.5 x 10⁻³</td>
</tr>
<tr>
<td>GSH→GSSG</td>
<td>6.7 x 10⁵</td>
<td>3.5 x 10⁻³</td>
</tr>
<tr>
<td>Cu, Zn-SOD (dismutation)</td>
<td>2.0 x 10⁹</td>
<td>8.0 x 10⁻⁶</td>
</tr>
</tbody>
</table>
3.4.1 Reactivity of Hydrogen Peroxide

Hydrogen peroxide and its anion, \( \text{HO}_2^- \), are powerful nucleophiles and act as potent oxidants towards organic substrates. However, these reactions are surprisingly slow, while other reactions of hydrogen peroxide proceed via hydroxyl radical intermediates or through complexation with transition metals (Hamilton, 1974). Perhaps the best known of these latter reaction types are the Fenton reagent mechanisms (Fenton, 1894), which generate the toxic hydroxyl radical (Eqs. 17 and 18)

\[
\begin{align*}
\text{Fe}^{2+} + \text{H}_2\text{O}_2 & \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^- \tag{17} \\
\text{OH}^- + \text{H}_2\text{O}_2 & \rightarrow \text{H}_2\text{O} + \text{O}_2^- + \text{H}^+ \tag{18}
\end{align*}
\]

The reduction of ferric ions could be mediated by superoxide or by hydrogen peroxide (Eqs. 19 and 20)

\[
\begin{align*}
\text{Fe}^{3+} + \text{O}_2^- & \rightarrow \text{Fe}^{2+} + \text{O}_2 \tag{19} \\
\text{Fe}^{3+} + \text{H}_2\text{O}_2 & \rightarrow \text{Fe}^{2+} + 2\text{H}^+ + \text{O}_2^- \tag{20}
\end{align*}
\]

The mixture of ferrous ions and hydrogen peroxide is known to be an extremely reactive system and can attack both aliphatic and aromatic compounds (Walling, 1975). Hydrogen peroxide is certainly toxic to living cells (Fridovich, 1976), but the reasons for this are not clearly understood. In many instances, the danger of hydrogen peroxide may lie in its ability to form Fenton-type reagents. In intact chloroplasts, \( \text{CO}_2 \) fixation is inhibited by hydrogen peroxide (Egneus et al., 1975; Allen, 1978a,b), possibly due to a leakage of Calvin cycle intermediates (Kaiser, 1976). Lipid peroxidation appears to be stimulated by hydrogen peroxide, but this is probably due to the indirect effect described above (Dodge, 1971, 1975).
3.4.2 Detection of Hydrogen Peroxide

The relative stability of hydrogen peroxide permits direct biochemical determinations to be made. A simple assay involves the addition of catalase which destroys hydrogen peroxide and releases oxygen which is detected with an oxygen electrode. Other biochemical assays which combine sensitivity with relative ease of operation include the decarboxylation of α-keto acids (Elstner and Heupel, 1973, 1976b) and the oxidation of NADH in the presence of an NADH-specific peroxidase (Elstner and Frommeyer, 1978a,b).

3.4.3 Protective Mechanisms against Hydrogen Peroxide

Although hydrogen peroxide per se may not be especially toxic, its presence combined with that of certain transition metal ions in living cells is potentially a significant hazard. Hydrogen peroxide production is often localised in similar areas to superoxide generation and thus the criteria for defence mechanisms bear close resemblances. The destruction of hydrogen peroxide can be catalysed by the enzyme catalase which is found in peroxisomes, a frequent contaminant of chloroplast preparations (Allen, 1977; Allen and Whatley, 1978; Halliwell, 1978b). Hydrogen peroxide may also be destroyed by peroxidase enzymes, with the concomitant oxidation of substrate. In addition to the absence of catalase in chloroplasts, peroxidase activity was originally believed to be lacking (Parish, 1972). However, Groden and Beck (1977, 1979) have reported the existence of a tightly bound ascorbate peroxidase in chloroplasts, as proposed
by Foyer and Halliwell (1976), which could detoxify hydrogen peroxide according to Eq. 21.

\[ \text{Ascorbate} + \text{H}_2\text{O}_2 \underset{\text{peroxidase}}{\xrightarrow{\text{ascorbate}}} \text{dehydroascorbate} + 2\text{H}_2\text{O} \] (21)

Dehydroascorbate may be reduced to ascorbate by glutathione, also present in chloroplasts (Eqn. 22)

\[ 2\text{GSH} + \text{dehydroascorbate} \rightarrow \text{GSSG} + \text{ascorbate} \] (22)

Reduced glutathione may be regenerated by NADPH in the presence of an NADPH-dependent glutathione reductase (Jocelyn, 1972; Halliwell and Foyer, 1978).

3.5 Production of Hydroxyl Radical

The hydroxyl radical is formed by homolytic or reductive cleavage of hydrogen peroxide and is one of the most potent oxidants known. It is highly reactive and can abstract protons or remove electrons from a substrate, resulting in a more stable free radical (Eqns. 23 and 24)

\[ \text{OH}^- + \text{RH} \rightarrow \text{OH}^- + \text{RH}^+ \] (23)

\[ \text{OH}^- + \text{RH} \rightarrow \text{H}_2\text{O} + \text{R}^- \] (24)

In biological systems, the hydroxyl radical was originally thought to be derived from the reaction of superoxide with hydrogen peroxide (Eqn. 25) (Haber and Weiss, 1934)

\[ \text{O}_2^- + \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + \text{OH}^- + \text{OH}^- \] (25)

On thermodynamic grounds, this reaction is feasible (Koppenol and Butler, 1977), but the failure to demonstrate its occurrence by various groups (McClune and Fee, 1976; Halliwell, 1976; Rigo, Stevanato, Finazzi-Agro and Rotilio, 1977; Gibian and
Ungermann, 1979), suggest that it does not take place at a significant rate. These studies have shown that the rate constant for the dismutation of superoxide is at least $10^7$ greater than that of the Haber-Weiss reaction (Pryor, 1976). However, catalysis by metal ions may allow the reaction to proceed at a significantly greater rate (Cohen, 1977; Halliwell, 1978c; Halliwell and De Ruyker, 1978). The hydroxyl radical may also be formed via a Fenton-type reaction as described in Section 3.4.1.

3.5.1 Reactivity of the Hydroxyl Radical

Due to its extreme reactivity, the hydroxyl radical is generally regarded as being unspecific in its attack (Fee and Valentine, 1977). Thus, in biological systems this species would lead to damage in the immediate vicinity of its generation. It possesses sufficient reactivity to attack most biological materials (Anbar and Neta, 1967) and instigate radical chain reactions, thus presenting a considerable danger to living systems.

3.5.2 Detection of the Hydroxyl Radical

The difficulty in detecting species with very short lifetimes has led to considerable controversy as to whether the hydroxyl radical is produced in biological systems. Early assays were based on the assumption that the hydroxyl radical was produced via the Haber-Weiss reaction. If this were the case, hydroxyl radical-dependent reactions would be inhibitiable by SOD and
catalase (Beauchamp and Fridovich, 1970; Elstner and Konze, 1974). The bleaching of p-nitrosodimethylaniline (PNDA) has been used as a probe for the hydroxyl radical in biological systems (Elstner and Zeller, 1978; Bors, Michel and Saran, 1979b). The specificity of this reaction had previously been demonstrated in chemical systems (Baxendale and Khan, 1969; Kraljic and El-Mohsni, 1978; Kraljic and Trumbore, 1965). From the biochemical investigations, it appears that freely diffusible hydroxyl radical cannot exist in biological systems. It is important to note, however, that the existence of a Fenton-type reagent is not ruled out by these investigations (Bors et al., 1979b).

3.5.3 Protective Mechanisms against the Hydroxyl Radical

The high reactivity of the hydroxyl radical and the equivalent Fenton-type oxidant infer that endogenous protective mechanisms must act at the site of possible radical production. The mechanisms for the production of both the oxidising species require hydrogen peroxide, thus the cell can control hydroxyl radical formation by regulating the generation and subsequent fate of this less toxic species. It is difficult to visualise specific scavengers of the hydroxyl radical because of its reactivity, although cells do contain relatively high levels of the antioxidant α-tocopherol (Baszynski, 1974; Hughes, Gaunt and Laidman, 1971).
3.6 Production of Singlet Oxygen

In contrast to the active oxygen species described in preceding sections, singlet oxygen is not an intermediate in the univalent reduction of dioxygen. It is however, highly reactive; a feature that is probably derived from the lack of a spin restriction (Fee and Valentine, 1977). Singlet oxygen may be generated by a variety of methods (see Krinsky, 1977), but only those reactions applicable to biological systems will be considered here.

One of the most widespread sources of singlet oxygen is through photosensitisation reactions, in which this reactive oxygen species may be formed through the reaction of excited triplet sensitisier and dioxygen (Krinsky, 1977). Excited chlorophyll has been shown to generate singlet oxygen by this method (Foote and Denny, 1968). The Haber-Weiss reaction (Haber and Weiss, 1934) has been proposed as a potential mechanism for the generation of singlet oxygen (Kellogg and Fridovich, 1975) and this has been shown to be thermodynamically feasible (Koppenol, 1976). However, despite numerous attempts to generate singlet oxygen via this reaction, no conclusive evidence has been obtained (Fee and Valentine, 1977; Foote, Shook, Abakerli, 1980). Another possible mechanism by which singlet oxygen may be generated from superoxide concerns the dismutation reaction. It has been proposed that the spontaneous dismutation reaction leads to the formation of singlet state oxygen (Arneson, 1970; Khan, 1970), while the enzymatic dismutation results in ground state oxygen (Goda, Chu, Kimura and Schaap, 1973; Halliwell, 1974). This is still a matter of some controversy (Poupko and Rosenthal, 1973; Fee and
Valentine, 1977). Hydrogen peroxide may also give rise to singlet oxygen, either through disproportionation (Smith and Kulig, 1976) or by a reaction with halides in the presence of a peroxidase (Piatt and O'Brien, 1979).

3.6.1 Reactivity of Singlet Oxygen

Singlet oxygen does not suffer from the spin restriction imposed upon dioxygen and is therefore much more reactive. It is not however, as reactive as the hydroxyl radical and is therefore more discriminating in its reactions with biological molecules. In particular, it has been shown to oxidise nucleic acids (Clagett and Galen, 1971; Rosenthal and Pitts, 1971), amino acids (Nilsson, Merkel and Kearns, 1972) and many other compounds (see Bors et al., 1974). Photodynamic attack of biological systems frequently results in membrane damage which is believed to be due to lipid peroxidation (Foote, 1976; Mead, 1976). Singlet oxygen has been shown to attack unsaturated lipids in vitro (Rawls and Van Santen, 1970) and there is evidence for its participation in in vivo reactions (Goda et al., 1973; Pederson and Aust, 1973; Bus, Aust and Gibson, 1974).

3.6.2 Detection of Singlet Oxygen

Several compounds react with singlet oxygen to form specific products, e.g. the reaction with cholesterol results in the formation of 5α-hydroperoxycholesterol and thus can be used as
indirect determinations of this species. The participation of singlet oxygen in reactions may also be investigated through the use of specific quenchers. The carotenoid pigments have been shown to quench singlet oxygen without themselves being destroyed in the process (Foote and Denny, 1968; Foote, Denny, Weaver, Young and Peters, 1970c). This is due to the carotenes possessing a sufficiently low triplet state energy (Foote, Chang and Denny, 1970b; Farmilo and Wilkinson, 1973). Other scavengers of singlet oxygen include the tocopherols, tertiary amines and azide (Krinsky, 1977).

3.6.3 Protective Mechanisms against Singlet Oxygen

The production of singlet oxygen in chloroplasts may occur via intermediates of oxygen reduction or through pigment sensitisation reactions. In a similar manner to hydroxyl radical production, control of singlet oxygen formation may be derived from regulating the generation and subsequent fate of superoxide and hydrogen peroxide. However, chlorophyll is a very effective sensitiser for photooxidations of organic substrates. In vivo, most of the energy of singlet excited chlorophyll is used in photosynthetic electron transfer reactions, but it has been calculated that about four excitations in $10^4$ undergo intersystem crossing to form triplet excited chlorophyll (Breton and Mathis, 1970), thus providing the potential for photodynamic damage. Carotenoids are found in close association with chlorophyll molecules in the photosynthetic membrane and β-carotene in this respect, relies on its ability to scavenge singlet oxygen formed from triplet chlorophyll
Fig 1.8 Excited pigment decay mechanisms
(Fig. 1.9) (Foote and Denny, 1968; Foote et al., 1970a,b,c; Anderson and Krinsky, 1973). Although the scavenging of singlet oxygen by the carotene pigments is a highly efficient process, the chloroplast also contains high levels of antioxidants (Halliwell, 1978a), which react preferentially with highly reactive oxygen species. More than 90% of the total α-tocopherol present in green leaves is localised in the chloroplast (Hughes et al., 1971) and is known to be photooxidised by singlet oxygen (Grams, Eskin and Inglett, 1973). It is also an important inhibitor of radical chain oxidations, such as lipid peroxidation (Green, 1972; Tappel, 1972; Anderson and Krinsky, 1973). Thus, the chloroplast would appear to be well protected against singlet oxygen and its possible deleterious consequences.
4. AIMS OF THE THESIS

The aim of the present investigation was to attempt to clarify the relevance of superoxide in various plant reactions and with particular reference to the mode of action of paraquat. The first part of the study deals with the generation of both superoxide and hydrogen peroxide in plant systems of increasing complexity. In parallel with these investigations, attempts were made to determine some physico-chemical parameters of redox compounds that mediated oxygen reduction. The aim of this study was to devise a system that could be used to predict the oxygen activating ability of other compounds.

The role of superoxide in the herbicidal action of paraquat was examined by using an artificial SOD complex PA-Cu, in a study which aimed to determine the relative importance of this radical as compared to other oxygen species. Both the hydrocarbon gases ethylene and ethane are known to be produced under a variety of stress conditions and oxygen is involved in the biosynthesis of each. The mechanisms of biosynthesis were investigated with respect to the role of various active oxygen species in a series of model reactions with chloroplast lamellae.

The existence of a plant biotype which showed resistance to paraquat afforded a further opportunity to investigate the role of superoxide in the phytotoxic action of the herbicide. This study involved the investigation of endogenous mechanisms protecting against paraquat and complemented the flax cotyledon experiments which entailed the use of an exogenous compound to provide protection.
Due to the disparate nature of the various areas of study, each section has been discussed separately. A synthesis of the major points from each section has been attempted in a concluding discussion.

Although the major part of this project was undertaken at the University of Bath, various periods were spent elsewhere. A period of three months was spent in 1978 with Professor E.F. Elstner in Munich, during which time a short study was made with Professor A. Trebst in Bochum. In late 1979, a period of three months was spent at Jealott's Hill with Dr. J.A. Farrington of ICI Plant Protection Ltd.
MATERIALS AND METHODS
1. PREPARATION OF EXPERIMENTAL MATERIAL

1.1 Growth of Plant Tissue

Pea seedlings (*Pisum sativum* var. Meteor) were used as the source for most chloroplast preparations. They were grown in seed trays containing Levington Universal Compost for 2-3 weeks in a greenhouse with a 14 h light regime of daylight supplemented by mercury vapour lamps and at a temperature of 21-24°C.

Spinach (*Spinacea oleracea* L.) was also used for isolation of chloroplasts. The plants were grown outdoors and fully expanded leaves were harvested after 6-8 weeks.

Flax seedlings (*Linum usitatissimum* var. Reina) were grown on waterlogged vermiculite in evaporating dishes for 7 days in a growth cabinet at a temperature of 20-22°C under constant illumination of 5.25 Wm⁻² provided by Warm White fluorescent tubes. The dishes were covered for the initial 48 h to maintain a high humidity, after which time the seedlings continued growth in a relative humidity of 70%.

Fronds from asparagus (*Asparagus officinalis* cv. Marche de Malines) were used as a source of mesophyll cells. The plants were grown in growth chambers with a 16 h/22°C photoperiod and a 8 h /18°C night regime with a constant relative humidity of 60%.
Conyza \((Conyza\_linefolia)\) were grown initially in individual pots under greenhouse conditions and were then transferred to a laboratory at 20-25°C with natural daylight conditions.

The range of plants used in the biological screen were grown in individual pots under greenhouse conditions.

1.2 Preparation of Experimental Compounds

The redox compounds used were of reagent grade or better. For use in chloroplast assays, stock solutions were prepared at a concentration of \(3 \times 10^{-3} \text{M}\). Most compounds were water soluble; those which were not were first dissolved in a minimal volume of methanol and then diluted with distilled water. The final concentration of methanol in the reaction mixture never exceeded 0.3% and was found to be without effect with respect to control values.

DCMU was initially dissolved in methanol and then an equal volume of distilled water was added. The final concentration of methanol in the reaction mixture never exceeded 2.5%.

The concentration of the redox compounds in the asparagus cell assay was 10 ppm which was added as a 10 μl sample of an emulsion containing the compound. The emulsion was composed as follows:
4 mg compound
0.4 cm³ JF 5969 (emulsifier)
1.6 cm³ H₂O

The compounds tested in the biological screen were applied at a concentration equivalent to 10 Kg ha⁻¹, in an emulsion of the same proportions as described above.

In uptake studies with labelled paraquat, (methyl⁻¹⁴C) paraquat chloride (specific activity 111 mCi mmol⁻¹) was dissolved in distilled water.

Penicillamine-copper complex (PA-Cu) was kindly provided by Dr. E. Lengfelder and was dissolved in distilled water.

1.2.1 Synthesis of DBMIB

DBMIB was synthesised from thymoquinone according to Trebst, Harth and Draber (1970). 5 g of thymoquinone was reacted with bromine water for 3 days with constant stirring. The reaction product was recrystallised four times from methanol. Melting point determinations and NMR analysis were performed on the synthesised material and the values were compared with those of authentic samples of DBMIB.
1.3 Treatment of Plant Tissue

1.3.1 Flax Cotyledons

Flax cotyledons were detached from 7 day old seedlings and floated on experimental solutions in 50 cm$^3$ screw-top conical flasks, which had been fitted with rubber septum material to facilitate analysis of the gaseous headspace. Streptomycin was routinely included in the incubation medium at a concentration of 100 ppm to inhibit fungal growth. The flasks were maintained in a growth cabinet at 20-22°C under continuous illumination of 5.25 Wm$^{-2}$ provided by Warm White fluorescent tubes.

Cotyledons for chloroplast fatty acid analysis were floated on experimental solutions in 50 cm$^3$ Medical Flat bottles, which were laid on their flat side and incubated as previously described.

1.3.2 Conyza Leaf Sections

Leaves were selected that were 5-10 cm in length and were then cut into 1-2 cm sections. These were floated on herbicide solutions in 50 cm$^3$ screw-top conical flasks, or in 5 cm Petri dishes in the case of CO$_2$ exchange studies.
2. ISOLATION OF CELLS, ORGANELLES AND SUBCELLULAR FRACTIONS

2.1 Asparagus Cell Isolation (Jealott's Hill Method)

Asparagus fronds were selected which were fully grown (approximately 3 cm in length) and dark green in colour. Mesophyll cells were isolated from the fronds by stroking them longitudinally with a plastic plant pot label into 0.01 M HEPES pH 7.6, while allowing the fronds to slip through the fingers. The fronds were collected together and the process repeated three times. The thick green suspension was filtered through fine nylon gauze to remove macroscopic plant debris. 2 cm³ of the cell filtrate was pipetted into each conical flask and illuminated in a water bath (Grant Instrument SS30) in the presence or absence of redox compound for 45 mins. The cells were then collected by centrifugation at 1500 g for 30 s. The pellet was resuspended in fresh buffer and used immediately.

2.2 Chloroplast Isolation Techniques

2.2.1 Chloroplasts for Electron Transport Studies

Apical leaves from 2-3 week old pea seedlings were used for most electron transport assays and chloroplast model systems. The method of isolation was modified from that described by Jensen and Bassham (1966).
A stock solution was prepared and contained the following:

- 0.33 M sorbitol
- $2 \times 10^{-3}$ M NaNO$_3$
- $2 \times 10^{-3}$ M EDTA
- $2 \times 10^{-3}$ M sodium isoascorbate
- $1 \times 10^{-3}$ M MnCl$_2$ • 4H$_2$O
- $1 \times 10^{-3}$ M MgCl$_2$ • 6H$_2$O
- $5 \times 10^{-4}$ M K$_2$HPO$_4$ • 3H$_2$O

Pea leaves were homogenised for two periods of 7 s with 30 s cooling, in an Ultra Turrax homogeniser in a buffered medium (10 cm$^3$ per 1 g tissue) of the following composition:

- $5 \times 10^{-2}$ M MES dissolved in the stock solution
- $2 \times 10^{-2}$ M NaCl above and adjusted to pH 6.1
- 0.1% BSA

The brei was filtered through four layers of muslin and the filtrate centrifuged at 1000 g for 5 mins in a MSE 18 centrifuge. The supernatant was discarded and the pellet was resuspended in the following buffer:

- $5 \times 10^{-2}$ M HEPES dissolved in the stock solution
- $2 \times 10^{-2}$ M NaCl above and adjusted to pH 6.7

The suspension was recentrifuged at 1000 g for 5 mins and the supernatant discarded. The pellet was dispersed in a buffer of the following composition:

- $5 \times 10^{-2}$ M HEPES dissolved in distilled water
- $5 \times 10^{-3}$ M Na$_4$P$_2$O$_7$ • 10H$_2$O and adjusted to pH 7.8
This was recentrifuged at 12,500 g for 10 mins and the pellet was resuspended in a few cm³ of this final buffer and maintained on ice until required for use (Type E chloroplasts; Hall, 1972).

Spinach leaves were used as the source of chloroplasts in some experiments. The isolation procedure was essentially the same as for pea chloroplasts except that prior to homogenisation in a Waring Blender, the leaves were deveined and washed in ice-cold distilled water.

2.2.2 Chloroplasts for Photophosphorylation Determinations

Spinach chloroplasts (Type C; Hall, 1972) were used in photophosphorylation studies and were isolated by a modification of the method of Ort and Izawa (1973). After washing in ice-cold distilled water, spinach leaves were deveined and homogenised for 5 s in an Omnimix homogeniser set on maximum speed, with the following buffer:

\[
\begin{align*}
0.3 \text{ M} & \quad \text{NaCl} \\
3 \times 10^{-2} \text{ M} & \quad \text{tricine - NaOH \quad pH 7.8} \\
3 \times 10^{-2} \text{ M} & \quad \text{MgCl}_2 \\
5 \times 10^{-4} \text{ M} & \quad \text{EDTA}
\end{align*}
\]

The homogenate was filtered through four layers of fine nylon gauze and centrifuged for 4 min at 2,500 g in a Sorvall RC-2B centrifuge. The pellet was resuspended with the aid of a paint brush in a buffer of the following composition:
After centrifuging at 2000 g for 15 s, the supernatant was decanted and recentrifuged at 2000 g for 4 mins. The chloroplast pellet was resuspended in a few cm³ of the above medium and used.

2.2.3 Chloroplasts for Fatty Acid Analysis

Following herbicide treatment, flax cotyledons were dipped in liquid nitrogen and ground with a precooled mortar and pestle and the following buffer was gradually added:

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Buffer Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3 M</td>
<td>NaCl</td>
</tr>
<tr>
<td>$5 \times 10^{-3}$ M</td>
<td>Tricine - NaOH</td>
</tr>
<tr>
<td>$3 \times 10^{-3}$ M</td>
<td>MgCl₂</td>
</tr>
<tr>
<td>$1 \times 10^{-3}$ M</td>
<td>Na$_3$N</td>
</tr>
</tbody>
</table>

The brei was filtered through four layers of muslin and centrifuged at 200 g for 2 mins. The supernatant was decanted and recentrifuged at 7000 g for 10 mins. The pellet was used for fatty acid analysis.
5 kg of spinach leaves were washed in distilled water, deveined and left overnight at 4°C. It was then homogenised with 0.02 M Tris buffer, pH 8.0 (1 dm³ Kg⁻¹) in a large Waring Blender. After filtering through fine nylon gauze, the homogenate was maintained at 4°C for 1 h. Acetone at -20°C was then slowly added to 40% by volume, with constant stirring on an ice bath to maintain the temperature below 0°C. After standing for 30 mins, the extract was centrifuged at 5000 g for 10 mins in a Sorvall RC-2B centrifuge, and the pellet discarded. Further cold acetone was added to bring the preparation to 80% v/v and was allowed to stand for a further 30 mins. After centrifugation at 5000 g for 10 mins, the supernatant was discarded and the pellet resuspended in a small volume of 0.05 M Tris buffer, pH 8.0 and dialysed overnight at 4°C against distilled water adjusted to pH 8.0 by the addition of solid Tris. The precipitated material was removed by centrifugation at 25000 g for 20 mins. The supernatant was applied to a Whatman DE 52 column (20 cm x 4 cm), which had previously been equilibrated with 0.05 M Tris buffer, pH 7.3. About 400 cm³ of 0.05 M Tris, pH 7.3 was then passed through the column, followed by similar volumes of 0.1 M Tris, pH 7.3 containing 0.1 M and 0.2 M NaCl. These eluted yellow flavin and greyish plastocyanin. Ferredoxin was eluted with 0.1 M Tris, pH 7.3 containing 0.4 M NaCl. The red-brown liquid was dialysed overnight as before. The extract was diluted four times with distilled water and applied to a second
Whatman DE 52 column (50 cm x 4 cm) which had been equilibrated as previously described. The column was washed with 0.1 M and 0.2 M Tris, pH 7.3 and ferredoxin was eluted with 0.2 M Tris, pH 7.3 and 0.4 M NaCl. The resultant fractions were subjected to spectro-photometric determination and those exhibiting a 420/280 nm absorbance ratio of less than 0.2 were discarded. The ferredoxin fractions were concentrated on a small Sephadex column (2 cm x 4 cm). The dark red ferredoxin eluate was stored frozen until required for use.

2.4 Extraction and Purification of SOD

The preparation of SOD extracts from Conyza leaves was based on the method of Sawada, Ohyama and Yamazaki (1972). 25 g of leaf tissue was homogenised in an Ultra Turrax homogeniser with 250 cm³ of the following buffer:

```
0.3 M NaCl
5 x 10⁻²M Tricine - NaOH  pH 7.8
3 x 10⁻³M MgCl₂
1% PVP
```

The homogenate was strained through four layers of muslin and solid (NH₄)₂SO₄ was added to give 35% saturation. After standing at 4°C for 1 h with occasional stirring, the extract was centrifuged at 1000 g for 15 mins. The pellet was discarded and further (NH₄)₂SO₄ was added to the supernatant to bring this to 55% saturation.
This was left for 1 h at 4°C as before and the precipitate was collected by centrifugation at 25000 g for 30 mins. The pellet was resuspended in a small volume of ice-cold distilled water and dialysed overnight at 4°C. The precipitate formed during dialysis was removed by centrifuging at 1000 g for 15 mins and then 0.5 volume of acetone (precooled to -20°C) was added to the supernatant and vigourously mixed. The precipitate was removed by centrifuging at 5000 g for 30 mins and 1.0 volume of acetone was added to the supernatant as before. The precipitate was collected by centrifuging at 20,000 g for 30 mins and the pellet was resuspended in a small volume of 2.5 x 10^{-3}M phosphate buffer, pH 7.8. The insoluble residue was removed by centrifugation at 1000 g for 15 mins. The supernatant was applied to a Sephadex G-75 column (20 cm x 1 cm), which had previously been equilibrated with 2.5 x 10^{-3}M phosphate buffer, pH 7.8. The column was eluted with the same buffer and fractions were diluted 1:4 with buffer before being subjected to spectrophotometric determination at 280 nm for protein. Fractions exhibiting an absorbance greater than 1.0 were pooled and concentrated by dialysis against polyethylene glycol 4000 for about 2 h. SOD extracts were stored at -20°C until required for use.
2.4.1 Gel Electrophoresis of SOD Extracts

Squares (2 cm x 2 cm) were cut from a single thickness of wet dialysis tubing and placed over the rubber grommets of the gel holder. The gel tubes (8.5 cm x 0.75 cm) were pressed into the grommets and were thus held vertically and sealed at their lower ends by the dialysis tubing. The tubes had previously been treated with a 5% solution of dichlorodimethylsilane (Fisons) in chloroform to aid final removal of the gels. The solutions required for forming the gels were prepared as follows:

Solution A: 36.3% w/v Tris buffer, pH 8.0
Solution B: 24.0% w/v urea
Solution C: 40.0% w/v acrylamide
   0.8% w/v N,N-methylenebisacrylamide
Solution D: 0.75% w/v ammonium persulphate
Solution E: TEMED

The proportions required to form the gels are given below:

<table>
<thead>
<tr>
<th>% GEL</th>
<th>cm³ SOLUTION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>15</td>
<td>5</td>
</tr>
</tbody>
</table>
After mixing, 3 cm$^3$ of the gel mixture was gently pipetted into each tube, so as to avoid air bubble formation. Water was layered onto the top of each gel to aid polymerisation and to ensure a flat loading surface. Polymerisation of the gels was complete within 45 mins and during this period, the bottoms of the gel tubes were immersed in water to prevent dehydration of the gels.

The polymerised gels were pre-electrophoresed at 0.5 mA per tube for 2 h prior to loading. The running buffer in both upper and lower chambers was composed as follows:

- $5 \times 10^{-2}$M Tris buffer pH 8.0
- $8 \times 10^{-2}$M glycine

SOD extracts were concentrated by dialysis against polyethylene glycol 4000 in order to reduce the volume of application. A drop of 2% bromophenol blue solution was added to each sample to act as a marker during electrophoresis. The extracts were densified by the addition of a few sucrose crystals and were then applied to the gel surface. Electrophoresis was performed at 5 mA per tube (200 mV) until the bromophenol blue marker dye had almost reached the bottom of the gels. To reduce the heating effect associated with the applied current, the gel apparatus was maintained at 4°C during electrophoresis.

The gels were then removed from the tubes, placed in test tubes and stained for SOD activity, according to Beauchamp and Fridovich (1971). The gels were first soaked in $2.45 \times 10^{-3}$M NBT for 20 mins in the dark and then briefly rinsed with distilled water.
prior to immersion in the following medium:

- $2.8 \times 10^{-2}$M TEMED
- $2.8 \times 10^{-5}$M riboflavin
- $3.6 \times 10^{-2}$M phosphate buffer pH 7.8

After 20 mins incubation in the dark, the gels were removed to dry test tubes and illuminated by a 500 W projector lamp at a distance of 30 cm for approximately 30 mins. The gels became uniformly blue-purple except where SOD was located. When maximum contrast had been achieved, the gels were photographed and then scanned in a Pye Unicam SP1800-1809 densitometer attachment at 600 nm.

2.5 Extraction of Crocin (Friend and Mayer, 1960)

Commercial saffron (Aldrich) was ground to a fine powder with a mortar and pestle. 3 g of the powdered saffron was extracted with diethyl ether in a Soxhlet extractor for 1 h to remove fats and lipid material. After the residue was dried briefly in a stream of nitrogen, it was re-extracted as before with methanol. The methanolic extract was evaporated to near dryness in a nitrogen stream and stored at -20°C in the dark until required for use.
3. QUANTITATIVE DETERMINATIONS

3.1 Chlorophyll Estimation

3.1.1 Leaf Tissue

Leaf sections or cotyledons were placed in 10 cm$^3$ of 80% acetone and maintained in the dark at -20°C for 7 days to allow chlorophyll to leach out of the tissue. A sample of the extract was determined spectrophotometrically at 645 nm and 663 nm. The amounts of total chlorophyll, chlorophylls $a$ and $b$ were calculated using the coefficients of Arnon (1949) as follows:

Total chlorophyll = $8.02 \ E_{663} + 20.2 \ E_{645} \ \text{mg dm}^{-3}$

Chlorophyll $a$ = $12.7 \ E_{663} - 2.69 \ E_{645} \ \text{mg dm}^{-3}$

Chlorophyll $b$ = $22.9 \ E_{645} + 4.68 \ E_{663} \ \text{mg dm}^{-3}$

3.1.2 Chloroplast Suspensions

0.2 cm$^3$ of chloroplast suspension was extracted with 9.8 cm$^3$ of acetone. The extract was filtered through Whatman No.1 filter paper and chlorophyll was determined as above. Occasionally, chlorophyll was determined by a single absorbance reading at 652 nm, according to the following equation:

Total chlorophyll = $\frac{E_{652} \times 1000}{34.5} \ \text{mg dm}^{-3}$

(Arnon, 1949)
3.2 Carotenoid Estimation (Modified from Bishop and Wong, 1971)

50 flax cotyledons were ground with a mortar and pestle in a small volume of 80% acetone and some acid-washed sand. The volume of acetone was gradually increased to 25 cm$^3$ and the extract was centrifuged at 2500 g for 3 mins. The supernatant was maintained in the dark while the sand pellet was re-extracted with two 15 cm$^3$ aliquots of 80% acetone. The total pigment extracts were combined and partitioned into 25 cm$^3$ diethyl ether in a 500 cm$^3$ separating funnel, in the presence of 200 cm$^3$ of saturated NaCl solution to aid partitioning. The upper ether layer containing the pigments was retained in the dark and the lower aqueous layer was re-extracted with 15 cm$^3$ of diethyl ether. The two ether fractions were combined and taken to dryness at 40°C on a rotary evaporator under vacuum. The pigment residue was redisolved in petroleum ether (60-80°C) and 1 cm$^3$ was applied as a streak to a 10 cm x 10 cm TLC plate, coated with silica gel (Kieselgel G Nach Stahl, Type 60) to a thickness of 0.5 mm. The TLC plate was developed in the dark for 45 mins, in a mixture of petroleum ether, isopropyl alcohol and water (100:10:0.5). The main carotenoid pigments were resolved in the order; α and β carotenes, lutein and zeaxanthin, violaxanthin and neoxanthin. They were quickly removed from the TLC plate and eluted from the silica gel with either petroleum ether (carotenes) or ethanol (xanthophylls). The silica gel was removed by centrifugation at 2500 g for 3 mins and the absorbance of the carotenoid pigments was determined at their respective wavelengths of maximum absorbance.
The pigment concentrations were calculated using the extinction coefficients of Jeffrey (1968), as given below:

<table>
<thead>
<tr>
<th>CAROTENOID</th>
<th>$\lambda_{\text{max}}$</th>
<th>$\varepsilon_{1% \text{ cm}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$ and $\beta$ carotene</td>
<td>450 nm</td>
<td>2505</td>
</tr>
<tr>
<td>Lutein and zeaxanthin</td>
<td>447 nm</td>
<td>2550</td>
</tr>
<tr>
<td>Violaxanthin</td>
<td>441 nm</td>
<td>2250</td>
</tr>
<tr>
<td>Neoxanthin</td>
<td>438 nm</td>
<td>2270</td>
</tr>
</tbody>
</table>

$$\text{Amount of pigment} = \frac{E \cdot y}{\varepsilon_{1\% \text{ cm}} = 100} \times 3 \text{ g}$$

where $E = \text{absorbance at } \lambda_{\text{max}}$

and $y = \text{volume of extract}$

3.3 Determination of Protein

Protein was determined according to the method of Lowry, Rosebrough, Farr and Randall (1957) as modified by Hartree (1972).

Solution A: 2 g potassium sodium tartrate
100 g sodium carbonate
500 cm$^3$ 1M NaOH
H$_2$O to 1L
Solution B : 2 g potassium sodium tartrate
1 g CuSO₄ • 5H₂O
90 cm³ 1M NaOH
10 cm³ H₂O

Solution C : 1 vol. Folin-Ciocalteu reagent
14 vols. H₂O

0.9 cm³ of solution A was added to 0.1 cm³ of protein sample and 1 cm³ of distilled water and heated to 50°C for 10 mins. After cooling to room temperature, 0.1 cm³ of solution B was added and the mixture allowed to stand for 15 cm³. 3 cm³ of freshly prepared solution C was added rapidly to ensure thorough mixing. The colour was developed at 50°C for 10 mins. After cooling to room temperature, the absorbance was determined at 650 nm.

A protein calibration curve was constructed using BSA as the protein standard was shown to be linear up to 100 µg protein.
3.4 Analysis of Fatty Acids

3.4.1 Lipid Extraction

Lipids were extracted from chloroplasts isolated from treated flax cotyledons by a modification of the method of Bligh and Dyer (1959). The chloroplast pellet obtained in Section 2.2.3 was resuspended in 15 cm$^3$ of methanol. This was vigourously shaken for 3 mins with 20 cm$^3$ of chloroform, 25 cm$^3$ of methanol and 10 cm$^3$ of distilled water, in a 500 cm$^3$ conical flask. The flask contents were then vacuum filtered through Whatman No.3 filter paper and the residue was washed with a further 20 cm$^3$ chloroform. The filtrate was transferred to a 1 dm$^3$ separating funnel containing 20 cm$^3$ of water and shaken. After standing for 5 mins, the lower fatty acid-rich layer (green) was removed by centrifugation at 4500 g for 4 mins. The aqueous phase was re-extracted with 20 cm$^3$ chloroform. The chloroform extracts were combined and taken to dryness under vacuum on a rotary evaporator at 30°C. The samples were stored at -20°C in the dark.

3.4.2 Preparation of Fatty Acid Methyl Esters

Methyl ester derivatives of the fatty acids prepared as described in the preceding section were obtained by a modification of the method of Metcalfe, Schmitz and Pelka (1966).
25 cm³ of 0.5 N methanolic NaOH was added to the lipid sample and refluxed for 20 mins. After cooling, 25 cm³ of 14% boron trifluoride methanol (Sigma) was added and refluxed for a further 20 mins. The extract was then transferred to a 1 dm³ separating funnel with 30 cm³ saturated NaCl solution and 50 cm³ petroleum ether (40-60°C). After shaking for 1 min, the upper, petroleum ether layer was removed and the lower, aqueous layer was re-extracted with a further 50 cm³ of petroleum ether. The extracts were combined and dried over anhydrous sodium sulphate for 30 mins. After vacuum filtering the extracts were dried on a rotary evaporator at 20°C under vacuum. The dried methyl esters were taken up in 3 cm³ of Aristar grade diethyl ether and stored in the dark under argon at -20°C.

3.4.3 Analysis of Fatty Acid Methyl Esters by GLC

A Pye Unicam GCD Chromatograph was used to separate and identify fatty acid methyl esters. It was fitted with a glass column with 10% DEGS as the stationery phase and 80/100 Chromosorb W-AW as the solid support. Both the column and detector oven were operated at 190°C and nitrogen was used as the carrier gas at a flow rate of 35 cm³ min⁻¹. Esters were identified by comparison of their retention times with those of pure methyl esters and also by an internal standards method.
3.5 Ethane and Ethylene Determination

Ethane and ethylene produced from both intact tissue and chloroplast model systems were detected by GLC. 1 cm$^3$ of the gaseous headspace of the reaction flasks was removed using a gas-tight syringe (Precision Sampling, St. Louis, Mo.) and analysed. Ethane and ethylene were identified by comparison of retention times with authentic samples. Two types of GLC apparatus were used for analysis.

3.5.1 Pye Unicam GLC

A Pye Unicam GCD Chromatograph equipped with an alumina column was used for most headspace analyses of intact tissue. The column and detector oven temperatures were 125°C and the gas flow rates were:

- $N_2$ : 40 cm min$^{-1}$
- $H_2$ : 15 lb in$^{-2}$
- air : 10 lb in$^{-2}$

3.5.2 Varian Aerograph GLC

Most headspace analyses of the chloroplast model experiments were determined using a Varian Aerograph 1400 GLC, fitted with a Poropak R column maintained at 70°C. The temperatures of both the injection chamber and detector oven were 125°C. The following gas flow rates were employed:
Quantitative determinations of the hydrocarbon gases were facilitated by the use of a Varian CDS 101 integrator.

3.6 CO₂ Exchange

The CO₂ exchange of Conyza leaf sections was monitored by an Infra-red Gas analyser (IRGA) supplied by Grubb Parsons Ltd., connected to an open circuit gas flow system. Compressed air (500 cm³ min⁻¹) was passed through a CaCl₂ drying tower and then divided into two equal streams. One stream was directed to the IRGA reference tube, while the other passed through the sample chamber to a second drying tube to remove water vapour from the leaf sections. The gas stream then passed to the IRGA sample tube. Identical flow rates in both the sample and reference tubes of the IRGA were achieved through the use of separate flowmeters.

The glass sample chamber was illuminated by a photoflood lamp giving a light intensity of 115 Wm⁻² at the chamber surface. A water-filled heat filter was introduced between the lamp and the sample chamber. CO₂ exchange was calculated from the following equation:
\[ y = \frac{x \cdot C_f}{M \cdot 10^{-6}} \]

where \( y \) = rate of CO\(_2\) exchange in \( \text{cm}^3 \text{ min}^{-1} \)
\( x \) = dark recorder value (evolution) or difference between light and dark recorder values (uptake)
\( C_f \) = calibration of one IRGA division
\( M \) = recorder/IRGA magnification factor.

3.7 Uptake of \( ^{14}\text{C}-\text{Paraquat} \) by Conyza

Leaf sections of Conyza were prepared as detailed in Section 1.3.2 and incubated with \( 10^{-5}\text{M} \) paraquat solution containing 0.2 \( \mu\text{Ci} \) of \( ^{14}\text{C} \)-labelled herbicide for 1 h, under a light intensity of 5.25 \( \text{Wm}^{-2} \). Treated tissue was solubilised in 1.5 \( \text{cm}^3 \) NCS (Amersham Corp.) for 24 h at 50°C. An aliquot of 0.1 \( \text{cm}^3 \) was added to 10 \( \text{cm}^3 \) of toluene-based scintillant of the following composition:

- 5 g PPO
- 0.2 g POPOP
- 500 \( \text{cm}^3 \) toluene
- 500 \( \text{cm}^3 \) Triton X-100

The samples were counted in a Packard Tri-Carb liquid scintillation counter.
4. EXPERIMENTAL ASSAY TECHNIQUES

4.1 Oxygen Electrode

4.1.1 Oxygen exchange in isolated chloroplasts

Oxygen exchange studies in chloroplast systems were conducted at 22°C in either a Rank electrode (Rank Bros., Cambridge), linked to a Servoscribe recorder, or in a Hansatech D.W. electrode (Bachofer, Reutlingen) coupled to a Phillips chart recorder. Both electrodes were calibrated using sodium dithionite, prior to each experiment. The basic 3 cm³ reaction mixture was composed as follows:

- \(2.5 \times 10^{-2} \text{M phosphate buffer, pH 7.8}\)
- \(1.7 \times 10^{-3} \text{M NH}_4\text{Cl}\)
- \(1.7 \times 10^{-3} \text{M MgCl}_2\)
- \(1 \times 10^{-3} \text{M NaN}_3\)

Chloroplast lamellae containing 100 µg chlorophyll

The chloroplast suspension was the last addition and was followed by a period of dark equilibration, before illuminating the reaction chamber. Details of other additions to the basic reaction mixture are described in the legends to the appropriate figures in the Results section.
4.1.2 Oxygen exchange by asparagus cells

A Hansatech oxygen electrode (Hansatech, King's Lynn) coupled to a Servoscribe recorder was used in oxygen exchange studies with isolated asparagus cells. In experiments to study the effect of compounds on net oxygen evolution (as a measure of CO₂ fixation), 2 cm³ of asparagus cells which had been preincubated in the absence of redox compound, were placed in the reaction chamber and allowed to equilibrate in the light for 1 min. before determining the basal rate of O₂ evolution. 10 µl of redox compound was added and its effect measured. The influence of preincubation of the cells with redox compound was determined similarly, with the exception that that oxygen exchange rates were followed in the presence and absence of fresh compound, added directly to the reaction chamber.

4.2 Determination of Superoxide (Elstner and Heupel, 1976)

Superoxide anion radical O₂⁻⁻, was assayed by its ability to oxidise hydroxylamine to nitrite according to the equation (Elstner, Heupel and Vaklinova, 1970; Elstner & Heupel, 1976)

\[ \text{NH}_2\text{OH} + 2\text{O}_2⁻⁻ + \text{H}⁺ \rightarrow \text{NO}_2⁻⁻ + \text{H}_2\text{O}_2 + \text{H}_2\text{O} \]  

(25)

The basic reaction mixture was as detailed in Section 4.1.1, except for the addition of 1 µmole of NH₂OH. Other conditions are described in the relevant figure legends in the Results section.

Nitrite formed during the incubation was determined following an
azo-coupling of sulphanilic acid with $\alpha$-naphthylamine. The following reagents were required:

- Sulphanilic acid reagent: 1.65 g sulphanilic acid
  - 125 cm$^3$ glacial acetic acid
  - 375 cm$^3$ H$_2$O

- $\alpha$-Naphthylamine reagent: 0.5 g $\alpha$-naphthylamine
  - 125 cm$^3$ glacial acetic acid
  - 375 cm$^3$ H$_2$O

On conclusion of the experiment, 1 cm$^3$ of the reaction mixture was added to 1 cm$^3$ of sulphanilic acid solution, followed by 1 cm$^3$ of $\alpha$-naphthylamine reagent. The colour was allowed to develop for 15 min at room temperature and the sample was centrifuged to remove chloroplast debris. The supernatant was subjected to spectrophotometric determination at 530 nm and the amount of nitrite formed was calculated from a standard curve obtained with KNO$_2$.

4.3 Determination of Hydrogen Peroxide

The assay used for the detection of hydrogen peroxide was based on its ability to oxidise NADH in the presence of a NADH-specific peroxidase from *Streptococcus faecalis* (Boehringer, Mannheim).

4.3.1 Hydrogen peroxide production by chloroplasts

The basic reaction mixture was as described in Section 4.1.1. On conclusion of the incubation period, the reaction mixture samples were centrifuged at 3000 g for 10 mins to remove chloroplast material.
The supernatant was assayed for hydrogen peroxide as follows:

- $6 \times 10^{-2}$ M acetate buffer, pH 5.5
- $1.67 \times 10^{-4}$ M NADH
- 1 cm$^3$ supernatant
- 1 μg NADH peroxidase (sp. act. 45 U/mg)

The enzyme was the last addition to the 3 cm$^3$ assay mixture and an initial absorbance was made after 15s, at 340 nm. The absorbance was redetermined after 30 mins. and the difference was proportional to the amount of hydrogen peroxide originally present.

4.3.2 Hydrogen peroxide production by flax cotyledons

30 treated flax cotyledons were dipped in liquid nitrogen and rapidly ground to a fine powder with a mortar and pestle. 20 cm$^3$ of the following buffer were added gradually and grinding was continued.

- $5 \times 10^{-2}$ M tricine-NaOH pH 7.8
- 0.3M NaCl
- $3 \times 10^{-3}$ M MgCl$_2$
- $1 \times 10^{-3}$ M NaN$_3$
- 0.1% BSA

The homogenate was centrifuged at 17,000 g for 15 mins and 1 cm$^3$ of the supernatant was taken and assayed for hydrogen peroxide as described in the preceding section.

4.4 Determination of SOD Activity

SOD isolated from Conyza as described in Section 2.4, was assayed
by two independent methods to compare the specific activity of the extracts.

4.4.1 Inhibition of nitrite formation from hydroxylamine

This assay was carried out essentially as described in Section 4.2 with the following differences. Superoxide was generated enzymatically from xanthine and xanthine oxidase in a 3 cm³ reaction mixture composed as follows:

- $2.5 \times 10^{-2}$M phosphate buffer, pH 7.8
- $5 \times 10^{-4}$M xanthine
- xanthine oxidase (containing 120 µg protein)
- SOD extract.

The reaction was started by the addition of xanthine oxidase and was conducted at 25°C for 20 mins. On conclusion of the reaction, 1 cm³ was taken and analysed for nitrite as described in Section 4.2. One unit of enzyme activity was defined as that amount which caused 50% inhibition of nitrite formation from hydroxylamine.

4.4.2 Stimulation of dianisidine photooxidation

In this assay, first described by Misra and Fridovich (1977), SOD stimulates the formation of oxidised dianisidine. Superoxide was generated photochemically and the 3 cm³ reaction mixture was composed as follows (Lengfelder and Elstner, 1979).

- $1 \times 10^{-2}$M phosphate buffer, pH 7.8
- $2 \times 10^{-4}$M dianisidine dihydrochloride
- $1.3 \times 10^{-5}$M riboflavin.

The reaction was started by the addition of riboflavin and the
sample cuvettes were illuminated by a 500 W projector lamp at a distance of 30 cm. At intervals, the absorbance was determined at 460 nm.

4.5 Bleaching of Crocin by Isolated Chloroplasts

The water soluble carotenoid crocin, was prepared as described in Section 2.5 and used in chloroplast model systems to investigate carotenoid destruction. The reaction mixture was as outlined in Section 4.1.1 with the exception that crocin was present at a concentration of $10^{-5} \text{M}$ and the chlorophyll concentration was reduced to 25 $\mu$g. The reaction was conducted at 20°C with a light intensity of 30 Klux and was terminated by the addition of 0.5 cm$^3$ of 0.3M ZnSO$_4$. After standing in the dark for 10 mins, the precipitate was removed by centrifugation at 2000 g for 5 mins. The absorbance of the supernatant was determined at 440 nm.

4.6 NADP Reduction

NADP reduction by isolated spinach chloroplasts was determined with a Zeiss PMQ 3 spectrophotometer fitted for cross illumination and linked to a Servoscribe recorder. The reaction mixture contained in 3 cm$^3$ at 22°C:

- $2.5 \times 10^{-2} \text{M}$ phosphate buffer, pH 7.8
- $1.7 \times 10^{-3} \text{M} \text{NH}_4\text{Cl}$
- $1.7 \times 10^{-3} \text{M} \text{MgCl}_2$
- $1.7 \times 10^{-3} \text{M} \text{NADP}$
- $3.3 \times 10^{-6} \text{M}$ ferredoxin

chloroplasts with 30 $\mu$g chlorophyll.
Other additions are described in the legends to the figures in the Results section.* The reaction was followed by the change in absorbance at 340 nm.

4.7 Determination of ATP\textsuperscript{32} Formation

Photophosphorylation by spinach chloroplast lamellae was determined according to Conover, Praire and Racker (1963), as the level of radioactivity remaining after extraction of \textsuperscript{32}P-labelled orthophosphate as phosphomolybdate in isobutanol-benzene. The reaction mixture was as follows:

- \(2.67 \times 10^{-2}\) M tricine-NaOH, pH 8.0
- \(3.3 \times 10^{-3}\) M MgCl\(_2\)
- \(3.3 \times 10^{-3}\) M ADP
- \(3.3 \times 10^{-3}\) M \textsuperscript{32}P
- \(1 \times 10^{-3}\) M NADP
- \(3.3 \times 10^{-6}\) M ferredoxin

chloroplasts with 200 \(\mu\)g chlorophyll.

The reaction volume was 3 cm\(^3\) and the vessels were illuminated for 15 mins with a light intensity of 30 Klux at 20\(^\circ\)C.

On completion of the reaction, chloroplast debris was removed by the addition of 0.2 cm\(^3\) of 20% TCA. After 5 min, the precipitate was removed by brief centrifugation. 0.8 cm\(^3\) of the supernatant

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Footnote*
In conjunction with the photophosphorylation studies, the absorbance of a sample of the reaction mixture was measured at 340 nm. Following the addition of 0.1 cm\(^3\) of \(1 \times 10^{-4}\) M PMS, the absorbance was redetermined; the difference between readings was proportional to the amount of reduced NADP present.
was added to 4 cm$^3$ of 1N perchloric acid and 1 cm$^3$ of 5% ammonium heptamolybdate solution. To this was added 5 cm$^3$ of isobutanol-benzene (1:1 v/v) and the sample was agitated for 10 mins and the organic phase discarded. The aqueous phase was reextracted with 5 cm$^3$ of isobutanol saturated with water and treated as before. The final extraction was made with 2.5 cm$^3$ of diethyl ether. From the residual aqueous phase, 0.4 cm$^3$ was pipetted onto a planchette, dried and determined for radioactivity with a Geiger-Müller tube for 1 min at 1KV.

4.8 Ethylene Formation in Isolated Chloroplasts

The role of active oxygen species in ethylene formation was determined in a series of model experiments using isolated chloroplasts. The basic reaction mixture of 3 cm$^3$ was composed as follows:

\[ 2.5 \times 10^{-2} \text{M phosphate buffer, pH 7.8} \]
\[ 1.7 \times 10^{-3} \text{M NH}_4\text{Cl} \]
\[ 1.7 \times 10^{-3} \text{M MgCl}_2 \]
\[ 1 \times 10^{-3} \text{M Na}_2\text{N}_3 \]
\[ 1 \times 10^{-2} \text{M L-methionine} \]
\[ 2 \times 10^{-4} \text{M pyridoxal phosphate} \]
\[ 50 \text{ mg BSA} \]

Details of other additions are given in the appropriate Results section.

The reactions were conducted in 15 cm$^3$ conical flasks with screw tops fitted with serum rubber to allow headspace sampling. They were illuminated for 30 min at 22°C at a light intensity of 30 Klux. The reaction was terminated by the addition of 0.1 cm$^3$
0.5N H₂SO₄. A 1 cm³ sample of the flask headspace was analysed by GLC as described in Section 3.5.

4.9 Ethane Evolution

The evolution of ethane from treated tissue was used as an indicator of lipid peroxidation in flax cotyledon experiments. Isolated chloroplasts were used to study the mechanism of ethane formation.

4.9.1 Ethane release from flax cotyledons

20 flax cotyledons were excised and incubated on 3 cm³ of medium in flasks as described in Section 1.3.1. The flask headspace was analysed by subjecting a 1 cm³ sample to GLC determination.

4.9.2 Ethane formation by isolated chloroplast lamella

Studies into the mechanism of ethane formation were made using the conicals flasks described in Section 4.8. The basic reaction mixture contained in 3 cm³:

- 2.5 x 10⁻² M phosphate buffer, pH 7.8
- 1.7 x 10⁻³ M NH₄Cl
- 1.7 x 10⁻³ M MgCl₂
- 1 x 10⁻³ M NaN₃
- 3.3 x 10⁻³ M α-linolenic acid
- 50 mg BSA

The flasks were incubated at 20°C with a light intensity of 250 Wm⁻².
On conclusion of the reaction, 1 cm$^3$ of the headspace was subjected to GLC analysis.

5. **ELECTRON MICROSCOPY**

5.1 **Sample Preparation for Transmission Electron Microscopy**

The following reagents were prepared:

a) 0.1 M phosphate buffer, pH 7.0 : diluted to 0.05M for washing leaf specimens

b) 3% Sorenson's Buffered Glutaldehyde : 12 cm$^3$ 25% GDA
   50 cm$^3$ 0.1M phosphate buffer
   38 cm$^3$ H$_2$O

c) 2% Osmium tetroxide (OsO$_4$) : 200 mg OsO$_4$ dissolved in 10 cm$^3$
   of 0.05M phosphate buffer and stored in a clean, stoppered bottle at 4°C.

d) Spurr's Low viscosity resin : (Spurr, 1969)
   10 cm$^3$ vinylcyclohexane dioxide (ERL 4206)
   6 cm$^3$ diglycidylether or polypropylene glycol (DER 736)
   26 cm$^3$ nonyl succinic anhydride (NSA)
   0.4 cm$^3$ dimethylaminoethanol (S-1)
e) Uranyl acetate : saturated solution in 70% methanol
f) Reynold's Lead Citrate : (Reynold, 1963)
   1.33g lead nitrate
   1.76g sodium citrate
   30 cm³ CO₂-free
distilled H₂O

The mixture was shaken vigorously for 1 min in a 50 cm³ volumetric flask and then allowed to stand with occasional shaking to ensure complete conversion of lead nitrate to citrate. After 30 mins, 8 cm³ of 1N NaOH was added and the solution was made up to 50 cm³ with further CO₂-free distilled water.

5.1.1 Method of preparation

Treated flax cotyledons were briefly dried on filter paper before being placed on dental wax with a few drops of GDA solution. The plant material was cut into 1 mm² sections and placed in specimen tubes with a few cm³ of GDA. The tubes were placed in a vacuum until all the sections sank, to ensure complete penetration of the fixative. The sections were then transferred to fresh fixative and were then incubated at room temperature for 30 min on a rotator. After removal from the GDA, the sections were washed with 3 x 5 min changes of 0.05M phosphate buffer, pH 7.0 and then post fixed in OsO₄ for 1 h at room temperature on the rotator. Following this incubation, OsO₄ was removed by 2 x 5 min changes of phosphate buffer and one 5 min wash in distilled water. The sections were then dehydrated in an acetone series (15%, 50%, 100%). The lower concentration washes were of 5 min
duration, but the sections were subjected to 3 x 5 min changes of 100% acetone. The lowest acetone concentration also contained 1% uranyl acetate.

The sections were transferred to Spurr’s resin and incubated overnight at room temperature on the rotator, to allow complete infiltration by the resin. The sections were then removed to flat embedding moulds with further resin and incubated at 60°C for 72h.

The resin blocks were trimmed with a razor blade to expose the leaf specimen and sections were cut on a Reichert (Austria) OMU ultramicrotome using a glass knife. The cut sections were released from the knife edge and were briefly stretched by chloroform vapour before mounting on copper grids.

The mounted sections were stained as follows, after centrifuging the stains immediately prior to use. Drops of uranyl acetate were placed on dental wax and the grids were inverted and placed on the stain for 7.5 min. After washing in 50% methanol, the grids were rinsed in three changes of CO₂-free distilled water. The sections were then stained with lead citrate for 7.5 min as described previously. Solid NaOH was present in the staining dish to prevent CO₂ reacting with the stain to form lead carbonate. After three washes in CO₂-free distilled water, the grids were blotted dry and stored in grid boxes.

5.1.2 Specimen examination

The sections were viewed in a Jeol 100CX transmission electron microscope and micrographs were recorded on Kodak 4489 electron microscope film (Estar thick base), which was developed in PQ
Universal developer diluted 1:15 with water and fixed in Hypam diluted 1:8 with water.

5.2 Sample Preparation for EDAX and STEM Analysis

Treated flax cotyledons were manipulated as described in the preceding sections with the following modifications. Vacuum infiltration by GDA was replaced by a longer incubation (1.5h) at room temperature. The post fixation step was omitted and the sections were dehydrated by 3 x 5 min changes of 2,2-dimethoxypropane (TAAB). The sections were then mounted in resin and sections were cut as before, but mounted on nylon grids impregnated with titanium.

5.2.1 Specimen examination and analysis

The sections were viewed using a Jeol 100 CX electron microscope fitted for STEM. Both stained and unstained sections were examined. The nylon grids were mounted on a beryllium holder. The distribution of PA-Cu was determined by electron dispersive X-ray analysis (EDAX) of the copper in the complex.
6. ELECTROCHEMICAL REDUCTION OF REDOX COMPOUNDS

The apparatus used to study the reduction of a number of redox compounds in the absence of oxygen is shown in Fig. 2.1. The function of the system was to allow the controlled reduction of a compound in the complete absence of oxygen and to permit any associated spectral differences to be detected. The apparatus was constructed almost entirely of glass with the minimum of plastic tubing, as oxygen was shown to pass through the plastic in a very short time.

The reduction of the material under investigation was performed in the reaction chamber and a positive pressure of oxygen-free argon (from reduced paraquat scrubbers) was used to expel a sample of the reaction mixture from the chamber, and via the spectrophotometric flow cell into the large reservoir. This was returned to the main reaction chamber after equalising the pressure in both vessels. Oxygen-saturated distilled water entered the chamber from a small, calibrated reservoir. This reservoir was also used in chemical reductions to accommodate solutions of sodium dithionite.

Electrochemical reductions were performed using a potentiostat linked to an integrator which indicated the total charge applied. The reference and secondary electrodes were contained in small plastic tubes dipping into the reaction mixture. A Vycor disc, permeable to electrons, was heat sealed into the end of each tube.
Fig. 2.1 Reduction apparatus
6.1 Chemical Reduction with \( \text{Na}_2\text{S}_2\text{O}_3 \)

The composition of the reaction mixture was as follows:

- \( 2.5 \times 10^{-4} \)M redox compound
- 1N KCl
- \( 1 \times 10^{-2} \)M buffer

Deoxygenated distilled water to 200 cm\(^3\)

Sodium dithionite was dissolved in 1 cm\(^3\) of 1N NaOH and diluted to 5 cm\(^3\) with deoxygenated distilled water. After adding the above components to the reaction chamber, the system was evacuated three times. Oxygen-free argon was admitted to return the system to atmospheric pressure after each evacuation. The sodium dithionite solution was added in aliquots of 4 drops (corresponding to 12 \( \mu \)moles \( \text{Na}_2\text{S}_2\text{O}_3 \)). After mixing, the spectrophotometric flow cell was flushed three times with reaction mixture prior to spectral scanning in the range 200-700 nm in a Pye Unicam SP1800 spectrophotometer. During chemical reduction studies, the electrodes shown in Fig. 2.1 were replaced by a probe which permitted the potential of the reaction mixture to be determined.

6.2 Potentiometric Reduction

The composition of the reaction mixture for potentiometric reduction studies is given in the preceding section. Oxygen was eliminated from the system and the potential across the reaction

*Bicarbonate buffer was used at high pH, otherwise phosphate buffer was employed.*
mixture was increased in increments of 25 mV. The corresponding cell current values were noted and plotted against the applied potential. The resultant curve indicated the reduction pattern of the compound under investigation and was used to determine the applied potential required to reduce the compound to its first stable reduction state.

Using a fresh reaction mixture, the applied potential was kept constant at the value determined above and the cell current was plotted against the total charge applied to the system. From this, the amount of charge required to half reduce the compound was established. During this reduction, the reaction mixture was scanned at 200-700 nm.

The reduction above was repeated with fresh reaction mixture until the total applied charge was equal to that required to half reduce the compound, i.e. the point at which maximum semiquinone formation occurred. Oxygen-saturated distilled water was then admitted to the system and the associated spectral changes were followed.

6.3 Analysis of Reduction Curves

The reduction curves were analysed with the aid of a Fortran IV computer program, based on a CSMP package. This optimised the experimental reduction values and calculated the extinction coefficients of the compound under investigation in its oxidised, semiquinone and reduced forms. The rate constant for the formation of semiquinone was also determined.
RESULTS AND DISCUSSION
RESULTS AND DISCUSSION

1. MEDIATION OF OXYGEN REDUCTION BY REDOX COMPOUNDS

1.1 Studies with Isolated Chloroplasts

A large range of compounds was investigated to determine their ability to stimulate chloroplastic oxygen reduction. Various chemical types were studied and selected according to their one-electron redox potential ($E_1$). The reducing side of PSI is believed to have a potential of about -0.55 V (Zweig and Awron, 1965; Zweig et al., 1965), while that of the $O_2/O_2^-$ couple is -0.16V. Thus, it would be expected that a potential mediator of photosynthetic oxygen reduction should possess a redox potential between these two values. It was hoped that a comparison of compounds with a range of $E_1$ values would indicate the relationship between redox potential and an ability to catalyse oxygen reduction in isolated chloroplasts.

The results from this survey are summarised in Table 2. The rates shown are the means of at least three experiments. Time courses of the formation of both products of oxygen reduction are given in the Appendix. It is apparent from the tabulated results that there is no direct relationship between the $E_1$ values of the compounds and the experimental data. However, those compounds which produced superoxide and hydrogen peroxide at high rates generally possessed $E_1$ values of between approximately -0.20V and -0.64V. This last value is of particular significance,
Table 2. Oxygen Reduction by Chloroplast Lamellae

<table>
<thead>
<tr>
<th>Compound</th>
<th>$E_1$</th>
<th>$\frac{O_2}{\text{umoles mg Chl}^{-1}\text{h}^{-1}}$</th>
<th>$\frac{O_2^-}{\text{umoles mg Chl}^{-1}\text{h}^{-1}}$</th>
<th>$\frac{H_2O_2}{\text{umoles mg Chl}^{-1}\text{h}^{-1}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trimethylnitrobenzene</td>
<td>-860mV</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Tetraquat</td>
<td>-640</td>
<td>80</td>
<td>27</td>
<td>65</td>
</tr>
<tr>
<td>p-Nitroacetanilide</td>
<td>-570</td>
<td>64</td>
<td>17</td>
<td>96</td>
</tr>
<tr>
<td>Triquat</td>
<td>-550</td>
<td>85</td>
<td>29</td>
<td>45</td>
</tr>
<tr>
<td>5-Nitouracil</td>
<td>-530</td>
<td>3</td>
<td>2</td>
<td>17</td>
</tr>
<tr>
<td>Nitrobenzene</td>
<td>-490</td>
<td>30</td>
<td>8</td>
<td>58</td>
</tr>
<tr>
<td>Paraquat</td>
<td>-440</td>
<td>82</td>
<td>27</td>
<td>120</td>
</tr>
<tr>
<td>m-Nitroacetophenone</td>
<td>-440</td>
<td>57</td>
<td>22</td>
<td>75</td>
</tr>
<tr>
<td>p-Nitrobenzoic acid</td>
<td>-420</td>
<td>55</td>
<td>17</td>
<td>33</td>
</tr>
<tr>
<td>Phthalic anhydride</td>
<td>-400</td>
<td>10</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Anthraquinone-2-sulphonate</td>
<td>-380</td>
<td>68</td>
<td>17</td>
<td>60</td>
</tr>
<tr>
<td>p-Nitroacetophenone</td>
<td>-360</td>
<td>23</td>
<td>2</td>
<td>23</td>
</tr>
<tr>
<td>m-Dinitrobenzene</td>
<td>-350</td>
<td>29</td>
<td>14</td>
<td>64</td>
</tr>
<tr>
<td>2,4-Dinitrobenzoic acid</td>
<td>-350</td>
<td>75</td>
<td>22</td>
<td>78</td>
</tr>
<tr>
<td>3,5-Dinitrobenzoic acid</td>
<td>-340</td>
<td>36</td>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td>Diquat</td>
<td>-340</td>
<td>65</td>
<td>15</td>
<td>84</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>-290</td>
<td>58</td>
<td>17</td>
<td>97</td>
</tr>
<tr>
<td>3,4-Dinitrobenzoic acid</td>
<td>-270</td>
<td>11</td>
<td>5</td>
<td>17</td>
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</tbody>
</table>
Table 2 continued

<table>
<thead>
<tr>
<th>Compound</th>
<th>Value 1</th>
<th>Value 2</th>
<th>Value 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Dinitrobenzene</td>
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<td>9</td>
<td>2</td>
</tr>
<tr>
<td>Duroquinone</td>
<td>-250</td>
<td>59</td>
<td>17</td>
</tr>
<tr>
<td>Indigo disulphonate</td>
<td>-250</td>
<td>59</td>
<td>17</td>
</tr>
<tr>
<td>Diphenyl bipyridium</td>
<td>-230</td>
<td>81</td>
<td>10</td>
</tr>
<tr>
<td>Dibenzoxquinone</td>
<td>-120</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>Menadione</td>
<td>-20</td>
<td>70</td>
<td>10</td>
</tr>
<tr>
<td>Benzoquinone</td>
<td>+330</td>
<td>34</td>
<td>12</td>
</tr>
<tr>
<td>Neutral Red</td>
<td>44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Janus Green</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Alizarin Red</td>
<td>15</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>DCPIP</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Safranine O</td>
<td>10</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Thionine</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>
as it implies that the reducing side of PSI has a potential of at least -0.64V, otherwise the compound with this \( E_\text{r} \) value (tetraquat) would not be photosynthetically reducible. Trimethyl benzene (\( E_\text{r} = -0.86V \)) was not reduced by isolated chloroplasts and thus the PSI reducing potential appears to lie between -0.64V and -0.86V, as deduced from this study.

The relationship between oxygen uptake by isolated chloroplasts and superoxide formation is shown in Fig. 3.1. A value for the stoichiometry of oxygen uptake to superoxide formation was calculated to be 2.03:1. This is about twice the predicted figure (Hall, 1976) and may reflect the occurrence of reactions competing with the assay. There does not appear to be a direct relationship between oxygen uptake and hydrogen peroxide production (Fig. 3.2). Similarly, superoxide formation did not correlate with hydrogen peroxide generation (figure not shown). It may be inferred from this that superoxide either underwent subsequent reaction of which hydrogen peroxide was not a product, or that this more reduced oxygen species was formed via reactions independent of a superoxide intermediate. The interaction of certain compounds such as DIMEB with photosynthetic electron transport has been demonstrated to result in the formation of hydrogen peroxide which was not derived from superoxide dismutation or dependent reactions (Elstner and Frommeyer, 1978a, b). Superoxide is known to reduce quinones and certain nitro compounds (Poupko and Rosenthal, 1973), becoming oxidised to dioxygen in the process. Additionally, in a chloroplast system, superoxide was shown to oxidise o-diphenols to the semiquinone form and was itself reduced to hydrogen peroxide (Elstner, Konze, Selman and
Fig. 3.1. Superoxide formation by isolated chloroplasts mediated by redox compounds.
Fig. 3.2. Hydrogen peroxide production by isolated chloroplasts mediated by redox compounds.
Stoffer, 1976; Elstner and Frommeyer, 1978b). Thus, in view of these possible side reactions involving superoxide and hydrogen peroxide and also the wide range of chemical types investigated, it is understandable that the stoichiometry of these products varied according to the compound under test.

1.2 Oxygen Exchange by Asparagus Mesophyll Cells

A number of the compounds described in the previous section were investigated for their effect on isolated mesophyll cells of asparagus. The compounds selected for further investigation were those which were active in the chloroplast studies on oxygen activation. PMS was also included as this has been shown to be reduced in vitro by NADH and was reoxidised to form superoxide (J.A. Farrington, personal communication). Although the bipyridyls showed high activity in the previous tests, they were omitted from this survey as they had previously been shown to require exceptionally long incubation times in this assay (M. Evans, personal communication). Oxygen exchange was determined in the presence of the compound under investigation, with and without a preincubation period with the compound. This procedure was adopted to determine immediate effects of the compound and also longer term effects due to a slow rate of uptake by the cells or a decreased rate of interaction with photosynthesis. A further study involved preincubation of the cells with the compound of interest, followed by sedimentation of the cells and addition of a fresh aliquot of the compound. The results of these various treatments on oxygen exchange by asparagus cells is shown in Table 3. The compounds
<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>-PREINCUBATION</th>
<th>+PREINCUBATION</th>
<th>PREINCUBATION +FRESH COMPOUND</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nitrobenzene</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>m-Nitroacetophenone</td>
<td>3</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>p-Nitrobenzoic acid</td>
<td>0</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Anthraquinone-2-sulphonate</td>
<td>3</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>m-Dinitrobenzene</td>
<td>3</td>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td>3,5-Dinitrobenzoic acid</td>
<td>1</td>
<td>4</td>
<td>4</td>
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<tr>
<td>3,4-Dinitrobenzoic acid</td>
<td>0</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Duroquinone</td>
<td>35</td>
<td>18</td>
<td>27</td>
</tr>
<tr>
<td>Indigo disulphonate</td>
<td>2</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>PMS</td>
<td>40</td>
<td>5</td>
<td>36</td>
</tr>
<tr>
<td>Menadione</td>
<td>141</td>
<td>22</td>
<td>24</td>
</tr>
<tr>
<td>Benzoquinone</td>
<td>3</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>Neutral Red</td>
<td>0</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Safranine O</td>
<td>0</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>2</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>
may be classed according to their effects on oxygen exchange as follows:

1) Compounds which exerted an immediate effect which was maintained throughout the preincubation period. The main example of this type was anthraquinone-2-sulphonate, which was not particularly active in this assay. Indigo disulphonate also appeared to act in a similar manner and again possessed low activity. These compounds thus appeared to be able to penetrate the cell and rapidly cross the chloroplast membranes, but were only slightly effective in stimulating oxygen uptake.

2) Compounds which produced an immediate effect, but which decreased upon prolonged incubation. The four compounds which fell into this category were m-nitroacetophenone, PMS, duroquinone and menadione, the last three of which were remarkably active. With the exception of menadione, these compounds initially stimulated oxygen uptake, but this was decreased by a preincubation period. However, the addition of fresh compound after the period of preincubation resulted in a similar stimulation to that initially observed. This may indicate that although these compounds initially affected oxygen exchange, the asparagus cell or chloroplast was capable of causing inactivation, metabolism to inactive products or by removal from the active site. Menadione caused the greatest stimulation of oxygen uptake which was also a short term effect, but in the case of this compound, a subsequent addition following preincubation was of little effect. It is possible that prolonged incubation of the cells with menadione led to a considerable impairment of the photosynthetic process. Thus, the decreased stimulation of oxygen uptake after a period of preincubation was not due to metabolism of the compound, but
reflected the reduced photosynthetic capability of the cells. In this case, further addition of the compound produced little effect as photosynthesis was the limiting factor. It is possible that duroquinone and PMS have a limited action in this way.

3) Compounds requiring a period of incubation with the asparagus cells before their effects become evident. This group comprised the majority of the compounds tested namely nitrobenzene, p-nitrobenzoic acid, 3,5-dinitrobenzoic acid, 3,4-dinitrobenzoic acid, benzoquinone, neutral red, safranine and riboflavin. Each of these compounds exhibited little or no initial activity, but appeared to stimulate oxygen uptake after the preincubation period. Further addition of the compound after preincubation had little effect. The most plausible explanation for these observations is that the uptake of these compounds by the cells was slow and hence initially they had little effect on oxygen exchange. The procedure for adding a second aliquot of compound to the cells involved removing the cells by brief centrifugation, discarding the supernatant (containing the compounds which had not penetrated the cells), and resuspension in buffer containing fresh compound. The fact that this treatment was without effect on oxygen exchange infers that the level of compound inside the cell and/or chloroplast remained constant. This also confirms the slow uptake of these compounds.

The only compound which did not fall readily into one of these categories was m-dinitrobenzene, which exerted its maximum effect after preincubation, followed by the addition of fresh compound. It is possible that this compound is rapidly complexed within the cell and thus greater concentrations need to be added before effects
on oxygen exchange are observable. It would also appear that m-dinitrobenzene was able to rapidly penetrate the asparagus cells.

1.3 Effects on a range of intact plants

The series of compounds previously tested for activity in asparagus cells was examined for general herbicidal effects on intact plants. A range of six crop plants was selected for the study and included both monocotyledonous and dicotyledonous species. From the results presented in Table 4, it is apparent that only three compounds possessed any significant herbicidal activity, namely m-dinitrobenzene, PMS and menadione. These compounds also appeared to be fairly uniform with regard to their species toxicity. Slight phytotoxic activity was observed with duroquinone, m-nitroacetophenone and 3,5-dinitrobenzoic acid. A qualitative analysis revealed striking similarities between the symptoms produced by all the active compounds and those associated with paraquat treatment. The less active compounds produced damage which was mainly restricted to the leaf margins. Chlorosis of leaf tissue was observed which was entirely restricted to those plants treated with nitro compounds, although it should be noted that not all nitro compounds produced chlorotic symptoms.

1.4 Physico-Chemical Considerations of Mediated Oxygen Production.

The studies into the mediation of oxygen reduction in isolated
### Table 4. Herbicidal Activity of Some Redox Compounds

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>SOYA</th>
<th>TOMATO</th>
<th>COTTON</th>
<th>LETTUCE</th>
<th>OATS</th>
<th>SUGAR BEET</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Nitrobenzene</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>m-Nitroacetophenone</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>p-Nitrobenzoic acid</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Anthraquinone-2-sulphonate</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>m-Dinitrobenzene</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>+++</td>
<td>++++</td>
</tr>
<tr>
<td>3,5-Dinitrobenzoic acid</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3,4-Dinitrobenzoic acid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>Duroquinone</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Indigo disulphonate</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>PMS</td>
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<td>++++</td>
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</tr>
<tr>
<td>Menadione</td>
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<td>+++</td>
<td>+</td>
</tr>
<tr>
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<td>-</td>
<td>+</td>
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</tr>
<tr>
<td>Neutral Red</td>
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<td>-</td>
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</tr>
<tr>
<td>Safranine O</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Key**

- + - 1-20% chlorosis
- ++ - 21-40% chlorosis
- +++ - 41-60% chlorosis
- ++++ - 61-80% chlorosis
- ++++++ - 81-100% chlorosis
chloroplasts indicated that the mechanism of reduction and subsequent reoxidation differed from one compound to another. This was demonstrated by the lack of correlation between the redox potential, $E_1$ and the products of oxygen reduction, superoxide and hydrogen peroxide. The redox characteristics of the compounds were investigated further using chemical and electrochemical methods of reduction.

In an initial series of experiments, sodium dithionite solution was used to chemically reduce the compounds. The use of this reductant was restricted to high pH values as it was observed to decompose at neutral pH resulting in a colloidal suspension of elemental sulphur. This prevented accurate spectrophotometric determination of the reduction curves for a given compound. This problem was overcome by substituting a potentiometric method of reduction for the chemical procedure. In addition to the advantage of permitting reduction over a much greater pH range, this particular method did not result in any change of volume to the reaction mixture and hence the concentrations of all components remained constant throughout the reduction.

The potentiometric reduction curves for several compounds are shown in Figs. 3.3 - 3.5. With the exception of the nitro compounds (Fig. 3.5), the general form of the curves is similar for each compound. Reduction commences with a relatively constant cell current at low applied potentials and after a small rise which indicates reduction of the compound, the cell current reaches a plateau which is maintained up to applied potentials of about 1000 mV. This second plateau represents the first stable reduction product of the compound. The increase in cell
Fig. 3.3. Potentiometric reduction of anthraquinone-2-sulphonate (a) and duroquinone (b).
Fig. 3.4. Potentiometric reduction of indigo disulphonate (a) and neutral red (b).
Fig. 3.5. Potentiometric reduction of p-nitroacetanilide (a) and m-nitroacetophenone (b).
current with applied potentials in excess of about 1000 mV is due to other complex reductions; possibly those of the buffer components.

The nitro compounds examined in this study did not show simple reduction characteristics. This was due to the nature of the molecules which possessed two or more groups that were reduced at or about the same applied potential. This phenomenon is particularly evident in the case of p-nitroacetanilide which exhibits four small plateaux over the applied potential range 675 - 950 mV. The aim of this initial series of experiments was to determine the minimum applied potential necessary to reduce the compounds to their first stable forms, i.e. the lowest applied potential which corresponded to the second cell current plateau. This value could not be determined for the nitro compounds investigated, due to the complexity of the reduction process. However, the relatively simple reduction characteristics of the non-nitro compounds permitted this applied potential value to be readily determined. Since this value was required for the subsequent experiments, only the non-nitro compounds could be investigated further.

The charge required to reduce the compound was determined by reduction at the minimum applied potential value ascertained as previously described. The cell current was plotted against the total applied charge and was extrapolated to determine the charge required to reduce the compound to its first stable intermediate (Figs. 3.6 - 3.7). Thus, the amount of charge to half reduce the compound was deduced. This level of reduction corresponded to the maximum amount of any semiquinone present
Fig. 3.6. Decrease in cell current during reduction of anthraquinone-2-sulphonate (a) and duroquinone (b).
Fig. 3.7. Decrease in cell current during reduction of indigo disulphonate (a) and neutral red (b).
and was therefore the optimum value to use for investigating the reaction between the semiquinone form of a compound and dioxygen. As can be seen from Fig. 3.7, the graphs for indigo disulphonate and neutral red were less than ideal and extrapolation to determine the total charge required for reduction was not possible. This was probably due to the low purity of the compounds (85%). The results obtained with duroquinone and anthraquinone-2-sulphonate followed the predicted trend more closely.

The course of reduction of the compounds was followed by discontinuous spectrophotometric determinations. The spectral scans for anthraquinone-2-sulphonate and duroquinone are shown in Figs. 3.8 and 3.9. Anthraquinone-2-sulphonate exhibited an isosbestic point in the visible region of the spectrum at 360 nm. In order to determine whether this was due to semiquinone formation occurring during the course of reduction, the experiment with anthraquinone-2-sulphonate was repeated at pH 11.55; at which pH the semiquinone should be more stable and therefore more easily detectable. It can be seen in Fig. 3.8, that as reduction proceeds, there is an initial absorbance increase at 360 nm followed by a decrease. This tends to indicate the formation and subsequent reduction of the semiquinone form of anthraquinone-2-sulphonate. However, this was only observed at elevated pH and thus the amount of semiquinone formed at more physiological pH values was exceedingly small.

1.5 Concluding Section

The aim of this investigation was to study the process of oxygen
Fig. 3.8 Reduction Spectra of Anthraquinone-2-sulphonate
Fig. 3.9 Reduction Spectra of Duroquinone
reduction in chloroplasts and to determine some characteristics of various mediators. It was hoped that it would be possible to predict the behaviour of possible mediators based on their \( E_1 \) values and observations from physico-chemical reduction.

A high proportion of the compounds tested were able to stimulate oxygen reduction in isolated chloroplasts, but this was not found to be in a direct relationship to their \( E_1 \) values. In addition to the reasons already mentioned, it is possible that the site of interaction of the mediators with the reducing side of PSI may not be quite as accessible as previously supposed. Thus, as well as requiring an \( E_1 \) value in the correct range, potential mediators might also need to possess a certain degree of lipophilicity in order to be able to associate more intimately with the thylakoid membrane. Thus, although the \( E_1 \) value of a given compound may be of major importance in determining the ability to mediate oxygen reduction, maximum superoxide and hydrogen peroxide formation can only occur if the compound can fully interact with PSI.

The series of experiments with asparagus cells also indicated that other factors beside \( E_1 \) values determined the reactivity of a compound. It was found that most compounds which were active in the chloroplast system also stimulated oxygen uptake in the asparagus cell assay. However, it was not possible to extrapolate the results in the chloroplast assay to predict the behaviour of compounds in the intact cells.

The results from the intact plant screen reflect to some extent the observations of the asparagus cell assay although direct
extrapolation is not possible. Although the phytotoxic symptoms were similar, the visual effects may be derived via different mechanisms.

It had been envisaged that the discrepancies between compounds observed in the biochemical assays would be resolved by investigating the actual mechanisms of reduction in a purely chemical system. It had also been anticipated that in the complete absence of oxygen, the semiquinone forms of the compounds would be sufficiently stable to permit a controlled reaction with oxygen, which would be slow enough to follow spectrophotometrically. Unfortunately, this was not found to be the case and although the apparatus employed was satisfactory for performing reductions, it was not suitable for detecting the very rapid reoxidation reactions of semiquinones.
2. HERBICIDAL ASPECTS OF THE MECHANISM OF PARAQUAT ACTION

Illuminated chloroplast lamellae produce superoxide via photosynthetic electron transport and this is stimulated by paraquat (Allen and Hall, 1973; Epel and Neumann, 1973) and other compounds possessing a low redox potential (see Section 1.1). This stimulation was abolished by SOD and a low molecular weight compound, penicillamine-copper complex (PA-Cu). The reaction of Cu(II) with L-penicillamine results in the formation of a mixed valence Cu(I), Cu(II) cluster with a molecular weight of about 2200 (Rupp and Weser, 1976; Birker and Freeman, 1977). This was previously shown to have similar properties to Cu, Zn and Mn SOD in chloroplast reactions involving superoxide (Lengfelder and Elstner, 1978; Lengfelder, Fuchs, Younes and Weser, 1979).

Due to its small molecular size and high water solubility, it might be expected to penetrate plant tissue with ease. Thus, PA-Cu might prove to be a useful tool in the determination of the role of superoxide in the herbicidal action of paraquat.

2.1 Pigment bleaching in flax cotyledons

Chlorophyll bleaching is one of the most obvious phytotoxic symptoms of paraquat treatment (Plate 1). Fig. 3.10 shows quantitatively that PA-Cu markedly delayed the breakdown of chlorophyll induced by paraquat. It should be noted that in initial experiments, PA-Cu and paraquat were applied to the flax cotyledons at the same time. This procedure occasionally gave variable results which were overcome in subsequent experiments by delaying the addition of paraquat by 24h. This allowed PA-Cu to penetrate the leaf tissue and thus be present at the
Plate 1. Treated flax cotyledons
Fig. 3.10 Chlorophyll levels in flax
site of paraquat action before the herbicide could exert its effect. All results shown with flax cotyledons were with this modified incubation.

The breakdown of carotenoid pigments in the water control was maintained at a very low level through the 72h illumination period (Fig. 3.11). In contrast, the presence of paraquat greatly increased pigment destruction. The additional presence of PA-Cu retarded the destruction of carotenoid pigments. Although the levels of all pigments decreased with paraquat treatment, the carotenes were more markedly affected than the xanthophylls and this was also observed in paraquat (PA-Cu treated tissue. The restraining action of PA-Cu was most evident in the levels of neoxanthin, lutein and zeaxanthin and to a lesser extent in the case of violaxanthin and the carotenes. In the presence of paraquat, 95% of the carotenes were destroyed, but the additional presence of PA-Cu limited the breakdown to 83%. In the combined case of the xanthophylls, 85% were destroyed by paraquat alone, but the decrease was reduced to 26% by the additional presence of PA-Cu.

2.2 Ethane Generation

The release of ethane from animal tissue was shown to be derived from lipid peroxidation reactions (Riely, Cohen and Lieberman, 1974). It has since been proposed as a marker of unsaturated fatty acid peroxidation in plants (Konze and Elstner, 1978; Montalbini, Koch, Burba and Elstner, 1978) and as an indication of cellular decompartmentalisation (Elstner and Konze, 1976).
Fig. 3.11. Carotenoid content of flax cotyledons treated with water (a), paraquat (b) and PA-Cu/paraquat (c).

\[ \alpha \text{ and } \beta \text{-carotenes}, \quad \text{lutein and zeaxanthin}, \quad \text{violaxanthin}, \quad \text{neoxanthin.} \]
One of the early effects of paraquat action is the initiation of lipid peroxidation reactions (Harris and Dodge, 1972b). Fig. 3.12 shows the release of ethane from flax cotyledons. No ethane was produced in the water control, but paraquat treatment resulted in early detection of the hydrocarbon, which increased exponentially during the course of the experiment. The effect of PA-Cu on paraquat-dependent ethane evolution was to conspicuously reduce the rate of evolution. Until about 60h illumination, the level of ethane in the presence of both paraquat and PA-Cu was approximately 50% of that resulting from cotyledons treated with paraquat alone. The additional presence of PA-Cu also prevented the rapid increase in the rate of ethane evolution observed after this time. As lipid peroxidation is known to involve free radical mediated chain reactions (Mead, 1976), only a small fraction of radicals need to be formed for lipid degradation to occur. Thus PA-Cu would have to be totally effective in scavenging all the superoxide produced as a consequence of paraquat treatment in order to prevent the instigation of lipid free radical generation.

2.3 Unsaturated Fatty Acid Levels in the Chloroplast

The demonstration of ethane evolution from paraquat treated flax cotyledons suggested that lipid peroxidation was occurring. The fatty acids from chloroplasts isolated from treated tissue were analysed to determine whether ethane evolution was truly indicative of lipid peroxidation induced by herbicide treatment. Flax cotyledons possess large quantities of the unsaturated linoleic and linolenic acids (18:2 and 18:3) and paraquat exerted its greatest effect on the levels of these fatty acids
Fig. 3.12 Ethane evolution from flax
in the chloroplast. After 48h, the 18:3 content of flax chloroplasts decreased by 50% (Fig. 3.13). PA-Cu decreased the rate of 18:3 breakdown and after 48h, only 24% had been destroyed. Over the same time period, the loss of this fatty acid in the control was 12%. After this time, PA-Cu appeared to have little effect on the rate of breakdown of 18:3 acid in the presence of paraquat. Herbicide treatment also appeared to result in a general decrease of other fatty acids including fully saturated types.

This effect was less marked in the presence of PA-Cu. These studies indicated that the decrease in triunsaturated fatty acid correlated with the rise in ethane evolution. Thus, the measurement of this hydrocarbon gas appears to be a useful non-destructive method for following herbicide induced plant damage.

2.4 Hydrogen Peroxide Production in Treated Flax Cotyledons

Hydrogen peroxide was originally believed to be the agent responsible for the herbicidal action of paraquat (Dodge, 1971). More recently, superoxide has been implicated (Farrington et al., 1973) and the previous sections have described studies in which paraquat action was inhibited by the use of a superoxide scavenger. The dismutation of superoxide results in the formation of hydrogen peroxide and so the action of SOD or compounds with SOD activity should stimulate hydrogen peroxide formation.

The treatment of flax cotyledons with paraquat led to an increased accumulation of hydrogen peroxide (Table 5) and the additional presence of PA-Cu slightly augmented this, as predicted. The
Fig. 3.13. Fatty acid levels of chloroplasts isolated from flax cotyledons treated with water (a), paraquat (b) and PA-Cu/paraquat (c). • 18:3, □ 18:2, △ 18:0, ▲ 16:0.
Table 5. Hydrogen Peroxide Content of Treated Flax Cotyledons

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>µmoles H$_2$O$_2$ g$^{-1}$ F.W.</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$O Control</td>
<td>0.34</td>
</tr>
<tr>
<td>Paraquat $10^{-5}$M</td>
<td>1.15</td>
</tr>
<tr>
<td>Paraquat + PA-Cu (50U)</td>
<td>1.31</td>
</tr>
</tbody>
</table>
relative levels of hydrogen peroxide in the various treatments did not correlate with other parameters of phytotoxicity and therefore it appears unlikely that this species is of primary importance in the mechanism of paraquat action. However, this does not eliminate the possibility that hydrogen peroxide plays a secondary role, perhaps after certain initial reactions have occurred.

2.5 Ultrastructure of Treated Flax Cotyledons

The ultrastructural appearance of 7 day old flax cotyledons (Plate 2), showed that the cells were highly vacuolated and the cytoplasmic components were confined to the cell wall regions. The chloroplasts were generally discoid in shape and contained prominent starch grains and thylakoid systems. The intergranal membrane network was clearly visible in addition to the granal stacks.

The effect of 12h paraquat treatment on the ultrastructure of flax chloroplasts is shown in Plate 3. The swollen appearance of the organelle is immediately apparent. The membranes forming the granal stacks have separated although it is still possible to discern the original granal locations. Numerous large starch grains are present and have resulted in a distortion in the shape of the chloroplast. This phenomenon bears a resemblance to water treated flax cotyledons incubated under high light intensity (Pallett, 1978). However, the granal stacks were shown to be still relatively intact even after 72h, unlike the paraquat treated tissue.
Plate 2. 7 day old Flax cotyledon
Plate 3. PQ treated flax
Plate 4. PQ/PA–Cu treated flax
Treatment of flax cotyledons with paraquat and PA-Cu caused significantly less visible damage to the chloroplast (Plate 4). Swelling of the organelle was considerably reduced and the thylakoid system remained intact with tight granal stacking. There were fewer starch grains present, although their size is approximately the same as those observed in paraquat treated tissue. Some distortion of the chloroplast shape is evident, but this is less severe than in the previous treatment. The chloroplast envelope and tonoplast were still intact, whereas treatment with the herbicide alone invariably resulted in disintegration of these membranes. This is in agreement with earlier observations that tonoplast and plasmalemma deterioration were among the first visible signs of paraquat damage (Harris and Dodge, 1972a).

2.6 Cellular Distribution of PA-Cu

The distribution of PA-Cu in flax cotyledonary tissue was investigated in order to determine movement characteristics of superoxide. Various regions of flax cells were scanned under the electron microscope coupled to an EDAX system, adjusted to detect copper atoms. An analysis of the resin background is shown in Plate 5. The peaks occurring at $K_\alpha = 0.45$ and $K_\alpha = 0.8$ correspond to titanium and copper respectively, both of which were present as components of the support grid. Plate 6 shows an analysis of the granal region of the chloroplast. The copper peak ($K_\alpha = 0.8$) is markedly increased and is the most abundant metal ion present. A specimen of flax tissue was examined which had previously been treated with D-penicillamine and no increase in the copper peak was observed. Thus, it would appear that the
Plate 5. EDAX of D-penicillamine treated flax

Plate 6. EDAX of PA–Cu treated flax
copper increase was due to the PA-Cu complex. Similar analyses were performed in either regions of the cell and the distribution of copper appeared fairly uniform throughout the tissue. This was verified by scanning complete cells of flax for copper using a narrow bandwidth of $K_0 = 0.8 \pm 0.01$. It can be concluded that the PA-Cu complex readily entered flax cotyledon tissue and distributed itself uniformly throughout the cell including the chloroplast. Thus, this study revealed the penetrating ability of PA-Cu, but was obviously unable to reveal any information regarding the mobility of superoxide.

2.7 Concluding Section

The phytotoxicity of paraquat originates in the chloroplast where it competes with NADP for electrons emanating from the primary acceptor of PSI. Although the lack of NADPH would ultimately lead to an inhibition of CO$_2$ fixation, it was realised in previous studies that plant starvation due to a paucity of elaborated carbon was not a primary factor in the action of paraquat (Dodge, 1971). Hydrogen peroxide and superoxide have both been implicated in the phytotoxic action of paraquat (Davenport, 1963; Dodge, 1971; Farrington et al., 1973) and the importance of each has been investigated here. The presence of PA-Cu markedly inhibited the effects of paraquat action, presumably by its ability to react readily and specifically with superoxide ($k = 10^9$ M$^{-1}$s$^{-1}$ (Lengfelder, Saran and Bors, 1977; Lengfelder and Elstner, 1978)). The only occasion when PA-Cu did not inhibit the action of paraquat was in the production of hydrogen peroxide. This
implies that hydrogen peroxide is not a primary agent in the mechanism of paraquat phytotoxicity. By contrast, superoxide appears to play a central role, as indicated by the protection afforded to flax cotyledon tissue by the superoxide scavenger, PA-Cu. This does not necessarily imply that superoxide is the causative species in the ensuing damage reactions, especially as it is believed to possess insufficient reactivity to act as an initiator (Bors et al., 1979a). It is thought that singlet oxygen and possibly the hydroxyl radical are more likely candidates in initiating lipid peroxidation (Rawls and Van Santen, 1970). Similarly, the photobleaching of both chlorophyll and carotenoid pigments has been shown to occur through the agency of singlet oxygen (Anderson and Krinsky, 1973), although evidence has been presented (Peiser and Yang, 1978), that chlorophyll is destroyed by alkoxy radicals without the direct involvement of oxygen. However, the results presented here indicate that superoxide does play a role in the photooxidation of the photosynthetic pigments, although it is possible that some bleaching is linked to reactions of lipid degradation. Oxygen is probably more important in the initial stages of pigment breakdown, but its effect may be augmented by lipid radicals once fatty acid peroxidation has been instigated.
3. DETERMINATION OF THE ROLE OF OXYGEN IN ETHYLENE AND ETHANE FORMATION

3.1 Oxygen and Ethylene Formation

The simple unsaturated hydrocarbon ethylene, is produced by plant tissue in response to a variety of stimuli (Lieberman, 1979; Yang and Pratt, 1978). Methionine has been shown to act as a substrate in model systems (Lieberman, Kunishi, Mapson and Wardale, 1965, 1966; Yang, Ku and Pratt, 1966) and in vivo (Burg and Clagett, 1967; Lieberman, 1975). The conversion of this amino acid (and analogues) to ethylene requires oxygen (Lieberman 1966) and is known to occur via free radical reactions involving superoxide and hydrogen peroxide (Beauchamp and Fridovich, 1970). It was originally believed that these species combined to form the highly reactive hydroxyl radical, but recent studies have shown that the oxidation of methionine or methional is not simply mediated by this radical (Bors, Lengfelder, Saran, Fuchs and Michel, 1976; Pryor and Tang, 1978). The role of active oxygen in ethylene formation was investigated in chloroplast model systems.

Ethylene production by isolated chloroplast lamellae was stimulated by the addition of methionine and pyridoxal phosphate and was further enhanced by the presence of ferredoxin (Table 6), in agreement with previous reports (Elstner and Konze, 1974a,b; Konze and Elstner, 1976). In the absence of methionine and pyridoxal phosphate, the effect of other additions was negligible except in the cases of PA-Cu and PA-Cu/paraquat, where a slight stimulation was evident. Similarly, the supplementary presence
Table 6. Ethylene Production by a Chloroplast Model System

<table>
<thead>
<tr>
<th>ADDITIONS</th>
<th>METHIONINE + PYRIDOXAL PHOSPHATE</th>
<th>FERREDOXIN</th>
<th>METHIONINE+PYRIDOXAL PHOSPHATE + FERREDOXIN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$C_2H_4$</td>
<td>$C_2H_6$</td>
<td>$C_2H_4$</td>
</tr>
<tr>
<td>None</td>
<td>0.2</td>
<td>0</td>
<td>15.0</td>
</tr>
<tr>
<td>PQ $10^{-5}$M</td>
<td>0.2</td>
<td>0</td>
<td>11.8</td>
</tr>
<tr>
<td>SOD 50 units</td>
<td>0.3</td>
<td>0</td>
<td>65.3</td>
</tr>
<tr>
<td>Catalase 800 units</td>
<td>0.1</td>
<td>0</td>
<td>8.4</td>
</tr>
<tr>
<td>PA-Cu 50 units</td>
<td>1.0</td>
<td>0</td>
<td>117.2</td>
</tr>
<tr>
<td>PQ + SOD</td>
<td>0.2</td>
<td>0</td>
<td>49.9</td>
</tr>
<tr>
<td>PQ + Catalase</td>
<td>0.2</td>
<td>0</td>
<td>10.6</td>
</tr>
<tr>
<td>PQ + PA-Cu</td>
<td>1.7</td>
<td>0</td>
<td>131.0</td>
</tr>
</tbody>
</table>

Conditions:
The reaction mixture contained in 3 cm$^3$: 75 μmoles phosphate buffer, pH 7.8, 5 μmoles NH$_4$Cl, 5 μmoles MgCl$_2$, 100 μg chlorophyll and where present, 32 μmoles methionine, 0.6 μmoles pyridoxal phosphate, 30 nmoles ferredoxin, 30 nmoles paraquat, 50 units SOD, 50 units PA-Cu, 800 units catalase.

The reaction was conducted for 45 mins at 22°C with a light intensity of 30 Klux Hydrocarbon gas concentrations expressed in nmoles mg$^{-1}$ chlorophyll h$^{-1}$.  

125.
of ferredoxin with other additions showed little effect on the formation of ethylene.

A comparison of the effects of various additions in the presence of methionine and pyridoxal phosphate revealed that a stimulation of ethylene production only occurred when SOD or PA-Cu was present. This feature has been used as a positive assay for compounds with SOD activity (Lengfelder and Elstner, 1978). Catalase, in contrast, inhibited ethylene production (Konze, 1977), as did paraquat, except when added in conjunction with PA-Cu when it enhanced formation. The effect of the various additions followed a similar pattern in the combined presence of methionine, pyridoxal phosphate and ferredoxin. However, in this case paraquat tended to inhibit ethylene production. This indicates that the endogenous electron acceptor is the preferred mediator of ethylene formation. SOD active compounds were also found to stimulate the ferredoxin-mediated reaction.

It was originally proposed that ethylene formation from methionine and methional involved the participation of hydroxyl radicals formed via the Haber-Weiss reaction (Eqn 25) (Beauchamp and Fridovich, 1970). This mechanism was based on the observation that ethylene generation was inhibited by both SOD and catalase. If this reaction occurs, it should be stimulated by low potential acceptors such as paraquat, as pointed out by Elstner, Saran, Bors and Lengfelder (1978). The results presented here indicate that the mechanism proposed by Beauchamp and Fridovich (1970) cannot explain ethylene production in a model system. The stimulation by SOD (or PA-Cu) and the inhibition by catalase
suggests that hydrogen peroxide plays a major role, while superoxide is not directly involved, apart from dismutating to form hydrogen peroxide. These results are in general agreement with those of Elstner et al. (1978), who proposed that the oxidant responsible for ethylene formation is derived from reduced ferredoxin and hydrogen peroxide and is probably a Fenton-type oxidant, \( \text{Fe}^{2+}.\text{H}_2\text{O}_2 \). This provides an explanation for the stimulation of ethylene formation by ferredoxin and the inhibitory effect of paraquat. It also accounts for the effects observed with SOD, PA-Cu and catalase.

3.2 Oxygen and Ethane Formation

The investigations described in Section 2.2 with flax cotyledons showed the evolution of ethane to be a sensitive, non-destructive indicator of membrane damage instigated by herbicide treatment. The role of superoxide was demonstrated using the scavenger, PA-Cu. Although other workers have investigated the mechanism of hydrocarbon gas production during lipid peroxidation, most of these studies have been confined to animal tissue (Dillard, Dumelin and Tappel, 1977; Dumelin, Dillard and Tappel, 1978) or non-biological systems (Dumelin and Tappel, 1977). The mechanism of ethane formation in a plant model system was investigated using isolated pea chloroplast lamellae.

Table 7 clearly shows that the presence of \( \alpha \)-linolenic acid stimulated ethane and ethylene formation irrespective of other additions. Other groups have also demonstrated that \( \alpha \)-linolenic acid can act as a substrate for ethane in plant homogenates and
### Table 7. Ethane Production by a Chloroplast Model System

<table>
<thead>
<tr>
<th>ADDITIONS</th>
<th>$C_2H_4$</th>
<th>$C_2H_6$</th>
<th>$\alpha$-Linolenic acid $C_2H_4$</th>
<th>$C_2H_6$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>0</td>
<td>0.8</td>
<td>1.3</td>
</tr>
<tr>
<td>Ferredoxin</td>
<td>0</td>
<td>0</td>
<td>1.3</td>
<td>1.4</td>
</tr>
<tr>
<td>Paraquat</td>
<td>0</td>
<td>0.4</td>
<td>0.9</td>
<td>5.0</td>
</tr>
<tr>
<td>DCMU</td>
<td>0</td>
<td>0.6</td>
<td>0.4</td>
<td>5.3</td>
</tr>
<tr>
<td>Crocin</td>
<td>0</td>
<td>0</td>
<td>0.8</td>
<td>1.3</td>
</tr>
<tr>
<td>PA-Cu</td>
<td>2.3</td>
<td>0.1</td>
<td>4.3</td>
<td>1.0</td>
</tr>
<tr>
<td>PQ + DCMU</td>
<td>0</td>
<td>1.0</td>
<td>0.8</td>
<td>4.6</td>
</tr>
<tr>
<td>PQ + Crocin</td>
<td>0</td>
<td>0.3</td>
<td>0.8</td>
<td>4.1</td>
</tr>
<tr>
<td>PQ + PA-Cu</td>
<td>3.3</td>
<td>0.1</td>
<td>4.5</td>
<td>2.9</td>
</tr>
<tr>
<td>DABCO</td>
<td>0.1</td>
<td>0.3</td>
<td>0.5</td>
<td>2.7</td>
</tr>
<tr>
<td>DCMU + DABCO</td>
<td>0.1</td>
<td>1.1</td>
<td>0.7</td>
<td>5.2</td>
</tr>
<tr>
<td>PQ + DABCO</td>
<td>0.2</td>
<td>1.5</td>
<td>1.3</td>
<td>5.1</td>
</tr>
</tbody>
</table>

**Conditions:**

The reaction mixture contained in 3 cm³: 75 μmoles phosphate buffer pH 7.8, 5 μmoles NH₄Cl, 5 μmoles MgCl₂, 3 μmoles NaN₃, 100 μg chlorophyll, 50 mg BSA and where present 10 μmoles α-linolenic acid, 30 nmole ferredoxin, 30 nmole paraquat, 50 units PA-Cu, 60 nmole DCMU, 0.3 nmole DABCO, 0.3 nmole crocin.

The reaction was conducted for 30 mins at 22°C with a light intensity of 30 Klux.

Hydrocarbon gas concentrations expressed in nmole mg⁻¹ chlorophyll h⁻¹.
organelles (John and Curtis, 1977; Konze and Elstner, 1976, 1978; Montalbini et al., 1978). In the absence of α-linolenic acid, there was little effect with the various other additions, although a slight stimulation of ethane formation was observed in the presence of paraquat, DCMU and DABCO. Ethylene was only detected in the presence of PA-Cu and with DABCO, to a lesser extent. In the presence of α-linolenic acid, the effects of the other additions became more apparent, as both ethane and ethylene were detectable in the control treatments. The greatest stimulation of ethane production was observed with paraquat or DCMU, although their combined effect was less than maximal.

The role of active oxygen species was investigated using the scavengers, PA-Cu, DABCO and crocin (the water soluble digentiobiose ester of the carotenoid acid, crocetin). The protection afforded by PA-Cu, both in the presence and absence of paraquat indicates the involvement of superoxide in the evolution of ethane from α-linolenic acid. This is in good agreement with results from paraquat-induced photooxidation in subchloroplast particles (Elstner, Lengfelder and Kwiatkowski, 1980). Carotenoid pigments are well known scavengers of singlet oxygen (Foote and Denny, 1968; Anderson and Krinsky, 1973) and it was found that crocin inhibited ethane formation in the presence of paraquat, as also reported for *Euglena gracilis* homogenates (Elstner and Osswald, 1980). Another proposed singlet oxygen quencher, DABCO (Ouannes and Wilson, 1968) was also examined for its effect. In the absence of other additions,
DABCO was found to stimulate ethane production, irrespective of the presence of the fatty acid substrate. However, in combination with DCMU or paraquat, no additive effect was observed, as compared to the herbicide alone. Thus, in this system, DABCO was unable to scavenge singlet oxygen, but exerted a stimulatory effect on ethane formation. The structure of the DABCO molecule suggests that it might interact with membranes and perhaps render them more susceptible to attack. The effect of DABCO reported here is in marked contrast to previous studies where it proved to be a potent inhibitor of lipid peroxidation (Takahama, 1978).

Ethylene formation by isolated chloroplast lamellae in the presence of α-linolenic acid was enhanced, although not to such an extent as ethane. It was noted that the additional presence of ferredoxin stimulated ethylene production, while ethane formation was unaffected. When ferredoxin was replaced by paraquat as the electron acceptor, the reverse situation was observed. Thus, although α-linolenic acid can serve as a substrate for both ethane and ethylene, the mechanisms of formation of each hydrocarbon gas are different as evinced by dissimilar electron acceptor requirements.

3.3 Concluding Section

Plants have long been known to produce small quantities of the simple hydrocarbons, ethylene and ethane (see Lieberman,
However, it is only comparatively recently that the relationship between ethane, ethylene and active oxygen species has been closely examined. Of particular importance, was the observation that ethylene production requires living tissue, while the release of ethane is proportional to the amount of damage to the plant (Elstner and Konze, 1976). This finding has led to the use of ethane measurement as an indication of plant damage induced by various factors such as herbicides, as described here.

The production of ethylene in model systems has been shown to be a ferredoxin-dependent process. Previous workers have shown that methionine is the major substrate in vivo (Baur, Yang, Pratt and Biale, 1971; Hanson and Kende, 1976; Lieberman et al., 1966) and the stimulation of ethylene formation observed in the absence of added ferredoxin suggests that a small amount of endogenous ferredoxin was still present. Paraquat was unable to substitute for ferredoxin in this stimulation. The small level of inhibition observed in the absence of added ferredoxin, with methionine as the substrate, indicates that the herbicide was competing with endogenous ferredoxin for PSI generated reducing power.

Although methionine was the preferred substrate for ethylene production, it was also formed from α-linolenic acid. In contrast, ethane generation only occurred in the presence of the lipid substrate. Chloroplasts contain high levels of α-linolenic acid (Mackender and Leech, 1974), which are released during ageing and inhibit electron transport.
(McCarty and Jagendorf, 1965; Siegenthaler and Rawyler, 1977). Ethane evolution has been shown to be increased in aged chloroplasts (Elstner et al., 1980) and thus α-linolenic acid would appear to be an endogenous substrate for ethane.

In contrast to ethylene production, the generation of ethane was unaffected by the addition of ferredoxin, but was markedly enhanced by paraquat, although exogenous α-linolenic acid was able to act as a substrate for both hydrocarbon gases. The requirement of a different electron acceptor for ethane formation suggests that the active oxygen species involved is also different. This is borne out by the observation that SOD-active compounds such as PA-Cu stimulated ethylene formation yet inhibited ethane production irrespective of the substrate. This infers that ethylene formation is dependent on the presence of hydrogen peroxide (confirmed by its inhibition by catalase), but that superoxide plays a major role in the mechanism of ethane production. Furthermore, it appears that superoxide itself is not the species directly responsible for ethane formation, as stimulation was also observed in the presence of DCMU, which does not produce this radical. Crocin scavenges singlet oxygen and thus this electron transport inhibitor gives rise to this highly toxic oxygen species by restricting energy dissipation between the photosystems (Ridley, 1977; Elstner and Pils, 1979).

The requirement of ethylene formation for hydrogen peroxide and ferredoxin implies a possible role for a Fenton-type reagent, Fe^{2+}.H_{2}O_{2} as proposed by Elstner et al. (1978).
However, in the case of ethane, the results presented here cannot be explained in terms of a Fenton complex. It appears more likely that the presence of paraquat stimulates superoxide production, which is responsible for the oxidation of \(\alpha\)-linolenic acid resulting in the generation of the hydrocarbon gas. However, superoxide probably does not possess sufficient reactivity to abstract protons in the initial stages of lipid peroxidation (Pryor, 1978; Bors et al., 1979a). The results shown here tend to implicate singlet oxygen in lipid peroxidation reactions and thus the toxicity of superoxide would appear to lie in its ability to generate this more reactive species. The observation that DCMU stimulated ethane production to a similar extent as paraquat is consistent with this view. Thus, singlet oxygen seems to be the active species and the mechanism of formation of this is immaterial with respect to an ethane forming ability.
The resistance of biotypes of *Conyza linefolia* towards paraquat developed under natural conditions in the field where the herbicide was employed in weed control. Paraquat was the sole herbicide used and was applied several times a year. It was observed after several years of this regime that certain plants were not killed by the herbicide at the concentrations routinely employed. These were identified as *Conyza* sp. and were found to tolerate paraquat concentrations several fold higher than those generally utilised for weed control. The present study was undertaken to elucidate the mechanism of paraquat resistance in these plants.

### 4.1 Chlorophyll Content

The initial observations that certain *Conyza* plants showed resistance to paraquat treatment was based on a retention of the green colour of the leaves. Fig. 3.14 shows the chlorophyll content of two *Conyza* biotypes, one of which was resistant to the herbicide. Bleaching of chlorophyll in the paraquat-susceptible type (I) increased with light intensity and the duration of illumination. The light intensity required to result in a 50% decrease in the pigment content after 24h was approximately 24 Wm$^{-2}$. The equivalent light intensity to produce similar results after 48h illumination was 12 Wm$^{-2}$, indicating that bleaching was proportional to the total radiation incident upon the treated leaves.
Fig. 3.14 Chlorophyll content of Conyza
The resistant Conyza (II) biotype showed no loss of chlorophyll except for a small amount of bleaching at the highest light intensity after 48h.

4.2 Ethane Evolution

The evolution of ethane from Conyza leaf sections was used as an indicator of herbicide induced membrane disruption (see Section 2.2). Fig. 3.15 shows the evolution of this hydrocarbon gas with increasing light intensity. The paraquat susceptible biotype produced ethane in a linear relationship with light intensity after 24h and 48h, with rates of 158 pmoles g$^{-1}$Wm$^{-2}$ and 456 pmoles g$^{-1}$Wm$^{-2}$ respectively. By contrast, no ethane was detected with the paraquat resistant Conyza even at the highest light intensity after 48h.

4.3 CO$_2$ Exchange

In normal susceptible plants, paraquat exerts its herbicidal effect by competing with NADP for electrons derived from PSI. Thus an early sign of phytotoxicity is the cessation of CO$_2$ fixation due to a lack of reduced pyridine nucleotide, required as a cofactor. Fig. 3.16 shows the effect of paraquat treatment on the Conyza biotypes. The susceptible line was unable to fix CO$_2$ after 4h of paraquat treatment. Over the first three hours, CO$_2$ fixation by the paraquat resistant biotype was reduced by rather less than 30%. After this initial period, there was a very marked increase in CO$_2$.
Fig. 3.15 Ethane evolution from Conyza
Fig. 3.16 CO₂ Fixation in Conyza
fixation capacity, which continued up until at least 7h. CO₂ exchange in the water treated controls remained fairly constant over the 7h incubation period.

4.4 Uptake of ¹⁴C-Paraquat

The studies on the CO₂ fixation ability of Conyza biotypes indicated that paraquat affected both the susceptible and resistant types. Nevertheless, the uptake of the herbicide was investigated using ¹⁴C-paraquat to ascertain whether similar levels were attained in both plant types. Table 8 shows that there was no difference in the uptake of paraquat between the biotypes. Thus, it appears that differences in the susceptibility of the leaf sections to paraquat was not related to a modification in the uptake of the herbicide. This was also found to be the case in paraquat resistant lines of perennial ryegrass (Harvey, Muldoon and Harper, 1978).

4.5 SOD Content of Conyza

The resistance of a Conyza biotype to paraquat did not appear to be due to reduced uptake by the plant and thus may be more directly related to biochemical aspects of the mechanism of action of the herbicides. Superoxide was shown to play a major role in the herbicidal action of paraquat in flax cotyledons (see Section 2) and thus it is possible that
Table 8. Uptake of $^{14}$C-Paraquat by *C. leucas* Leaf Sections

<table>
<thead>
<tr>
<th>Conyza Biotype</th>
<th>cpm g$^{-1}$ F.W.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible</td>
<td>83478</td>
</tr>
<tr>
<td>Resistant</td>
<td>86053</td>
</tr>
</tbody>
</table>
increased endogenous levels of SOD might confer some resistance upon the paraquat resistant biotype.

The aerobic photooxidation of dianisidine mediated by flavins generates superoxide, but is also inhibited by its presence (Misra and Fridovich, 1977). The removal of this oxygen radical stimulates the reaction and has been used as an assay for SOD (Misra and Fridovich, 1977; Lengfelder and Elstner, 1979). Fig. 3.17 compares the ability of extracts of paraquat resistant and susceptible biotypes of Conyza to stimulate dianisidine photooxidation. Both extracts appeared to possess SOD activity in this assay. However, the extract from the paraquat resistant type possessed greater specific activity.

The specific activity of the SOD extracts were quantified using the ability of SOD to inhibit the oxidation of hydroxylamine by superoxide anions generated by xanthine oxidase acting on xanthine (Elstner and Heupel, 1976). The inhibition curves for the extracts are shown in Fig. 3.18 One unit of enzyme activity (that which caused 50% inhibition) corresponded to 4.7 µg protein and 13.7 µg protein from the resistant and susceptible biotypes respectively. From this the following specific activities were calculated:

\[
\begin{align*}
\text{Resistant biotype extract} & \quad 213 \text{ Units mg}^{-1} \text{ protein} \\
\text{Susceptible biotype extract} & \quad 73 \text{ Units mg}^{-1} \text{ protein}
\end{align*}
\]

Work of Harper and Harvey (1978) showed that paraquat tolerant cultivars of perennial ryegrass also possessed increased SOD activity. However, as seen in Table 9, the differences
Fig. 3.17. Photooxidation of dianisidine in the presence of 50 units of bovine SOD (●–●), Conyza biotype I SOD extract (○–○), Conyza biotype II SOD extract (■–■) and a control (△–△). Extracts from biotypes I and II contained 32 µg and 28 µg protein respectively.
Fig. 3.18. Inhibition of nitrite formation from hydroxylamine by SOD extracts from Conyza biotypes I (■—■) and II (○—○).
were almost five times greater for Conyza than for ryegrass.

The increased levels of SOD detected in the studies of paraquat resistant Conyza lines could be derived from two sources. Firstly, the increased activity could be due to the presence of another isozyme (s) of SOD or secondly, it is possible that the levels of all SOD isozymes present in the susceptible biotype are increased in the resistant line. These possibilities were investigated by subjecting the extracts to polyacrylamide gel electrophoresis. Fig. 3.19 shows the appearance of the gels after staining in the presence and absence of KCN. The extract from the paraquat susceptible biotype possessed two SOD bands which disappeared in the presence of KCN, indicating them to be the Cu, Zn enzyme. These two bands were also observed in the paraquat resistant extract, together with two other bands. These two extra bands were shown to be cyanide-insensitive, indicating that they were Mn (or Fe) containing enzymes. In earlier studies with SOD extracts from wheat, Beauchamp and Fridovich (1973) observed that one cupro-zinc isozyme retained its activity in the presence of 1% SDS. The Conyza extracts were subjected to SDS treatment which resulted in the elimination of all bands in both the resistant and susceptible biotypes.

4.6 Concluding Section

The investigation of the mechanism by which a certain biotype of Conyza was resistant to paraquat indicated that this is a
Table 9. Comparison of SOD Levels in *Conyza* and Ryegrass Biotypes

<table>
<thead>
<tr>
<th>Biotype</th>
<th>SOD Levels in Ryegrass as % of susceptible type</th>
<th>SOD levels in <em>Conyza</em> as % of susceptible type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Resistant</td>
<td>142</td>
<td>292</td>
</tr>
</tbody>
</table>

* Data of Harper and Harvey (1978)
Fig. 3.19. SOD Content of Conyza Biotypes
relatively complex process and not based simply on one modification that restricts the toxicity of the herbicide. Paraquat exerts its herbicidal effect through the agency of active oxygen species, notably superoxide (see Section 2). Thus, in the case of Conyza, it is possible that the observed resistance to paraquat is based on an increase in the endogenous SOD levels which prevents superoxide from instigating various deleterious reactions. However, this does not account for the observed stimulation of CO₂ fixation. It would appear that in the resistant biotype, paraquat was not as readily reduced as in the susceptible type, so that most of the PSI derived reducing power resulted in NADP production. However, if a small amount of paraquat reduction occurred, this would prevent NADPH formation without affecting photophosphorylation (Arnon et al., 1961). Thus, the ATP/NADPH ratio would be shifted in favour of that required for optimal CO₂ fixation (Krause and Heber, 1976), and hence could account for the increased CO₂ uptake observed. It was previously demonstrated (Elstner and Kramer, 1973; Elstner and Heupel, 1973), that in chloroplasts, reduction of oxygen occurred when the NADP pool was about 80% reduced, thus providing an endogenous mechanism for increasing the ATP/NADPH ratio. The reduction of paraquat would result in increased superoxide formation upon reoxidation of the cationic radical and thus the increased SOD levels observed could prevent toxic reactions caused by this oxygen species.

In summary, the resistance of a Conyza biotype to paraquat may be based on a decreased reduction of paraquat, either by
restriction of its ability to penetrate the chloroplast or by a decrease in its capability to compete with NADP for PSI reducing power. The increased SOD levels tend to suggest that some paraquat was reduced and that this presumably occurred in the chloroplast due to the high negative redox potentials involved.
5. Oxygen Activating Ability of 4-Dimethylaminophenol

4-Dimethylaminophenol (DMAP) is known to rapidly transform haemoglobin into ferrihaemoglobin (methaemoglobin) in dogs and humans (Kiese, Rauscker and Weger, 1966; Kiese and Weger, 1969). This only occurs in the presence of oxygen and it appears that a product of DMAP autoxidation is responsible for the oxidation of ferrohaemoglobin. The reactions of DMAP are thought to involve one-electron transfer and thus, together with the absolute requirement for oxygen, suggests an oxygen activating system (Eyer, Kiese, Lipowsky and Weger, 1974). It was therefore decided to examine the effects of DMAP on chloroplast mediated reactions. Additionally, DMAP bears a structural resemblance to established electron donors to PSI such as DAD and TMPD (Fig. 3.20) and thus might be expected to also donate electrons.

The ability of DMAP to act as an electron donor is shown in Table 10. In the presence of a DCMU block, DMAP donated electrons to PSI, thereby causing the reduction of NAD. This was stimulated by ascorbate in a similar manner to the traditional PSI donors, DAD and TMPD (Trebst, 1974). A comparison of DMAP with these other donors revealed that DMAP could catalyse phosphorylation similar to DAD (Table 11). The ATP/2e ratio in the presence of DMAP increased from 0.6 (the value expected from one proton release upon 2e oxidation), to more than 1.0 with increasing DMAP concentration. Since DMAP can oxidise NADPH during autoxidation reactions (P. Eyer, personal communication), the observed NADPH concentration
Fig. 3.20 Structures of some electron donors

TMPD

DMAP

DAD
Table 10. NADP Production in Isolated Chloroplasts

<table>
<thead>
<tr>
<th>Order of Addition</th>
<th>NADP Reduction μmoles mg⁻¹ Chl.h⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroplasts</td>
<td>28</td>
</tr>
<tr>
<td>DCMU</td>
<td>0</td>
</tr>
<tr>
<td>DMAP</td>
<td>10</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>30</td>
</tr>
<tr>
<td>Chloroplasts</td>
<td>23</td>
</tr>
<tr>
<td>DCMU</td>
<td>0</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>0</td>
</tr>
<tr>
<td>DMAP</td>
<td>28</td>
</tr>
<tr>
<td>Paraquat</td>
<td>13</td>
</tr>
</tbody>
</table>

Conditions:

Reaction mixture contained in 3 cm³: 75 μmoles phosphate buffer pH 7.8, 5 μmoles NH₄Cl, 5 μmoles MgCl₂, 5 μmoles NADP, 10 nmol ferredoxin, 30 μg chlorophyll and where added, 3 μmoles DMAP, 60 nmoles DCMU, 10 μmoles ascorbate, 30 nmoles paraquat.

The reaction was conducted in a thermostated spectrophotometric cell at 22°C.
Table 11. Effect of DMAP on the ATP/2e Ratio in Chloroplasts

<table>
<thead>
<tr>
<th>ADDITIONS</th>
<th>µmoles NADPH mg⁻¹ Chl h⁻¹</th>
<th>µmoles ATP³² mg⁻¹ Chl h⁻¹</th>
<th>ATP³²/2e</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>35</td>
<td>27</td>
<td>0.8</td>
</tr>
<tr>
<td>DCMU</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>DCMU + ascorbate/DAD</td>
<td>29</td>
<td>28</td>
<td>1.0</td>
</tr>
<tr>
<td>DCMU + ascorbate/TMPD</td>
<td>13</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DCMU + ascorbate/DMAP (5 x 10⁻⁴ M)</td>
<td>13</td>
<td>8</td>
<td>0.6</td>
</tr>
<tr>
<td>DCMU + ascorbate/DMAP (10⁻³ M)</td>
<td>15</td>
<td>18</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Conditions:

The reaction mixture contained in 3 cm³: 80 µmoles tricine buffer pH 8.0, 10 µmoles MgCl₂, 10 µmoles ADP, 10 µmoles ³²pᵢ, 3 µmoles NADP, 10 mM ferredoxin, 200 µg chlorophyll and where present, 0.3 µmoles DCMU, 0.3 µmoles DAD, 0.3 µmoles TMPD, 40 µmoles sodium isoascorbate. DMAP concentrations as indicated.

The reaction was conducted at 20⁰C for 15 mins at a light intensity of 30 Klux.
reflected its steady state level rather than its rate of formation and thus the ATP/2e ratio may have been erroneously high.

Although DMAP acted as a PSI acceptor in oxygen electrode studies (Table 12), it did not stimulate superoxide formation as measured by its ability to oxidise hydroxylamine to nitrite (Table 13). However, it was observed to inhibit nitrite formation in the presence of paraquat which suggests that DMAP reacted with superoxide and was consequently in competition with the assay system for this oxygen radical. In contrast to superoxide formation, the production of hydrogen peroxide was markedly stimulated by DMAP (Table 14).

These results indicate that DMAP can act both as an acceptor and as a donor for PSI, depending on whether a DCMU block is present. As a donor, it behaved like established PSI donors such as DAD and TMPD. The phosphorylating ability of DMAP indicated that it could transport protons as well as electrons across the thylakoid membrane.

Although the determination of the products of oxygen reduction in the presence of DMAP indicated that the compound reacted with superoxide to form hydrogen peroxide, studies with superoxide generated by pulse radiolysis showed no reaction between the radical and DMAP (E. Lengfelder and P. Eyer, personal communication). In the same study, DMAP was found to be reduced at the expense of hydroxylamine, which became oxidised to nitrogen. Thus, although DMAP appears to interact with chloroplast electron transport, its ability to activate oxygen could not be investigated in this system due
### Table 12. Oxygen Uptake in Isolated Chloroplasts

<table>
<thead>
<tr>
<th>Order of Additions</th>
<th>$O_2$ uptake $\mu$moles mg$^{-1}$Chl h$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroplasts</td>
<td>2</td>
</tr>
<tr>
<td>DMAP</td>
<td>35</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>17</td>
</tr>
<tr>
<td>Paraquat</td>
<td>127</td>
</tr>
<tr>
<td>Chloroplasts</td>
<td>2</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>4</td>
</tr>
<tr>
<td>Paraquat</td>
<td>43</td>
</tr>
<tr>
<td>DCMU</td>
<td>4</td>
</tr>
<tr>
<td>DMAP</td>
<td>28</td>
</tr>
</tbody>
</table>

**Conditions:**

The basic reaction mixture was that detailed in the legend to Table 6 with the additional presence of 3 $\mu$mol NaN$_3$.

The concentrations of the addition were as for Table 10.

The reaction was conducted at 22°C.
Table 13. Superoxide Formation in Isolated Chloroplasts

<table>
<thead>
<tr>
<th>Addition</th>
<th>μmoles NO$_2^-$ mg$^{-1}$ Chl.h$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.4</td>
</tr>
<tr>
<td>DMAP</td>
<td>0.3</td>
</tr>
<tr>
<td>DCMU</td>
<td>0</td>
</tr>
<tr>
<td>DCMU + DMAP</td>
<td>0.1</td>
</tr>
<tr>
<td>PQ</td>
<td>3.5</td>
</tr>
<tr>
<td>PQ + DMAP</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Conditions:
The basic reaction mixture was as described for Table 12 with the additional presence of 1 μmole NH$_2$OH. The concentrations of the additions were as for Table 10.

The reaction was conducted at 22°C for 15 mins with a light intensity of 30 Klux.
Table 14. Hydrogen Peroxide Production by Isolated Chloroplasts

<table>
<thead>
<tr>
<th>Additions</th>
<th>μmoles H₂O₂ mg⁻¹ Chl h⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5.1</td>
</tr>
<tr>
<td>DMAP</td>
<td>8.7</td>
</tr>
<tr>
<td>PQ</td>
<td>11.1</td>
</tr>
<tr>
<td>PQ + DMAP</td>
<td>8.4</td>
</tr>
<tr>
<td>DCMU</td>
<td>0</td>
</tr>
<tr>
<td>DCMU + DMAP</td>
<td>2.1</td>
</tr>
<tr>
<td>DCMU + PQ</td>
<td>0</td>
</tr>
<tr>
<td>DCMU + PQ + DMAP</td>
<td>5.4</td>
</tr>
<tr>
<td>DBMIB</td>
<td>0</td>
</tr>
<tr>
<td>DBMIB + DMAP</td>
<td>8.4</td>
</tr>
</tbody>
</table>

Conditions:

The basic reaction mixture was as described for Table 12 and the concentrations of additions was as detailed in Table 10. DBMIB was used at a concentration of 30 nmoles per reaction.

The reaction was conducted at 22°C for 20 mins with a light intensity of 30 Klux.
to interference of DMAP or its autoxidation product(s) with assay system.
CONCLUDING DISCUSSION
CONCLUDING DISCUSSION

The discovery of the superoxide dismutating function of a copper protein, erythrocuprein (subsequently known as SOD) in 1969 (McCord and Fridovich, 1969a) led to the realisation of the importance of this enzyme in protecting most, if not all organisms that inhabit an aerobic environment for all or part of their lives. Superoxide has been shown to be toxic in a variety of biological systems and the importance of SOD as a tool for investigating superoxide production cannot be overemphasised. During the last decade, many workers have reexamined various reactions involving oxygen radicals to determine the role of superoxide and other oxygen species. This present study was undertaken to investigate oxygen activation in chloroplasts and attempts to define a role for the various active species involved in the mechanism of paraquat action.

Although superoxide production via mediated photosynthetic electron transport did not readily correlate with electrochemical data for the compounds, it is noteworthy that a large number of compounds of various chemical types stimulated the generation of this radical. Other work with E. coli has also demonstrated the ability of many compounds to stimulate superoxide production (Hassan and Fridovich, 1979a).

Paraquat was particularly efficient at stimulating superoxide production in chloroplasts and in E. coli (Hassan and Fridovich, 1977, 1978) and therefore it was decided to investigate the involvement of this species in the herbicidal reactions
of paraquat. It is clear from the use of the SOD-active compound PA-Cu, that superoxide is of primary importance in the phytotoxic effect. Although all the symptoms of paraquat action appeared to involve superoxide, the most important effects related to those reactions of the herbicide which resulted in membrane damage. Superoxide clearly plays an important role in lipid peroxidation reactions and ethane formation. However, ultrastructural studies have demonstrated that among the first signs of paraquat damage are the disruption of tonoplast and plasmalemma membranes (Harris and Dodge, 1972a). The site of superoxide production is the reducing side of PSI, located on the outer thylakoid surface. Thus, superoxide must either be sufficiently stable to diffuse to the sites of potential damage or give rise to other species with this property. However, the relative stability of the migrating species implies a decreased reactivity and thus it would be an unlikely candidate as the instigator of lipid peroxidation. Previous calculations (Farrington et al., 1973) have shown that superoxide is sufficiently stable to diffuse to the tonoplast or plasmalemma. It has been shown that the reactivity of the radical is restricted and superoxide is not believed to be able to initiate lipid peroxidation (McCay et al., 1976; Bors et al., 1979a). Thus, the initiation of lipid peroxidation reaction probably involves a more reactive species.

Ethane evolution in both intact tissue and chloroplast models showed the involvement of superoxide. α-Linolenic acid was able to act as a substrate for in vitro ethane production
and its relative abundance in plant membranes indicated that it was a likely candidate as the \textit{in vivo} substrate. The model experiments also confirmed that oxygen species other than superoxide were able to initiate lipid peroxidation. In the presence of DCMU, chloroplasts do not produce superoxide, but ethane evolution was still shown to occur at rates similar to those with paraquat. Both types of stimulation were inhibited by crocin, indicating the involvement of singlet oxygen (Elstner and Osswald, 1980). It has been suggested (Pallett, 1978) that treatment with CMU (which acts in an identical manner to DCMU), results in restricted energy dissipation and that excited pigment molecules of PSII react with dioxygen to form singlet oxygen. By contrast, singlet oxygen generated as a result of paraquat treatment, would appear to be derived from reactions of superoxide. Singlet oxygen is highly reactive and is capable of attacking polyunsaturated fatty acids (Rawls and Van Santen, 1970; Anderson, Krinsky, Stone and Clagett, 1974). It is therefore unlikely to migrate far from its site of generation. Although both CMU and paraquat treatment of plant tissue result in pigment breakdown and lipid peroxidation as determined by ethane evolution, there are marked differences in the ultrastructural changes. In contrast to paraquat, CMU treatment resulted in initial damage to the chloroplast membranes. This gives further clues to possible differences in the mechanisms of action of the two compounds. CMU inhibits electron transport at PSII and singlet oxygen would be generated in close proximity to the chloroplast membrane. Paraquat treatment
leads to superoxide formation which, being more stable, migrates from the chloroplast membranes to the tonoplast and plasmalemma where it participates in other reactions which form singlet oxygen. Although the mechanism by which singlet oxygen could be generated from superoxide has not yet been elucidated, it has been demonstrated to occur in non-aqueous systems (Khan, 1970).

The hypothesis proposed here implies that either the reduced paraquat radical or superoxide can pass through the chloroplast envelope without causing damage. Work with erythrocyte membranes suggests that superoxide can traverse this barrier without causing damage (Lynch and Fridovich, 1978). However, in extensive studies with *E. coli* and paraquat reduction, Hassan and Fridovich (1979b) proposed that the species which crossed the cell membrane was the paraquat radical and not superoxide. In the illuminated chloroplast, the concentration of dioxygen would be sufficiently great to ensure that reduced paraquat would be immediately reoxidised, forming superoxide. Thus, in this system, it is the oxygen radical which appears to cross membranes without instigating deleterious reactions. However, the reactivity of singlet oxygen dictates that it reacts at its site of production and thus the DCMU-generated species causes chloroplast damage, while that derived from paraquat attacks the tonoplast and plasmalemma (Fig. 5.1).

The hydroxyl radical has also been proposed as the primary oxidant in lipid peroxidation (Fong et al., 1973; McCay 1976; Fong et al., 1976), although the earlier proposal
Fig. 5.1 Sequences of herbicide action

C - Chloroplast envelope
T - Tonoplast
P - Plasmalemma
that the radical was formed via the Haber-Weiss reaction is now understood to be incorrect (see Bus and Gibson, 1979). It is thought however, that this reaction may occur through the catalysis of chelated iron (III) (Pederson and Aust, 1972; Tyler, 1975). The hydroxyl radical is one of the most reactive species known (Anbar and Neta, 1967) and reacts with almost all biological molecules. Thus, it is highly unlikely that specific damage would occur if this radical mediated the action of paraquat. In addition to this, attempts to demonstrate the existence of the hydroxyl radical in vivo have been unsuccessful (Elstner and Zeller, 1978).

The studies described here clearly demonstrate the central role of superoxide in the action of paraquat. They do not however, unequivocally indicate which active oxygen species is directly responsible for initiating the attack on unsaturated fatty acids in the cell membranes, although singlet oxygen would appear to be the most plausible candidate. The hypothesis proposed here might be difficult to substantiate because direct determination of the instigating species may not be possible using conventional biochemical techniques, as the formation of the species may occur in close contact with the membrane. It is clear from the scheme presented in Fig. 5.2, that superoxide is the important control point in the mechanism of action of paraquat. If the generation and/or fate of this radical species can be controlled, then the normal herbicidal properties of paraquat will not be manifested. Although the biochemistry and structure of the chloroplast are such, that under normal circumstances,
Fig. 5.2 Scheme showing herbicide action
superoxide presents little danger to the plant, it appears that paraquat produces levels of superoxide in excess of the endogenous defence mechanisms. The following reactions may outline the mechanism of paraquat action, resulting in lipid peroxidation and ethane formation.

\[
PQ^{2+} + e^- \rightarrow PQ'^+ \\
PQ'^+ + O_2 \rightarrow PQ^{++} \\
O_2^- \text{ exact mechanism unknown } \rightarrow O_2 \\
\alpha-\text{lin} + O_2 \rightarrow \alpha-\text{lin} OOH \\
\alpha-\text{lin} OOH + RH \rightarrow \alpha-\text{lin} OOH + R' \\
\alpha-\text{lin} OOH + M^{n+} \rightarrow \alpha-\text{lin} O' + M^{(n+1)} + OH^- \\
\alpha-\text{lin} O' \text{ } \beta\text{-scission} \rightarrow CH_3CH_2\cdot + \text{CHO(CH}_2)_2R \\
CH_3CH_2\cdot \text{ } \text{reduction} \rightarrow C_2H_6
\]

The photooxidation of the chloroplast pigments appears to need similar factors as lipid peroxidation, namely superoxide or singlet oxygen. Earlier studies by Peiser and Yang (1977), indicated a major role for superoxide in chlorophyll destruction during bisulphite oxidation. However, in the same study, singlet oxygen was shown not to be involved. In a subsequent investigation (Peiser and Yang, 1978), these workers showed that chlorophyll destruction in the presence of bisulphite and lipid hydroperoxides was independent of a requirement for oxygen species. The inhibition in the present study with PA-Cu, of both lipid peroxidation and pigment bleaching is strong evidence that oxygen is involved in these reactions. As mentioned previously, this does not exclude the possibility that some pigment photooxidation occurs through the agency of lipid free radicals, but this would probably be of relatively
minor importance in the initial stages of damage.

In conclusion, the importance of superoxide in the mode of action of paraquat has been unequivocally demonstrated and this would appear to be the primary mechanism of the phytotoxic effect. Although this oxygen free radical is important in the reactions leading to fatty acid destruction and pigment breakdown, other more reactive species, notably singlet oxygen, are more likely instigators. The actual mechanism by which these may be formed from superoxide is still a matter of some speculation, although it is arguably of less importance than the overall scheme of events which results in plant death.

Note
Fridovich and co-workers have just recently shown (personal communication, August, 1980), that PA-Cu may not itself be active as an artificial SOD. Their results indicate that the complex breaks down to SOD-active products, which are responsible for the observed effects. These results are compatible with the work presented here and do not affect the validity of the data.
Fig. 1. Superoxide and hydrogen peroxide production by tetraquat (●—●), triquat (■—■), paraquat (▲—▲) and diquat (△—△).

\[ \text{NO}_2^-, \text{H}_2\text{O}_2 \text{ in } \mu\text{moles mg}^{-1}\text{Chl} \]

N.B. All compounds present at a concentration of \(10^{-4}\text{M}\).
Fig. 2. Superoxide and hydrogen peroxide production by nitrobenzene (■—■), m-nitroacetophenone (●—●), p-nitrobenzoic acid (▲—▲) and anthraquinone-2-sulphonate (○—○).
Fig. 3. Superoxide and hydrogen peroxide production by 5-nitouracil (■—■), p-nitroacetophenone (●○), m-dinitrobenzene (▲▲) and 2,4-dinitrobenzoic acid (△△).
Fig. 4  Superoxide and hydrogen peroxide production by  
3,5-dinitrobenzoic acid (■—■), riboflavin (●—●),  
3,4-dinitrobenzoic acid (▲—▲) and p-dinitrobenzene  
(△—△).
Fig. 5. Superoxide and hydrogen peroxide production by indigo disulphonate (■—■), duroquinone (▲—▲), menadione (●—●) and \( \left[ \begin{array}{c} & C_6H_5 \\ \end{array} \right]_2 (\triangle—\triangle). \)
Fig. 6. Superoxide and hydrogen peroxide production by trimethyl-nitrobenezene (■–■), dibenzoquinone (○–○), benzoquinone (▲–▲) and safranine 0 (△–△).
Fig. 7. Superoxide and hydrogen peroxide production by a control (■—■), Janus Green (△—△), Alizarin Red (●—●) and phthalic anhydride (▲—▲).
Fig. 8. Superoxide and hydrogen peroxide production by DCPIP (●●), thionine (■■), neutral red (▲▲) and p-nitroacetanilide (▲▲).
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Diquat (1,1'-ethylene-2,2'-dipyridylium dibromide) in photoreactions of isolated chloroplasts.
Oxygen Activation in Chloroplasts: Models for “in vivo” Observations

By

ERICH F. ELSTNER

Institut für Botanik und Mikrobiologie, Techn. Universität München

RICHARD YOUNGMAN

School of Biological Sciences, University of Bath, England

(Vorgetragen auf der Botaniker-Tagung in Marburg am 13. September 1978)

Sauerstoffaktivierung in Chloroplasten

Chloroplasten setzen im Licht nicht nur Sauerstoff frei, sondern „photoaktivieren“ ihn auch. Unter „aktivem“ Sauerstoff versteht man reaktive Sauerstofformen oder -verbindungen wie Singulett-Sauerstoff (\(\text{O}_2\)), Superoxidanion (\(\text{O}_2^-\)) oder dessen Säure (\(\text{HO}_2\)), Wasserstoffperoxid (\(\text{H}_2\text{O}_2\)), OH-Radikal (\(\text{OH}\)), org. Peroxide und deren Radikalformen (ROOH, ROO') sowie Metall-O\(_2\)-Komplexe wie das Fenton-Reagenz oder das Perferrylion (Fe-\(\text{H}_2\text{O}_2\), Fe\(^{3+}\)-\(\text{O}_2\)).

Es gibt Hinweise für die lichtabhängige Bildung aller genannten \(\text{O}_2\)-Spezies, ausgenommen das \(\text{OH}\)-Radikal.

Bei der Aktivierung spielen sowohl Photosystem II (\(\text{O}_2\), \(\text{H}_2\text{O}_2\), ROOH) als auch Photosystem I (\(\text{O}_2^-\), \(\text{H}_2\text{O}_2\), \(\text{O}_2\)-Metallokomplexe, ROOH) eine Rolle.

Die beschriebenen Reaktionen spielen möglicherweise eine bedeutende Rolle nach Herbizidbehandlungen (DCMU, Paraquat) oder nach Infektionen.

Introduction

Oxygen activation is one of the most important metabolic reactions in cellular metabolism of aerobically living organisms. Since molecular oxygen exists in the triplet ground state its rates of reaction with organic compounds are extremely low. Oxygen thus has to be activated in order to become reactive. Besides other cellular compartments in plants (1) chloroplasts have been shown to exhibit oxygen activating activities (2).

In this communication we describe “in vitro” models for some reactions observed “in vivo” involving activated oxygen species. Certainly, most of the described reactions ‘per se’ are physiological irrelevant; they might mimic, however, reactions occuring in the intact plant during herbicide-induced symptom expression.
Materials and Methods

All applied methods and materials have been described elsewhere (1—4). The basic methods are summarized in Figure 1, where SA stands for sulfanilic acid and α-NA for α-naphthylamine.

Results and Discussion

I. Oxygen Activation by Photosystem II (PS-II)

A. Ethane formation and carotene bleaching

The importance of activated oxygen during symptom expression after paraquat application on whole flax leaves is shown in Table 1:

Table 1
Effects of DCMU and Penicillamine-copper (PA-Cu) on Chlorophyll bleaching and ethane formation by paraquat-treated flax leaves. Time of experiment: 72 hours. Temperature: 20°C. Light intensity: 5.500 lx. 20 flax leaves with ca. 0.2 g were used per flask; ethane is expressed as nmol/g fresh weight; chlorophyll as μg/g fresh weight.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ethane formed</th>
<th>Chlorophyll content</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Control (or paraquat in the dark)</td>
<td>0</td>
<td>540</td>
</tr>
<tr>
<td>b) Paraquat (10⁻⁶ M)</td>
<td>18</td>
<td>300</td>
</tr>
<tr>
<td>c) Paraquat + PA-Cu (50 SOD-units)</td>
<td>5</td>
<td>430</td>
</tr>
<tr>
<td>d) Paraquat + DCMU (10⁻⁶ M)</td>
<td>11</td>
<td>480</td>
</tr>
</tbody>
</table>

Identification of Activated Oxygen Species

1) \( \text{NH}_2\text{OH} + \text{H}^+ + 2\text{O}_2^- \rightarrow \text{NO}_2^- + \text{H}_2\text{O}_2 + \text{H}_2\text{O} \)  
(red colour with \( \text{SA} + \alpha\text{NA} \))

2) \( \frac{\text{NADH}_2 + \text{H}_2\text{O}_2}{\text{Peroxidase}} \rightarrow \frac{\text{NAD} + 2\text{H}_2\text{O}}{(\Delta E_{340})} \)

\( \text{R} - \text{C} - 14\text{COOH} + \text{H}_2\text{O}_2 \rightarrow \text{RCOOH} + 14\text{CO}_2 \)  
(Scintillation counting)

3) Fenton's oxidant:

\( \text{Methionine} + \text{Pyridoxal phosphate} \rightarrow \text{ethylene} + \text{Products} \)  
(Gaschromatograph)

Fig. 1
Chlorophyll bleaching in the presence of paraquat is delayed by a copper-penicillamine complex (PA-Cu) or by DCMU. Ethane formation as an indication for cellular compartmentalization (3) and unsaturated fatty acid peroxidation (5, 6) is inhibited by ca. 60% by PA-Cu and by ca. 30% by DCMU. Since we showed that PA-Cu with a molecular weight of ca. 2200 has strong superoxide dismutase (SOD) activity (7) and superoxide anion is not formed in chloroplasts in the presence of DCMU (4), we have to assume that light-dependent ethane formation can proceed by two different mechanisms, one being dependent on PS-I involving $O_2^-$, the other one driven by PS-II not involving $O_2^-$. 

In vitro experiments with isolated chloroplast lamellae in the presence or absence of $\alpha$-linolenic acid ($\alpha$-lin) may represent models of two different "in vivo" reactions involved in ethane formation. In the absence of $\alpha$-lin, both paraquat and DCMU stimulate ethane formation; in the presence of $\alpha$-lin, DCMU inhibits paraquat-stimulated ethane formation. This result indicates different mechanisms of ethane formation from internal and external substrate ($\alpha$-lin, see Ref. 5), one involving oxygen activation by PS-I via paraquat and the other one activated by PS-II in the presence of DCMU (Table 2).

Since PA-Cu has no significant effect on either one of these in vitro reactions the in vivo effects (see Table 1) suggest yet an additional reaction involved in paraquat-induced ethane formation in whole leaves.

![Diagram of gentiobiose and "crocin" structure](image)

**Fig. 2**

**Crocin Bleaching by Chloroplast Lamellae**

(p mol/min)
Effects of DCMU on ethane formation by isolated chloroplast lamellae. Reaction conditions: Total vol.: 3 ml; time: 30 min; temperature: 22°C. Additions: Isolated chloroplast lamellae with 100 µg chlorophyll; 75 µmol phosphate buffer pH 7.8; 5 µmol NH₄Cl; 5 µmol MgCl₂; 3 µmol NaN₃; 50 mg bovine serum albumine; 1 µmol α-linolenic acid (where indicated); paraquat (10⁻⁶ M) and DCMU (10⁻⁶ M), where indicated; penicillamine-copper (PA-Cu): 50 SOD units.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Ethane formed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>— α-lin</td>
</tr>
<tr>
<td>a) Control</td>
<td>0</td>
</tr>
<tr>
<td>b) Paraquat</td>
<td>0.6</td>
</tr>
<tr>
<td>c) Paraquat + DCMU</td>
<td>1.2</td>
</tr>
<tr>
<td>d) Paraquat + PA-Cu</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Another experiment supports also the idea of the formation of “activated” oxygen by PS-II in the presence of DCMU: the water soluble carotene crocin is reductively photobleached by electrons from PS-I (8); in the presence of DCMU a second reaction seems to start after a lag period of several minutes (Fig. 2).

Several mechanisms have recently been suggested for crocin bleaching (6). In the light of the results presented in Tables 1 and 2 and from other observations we suggest that, in the presence of DCMU, restricted energy dissipation...
in PS-II yields activated oxygen (possibly $^{1}\text{O}_2$) which adds onto double bonds of unsaturated fatty acids yielding hydroperoxides.

These hydroperoxides decompose under malondialdehyde- and ethane formation (5, 6, 8, 9), cooxidizing carotenes:

Primary reactions:

\[
\begin{align*}
\text{PS-II} & \rightarrow ^{1}\text{PS-II} (^{1}\text{PS-II} = \text{excited PS-II}) \\
^{1}\text{PS-II} + \text{O}_2 & \rightarrow ^{1}\text{O}_2 + \text{PS-II} \\
^{1}\text{O}_2 + \alpha\text{-lin} & \rightarrow \alpha\text{-lin-OOH}
\end{align*}
\]

Secondary reactions:

\[
\begin{align*}
\alpha\text{-lin-OOH} & \rightarrow \text{ethane + products} \\
\alpha\text{-lin-OOH} + \text{crocin} & \rightarrow \text{degradation products (bleached)}
\end{align*}
\]

The involvement of singlet oxygen ($^{1}\text{O}_2$ in our scheme) is not yet proven. Pulse radiolysis experiments with crocin showed, however that, besides the well known effect with $^{1}\text{O}_2$, only $\text{OH}^-$ and $e_{aq}$ (hydrated electron) significantly bleach crocin (8).

We determined the possibility of the formation of the $\text{OH}^-$ by PS-II. A clearly negative result has been obtained with the aid of the specific reaction of p-nitrosodimethylaniline (PND) with $\text{OH}^-$. As shown in Figure 3, both

### Products of Photosynthetic Oxygen Reduction

<table>
<thead>
<tr>
<th>Electron Acceptor</th>
<th>Products</th>
<th>[μmol x mg Chl$^{-1}$ x h$^{-1}$]</th>
<th>DCMU</th>
<th>DBMIB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$\text{H}_2\text{O}_2$</td>
<td>$\text{O}_2^-$</td>
<td>$\text{H}_2\text{O}_2$</td>
</tr>
<tr>
<td>Paraquat</td>
<td></td>
<td>40</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>MPT</td>
<td></td>
<td>75</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>DIMEB</td>
<td></td>
<td>30</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Caffeic Acid</td>
<td></td>
<td>35</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

**MPT:** 2-[4-Methyl-4-pyridinio] 1.3.5 triazonium bromide

**DBMIB:** Dibromothymoquinone

**DIMEB:** 2,3-Dimethyl-5,6-methylenedioxy p-benzoquinone

**Conclusion:** Paraquat, MPT: 1e-transfer to $\text{O}_2$

DIMEB,

Caffeic acid: 2e-transfer to $\text{O}_2$

Fig. 4
electron acceptors for PS-I and electron transport inhibitors like DCMU or DBMIB completely inhibit PNDA bleaching. This implies (for discussion see Ref. 10) that under the applied conditions OH\(^-\) is not formed, rendering photosynthetic PNDA bleaching a specific reaction for assaying the reducing side of PS-I. This result also proves that a spontaneous “Haber-Weiss”-reaction according to eqn. 1:

\[
\text{eqn. 1: } \text{O}_2^- + \text{H}_2\text{O}_2 \rightarrow \text{OH}^- + \text{OH}^- + \text{O}_2
\]
does not occur in chloroplasts in the presence or absence of autooxidizable electron acceptors.

**Conclusion**

Since OH\(^-\) is apparently not formed in isolated chloroplasts in the light we assume that \(^1\text{O}_2\) is a good candidate for initiating both ethane formation and crocin bleaching in the presence of DCMU.

**B. Hydrogen peroxide formation**

We recently reported on the differentiation of several mechanisms of oxygen reduction by different autooxidizable compounds (4). As shown in Figure 4, paraquat and MPT produce both \(\text{O}_2^-\) and \(\text{H}_2\text{O}_2\), while DIMEB and caffeic acid mainly produce \(\text{H}_2\text{O}_2\) in the presence of illuminated chloroplast lamellae. DCMU inhibits the production of both \(\text{O}_2^-\) and \(\text{H}_2\text{O}_2\) by all tested compounds, DBMIB inhibits all reactions except \(\text{H}_2\text{O}_2\) formation by DIMEB. This result and the differential effects of SOD

**Photosynthetic \(\text{H}_2\text{O}_2\) Formation:**
**Effects of DBMIB and Superoxide Dismutase [SOD]**

<table>
<thead>
<tr>
<th>Electron Acceptor</th>
<th>(10^{-5}\text{M DBMIB})</th>
<th>60 units SOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIMEB</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Paraquat</td>
<td>70</td>
<td>0</td>
</tr>
<tr>
<td>Caffeic Acid</td>
<td>75</td>
<td>65</td>
</tr>
</tbody>
</table>

Conclusions:

\[
\text{Fig. 5}
\]
Oxygen Activation in Chloroplasts  

(Fig. 5) led us to assume mechanisms for oxygen reduction as shown under “Conclusions” in Figure 5: Production of $\text{H}_2\text{O}_2$ katalyzed by DIMEB involves an electron donor for DIMEB located after PS-II between the inhibition sites of DCMU and DBMIB (possibly plastoquinone). The transfer of electrons to oxygen is a two-electron step not involving intermediately formed $\text{O}_2^-$ as in the case of $\text{H}_2\text{O}_2$ production driven by PS-I.

II. Oxygen Activation by Photosystem I (PS-I)

Production of $\text{O}_2^-$ by low potential redox compounds like ferredoxin or bipyridinium salts are driven by PS-I (4). This is mainly due to thermodynamic reasons, since only the reducing side of PS-I is electronegative enough to drive monovalent oxygen reduction ($E'_0$ for $\text{O}_2/\text{O}_2^-$ = $-0.33$ V, Ref. 11). Besides one electron oxygen reduction by PS-I, two other oxygen activating reactions are observed as PS-I dependent. We thus differentiate three PS-I driven oxygen activations, exhibiting different sensitivity towards SOD:

a) $\text{H}_2\text{O}_2$ production mediated by paraquat is not influenced by SOD (Fig. 5),
b) $\text{H}_2\text{O}_2$ production mediated by o-diphenols is inhibited by SOD,
c) ethylene formation from methionine is stimulated by SOD.

### Photosynthetic Ethylene Formation from Methionine

<table>
<thead>
<tr>
<th>I) Products</th>
<th>formed:</th>
<th>(\text{µmol})</th>
<th>(\text{nmol})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electron Acceptor [10 (\mu\text{M})]</td>
<td>$\text{H}_2\text{O}_2$</td>
<td>$\text{O}_2^-$</td>
<td>$\text{C}_2\text{H}_4$</td>
</tr>
<tr>
<td>paraquat</td>
<td>16</td>
<td>13</td>
<td>1</td>
</tr>
<tr>
<td>ferredoxin</td>
<td>8</td>
<td>4</td>
<td>26</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>II) Stimulation by SOD, Inhibition by Catalase</th>
<th>% Ethylene formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>ferredoxin</td>
<td>100</td>
</tr>
<tr>
<td>&quot; + 200 U SOD</td>
<td>300</td>
</tr>
<tr>
<td>&quot; + &quot; Catalase</td>
<td>20</td>
</tr>
</tbody>
</table>

### III) Conclusions:

Reduced ferredoxin and $\text{H}_2\text{O}_2$ are necessary for ethylene formation - $\text{O}_2^-$ reoxidizes reduced ferredoxin; SOD removes $\text{O}_2^-$.  

Fig. 6
As shown in Figure 6, paraquat does not stimulate the production of an oxygen species responsible for ethylene formation from methionine; the inhibition by catalase of ferredoxin-stimulated ethylene formation from methionine together with the stimulation by SOD makes it likely that a Fenton-type reagent is produced from reduced ferredoxin and \( \text{H}_2\text{O}_2 \) which finally is the oxidant for methionine, producing ethylene.

The presented models (summarized in Fig. 7) are not thought to represent in vivo conditions; they might help to understand certain underestimated aspects of “the chloroplast at work” (12) and the “mode of action of well known herbicides” (13).

**Summary**

Chloroplasts not only produce molecular oxygen from water in the light but also “photoactivate” oxygen. As photoactivated oxygen we address reactive oxygen species as singlet oxygen (\( ^1\text{O}_2 \)), superoxide ion (\( \text{O}_2^- \)), hydrogen peroxide (\( \text{H}_2\text{O}_2 \)), OH-radical (\( \text{OH}^- \)), organic peroxides and their radicals (\( \text{ROOH}, \text{ROO}^- \)) and metallo-\( \text{O}_2 \)-complexes as Fenton-type reagents or per-
ferryl ion (Fe-H₂O₂, Fe²⁺-O₂⁻). There is evidence for the light dependent production of all of the above oxygen species, except the OH-radical.

Both photosystem II (¹O₂, H₂O₂, ROOH) and photosystem I (O₂⁻, H₂O₂, O₂-metallo-complexes, ROOH) are involved in the formation of the above species. The described reactions may also play an important role "in vivo," especially after herbicide treatment (DCMU, paraquat) or after infections.

References


E. F. Elstner
Institut für Botanik und Mikrobiologie
Technische Universität München
Arcisstraße 21
D-8000 München 2
Mechanism of Paraquat Action: Inhibition of the Herbicidal Effect by a Copper Chelate with Superoxide Dismutating Activity

Richard J. Youngman and Alan D. Dodge
School of Biology, University of Bath, Bath, Avon, BA2 7AY, U.K.

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Paraquat, Plant Pigments, Fatty Acids, Superoxide, Copper Chelate, Flax Cotyledons

The treatment of flax cotyledons (Linum usitatissimum) with paraquat was shown to decrease the levels of chlorophyll and carotenoid pigments. The fatty acid content of chloroplast fragments isolated from treated tissue was determined and shown to be greatly decreased by paraquat treatment. The superoxide radical was demonstrated to play an important role in the phytotoxic action of paraquat by the use of a copper chelate of D-penicillamine, which has a high superoxide dismutating activity. The action of paraquat was inhibited by this compound. The role of superoxide is discussed with reference to the generation of more toxic species, such as singlet oxygen.

Introduction

The bipyridylium herbicides e. g., paraquat and diquat, initiate their phytotoxic action by successfully competing with NADP for electrons emanating from the P 700 acceptor [1]. They are reduced univalently and undergo immediate reoxidation by molecular oxygen [2]. The superoxide anion and hydrogen peroxide are both formed during the autoxidation of the reduced compounds. It was originally believed that hydrogen peroxide was the initial toxic species responsible for the action of paraquat [3], more recently however, superoxide has been shown to be an earlier, more reactive intermediate [2]. Hydrogen peroxide is formed via both the enzymatic and nonenzymatic dismutation of superoxide. Superoxide dismutase (SOD) is present in the chloroplast [4] and is found associated with the thylakoid lamellae and free in the stroma [5, 6] and rapidly catalyses the harmless dismutation of superoxide [7]. Asada et al. [8] have calculated the concentration of SOD in the chloroplast to be about $10^{-5}$ M, which is sufficient to deal with steady-state concentrations of superoxide of about $10^{-5}$ M. However, the presence of paraquat can increase the superoxide concentration up to $10^{-8}$ — $10^{-7}$ M, which is in excess of the enzymatic defences and so will lead to cellular damage.

Earlier work showed that paraquat treatment caused the loss of chlorophyll and disruption of the tonoplast and plasmalemma membranes [9]. In the present study, the role of superoxide in the herbicidal action of paraquat has been investigated with the aid of a low molecular weight copper chelate of D-penicillamine (PA-Cu) [10, 11], which is known to have a high superoxide dismutating activity [12].

Materials and Methods

Flax seedlings (Linum usitatissimum var. Reina) were grown under continuous illumination of 5.25 W/m² on waterlogged vermiculite for seven days at 25 °C ± 3 °C. The cotyledons were removed and floated on solutions as indicated under the same conditions of temperature and illumination as described above. The final concentration of paraquat was $10^{-8}$ M and 50 units of PA-Cu were used per treatment. The addition of paraquat was delayed for 24 hours to allow PA-Cu to infiltrate the cotelodon tissue.

Chloroplast fragments for fatty acid analysis, were isolated from treated cotyledons by a modification of the procedure of Izawa and Good [13]. Total lipids were extracted with a chloroform-methanol mixture [14] and then refluxed with methanolic NaOH and boron-trifluoride-methanol to form methyl ester derivatives of the fatty acids [15, 16]. These were separated and identified using a Pye Unicam GCD chromatograph with 10% diethylene glycol succinate as the stationary phase. The column temperature was 190 °C.

The carotenoid pigments of treated cotyledons were extracted and the levels determined according to the method of Bishop and Wong [17]. Chlorophyll was measured as described by Arnon [18].

Abbreviations: Paraquat, 1,1’dimethyl-4,4’bipyridylium dichloride; SOD, superoxide dismutase; PA-Cu, copper chelate of D-penicillamine.

Reprint requests to R. J. Youngman.
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Results

The decrease in chlorophyll content is one of the most obvious symptoms of the phytotoxic action of paraquat. Chlorophyll breakdown in paraquat treated cotyledons was markedly reduced by PA-Cu (Fig. 1) during an illumination period of 72 hours. Similarly the breakdown of carotenoid pigments in the presence of paraquat was retarded by PA-Cu over the same time period (Fig. 2). Although the levels of all carotenoid pigments decreased following paraquat treatment, α- and β-carotenes were more markedly affected than the xanthophylls. In addition, the restraining effect of PA-Cu was most evident on the xanthophyll pigment levels, α- and β-carotenes were completely destroyed by paraquat treatment, but the additional presence of PA-Cu reduced the breakdown to 87%. In the case of the xanthophylls, 89% were destroyed by paraquat alone, but the decrease was only 26% in the presence of PA-Cu.

One of the early effects of paraquat treatment is the initiation of lipid peroxidation reactions which

**CHLOROPHYLL CONTENT OF FLAX COTYLEDONS**

Fig. 1. Chlorophyll content of paraquat treated flax cotyledons in the presence and absence of PA-Cu. Incubation conditions were as described under Materials and Methods.

**CAROTENOID CONTENT OF FLAX COTYLEDONS**

Fig. 2. Changes in carotene and xanthophyll levels of treated flax cotyledons. Incubation conditions are detailed in Materials and Methods.

**FATTY ACID CONTENT OF CHLOROPLASTS**

Fig. 3. Effect of paraquat on the fatty acid content of chloroplasts from paraquat treated flax cotyledons, in the presence and absence of PA-Cu. Experimental conditions are given in Materials and Methods.
primarily involve unsaturated fatty acids and lead to membrane damage and eventual loss of cell integrity. Fig. 3 shows the effect of paraquat in the presence and absence of PA-Cu, on the levels of some important chloroplastic fatty acids. After 72 hours of paraquat treatment, the greatest decrease was observed in the levels of linoleic and linolenic acids (18:2 and 18:3 acids, respectively). Paraquat treated flax cotyledons have been shown to release ethane [19, 20] and both these fatty acids probably act as substrates for this hydrocarbon gas [21—23]. In general, PA-Cu appeared to retard the breakdown of these fatty acids.

Discussion

The treatment of flax cotyledons with paraquat led to a breakdown of chlorophyll and carotenoid pigments and a decrease in the levels of fatty acids, notably linoleic and linolenic. A concomitant release of ethane has also been demonstrated [19, 20]. All these parameters of paraquat action were inhibited by PA-Cu. This showed that PA-Cu was able to enter the cotyledonary tissue and scavenge superoxide which appeared to play a role in each of the degradative processes. It is thought unlikely that superoxide itself possesses sufficient reactivity to abstract protons from unsaturated fatty acids in membranes to instigate lipid peroxidation reactions. It is probable that the importance of superoxide lies in its ability to give rise to more reactive species such as singlet oxygen, which have been shown to initiate lipid peroxidation reactions [24, 25].

Chlorophyll and carotenoid photobleaching has been shown to occur through the agency of singlet oxygen, and β-carotene is known to be an effective quencher of this toxic oxygen species [26]. Singlet oxygen may be formed from the superoxide radical or via the decomposition of lipid hydroperoxides. Peiser and Yang [27] have provided in vitro evidence that chlorophyll is destroyed by alkoxy radicals without the direct involvement of oxygen. However, our results indicate that superoxide does play a role in the bleaching reactions, although this does not rule out the possibility that some bleaching is unrelated to lipid peroxidation reactions. Oxygen is probably more important in the initial stages of pigment breakdown, but its effect may be augmented by lipid radicals once fatty acid breakdown has been instigated.

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Inhibition of paraquat phytotoxicity by a novel copper chelate with superoxide dismutating activity

R. J. Youngman, A. D. Dodge, E. Lengfelder and E. F. Elstner

School of Biological Sciences, University of Bath, Bath BA2 7AY (England); and Strahlenbiologisches Institut, Universität München, D–8000 München 2 (Federal Republic of Germany); and Institut für Botanik und Mikrobiologie, Technische Universität München, D–8000 München 2 (Federal Republic of Germany), 11 December 1978

Summary. A chelate with superoxide dismutase activity, D-penicillamine copper complex, was shown to inhibit paraquat toxicity in flax cotyledons (Linum usitatissimum var. Reina). Paraquat-stimulated chlorophyll loss and ethane production were markedly reduced by this complex. The role of superoxide in the action of paraquat is briefly discussed.

It is well-known that the action of the herbicide paraquat is dependent upon light and oxygen. In treated plants as in the normal photosynthetic reaction, light induces chloroplast electron transport which leads to a reduction of paraquat by a one electron transfer process. The immediate reoxidation of this free radical by oxygen was shown to generate hydrogen peroxide, which was thought to be the primary toxic agent. However, more recent work has suggested that one electron transfer to oxygen initially gives rise to the superoxide free radical. Superoxide production mediated by paraquat (=methyl viologen) has been demonstrated in experiments with isolated chloroplasts. Although superoxide dismutase enzymes are present within the chloroplast, it is assumed that the level of superoxide formed in vivo following treatment of the leaves with paraquat is in excess of the capabilities for enzymic dismutation and this leads to cellular damage. In the present study, we have provided evidence for the generation of superoxide in vivo by the use of a superoxide dismutating copper chelate of D-penicillamine.

The reaction of Cu(II) with D-penicillamine results in the formation of a mixed valence Cu(I) Cu(II) cluster with a mol wt of about 2200. Ethane generation by flax cotyledons. The conditions were as detailed for the table. Ethane was determined as described previously in a Varian Aerograph model 1400 gas chromatograph.

![Graph](image-url)
chloroplasts, have shown that this D-penicillamine copper chelate (PA-Cu) has an action similar to the Cu-Zn and Mn superoxide dismutase enzymes in a range of reactions involving superoxide.

Flax cotyledon leaves were floated on solutions of paraquat and PA-Cu and the herbicidal effect was initially followed by assessing the breakdown of chlorophyll, the most obvious phytotoxic symptom. The table shows that after 72 h illumination, the breakdown of chlorophyll in paraquat treated leaves was significantly retarded by the additional presence of PA-Cu.

Previous work has shown that disruption of the tonoplast and plasmalemma were the earliest structural changes observed in leaf cells following paraquat treatment. This is brought about by various reactions generally known as lipid peroxidation which are initiated by free radicals. It is unlikely that superoxide possesses the necessary reactivity to abstract protons from unsaturated fatty acids in the membrane to instigate this deteriorative chain reaction, it is more probable that initiation is by a more reactive species derived from superoxide, such as singlet oxygen or .OH radicals. The process of lipid peroxidation can be monitored by the release of ethane from the damaged tissue. The figure shows that the release of this simple hydrocarbon from flax cotyledon leaves was considerably promoted by paraquat treatment, however this was minimised when PA-Cu was also present. Although PA-Cu has a high superoxide dismutating capability ($K = 10^3 M^{-1} S^{-1}$), it failed to completely prevent paraquat induced damage.

Chlorophyll levels in treated flax cotyledons

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Chlorophyll content (µg/g fresh weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>540</td>
</tr>
<tr>
<td>Paraquat (10^{-6} M)</td>
<td>300</td>
</tr>
<tr>
<td>Paraquat + PA-Cu</td>
<td>430</td>
</tr>
</tbody>
</table>

20 cotyledon leaves from 7-day-old flax seedlings were used for each treatment and were incubated in 70-ml screw top flasks fitted with serum rubber material to allow sampling of the flask atmosphere. The addition of paraquat was delayed for 24 h to allow PA-Cu to penetrate the leaf tissue. A concentration of 50 nmoles of PA-Cu in 3 ml was equivalent to 50 superoxide dismutase units, as determined by the ability to inhibit nitrite formation from hydroxylamine. Chlorophyll was determined upon conclusion of the experiment (72 h illumination with 5500 lux), according to the method of Arnon.

This can be explained by the fact that only a very small proportion of the superoxide produced need escape dismutation for the damage process to assume a more complex nature which is then more difficult to control. These experiments have provided good evidence for the actual generation of superoxide in vivo in paraquat treated leaves. They also demonstrate that PA-Cu, which is already in pharmaceutical use in the nonchelated form, might be efficacious in the reduction of paraquat poisoning in humans.
CORRIGENDA

Abbreviations

CSMP - continuous systems modelling programme
ICI - Imperial Chemical Industries Ltd.
PUPA - polyunsaturated fatty acids

References


p66 - line 18. Add after '....reaction chamber,' "with saturating light of 30 Klux at the electrode surface."