Aspects of chloroplast protection against photo-oxidative damage

Gillham, David J.

Award date:
1986

Awarding institution:
University of Bath

ASPECTS OF CHLOROPLAST PROTECTION AGAINST PHOTO-OXIDATIVE DAMAGE

Submitted by David J. Gillham

for the degree of Ph.D.

of the University of Bath

1986

Copyright

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

CONTENTS

Acknowledgements

I would like to thank my supervisor, Dr. Alan Dodge for initiating my interest in this project, and for helpful discussions during the period of this work. I am also very grateful to the University of Bath for the award of a University Research Bursary and also for providing laboratory facilities. I would like to thank Dr. Ken Wright of FBC Limited for providing samples of diphenyl ether herbicides used in this study, and thanks also to Martin Huxley of Long Ashton Research Station for the irradiance data used in Figure 19.

Finally, I would like to thank my wife, Penny, for her support and encouragement throughout this project, and for her unquestioning faith in my fascination for the garden pea.

Abstract

Chloroplasts from higher plants contain the enzymes superoxide dismutase, ascorbate peroxidase and glutathione reductase that scavenge $O_2^{\cdot -}$ and H_2O_2, and the antioxidants ascorbate and glutathione together with carotenoid pigments. A high proportion of the activity of these enzymes was detected in chloroplasts from pea leaves, although they only contained 30% of leaf ascorbate and 10% of leaf glutathione. Chloroplasts contained no detectable catalase or peroxidase activity.

Carotenoid levels and chloroplast enzymes that scavenge $O_2^{\cdot -}$ and H_2O_2 were monitored during leaf development (greening) and senescence. Etiolated leaves contained antioxidants and enzymes, and the levels of ascorbate and ascorbate peroxidase increased during greening. Levels of chloroplast antioxidant enzymes declined during the senescence of flax cotyledons and pea leaf discs. Levels of these antioxidants and of enzymes that scavenge $O_2^{\cdot -}$ and H_2O_2 were monitored in the leaves and chloroplasts of pea plants grown under glasshouse conditions at different times of the year, and in response to different growth light intensities. Plants grown in summer contained higher levels of ascorbate, ascorbate peroxidase and glutathione reductase than plants grown in winter. Chloroplasts isolated from plants grown at a low light intensity contained less ascorbate, ascorbate peroxidase and glutathione reductase than plants grown at a higher light intensity. Light may therefore be an important factor that influences the levels and activity of chloroplast antioxidant mechanisms.

A number of herbicides or stress conditions that promote photo-oxidative injury to plants have been investigated. Damage induced by an electron flow inhibitor (monuron),a bipyridyl (paraquat) and a photosensitizer (rose bengal) has been compared with that induced by nitrodiphenyl ether herbicides. All four classes of compound induced

similar light dependent chlorophyll bleaching, inhibition of photosynthesis and membrane lipid peroxidation to leaf material. The effect of these herbicides on electron flow reactions of isolated thylakoids was used to distinguish between different sites of activation or modes of action. The damage induced probably occurred because the increased formation of free radicals, $O_2^{\cdot -}$, H_2O_2 or $^1O_2$ exceeded the capacity of endogenous chloroplast antioxidant mechanisms to scavenge them. Photoinhibition of photosynthesis in pea leaf discs incubated at chilling temperatures, or of thylakoid membranes incubated in the absence of electron acceptors, indicated that damage was induced by a mechanism analogous to the mode of action of photosynthetic inhibitor herbicides. Injury was light dependent and resulted in the loss of photosystem II function.

Peroxidation of illuminated thylakoid membranes induced by herbicides was strongly retarded by ascorbate, carotenoids and α-tocopherol. These antioxidants are normally present in chloroplasts, indicating their possible protective function *in vivo*. Chlorophyll bleaching of pea leaf discs induced by the herbicide paraquat was promoted if endogenous superoxide dismutase activity was lowered using the inhibitor diethyl dithiocarbamate. Leaf discs containing chloroplasts with reduced levels of ascorbate, ascorbate peroxidase and glutathione reductase, achieved by growth of plants at low light intensity, also showed enhanced rates of paraquat induced bleaching. Control of the levels of chloroplast mechanisms that scavenge free radicals, $O_2^{\cdot -}$, H_2O_2 or $^1O_2$ may be one important factor by which increased or decreased tolerance to stress is achieved.

Abbreviations

AA	ascorbic acid
Chl	chlorophyll
^{3}Chl	triplet chlorophyll
CF_1	coupling factor 1
CMU	monuron 3-(4-chlorophenyl)-1,1-dimethylurea
Cyt B	cytochrome B
Cyt F	cytochrome F
DABCO	1,4-diazabicyclo(2,2,2)octane
DCPIP	dichlorophenol indophenol
DDTC	diethyl dithiocarbamate
DHA	dehydroascorbate
DPC	diphenylcarbazide
DPE	diphenyl ether
Fd	ferredoxin
FeCN	ferricyanide
GSH	reduced glutathione
GSSG	oxidised glutathione
H_2O_2	hydrogen peroxide
Hepes	4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid
IRGA	infra red gas analyser
LHCP	light harvesting chlorophyll protein
NADPH-GPD	NADPH glyceraldehyde 3-phosphate dehydrogenase
$O_2^{\cdot -}$	superoxide
$^1O_2$	singlet oxygen
$OH^{\cdot}$	hydroxyl radical
PaCu	penacillamine-copper complex
Paraquat	1,1-dimethyl-4,4-bipyridylium dichloride

P/C	plastocyanin
PQ	plastoquinone
PS I	photosystem I
PS II	photosystem II
SOD	superoxide dismutase
Tricine	N-Tris (hydroxymethyl) methylglycine
Tris	2-amino-2-(hydroxymethyl)propane-1,3-diol

Publications

Gillham, D.J., Dodge, A.D. (1984). Aspects of paraquat action. Plant Physiology 71; S-51.

Gillham, D.J., Evans, M.W., Dodge, A.D. (1985). Oxyfluorfen activation by photosynthetic electron transport. Proceedings of British Crop Protection Conference - Weeds 1195-1201.

Gillham, D.J., Dodge, A.D. (1985) Chloroplast protection in greening leaves. Physiologia Plantarum 65; 393-398.

Gillham, D.J., Dodge, A.D. (1986) Hydrogen peroxide scavenging systems in chloroplasts: A quantitative study. Planta 167; 246-251.

Gillham, D.J., Dodge, A.D. (1986) Studies into the action of the diphenyl ether herbicides acifluorfen and oxyfluorfen. 1. Activation by light and oxygen in leaf tissue. Pesticide Science In press.

Gillham, D.J., Dodge, A.D. (1986) Studies into the action of the diphenyl ether herbicides acifluorfen and oxyfluorfen. 11. The interaction with photosynthetic electron transport. Pesticide Science In press.

Dodge, A.D., Gillham, D.J. (1986) Methods for investigating the action of light activated herbicides. 1. The assessment of lipid peroxidation. Aspects of Applied Biology 11; 97-104.

Gillham, D.J., Dodge, A.D. (1986) Methods for investigating the action of light activated herbicides. II. Discrimination between sites of activation. Aspects of Applied Biology 11; 105-110.

INTRODUCTION

1. Photosynthesis

Photosynthesis is defined as the assimilation of CO_2 in light to form carbohydrates and oxygen (Trebst and Avron, 1977). Experiments in the 1930's by Hill (1937, 1939) first demonstrated that isolated chloroplasts could reduce artificial electron acceptors in light with the evolution of oxygen. Improved techniques of chloroplast isolation from higher plants have subsequently shown that the entire process of photosynthesis occurs within chloroplasts.

1.1 Chloroplast structure and function

Chloroplasts from higher plants consist of a double membraned envelope enclosing a stroma containing enzymes necessary to convert CO_2 into carbohydrates in the photosynthetic Calvin cycle. The most prominent feature of chloroplasts is their complex internal membrane structure which can be resolved as stacked (granal) and unstacked (stromal) thylakoid lamellae. Chloroplast thylakoids contain a high proportion of unsaturated lipid in their membrane structure, together with the light harvesting pigment proteins, photosystem I and II and other protein complexes required for photosynthetic electron flow (Barber, 1983).

The most widely accepted model of photosynthetic electron flow is based on the scheme proposed by Hill and Bendall (1960), which requires two photosystems linked by a series of electron carriers arranged in order of decreasing electronegativity. The two reaction centres, P_{680} and P_{700}, detected in higher plant thylakoids contain chlorophyll and are closely associated with further light harvesting chlorophylls. The complex containing P_{700} is known as photosystem I, while that containing P_{680} is photosystem II. Light energy absorbed

by chlorophyll is channelled into the reaction centres. Excitation of chlorophyll molecules within the reaction centres initiates the loss of an electron to a neighbouring acceptor molecule. Charge separation in photosystem II causes the reduction of a primary acceptor, Q, and the formation of an oxidant, Z. Reduced Q, possibly a form of plastoquinone, transfers electrons to further plastoquinone molecules in the thylakoid membrane. Electrons pass from plastoquinone to cytochrome f and plastocyanin, a copper containing protein loosely attached to the inner face of the thylakoid membrane.

Light absorbed by the reaction centre chlorophyll in photosystem I causes charge separation, and the reduction of an acceptor molecule, X. The photosystem I electron acceptor can reduce the iron sulphur protein ferredoxin. Chloroplast ferredoxin $NADP^+$ reductase catalyses the reduction of $NADP^+$.

Electrons ejected from photosystem II are replaced by the photolysis of water, accompanied by oxygen evolution. Experiments by Jolliot and Kok (1975) demonstrated that the water oxidising complex accumulated four positive charges, each representing one quantum of light, prior to the rapid splitting of two molecules of water. The oxidising complex, Z, contains manganese as an essential co-factor. An outline of an updated Hill-Bendall scheme of photosynthetic electron flow is shown in Figure 1.

1.2 Photophosphorylation

The fixation of CO_2 by photosynthesis requires both NADPH and ATP. The synthesis of ATP may be associated with photosynthetic electron flow from water to $NADP^+$ (non cyclic photophosphorylation), or may occur as a consequence of electron flow around photosystem I (cyclic

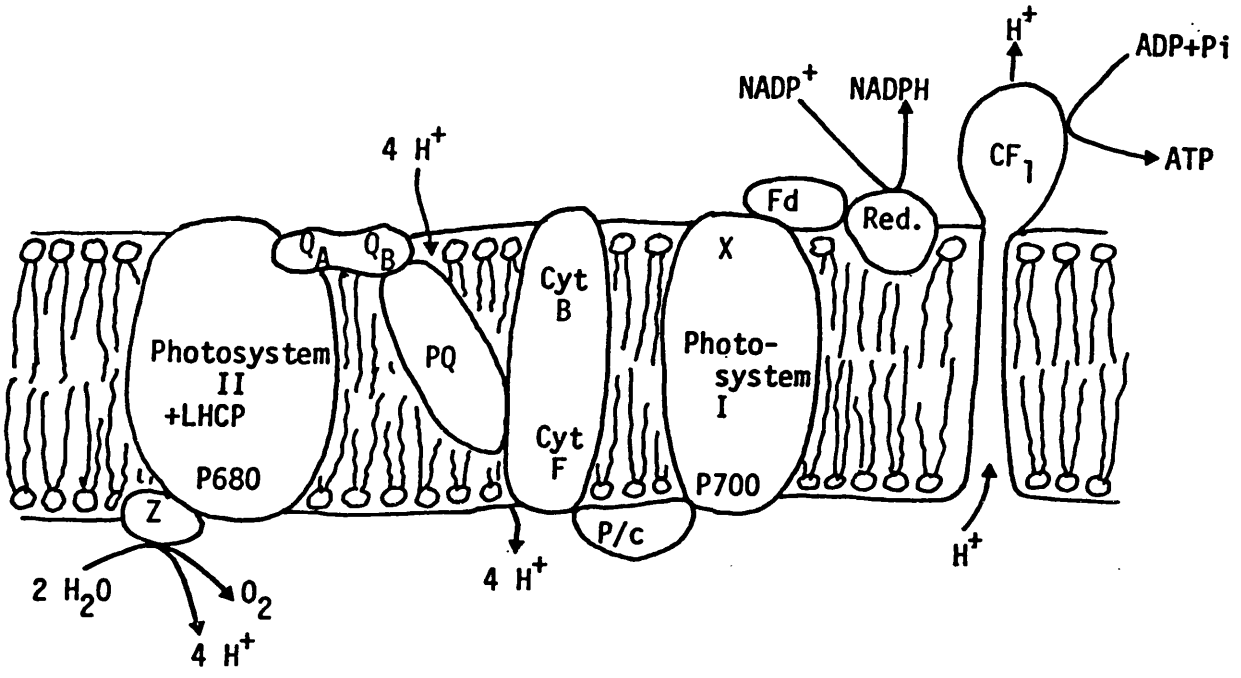


Fig 1. The electron transport pathway of higher plant chloroplasts. (based on Foyer 1984)

photophosphorylation). Photophosphorylation in chloroplasts may also occur as a consequence of electron flow to oxygen catalysed by electron acceptors such as methyl viologen (pseudo cyclic photophosphorylation). In the latter example ATP synthesis is accompanied by significant rates of oxygen uptake by chloroplasts as oxygen is reduced by photosystem I electron flow. Both cyclic or pseudo cyclic photophosphorylation may be significant reactions *in vivo* for the formation of ATP in the absence of $NADP^+$ reduction.

Two models to explain the mechanism of phosphorylation have been proposed. The chemical coupling hypothesis proposes the formation of an energy rich intermediate which is coupled to ATP synthesis (Chance and Williams, 1956). The chemiosmotic hypothesis is based on the accumulation of a proton gradient across the thylakoid membrane during electron flow, which drives ATP synthesis (Mitchell, 1966). For chemiosmotic phosphorylation in chloroplasts it is proposed that the arrangement of electron carriers in the thylakoid membrane promotes proton uptake on one side of the membrane and proton release into the intrathylakoid space during photosynthetic electron flow. This hypothesis requires that thylakoid membranes are impermeable to protons except at certain sites, the chloroplast coupling factor. Coupling factors are ATPases which allow for the controlled release of the proton gradient, generating ATP. Uncouplers such as amines or NH_4Cl increase the permeability of thylakoids to protons, preventing the build up of a proton gradient across the membrane. Rates of electron flow are thus stimulated by uncouplers as electron flow is no longer dependent on the rate of photophosphorylation. The chemiosmotic hypothesis accounts for many of the observed properties of photophosphorylation in chloroplasts (Halliwell, 1981).

1.3 Carbon dioxide fixation

The ATP and NADPH generated by the light reactions of photosynthesis are used by chloroplasts to drive the incorporation of CO_2 into sugars (Bassham, 1977). The initial stage in the photosynthetic reductive carbon cycle is carboxylation of ribulose 1,5-bisphosphate by CO_2, catalysed by the enzyme ribulose 1,5-bisphosphate carboxylase, forming two molecules of 3-phosphoglycerate. Phosphoglycerate is converted to 3-phosphoglyceraldehyde in a reaction that requires both NADPH and ATP. These three carbon compounds may be exported from the chloroplast into the cytosol or further metabolised to regenerate ribulose 1,5-bisphosphate. ATP is required in this cycle for the phosphorylation of ribulose 5-phosphate into ribulose 1,5-bisphosphate. This cycle of reactions is drawn in Figure 2.

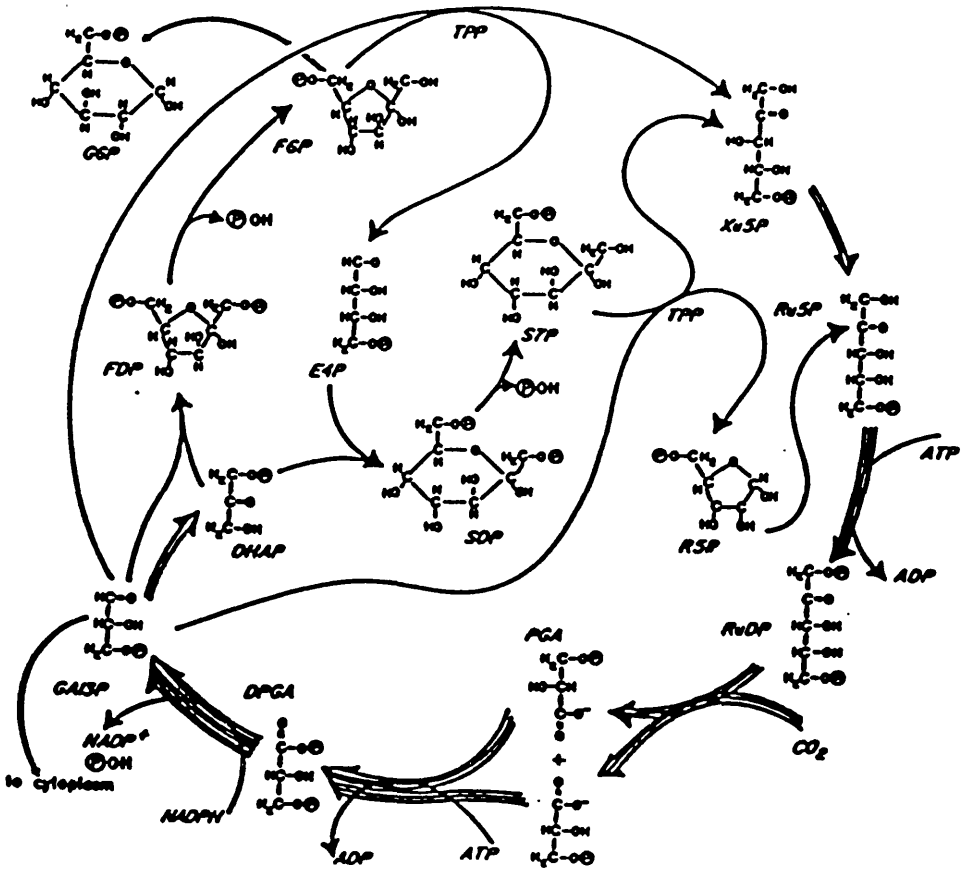


Fig 2. The reductive pentose phosphate pathway of CO_2 incorporation. (from Bassham, 1977)

2. Oxygen Activation

2.1 Properties of Oxygen

Oxygen is essential for the life of aerobic organisms. However, oxygen, and more reactive products derived from oxygen, can be toxic to biological systems. The oxygen molecule, shown in Figure 3, contains two unpaired electrons each with a parallel spin, and therefore occupying different π^* antibonding orbitals. This form of oxygen, the triplet state, is the state of lowest energy, and is therefore the ground state. Most organic molecules contain electrons arranged in spin opposed pairs, referred to as the singlet state. The addition of a pair of electrons to dioxygen would be prevented according to the Pauli Exclusion Principle, as this would result in two electrons with parallel spin occupying the same orbital. Ground state oxygen is therefore relatively unreactive towards biological molecules due to the spin restriction encountered when triplet oxygen reacts with a singlet substrate (Taube, 1965).

The reactivity of oxygen can however be increased by the excitation of triplet ground state oxygen to a singlet state (Foote, 1976). Two singlet states of oxygen are known, designated $^1\Delta g$ and $^1\Sigma g^+$. Either singlet state can revert to the ground state by emitting radiation, or by the transfer of energy to a quencher molecule. The $^1\Sigma g^+$ singlet state is unstable and there is no evidence for its formation in biological systems. The $^1\Delta g$ singlet state of oxygen is more stable and probably occurs during the dye sensitized transfer of light energy to oxygen termed photodynamic action (Nilsson and Kearns, 1973).

The spin restriction of oxygen can also be circumvented by oxygen reduction. Direct divalent reduction of ground state oxygen is not

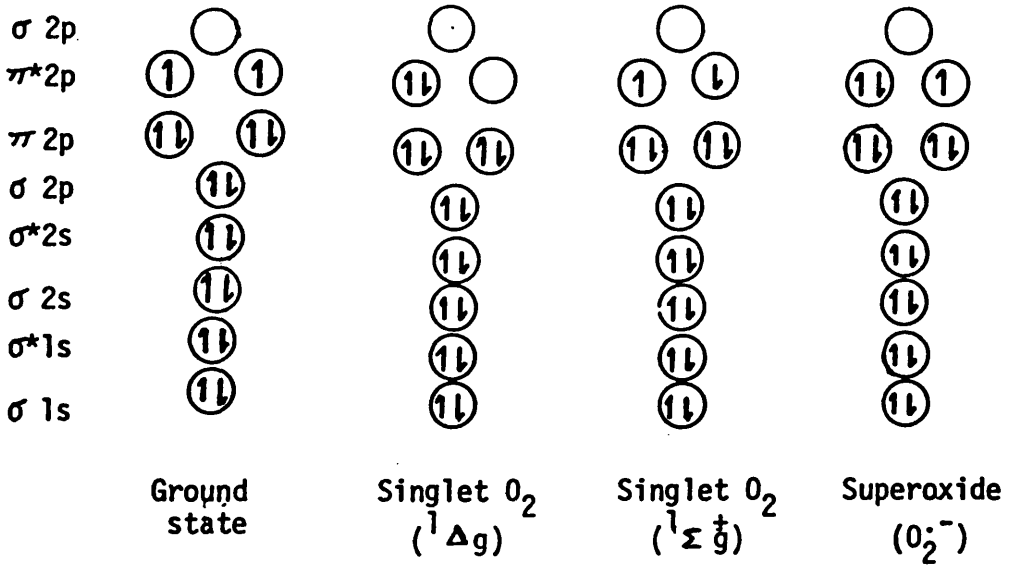


Fig 3a. Bonding in the diatomic oxygen molecule.
(Halliwell and Gutteridge, 1985)

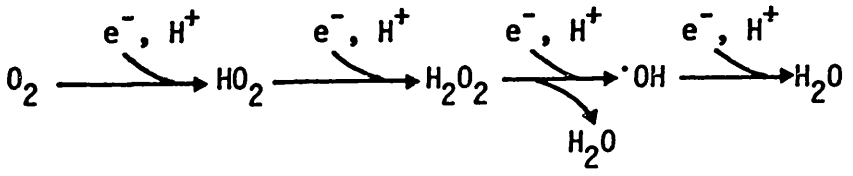


Fig 3b. Univalent pathway of oxygen reduction.
(Elstner,1982)

a feasible reaction due to the spin restriction. The addition of electrons one at a time in the univalent pathway of oxygen reduction does however avoid the spin restriction (Elstner, 1982). The complete reduction of oxygen to water requires four electrons, although the intermediates of this pathway are more reactive than ground state oxygen. This presents a potential hazard whenever univalent oxygen reduction occurs in biological systems. The states of oxygen, and the pathway of univalent oxygen reduction is outlined in Figure 3.

2.2 Oxygen reduction in chloroplasts

The univalent pathway is the most feasible route of oxygen reduction in biological systems. The addition of one electron to oxygen forms the hydroperoxy radical ($HO_2^{\cdot}$) or its conjugate base, superoxide ($O_2^{\cdot -}$). The formation of $O_2^{\cdot -}$ has been demonstrated in chloroplasts (Asada and Kiso, 1973) and mitochondria (Rich and Bonner, 1978), and may be formed during a variety of enzyme catalysed oxidative reactions (Fridovich, 1976).

Potentially the most important site of $O_2^{\cdot -}$ formation in the leaves of higher plants is the reduction of oxygen by photosynthetic electron flow. Isolated chloroplast lamellae have been widely demonstrated to produce $O_2^{\cdot -}$ on illumination (Asada and Kiso, 1973; Allen and Hall, 1973; Elstner and Heupel, 1974). Superoxide formation has also been demonstrated from intact chloroplasts or photosynthetic algae, or by chloroplasts *in vivo* (Egneus *et al.*, 1975; Radmer and Kok, 1976; Glidewell and Raven, 1977). The site of $O_2^{\cdot -}$ formation by photosynthesis has been established as the reducing site of photosystem I (Asada *et al.*, 1974a; Miller and MacDowall, 1975). Oxygen reduction by photosystem I can probably be mediated by the photosystem I acceptor, or ferredoxin. Isolated chloroplast lamellae take

up oxygen under illumination, although the rate of oxygen uptake is significantly enhanced if ferredoxin is added (Halliwell, 1981). Up to 15% of photosystem I electron flow may be diverted to oxygen, forming $O_2^{\cdot -}$, *in vivo* (Allen, 1976; Asada *et al.*, 1977).

Two important physiological roles for the photoreduction of oxygen have been proposed. Efficient CO_2 assimilation requires the light driven generation of NADPH and ATP in the correct stoichiometric amounts. Pseudocyclic electron flow to oxygen in chloroplasts may support ATP synthesis without $NADP^+$ reduction, and may be a mechanism of varying the ratio of ATP to NADPH formed (Allen, 1976). An alternative role of oxygen reduction *in vivo* is that it allows dissipation of light energy from light harvesting chlorophyll under conditions of low rates of CO_2 fixation (Furbank, 1984). Under such conditions electron flow to oxygen forming $O_2^{\cdot -}$ would prevent the over-reduction of electron transport intermediates.

Further reduction of oxygen in the univalent pathway shown in Figure 3 generates H_2O_2. Hydrogen peroxide can be formed *in vivo* by the dismutation of $O_2^{\cdot -}$ either spontaneously (Bielski, 1978) or catalysed by superoxide dismutase (McCord and Fridovich, 1969) or by manganese (Lumsden and Hall, 1975; Kono *et al.*, 1976). Additionally, enzymes such as glycollate oxidase can transfer two electrons to oxygen directly forming H_2O_2 (Halliwell, 1981). Experiments by Mehler (Mehler, 1951 a, b; Mehler and Brown, 1952) demonstrated oxygen uptake by illuminated chloroplasts and the accumulation of H_2O_2. Photosynthetic formation of H_2O_2 has been widely demonstrated (Good and Hill, 1955; Egneus *et al.*, 1975; Radmer and Kok, 1976), and may occur through the reduction of $O_2^{\cdot -}$ (Allen, 1977a) or $O_2^{\cdot -}$ dismutation (Asada *et al.*, 1974a).

2.3 Singlet oxygen

The most important mechanism of $^1O_2$ formation in biological systems is by dye sensitized photodynamic action (Foote, 1976; Krinksky, 1977). The absorption of light by sensitizers results in a singlet excited state (1) which can be converted to a longer lived triplet state by spin inversion (2). Molecular oxygen can react with triplet sensitizers generating singlet oxygen (3):

$$S \longrightarrow {}^1S \qquad (1)$$

$$^1S \longrightarrow {}^3S \qquad (2)$$

$$^3S + {}^3O_2 \longrightarrow S + {}^1O_2 \qquad (3)$$

Singlet oxygen is much more reactive than triplet ground state oxygen as the spin restriction has been removed (Figure 3). Both chlorophyll a and b have been demonstrated to generate $^1O_2$ by dye sensitized reactions (Foote, 1968, 1976). In chloroplasts photodynamic formation of $^1O_2$ from chlorophyll may be a significant aspect of oxygen toxicity (Rabinowitch and Fridovich, 1983). Photodynamic reactions involving $^1O_2$ are probably enhanced when normal photosynthetic energy dissipation from chlorophyll is prevented by the inhibition of photosynthetic electron flow with certain herbicides (Dodge, 1983). Singlet oxygen may also be generated during the non-enzymic dismutation of $O_2^{\cdot -}$ (Khan , 1970), or during the breakdown of lipid peroxides (Lai *et al.*, 1978).

2.4 Hydroxyl radical

It has been realised for many years that the ability of H_2O_2 to oxidise organic compounds was greatly enhanced by iron salts. Haber and Weiss (1934) proposed that the catalytic decomposition of H_2O_2 by iron salts initiated a chain reaction forming $O_2^{\cdot -}$ and hydroxyl radicals ($OH^{\cdot}$). Considerable evidence has accumulated to show that

$O_2^{\cdot -}$ and H_2O_2 can react together in biological systems to form $OH^{\cdot}$ (Halliwell and Gutteridge, 1985). The reactions involve the interaction of iron and $O_2^{\cdot -}$ (4), followed by oxidation with H_2O_2 (5). The net reaction is shown in Equation 6.

$$Fe^{3+} + O_2^{\cdot -} \longrightarrow Fe^{2+} + O_2 \qquad (4)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\cdot} + OH^{-} \qquad (5)$$

$$O_2^{\cdot -} + H_2O_2 \xrightarrow{Fe^{3+}} O_2 + OH^{-} + OH^{\cdot} \qquad (6)$$

Although several of the early attempts to demonstrate these reactions were unsuccessful (Halliwell, 1976; McClune and Fee, 1976), recent observations have shown the formation of $OH^{\cdot}$ in the presence of iron or copper salts (Rowley and Halliwell, 1983; Halliwell and Gutteridge, 1985). In chloroplasts the formation of $OH^{\cdot}$ may occur if $O_2^{\cdot -}$ and H_2O_2 are not scavenged efficiently.

3. Oxygen toxicity in chloroplasts

3.1 Lipid peroxidation

Lipids form approximately 35% of the dry weight of chloroplasts. A high proportion (75%) of chloroplast lipid is mono and digalactosyl-diacylglycerol (Barber, 1983). Thylakoid lipids contain a high proportion of double bonds; unsaturation promoting membrane fluidity. Linolenic acid, containing three double bonds can account for up to 90% of the fatty acid present in chloroplast lipids of some species (Quinn and Williams, 1978). Polyunsaturated fatty acids are particularly susceptible to peroxidation. Lipid peroxidation is initiated by hydrogen abstraction from an unsaturated fatty acid forming a fatty acid radical (Figure 4). Attack by oxygen generates a lipid peroxide which can abstract a hydrogen atom from an adjacent unsaturated fatty acid, forming a lipid hydroperoxide and thus initiating a chain reaction. Lipid hydroperoxides decompose to give a range of products including aldehydes, such as malondialdehyde (Pryor, 1978), or hydrocarbons such as ethane (Sandmann and Böger, 1982). Triplet sensitizers such as chlorophyll can initiate hydrogen abstraction from unsaturated fatty acids in a type I reaction (Foote, 1976). Alternatively triplet sensitizers can react with oxygen forming $^1O_2$ that can react with unsaturated fatty acids directly to form lipid hydroperoxides in a type II reaction (Figure 4b). Hydroxyl radicals may also induce hydrogen abstraction, and therefore the peroxidation of unsaturated fatty acids (Halliwell, 1981).

3.2 Inhibition of photosynthesis

Photosynthetic reactions can be suppressed or inhibited by oxygen or activated oxygen species. Oxygen can inhibit CO_2 fixation because

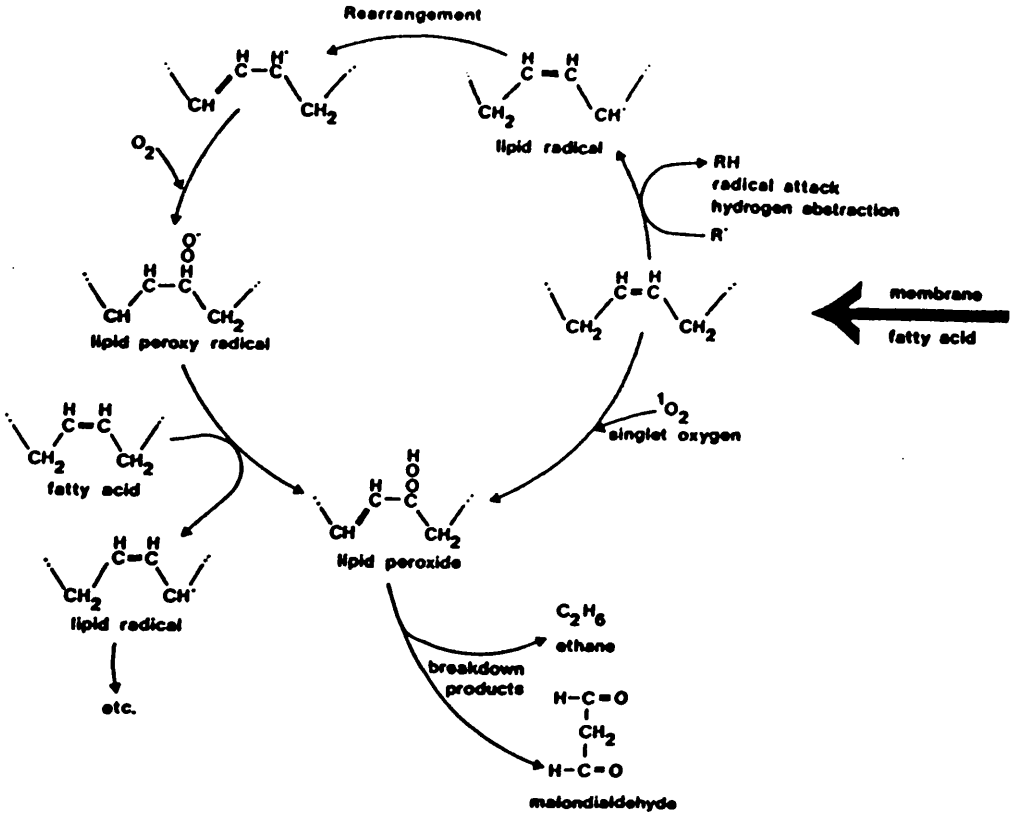


Fig 4. The mechanism of peroxidation of membrane lipids.

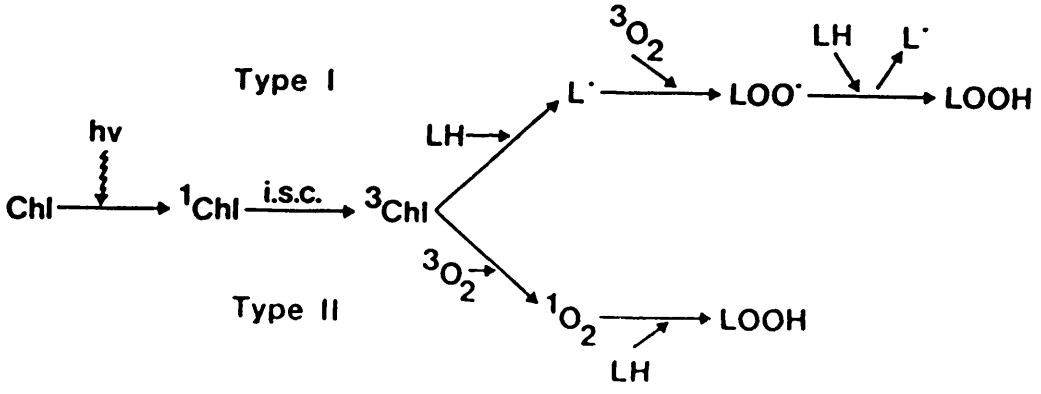


Fig 4b. Type I and Type II mechanisms of lipid peroxidation initiated by chlorophyll. (i.s.c. -intersystem crossing, Chl-chlorophyll, LH- unsaturated membrane lipid.)

O_2 is itself a substrate for the enzyme ribulose 1,5-bisphosphate carboxylase-oxygenase. Oxygen and CO_2 compete for the same active site on the enzyme in either photorespiration or photosynthesis (Foyer and Hall, 1980b). The diversion of photosynthetic electron flow to oxygen forming $O_2^{\cdot -}$ in pseudocyclic photophosphorylation may reduce the capacity for CO_2 assimilation. The accumulation of H_2O_2 in chloroplasts has more serious consequences for photosynthetic activity. Kaiser (1976) demonstrated that 10 μM H_2O_2 was sufficient to inhibit photosynthesis by 50% in intact chloroplasts. The site of inhibition appeared to be certain Calvin cycle enzymes including fructose and sedoheptulose bisphosphatases and glyceraldehyde 3-phosphate dehydrogenase (Heldt *et al.*, 1978; Kaiser, 1979; Tanaka *et al.*, 1982b). Oxidation of these enzymes by H_2O_2 probably prevents their participation in the Calvin cycle (Charles and Halliwell, 1980).

A common feature of damage to the photosynthetic apparatus induced by environmental stress or certain air pollutants such as SO_2 in the presence of high light intensities is loss of photosystem II function (Shimazaki *et al.*, 1984; Powles, 1984). Increased formation of damaging oxygen species ($O_2^{\cdot -}$, H_2O_2, $OH^{\cdot}$ or $^1O_2$) under conditions of drought stress, chilling or high light intensities (Osmond, 1981; Powles, 1984) or induced by air pollutants (de Kok *et al.*, 1983; Sakaki *et al.*, 1983) may contribute to the damage to photosystem II. Photosensitizers that induce the formation of $^1O_2$ also promote damage to photosystem II in isolated thylakoids (Knox and Dodge, 1985).

Damage to the photosynthetic assembly, whether through inhibition of electron flow, loss of Calvin cycle activity or as a consequence of fragmentation of chloroplast membranes, is probably a significant aspect of the toxicity of active oxygen species in chloroplasts.

3.3 Other damage reactions

One of the earliest proposals to account for the toxic effects of oxygen was that oxygen inactivates enzymes (Haugaard, 1968). Many enzymes from anaerobic organisms are inhibited by oxygen, and similar effects have been observed with enzymes from aerobes. Particularly sensitive are those enzymes that contain reduced thiol (-SH) groups (Halliwell, 1981), which are readily oxidised with a resulting loss of enzyme activity. The chloroplast Calvin cycle enzyme $NADP^+$ glyceraldehyde 3-phosphate dehydrogenase is inhibited when chloroplasts are exposed to high oxygen tensions (Ellyard and Gibbs, 1980). Other Calvin cycle enzymes are inhibited by H_2O_2 through oxidation of the enzyme thiol group (Charles and Halliwell, 1980). The accumulation of H_2O_2 in chloroplasts may also inhibit CuZn SOD (Asada *et al.*, 1975).

Nilsson and Kearns (1973) demonstrated that photodynamic oxidation of certain amino acids, and the inhibition of the enzyme alcohol dehydrogenase was caused by $^1O_2$. Proteins that contain methionine, tryptophan, histidine or cysteine residues are probably particularly susceptible to damage by $^1O_2$ (Halliwell and Gutteridge, 1985). Hydroxyl radicals, due to their extreme reactivity can attack most biological molecules (Anabar and Neta, 1967; Halliwell, 1981). The damaging action of $O_2^{\cdot -}$ and H_2O_2 in biological systems is probably to a large extent due to the formation of $OH^{\cdot}$.

4. Protective mechanisms against oxygen toxicity

4.1 Superoxide

Probably the best defence against damaging oxygen species is to avoid their formation. Enzymes such as cytochrome oxidase, the terminal oxidase of respiratory electron transport, can reduce oxygen by a di-valent or tetravalent mechanism, thus avoiding the formation of $O_2^{\cdot -}$ (Fridovich, 1978). The photosynthetic apparatus of plants can adapt to some degree to match environmental growth conditions (Boardman, 1977; Berry and Björkman, 1980). This ensures that the size of the light harvesting apparatus and the capacity for electron transport reactions match the capacity for CO_2 assimilation. Thus much of the light energy absorbed by chlorophyll would be dissipated through photo-synthetic reactions. However chloroplasts can form $O_2^{\cdot -}$ as a normal reaction of photosynthesis. Indeed its formation may be an important physiological reaction of photosynthesis to allow for the generation of ATP in the absence of $NADP^+$ reduction in pseudocyclic photophos-phorylation (Furbank, 1984).

One important mechanism for removing $O_2^{\cdot -}$ is the dismutation reaction (7), which can occur spontaneously (Bielski, 1978) or catalysed by superoxide dismutase (McCord and Fridovich, 1969).

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \longrightarrow H_2O_2 + O_2 \qquad (7)$$

The rate constant for this reaction has been calculated at 2×10^5 $M^{-1}s^{-1}$ for the spontaneous dismutation of $O_2^{\cdot -}$ (Bielski, 1978), or 2×10^9 $M^{-1}s^{-1}$ for the reaction catalysed by SOD (Asada *et al.*, 1977). The high concentration of SOD in cells, and the increased rate of $O_2^{\cdot -}$ decay catalysed by SOD favours enzymic rather than spontaneous dismutation (Fridovich, 1975).

SOD has been isolated from a wide range of organisms including nearly all aerobes and some anaerobic organisms (Fridovich, 1975). Three classes of SOD have been isolated which are all metaloproteins containing either copper/zinc (CuZn SOD), iron (Fe SOD) or manganese (Mn SOD) as the prosthetic group. CuZn SOD is associated with eucaryotic organisms, whereas Mn SOD is found in procaryotes and the mitochondria of eucaryotes (Fridovich, 1975). Fe SOD has been isolated from bacteria and algae and from three families of higher plants (Fridovich, 1975; Bridges and Salin, 1981). Amino acid sequencing studies indicate close similarities between Mn SOD and Fe SOD, although CuZn SOD is distinct (Harris *et al.*,, 1980). CuZn SOD is also inhibited by cyanide, whereas both Mn SOD and Fe SOD are not, and this is often used to distinguish between these families of SOD in crude extracts (Rabinowitch and Fridovich, 1983). The CuZn SOD is also inactivated by H_2O_2 (Asada *et al.*, 1975).

In the leaves of higher plants Mn SOD is located in mitochondria (Giannopolitis and Ries, 1977; Jackson *et al.*, 1978; Rabinowitch and Fridovich, 1983), although this probably only represents 3-5% of the total leaf SOD (Jackson *et al.*, 1978). The majority of SOD in green leaves is present in chloroplasts (Asada *et al.*, 1973; Lumsden and Hall, 1974; Jackson *et al.*, 1978). Chloroplast SOD is of the CuZn type located in the stroma, and can be released on rupture of the chloroplast envelope (Jackson *et al.*, 1978). Foyer and Hall (1980a) demonstrated that osmotic shock of intact chloroplasts released over 90% of the SOD present. Some SOD may also be present in chloroplasts associated with thylakoid membranes or the intrathylakoid space (Lumsden and Hall, 1974; Hayakawa *et al.*, 1984, 1985).

Thylakoid membranes may also be protected against $O_2^{\cdot -}$ by manganese which catalyses the dismutation reaction (Lumsden and Hall, 1975; Kono *et al.*, 1976). Dismutation of $O_2^{\cdot -}$ by manganese is probably particularly important in a number of aerobic Lactobacillus organisms which do not contain SOD (Archibald and Fridovich, 1981). The chloroplast stroma also contains millimolar concentrations of ascorbate (Foyer *et al.*, 1983; Law *et al.*, 1983) that can react with $O_2^{\cdot -}$ at a considerable rate (Epel and Neuman, 1973; Nishikimi, 1975).

$$\text{Ascorbate} + 2H^+ + 2O_2^{\cdot -} \longrightarrow \text{Dehydroascorbate} + H_2O_2 \qquad (8)$$

Although the rate constant for this reaction (2.7×10^5 $M^{-1}s^{-1}$) is lower than for SOD catalysed dismutation of $O_2^{\cdot -}$, the ascorbate concentration of the stroma (10-50 mM) is higher than the 10 μM concentration of SOD (Asada *et al.*, 1977; Halliwell, 1981). Ascorbate may therefore intercept a significant proportion of the $O_2^{\cdot -}$ formed in chloroplasts *in vivo*.

4.2 Hydrogen peroxide

Many aerobic organisms contain catalase which degrades H_2O_2 according to Equation 9:

$$2H_2O_2 \longrightarrow 2H_2O + O_2 \qquad (9)$$

Catalase can break down high concentrations of H_2O_2 very rapidly, although it is less effective at scavenging low H_2O_2 concentrations, because of its low affinity for this substrate (Jones and Suggett, 1968; Scandalios *et al.*,, 1972). Much of the catalase activity in the leaves of higher plants is present in peroxisomes (Tolbert *et al.*, 1968), where it probably scavenges H_2O_2 generated by photorespiration. Chloroplasts from higher plants contain little if any catalase (Allen, 1977b; van Ginkel and Brown, 1978). Although catalase may be a common

contaminant of chloroplast preparations (Allen, 1977b),it probably has no significant function in scavenging H_2O_2 in that organelle *in vivo*.

Glutathione peroxidase is present in the cytosol and mitochondria of animal cells, and catalyses glutathione dependent decomposition of H_2O_2 (Halliwell, 1981). Glutathione peroxidase has not however been detected in higher plants (Smith and Schrift, 1979; Halliwell, 1981). Plant extracts do contain peroxidases that can act on a wide range of substrates, and are usually assayed *in vitro* by the H_2O_2 dependent oxidation of guiacol. Since the identity of their natural substrates are largely unknown, their role in scavenging H_2O_2 *in vivo* is difficult to assess (Halliwell, 1981).

Chloroplasts from higher plants scavenge H_2O_2 in light at appreciable rates (Nakano and Asada, 1980). Chloroplasts contain the enzyme ascorbate peroxidase which may be bound to the thylakoid membranes (Groden and Beck, 1979) or present in the stroma (Nakano and Asada, 1981; Jablonski and Anderson, 1982). The enzyme catalyses the oxidation of ascorbate by H_2O_2 (10).

$$2H^+ + \text{Ascorbate} + H_2O_2 \longrightarrow \text{Dehydroascorbate} + 2H_2O \quad \cdot \quad (10)$$

Chloroplast ascorbate peroxidase has a high affinity for H_2O_2 (K_m = 5-50 μM) and shows optimum activity at alkaline pH values present in the chloroplast stroma during illumination (Nakano and Asada, 1981; Gerbling *et al.*, 1984). Enzyme activity is also saturated by ascorbate concentrations below those present in the chloroplast stroma (Nakano and Asada, 1981; Jablonski and Anderson, 1982). Ascorbate peroxidase, which can scavenge H_2O_2 at rates up to 2000 μmol mg $chl^{-1}h^{-1}$ (Groden and Beck, 1979), may be an important enzyme in degrading low concentrations of H_2O_2 in chloroplasts during illumination.

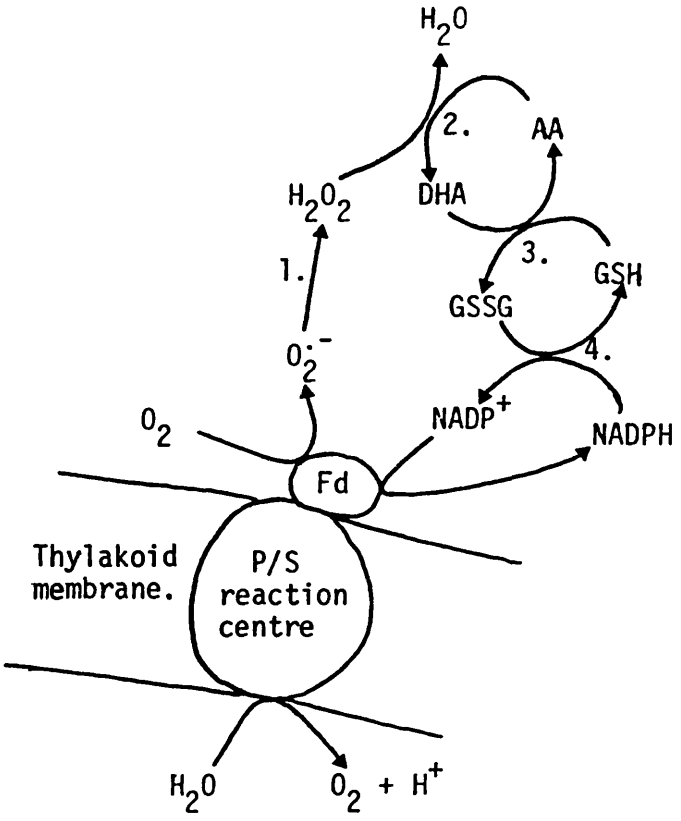


Fig 5. The mechanism of oxygen reduction to superoxide by photosynthetic electron flow, and the scavenging of $O_2^{\cdot-}$ and H_2O_2 by SOD (1), ascorbate peroxidase (2), dehydroascorbate reductase (3) and glutathione reductase (4) in chloroplasts.

Dehydroascorbate, formed by reaction 10, can be reduced to ascorbate by glutathione (GSH) which is present in the chloroplast stroma at millimolar concentrations (Foyer and Halliwell, 1977; Foyer *et al.*, 1983). This reaction (11) proceeds non enzymically at pH 8.0 at an appreciable rate.

$$\text{Dehydroascorbate} + 2\text{GSH} \longrightarrow \text{Ascorbate} + \text{GSSG} \qquad (11)$$

Chloroplasts also contain the enzyme dehydroascorbate reductase that can catalyse this reaction (Jablonski and Anderson, 1981; Nakano and Asada, 1981; Hossain and Asada, 1984).

Oxidised glutathione (GSSG) can be reduced in chloroplasts by a reaction that consumes NADPH catalysed by the enzyme glutathione reductase (12):

$$\text{GSSG} + \text{NADPH} + \text{H}^+ \longrightarrow 2\text{GSH} + \text{NADP}^+ \qquad (12)$$

Glutathione reductase has been isolated from pea and spinach chloroplasts (Foyer and Halliwell, 1976; Schaedle and Bassham, 1977; Jablonski and Anderson, 1978). Chloroplast activities of glutathione reductase are probably adequate to reduce GSSG formed by reaction 11. This cycle of reactions to scavenge H_2O_2 in chloroplasts is drawn in Figure 5.

Considerable evidence in support of a cycle of reactions involving ascorbate and glutathione to scavenge H_2O_2 in chloroplasts has emerged. Chloroplasts contain millimolar concentrations of ascorbate and glutathione (Law *et al.*, 1983; Foyer *et al.*, 1983), sufficient to ensure that high activities of ascorbate peroxidase, dehydroascrobate reductase and glutathione reductase are maintained. The breakdown of H_2O_2 by this cycle is dependent on photosynthetic electron flow to form NADPH. The scavenging of H_2O_2 by chloroplasts has been demonstrated to be light dependent and inhibited by compounds that prevent photosynthetic

electron flow (Nakano and Asada, 1980, 1981; Jablonski and Anderson, 1982; Anderson *et al.*, 1983). Studies with radio labelled H_2O_2 and O_2 have demonstrated that the decomposition of H_2O_2 by illuminated chloroplasts does not result in O_2 evolution, and is therefore consistent with the reaction in equation 10 (Asada and Badger, 1984).

4.3 Singlet oxygen

One mechanism by which energy spillover from light harvesting chlorophyll to oxygen can be avoided is to adjust the size of the light harvesting assembly to match the capacity of chloroplasts for photosynthetic reactions. Thus plants grown at high light intensities contain less chlorophyll but a higher capacity for photosynthetic reactions than plants grown at low light intensity (Boardman, 1977). Damage that occurs to the photosystem II reaction centre when low light adapted plants are transferred to strong light (Powles and Critchley,1980; Critchley, 1981) is probably due to photodynamic effects as a result of energy spillover from chlorophyll. Adaptation of the size of the light harvesting assembly to match the light environment ensures that much of the light absorbed by chlorophyll is utilised to drive photosynthetic reactions.

At high light intensities when the rates of CO_2 assimilation are low, photoreduction of oxygen forming $O_2^{\cdot -}$ may be one mechanism of maintaining photosynthetic electron flow and thus preventing the over-reduction of electron carriers and energy spillover from chlorophyll to form $^1O_2$ (Furbank, 1984). Superoxide can be metabolised in chloroplasts by the enzyme SOD and through the chloroplast ascorbate-glutathione cycle. Thus the formation of $O_2^{\cdot -}$, which is less reactive than $^1O_2$, may restrict the formation of $^1O_2$.

Chloroplast membranes contain quenchers such as α-tocopherol and carotenoid pigments that can scavenge $^1O_2$. Some 90% of α-tocopherol present in leaves is found in chloroplast membranes (Bucke, 1968; Hughes *et al.*, 1971). Tocopherols can react with free radicals, $^1O_2$ and can quench lipid peroxidation (Fahrenholtz *et al.*, 1974; Yamauchi and Matsushita, 1979). The radical generaged by α-tocopherol reacting with $^1O_2$ can be reduced back to α-tocopherol by ascorbate (Packer *et al.*, 1979). Ascorbate and glutathione present in the chloroplast stroma can also quench $^1O_2$ directly (Bodannes and Chan, 1979; Chou and Khan, 1983).

Carotenoid pigments are also important in preventing photo-oxidative damage to chloroplast membranes. Mutants which lack carotenoids bleach rapidly on exposure to light (Anderson and Robertson, 1960). Similar observations have been made with plants treated with herbicides that inhibit carotenoid synthesis (Bartels and Watson, 1978; Ridley and Ridley, 1979). The protective function of carotenoid pigments has also been demonstrated in experiments into the peroxidation of liposome membranes. Incorporation of β-carotene protected such membranes against dye sensitized peroxidation (Anderson and Krinsky, 1973; Anderson *et al.*, 1974).

Carotenoids are present in chloroplasts as carotenes and xanthophylls. An important feature of the carotenoid molecule is that it contains several conjugated double bonds. Carotenoids that contain nine or more double bonds are effective quenchers of $^1O_2$, while carotenoids with seven or less double bonds or carotenoid synthesis precursors such as phytoene or phytofluene are ineffective quenchers (Krinsky, 1979). Carotenoids can quench both triplet chlorophyll directly or they can quench $^1O_2$ (Foote, 1968; Foote and Denny, 1968; Krinsky, 1979).

4.4 Hydroxyl radical

The extreme reactivity of $OH^{\bullet}$ means it will react with most biological molecules in its path. Quenchers like α-tocopherol in thylakoid membranes or ascorbate and glutathione in the chloroplast stroma may scavenge $OH^{\bullet}$. The most effective protection against $OH^{\bullet}$ is to prevent its formation. Chloroplast enzymes that scavenge $O_2^{\bullet -}$ and prevent the accumulation of H_2O_2 thus have an added importance.

5. Concluding section: Aims of this study

Chloroplasts from higher plants have the potential to generate several forms of activated oxygen species that may be toxic to photosynthetic reactions if their formation and subsequent scavenging is not carefully controlled. High internal oxygen concentrations during photosynthesis, and the presence of compounds such as ferredoxin that can reduce oxygen to $O_2^{\cdot -}$ may lead to the formation of more toxic H_2O_2 or $OH^{\cdot}$. Chloroplast pigments can sensitize the formation of $^1O_2$, especially if photosynthetic electron flow is inhibited. The damaging effects of these species are restricted under normal conditions by enzymes such as SOD, ascorbate peroxidase and glutathione reductase, and antioxidants that can scavenge activated oxygen species. However under conditions when normal photosynthetic reactions are impaired such as during drought stress or chilling injury or treatment with certain herbicides, the formation of these active oxygen species may be enhanced (Osmond, 1981; Dodge, 1983; Powles, 1984). Air pollutants such as O_3 or SO_2 may also promote the formation of $O_2^{\cdot -}$, H_2O_2 or $^1O_2$ in chloroplasts (Shimazaki *et al.*, 1980; Sakaki *et al.*, 1983; de Kok *et al.*, 1983; Tanaka and Sugahara, 1980; Tanaka *et al.*, 1982a). Chloroplast enzymes and antioxidants that scavenge these damaging oxygen species may therefore have an important role in the tolerance of plants to such stresses.

Following the isolation of SOD (McCord and Fridovich, 1969), a number of workers have investigated the role of this enzyme in protecting organisms against oxidative stress. Studies with bacteria, yeast and algae have indicated that tolerance to high oxygen tensions, paraquat, SO_2, chilling and photo-oxidative conditions could be associated with endogenous SOD activity (Gregory and Fridovich, 1973; Gregory *et al.*,

1974; Pullich, 1974; Hassan and Fridovich, 1977a, 1978; Steinitz *et al.*, 1979; Rabinowitch *et al.*, 1983; Clare *et al.*, 1984; Rabinowitch and Fridovich, 1985). In higher plants SOD activity has been monitored during development and senescence, in response to chilling and drought stress, and in tolerance to paraquat, SO_2 and O_3 (Beauchamp and Fridovich, 1973; Simon *et al.*, 1974; Giannopolitis and Ries, 1977; Foster and Hess, 1980, 1982; Tanaka and Sugahara, 1980; Dhindsa *et al.*, 1981; Youngman and Dodge, 1981; Michalski and Kaniuga, 1981; Lee and Bennett, 1982; McRae and Thompson, 1983). Studies of the role of SOD in plant tolerance to stresses that may promote $O_2^{\cdot -}$ formation in chloroplasts must be coupled with assessments of the activity of enzymes that scavenge H_2O_2. In many of these studies the activity of SOD has been monitored in parallel to changes in catalase or peroxidase (Simon *et al.*, 1974; Dhindsa *et al.*, 1981; McRae and Thompson, 1983). The activity of chloroplast enzymes that scavenge H_2O_2 in response to such stresses has been largely ignored.

The aim of this investigation was to examine the role of activated oxygen species, particularly $O_2^{\cdot -}$ and H_2O_2, in photo-oxidative damage to plants, and to assess the role of chloroplast antioxidant mechanisms in restricting injury. In the first part of this study the enzymes that scavenge $O_2^{\cdot -}$ and H_2O_2 have been assessed in leaf homogenates and chloroplasts to determine the proportion and activity present in chloroplasts. Enzyme and antioxidant levels have been monitored during leaf development and senescence, in leaves and chloroplasts from plants grown at different light intensities and in plants grown under glass-house conditions at different times of the year. In the second part of this study photo-oxidative injury has been induced by a number of herbicides that may promote the formation of free radicals, $O_2^{\cdot -}$,

H_2O_2 and $^1O_2$ in chloroplasts. The role of chloroplast antioxidant mechanisms in preventing such injury has also been considered.

MATERIALS AND METHODS

MATERIALS AND METHODS

1. Preparation of experimental materials

1.1 Growth of plant material

Flax (*Linum usitatimum* var. Reina) seedlings were grown on moist vermiculite in crystallising dishes in a constant environment cabinet under continuous illumination of 300-400 μmol $m^{-2}s^{-1}$ photon flux density provided by warm white fluorescent tubes, and a mean air temperature of 23°C.

Pea (*Pisum sativum* var. Meteor) seedlings were grown in trays of moist Levington Universal Compost in a glasshouse under natural daylight conditions or natural daylight extended to a 14 h photoperiod (Thorn 400W mercury vapour lamps) and a mean air temperature of 22°C. Plants were grown for 14-21 days prior to transfer to the laboratory. For experiments involving plants grown at different light intensities, pea plants were grown (as above) in a controlled environment cabinet for 14-21 days. The light intensity in the cabinet was varied between 100 and 400 μmol $m^{-2}s^{-1}$ photon flux density using layers of Kodak neutral density filter mounted on a stage 200 mm above the plants. For etiolated plants, peas were grown in trays of moist compost in total darkness in a growth room at 20-23°C for 8 days.

Maize (*Zea mays* var. Pioneer) plants were grown in trays of Levington Universal Compost under glasshouse conditions and a mean air temperture of 22°C, for 14-21 days.

Spinach plants used in the isolation of ferredoxin and superoxide dismutase were purchased from a local market.

1.2 Preparation of experimental compounds

Stock solutions of monuron (1 mM) were prepared by initially dissolving the chemical in methanol and then refluxing for 2-3 hours

until a clear solution was obtained. Paraquat was dissolved in distilled water. The diphenyl ether herbicides were dissolved in acetone and fluridone dissolved in ethanol, and diluted with distilled water. Final concentrations of solvent in experimental test solutions did not exceed 0.5%.

Penacillamine copper complex was prepared according to the method described by Birker and Freeman (1977). Penacillamine (100 mg) was dissolved in 15 ml of 0.5 M sodium acetate, pH 6.2, and mixed with 2 ml $CuCl_2.2H_2O$ (containing 85 mg $CuCl_2$). After adding an approximately equal volume of ethanol, the precipitate formed was removed by filtering, washed with alcohol and redissolved in 5.0 ml distilled water. The concentration of the complex was determined from the absorbance at 518 nm, corresponding to 1820 $M^{-1}cm^{-1}$ per atom of copper.

Crocetin was prepared from commercial saffron according to the method of Friend and Mayer (1960). Approximately 2-3 g of finely ground saffron was extracted for 1 hour with diethyl ether in a Soxhlet extractor to remove fats and lipids. The residue was dried under a stream of nitrogen and re-extracted with methanol. After reducing the volume by evaporation under a stream of nitrogen, methanolic extracts were stored in darkness at -20°C until required. Prior to use, an aliquot of the extract was dried under a stream of nitrogen and redissolved in a small volume of distilled water.

1.3 Treatment of plant material

Pea leaf discs were routinely used to test the effects of chemical treatment on plant material. Pea plants grown in a glasshouse or a constant environment chamber were transferred to the laboratory, and 15 mm diameter leaf discs cut from sub-apical leaves with a sharp

cork borer and floated on the appropriate test solution in glass petri dishes. For experiments on the effect of chilling on plants, discs cut from pea leaves or segments of the second leaf of maize seedlings were floated on distilled water in crystallising dishes and transferred to either a constant environment chamber or a dark growth room. Dishes were maintained at 20°C or cooled and kept at 5°C by standing in an ice bath.

For incubation of leaf discs under altered gas atmospheres or for ethane determinations, discs were floated on test solutions in 25 ml glass vials, or 20 ml glass tubes, in each case fitted with a screw cap containing a rubber septum. Tubes or vials were kept under air, or flushed with oxygen or nitrogen using a hypodermic needle attached to the gas supply for 5 min prior to incubation. Details of the experimental conditions for each experiment are listed in the Results section.

1.4 Replication and statistics

All results represent the mean of at least three replicate samples. Each experiment was repeated twice, and the data pooled. The standard error of the means were less than 10% of the mean for each experiment, unless indicated otherwise in the Results section.

2. Subcellular fractionation techniques

2.1 Leaf homogenates

Crude enzyme extracts from pea leaves or treated leaf discs were used for many of the assays.Leaf material was ground in a cold mortar or a glass homogeniser, in 5-10 ml of cold 50 mM Tricine-NaOH, pH 7.8. The homogenate was squeezed through 4 layers of muslin and centrifued at 3000 g for 5 min in an M.S.E. Chillspin centrifuge at 4°C. The supernatant was used for enzyme determinations.

2.2 Chloroplast membranes for electron transport studies

Leaf material was ground in a cold mortar with cold 50 mM Tricine-NaOH, pH 7.6, containing 0.3 M NaCl and 5 mM $MgCl_2$, using approximately 10 ml buffer per gram of leaf material. The homogenate was squeezed through 4 layers of muslin and centrifuged at 200 g for 1-2 min at 4°C. The pellet was discarded and the supernatant centrifuged at 3000 g for 10 min at 4°C. The pellet consisting of chloroplast membranes was resuspended in 50 mM Tricine-NaOH, pH 7.6,containing 0.03 M NaCl and 5 mM $MgCl_2$.

2.3 Intact chloroplasts

Intact chloroplasts (Type A, Hall, 1972) were prepared according to the method described by Walker (1980). Approximately 25 g of leaf material were homogenised in a domestic blender (3 x 2 s bursts) in 100 ml ice slush of grinding media. The extract was squeezed through 2 layers of muslin and filtered through 8 layers of muslin plus 1 layer of cotton wool. Chloroplasts were recovered by centrifugation at 4 500 rpm for 20 s in a M.S.E. Chillspin centrifuge (maximum radius 16.8 cm). The pellet was superficially washed with 50 ml washing medium, and

resuspended in 1-2 ml incubating buffer. The respective composition of buffers were:

Grinding buffer	0.33 M	sorbitol
	50 mM	Na_2HPO_4 adjusted to pH 6.5 with KOH
	50 mM	KH_2PO_4
	5 mM	$MgCl_2$
	0.1% (w/v)	Bovine serum albumin type V
Washing buffer	0.33 M	sorbitol
	5 mM	$MgCl_2$
	0.1% (w/v)	BSA
	4 ml incubation buffer per 100 ml	
Incubation buffer	0.33 M	sorbitol
	50 mM	Hepes-KOH pH 7.6
	2 mM	EDTA
	1 mM	$MgCl_2$
	0.1% (w/v)	BSA

This technique routinely yielded chloroplasts that were approximately 60% intact. Further purification was achieved by layering the chloroplast preparation over 10 ml of 40% Percoll containing 0.33 M sorbitol and 50 mM Hepes-KOH, pH 7.6, and centrifuging at 4 500 rpm for 1-2 min. After washing, the pellet was resuspended in 2 ml of incubating buffer and contained chloroplasts of greater than 75% intactness (Edwards and Walker, 1983). Alternatively the modified Percoll gradient method described by Nakano and Asada (1980) was used to purify chloroplasts. The chloroplast preparation was layered above a Percoll density gradient prepared by pipetting successively 1.0 ml 90%, 3.0 ml 70%, 4.0 ml 40% and 2.0 ml 10% Percoll containing 50 mM Hepes-KOH, pH 7.6, 0.33 M sorbitol, 10 mM NaCl, 1 mM $MgCl_2$, 2 mM EDTA

and 0.5 mM KH_2PO_4. After centrifuging at 6 500 rpm for 15 min the intact chloroplasts were removed from between the third and bottom layers. Chloroplasts were washed to remove Percoll and were usually greater than 80% intact.

2.4 Subcellular fractionation

The distribution of enzymes between organelles and the leaf soluble fraction of pea leaves was examined. For the chloroplast fraction, intact chloroplasts were prepared as described in Section 2.3. The pellet from the first centrifugation contained both intact and broken chloroplasts, nuclei and cell debris. The supernatant from this stage was centrifuged at 6000 g for 15 min at 4°C. The pellet from this step containing mitochondria, peroxisomes and broken chloroplasts was taken as the crude mitochondrial fraction and the supernatant was referred to as the leaf soluble fraction. Total activity of enzymes assayed in each fraction was calculated. This was compared with the activity of those enzymes in a total leaf homogenate prepared by grinding 0.5-1.0 g leaf material in 10 ml of grinding buffer (Section 2.3) containing no osmoticum. This was squeezed through 2 layers of muslin to remove coarse cellular debris.

2.5 Preparation of spinach ferredoxin

Spinach ferredoxin was prepared according to the method described by Buchanan and Arnon (1969). Spinach leaves were purchased from a local market, and were deveined and washed in distilled water. These were stored in plastic bags at -20°C until required. Approximately 5 kg of leaves were crushed by hand and then passed through a mincer into 0.02 M Tris-HCl, pH 8.0 (1000 ml/kg). The mixture was allowed

to thaw, squeezed through 2 layers of muslin, and acetone was added to give a final concentration of 75% (v/v). After standing for 1 hour at -20°C, the supernatant was removed and the precipitate centrifuged at 10 000 g for 10 min to remove excess acetone. The precipitate was dried with a hair drier, redissolved in 0.15 M Tris-HCl, pH 7.3 (50 ml/kg leaves) and dialysed overnight against 10 volumes of 1 mM Tris-HCl, pH 7.3. Following centrifugation (10 000 g for 30 min)the clear supernatant was absorbed onto a DEAE cellulose column (4 cm x 2 cm high per kg of leaf material) pre-equilibrated with 0.15 M Tris-HCl, pH 7.3, containing 0.08 M NaCl. The column was washed with 0.15 M Tris-HCl, pH 7.3, containing 0.08, 0.11 and 0.14 M NaCl successively to elute flavins and plastocyanin. Ferredoxin was eluted from the column with 0.30 M Tris-HCl, pH 7.3, containing 0.55 M NaCl. After diluting 2.5 times with distilled water, ferredoxin was applied to a second DEAE cellulose column (3 cm x 50 cm high) pre-equilibrated as before. Ferredoxin was eluted with 0.15 M Tris-HCl, pH 7.3, containing 0.25 M NaCl,and fractions with a 420/276 nm absorbance ratio of greater than 0.4 were pooled and retained. Ferredoxin preparations were stored at -20°C until required.

2.6 Ferredoxin $NADP^+$ reductase

The eluate from the first chromatography step in the preparation of spinach ferredoxin contained ferredoxin $NADP^+$ reductase. This was further purified by ammonium sulphate precipitation. Sodium pyrophosphate (0.1 M) was added to the eluate (10% v/v) to protect the enzyme against denaturation. This solution was then fractionated with ammonium sulphate, and the fraction that precipitated between 50-66% salt saturation was retained (Shin, 1969). The precipitate was redissolved in 0.1 M Tris-HCl, pH 7.3, and was taken as crude enzyme.

Ferredoxin $NADP^+$ reductase activity in the preparation was assayed according to the method of Avron and Jagendorf (1956), using the enzyme to catalyse the reduction of DCPIP by NADPH. The 2.0 ml reaction mixture contained 0.1 M Tris-HCl, pH 7.3, 1 mM NADPH, 50 µM DCPIP and an aliquot of enzyme preparation. The reaction was followed as the change in absorbance at 620 nm as DCPIP was reduced. One unit of enzyme activity was defined as the amount of enzyme causing a decrease in absorbance at 620 nm of 1.0 per minute.

2.7 Extraction and purification of superoxide dismutase

SOD from spinach leaves was extracted and purified by a method based on that described by Vaughan *et al.* (1982). Samples of 25 g of leaf material were homogenised in 250 ml 0.1 M phosphate buffer, pH 7.8, and the debris removed by centrifugation at 10 000 g for 15 min. SOD in the supernatant was partially purified by adding ammonium sulphate to 35% saturation, standing for 1 h at 5°C, and centrifuging at 2 500 g for 10 min. More ammonium sulphate was added to the supernatant to bring this to 55% saturation, and after standing for 1 h and centrifuging as before, the precipitate containing SOD was dissolved in 25 ml phosphate buffer, pH 7.8. A 5.0 ml aliquot of the enzyme solution was applied to a G-75 Sephadex column (2.5 x 30 cm), previously washed with 0.1 M phosphate buffer, pH 7.8, and eluted with the same buffer using a flow rate of 20 ml per hour. Fractions (5.0 ml) were collected, and those showing SOD activity were pooled, and concentrated by dialysis against polyethylene glycol 4000. SOD extracts were stored at -20°C until required.

3. Quantitative determinations

3.1 Chlorophyll and carotenoids

Chlorophyll and carotenoid levels in leaf discs were determined in acetone extracts obtained by soaking discs in 80% acetone in darkness for 5 days. Chlorophyll was quantified according to Arnon (1949):

$$\text{Chlorophyll } (\mu g\ ml^{-1}) = 8.02\ (A_{663}) + 20.2\ (A_{645})$$

Carotenoid levels in etiolated or greening leaves were estimated from the absorbance of the acetone extract at 480 nm after correction to account for chlorophyll according to Kirk and Allen (1965):

$$\text{Carotenoid} = A_{480} + (0.114\ A_{663} - 0.638\ A_{645})$$

Alternatively leaf discs were soaked for 1-2 days in ethanol, and pigments estimated from the absorbance of the ethanol extract at 470, 649 and 665 nm according to Lichtenthaler and Wellburn (1983):

$$\text{Chlorophyll a } (\mu g\ ml^{-1}) = 13.95\ (A_{665}) - 6.88\ (A_{649})$$

$$\text{Chlorophyll b } (\mu g\ ml^{-1}) = 24.96\ (A_{649}) - 7.32\ (A_{665})$$

$$\text{Carotenoid } (\mu g\ ml^{-1}) = \frac{1000\ (A_{470}) - 20.5\ C_a - 114.8\ C_b}{245}$$

Chlorophyll concentration of chloroplast preparations was determined by adding 0.1 ml of chloroplasts to 3.9 ml 80% acetone, centrifuging (2 500 g x 5 min) and determining the absorbance at 663 and 645 nm as above.

3.2 Protein

Protein was determined according to the method of Hartree (1972).

Solution A. 2 g potassium sodium tartrate

100 g sodium carbonate

500 ml 1 N NaOH

made up to 1 litre with distilled H_2O.

Solution B.	2 g potassium sodium tartrate
	1 g copper sulphate
	10 ml 1N NaOH
	H_2O to 100 ml.
Solution C.	1 vol. Folin Ciocalteu reagent
	14 vol. H_2O

0.9 ml of solution A was added to 1.0 ml of protein sample, and heated to 50°C for 10 min. After cooling to room temperature, 0.1 ml solution B was added, and the mixture left to stand for at least 10 min. Freshly mixed solution C (3.0 ml) was added rapidly and the colour was developed by heating to 50°C for 10 min. After cooling to room temperature the absorbance was determined at 650 nm.

A calibration curve was constructed with BSA as the protein standard and was linear between 15 and 100 µg protein.

3.3 Ascorbate

Ascorbate was determined by the method of Oser (1979) as described by Mukherjee and Choudhuri (1983), based on spectrophotometic determination of the red colour formed between dehydroascorbate and 2,4-dinitrophenylhydrazine. A sample of plant extract containing approximately 100 mg ascorbate was mixed with 6% TCA to give a final volume of 10 ml, left to stand for 5 min and centrifuged (200 g x 5 min). Ascorbate in the supernatant was oxidised by the addition of 0.3 g acid washed activated charcoal, mixed thoroughly, and filtered through No. 1 Whatman filter paper. To a 2.0 ml aliquot of the filtrate 1.0 ml 2,4-dinitrophenylhydrazine (2% in 9 N H_2SO_4) and 1 drop 6% thiourea (in 70% ethanol) was added. After heating for 15 min in a boiling water bath and cooling, 2.5 ml 80% H_2SO_4 was added slowly, mixed, and

the absorbance determined at 530 nm. Ascorbate was calculated from a calibration curve,constructed using 0.5 - 40 µg ascorbate.

3.4 Glutathione

Determination of glutathione was based on the method described by Law *et al.* (1983), based on the glutathione reductase specific reduction of glutathione by NADPH. The reaction mixture contained 330 mM sorbitol, 50 mM Hepes-KOH, pH 7.6, 0.5 mM NADPH, 0.3 mM DTNB and glutathione reductase (1 unit). The rate of increase in absorbance at 412 nm obtained with leaf extracts or chloroplasts was compared to standards containing known amounts of glutathione.

3.5 Ethane

Ethane evolved from intact leaf discs was determined by GLC. Leaf material was incubated under constant illumination in 25 ml screw top vials fitted with air tight rubber seals. After incubation for varying lengths of time 1 ml samples of the gas headspace were removed using an air tight syringe, and analysed in a Pye-Unicam chromatograph with an alumina column. The column oven temperature was 125°C and the gas flow rate set to 40 ml min^{-1}. Ethane was identified and quantified by comparing the retention time and peak height of the sample with authentic standards.

3.6 Carbon dioxide exchange

The CO_2 exchange of leaf segments of discs was measured in an Infra-red Gas analyser (Model 225; Analytical Development Co., Hoddesdon, Herts), connected to an open circuit gas flow system. Compressed air (800 ml min^{-1}) was passed through a $CaCl_2$ drying chamber

and divided into two streams. One stream passed through the sample chamber to a second drying chamber into the IRGA sample tube. The other stream went directly into the IRGA reference tube. The flow rate of gas through both streams was identical.

Leaf material in the sample chamber was incubated in darkness, and then illuminated with a photoflood lamp (Thorn 400W) giving a light intensity of 250 µmol $m^{-2} s^{-1}$ photon flux density at the chamber surface. CO_2 exchange by leaf material was calculated by comparing the CO_2 content of the air flow through the sample with that of the reference stream. The IRGA was calibrated using a gas supply with a known CO_2 concentration.

3.7 Leaf disc chlorophyll fluorescence

A portable Kautsky apparatus (Plant Productivity Meter SF-10; Richard Branker Research, Ottawa, Canada) was used to monitor the fluorescence kinetics from chlorophyll. The fluorimeter induced chlorophyll fluorescence with radiation at 670 nm and detected fluorescence at wavelengths > 710 nm. The output was monitored on a chart recorder. All plant material was maintained in darkness for at least 1 hour prior to measurement of chlorophyll fluorescence. Fluorescence kinetics were assessed on 10 replicate samples of leaf material, and the change in variable fluorescence analysed (see Results Section 7).

3.8 Electrolyte leakage

Electrolyte leakage as an assessment of herbicide induced injury to plant membranes has become widely used (Vanstone and Stobbe, 1977). Leaf discs (15 mm diameter) were cut from glasshouse grown pea plants and floated on 20 ml of herbicide solution, prepared using deionised water, in glass petri dishes (15 leaf discs per 20 ml), and

incubated under continuous illumination. Conductivity changes in the solutions were assessed using a Data Scientific PT1-18 Digital Conductivity meter, both prior to and following various periods of illumination.

4. Experimental assay techniques

4.1 Photosynthetic electron transport

Photosynthetic electron transport by isolated chloroplast lamellae was assessed by measuring electron flow from water to a variety of electron acceptors in a Hansatech oxygen electrode, coupled to a Phillips chart recorder. The electrode was calibrated with sodium dithionite prior to each experiment. The basic 3.0 ml reaction mixture contained:

30 mM Tricine-NaOH, pH 8.0

5 mM $MgCl_2$

1.7 mM NH_4Cl

Chloroplast lamellae containing 50-100 µg chlorophyll In all cases the chloroplast lamellae were the last addition, and the reaction mixture was allowed to equilibrate at 20°C in darkness before being illuminated (500 µmol $m^{-2}s^{-1}$ photon flux density provided by projector lamp).

Additions to this basic reaction mixture were:

Photosystem I 6.6 µM DCPIP

13.3 mM ascorbate

66 µM monuron

6.6 µM paraquat

Photosystem II (ferricyanide reduction)

1.33 mM $K_3Fe(CN)_6$

Photosystem II (silicomolybdate reduction)

1.0 mg silicomolybdate

Photosystem I and Photosystem II (Mehler reaction)

6.6 µM paraquat

Photosystem I and Photosystem II (using DPC as an electron donor to PSII)

1.0 mM 1,5-diphenylcarbazide (DPC)

6.6 µM paraquat

Differences in oxygen uptake or evolution were calculated between the light and dark reactions and results expressed as µmol O_2 mg^{-1}chl h^{-1}.

4.2 Chloroplast intactness

Chloroplast intactness was assayed by the method described by Edwards and Walker (1983). Photosynthetic electron transport activity of intact and osmotically shocked chloroplasts was assessed in an oxygen electrode using FeCN as an electron acceptor. The 2.0 ml reaction mixture contained:

0.33 M sorbitol

50 mM Hepes-NaOH, pH 7.6

1 mM $MgCl_2$

1 mM $MnCl_2$

1 mM EDTA

1.5 mM $K_3Fe(CN)_6$

5 µM NH_4Cl

Chloroplasts containing 50-100 µg chlorophyll.

Chloroplast intactness was assayed as the rate of FeCN reduction in intact chloroplast as a percentage of the rate in osmotically shocked chloroplasts.

4.3 $NADP^+$ reduction

This was determined by following the absorbance change at 340 nm, due to the reduction of $NADP^+$ to NADPH, in a Shimadzu UV 260 recording spectrophotometer. The 3.0 ml reaction volume contained 30 mM Tricine-NaOH,

pH 8.0, 5 mM $MgCl_2$, 1.7 mM NH_4Cl, 13.3 µM $NADP^+$, 100 µg ferredoxin and chloroplast lamellae containing 30 µg chlorophyll. This was prepared in a silica-glass cuvette and illuminated (500 µmol $m^{-2}s^{-1}$ photon flux density). At set time intervals the cuvette was transferred to the spectrophotometer and the absorbance determined at 340 nm.

4.4 NADPH oxidation

Oxidation of NADPH in darkness, catalysed by ferredoxin and ferredoxin $NADP^+$ reductase was based on the method described by Wessels (1965). The basic 3.0 ml reaction mixture contained 0.33 M Tris-HCl, pH 8.0, and 80 µg NADPH. Other additions, listed in the Results section, included chloroplast membranes containing 30 µg chlorophyll, 150 µg ferredoxin, 0.5 units ferredoxin $NADP^+$ reductase, 25 µM myoglobin, 25 µM cytochrome C, and 25 µM acifluorfen or oxyfluorfen. The reaction was followed as the fall in absorbance at 340 nm as NADPH was oxidised.

4.5 Superoxide formation by illuminated chloroplasts

Superoxide was assayed by its ability to oxidise hydroxylamine to nitrite according to the method described by Elstner *et al.* (1975). The 3.0 ml reaction mixture contained:

25 mM Tris-HCl, pH 8.0

1.0 mM KCN

66 µM NH_4Cl

1.0 µM NH_2OH

Chloroplast membranes containing 100 µg chlorophyll.

The reaction mixture was illuminated in a water bath (20°C), illuminated from below (Thorn 400 W photoflood lamps giving 500 µmol photon

flux density at the samples). Aliquots were analysed for nitrite by adding 1.0 ml of sulphanilamide (1% w/v in 25% HCl) and 1.0 ml of naphthylethylene diamine dihydrochloride (0.02% w/v) to 1.0 ml of sample. The colour was allowed to develop for 20 min at room temperature, and the absorbance determined at 540 nm. Nitrite present was calculated from a calibration curve obtained with potassium nitrite.

4.6 Enzyme assays

4.6.1 Superoxide dismutase

Superoxide dismutase was assayed by the method described by Elstner and Heupel (1976), based on SOD inhibiting the oxidation of hydroxylamine by superoxide:

$$NH_2OH + 2O_2^{\cdot -} + H^+ \longrightarrow NO_2^- + H_2O + H_2O_2$$

Superoxide was generated enzymically by xanthine/xanthine oxidase. The 3.0 ml reaction mixture contained 22 mM potassium phosphate, pH 7.8, 0.33 mM hydroxylamine, 0.5 mM xanthine, and an aliquot of enzyme extract. The reaction was initiated by adding xanthine oxidase (Sigma) containing 100 µg protein. After incubation at 25°C for 20 min, an aliquot (1.0 ml) was analysed for nitrite (see section 4.5). One unit of enzyme activity was defined as the amount of enzyme that causes a 50% inhibition of nitrite formation from hydroxylamine.

Alternatively SOD was assayed by the inhibition of nitro blue tetrazolium oxidation to formazan by superoxide, based on the method described by Beauchamp and Fridovich (1971). The reaction mixture (3.0 ml) contained phosphate buffer as above, containing 0.1 mM xanthine, 25 µM nitro blue tetrazolium, and an aliquot of SOD. The reaction was initiated by the addition of xanthine oxidase containing 50-60 µg

protein. After incubation at 20°C for 25 min, the absorbance of the solutions was determined at 560 nm, and SOD activity calculated. One unit of activity represented the amount of enzyme that inhibited formazan production by 50%.

4.6.2 Ascorbate peroxidase

Two methods were used in determining activity of this enzyme. In the method described by Groden and Beck (1979), ascorbate peroxidase was determined by following H_2O_2 breakdown in an oxygen electrode. The 3.0 ml reaction volume contained an aliquot of enzyme extract, 0.1 M Tris-HCl, pH 8.0, and 8.5 mM ascorbate. The peroxidative reaction was initiated by adding 0.05 ml of 8 mM H_2O_2. The H_2O_2 remaining after 30 s was determined polargraphically following the addition of excess catalase (2500 units). H_2O_2 consumed by the peroxidative reaction was calculated from the difference between added and unreacted H_2O_2, after accounting for controls in which either ascorbate or the enzyme extract had been omitted. Peroxide consumed was proportional to the amount of enzyme extract added, and to the reaction time.

Alternatively, ascorbate peroxidase was determined by following the change in absorbance at 290 nm as ascorbate was oxidised (Nakano and Asada, 1981).The 3.0 ml reaction volume contained 50 mM Hepes-NaOH, pH 7.6, 0.1 mM EDTA, 0.5 mM ascorbate, 0.1 mM H_2O_2 and an aliquot of enzyme extract. Correction was made for the oxidation of ascorbate in the absence of H_2O_2 or enzyme. Enzyme activity was expressed as µmol ascorbate oxidised.

4.6.3 Glutathione reductase

Glutathione reductase was assayed by measuring the enzyme catalysed reduction of glutathione by NADPH, by following the loss of absorbance at 340 nm as NADPH was oxidised (Jablonski and Anderson, 1978). The 3.0 ml reaction volume contained enzyme extract, 0.4 M potassium phosphate, pH 8.0, 0.4 mM EDTA, 0.5 mM NADPH and 5 mM GSSG. The reaction was followed by the change in absorbance at 340 nm and enzyme activity, expressed as µmol NADPH oxidised, was calculated after accounting for controls that lacked either GSSG or enzyme extract.

4.6.4 Dehydroascorbate reductase

This enzyme was assayed by the increase in absorbance at 265 nm as ascorbate was formed from dehydroascorbate according to the method described by Nakano and Asada (1981). The 3.0 ml reaction volume contained 50 mM potassium phosphate, pH 7.0, 0.1 mM EDTA, 2.5 mM GSH and 0.2 mM dehydroascorbate. The rate of reduction of dehydroascorbate to ascorbate was calculated, and corrected for the rate of reduction in the absence of enzyme, and is expressed as µmol ascorbate formed.

4.6.5 Glutathione peroxidase

Glutathione peroxidase was assayed by the method described by Overbaugh and Fall (1982). The 3.0 ml reaction volume contained enzyme extract, 50 mM potassium phosphate, pH 8.0, 0.1 mM EDTA, 0.1 mM GSH, 0.1 mM NADPH, glutathione reductase (7.5 units) and 0.4 mM H_2O_2 or 0.4 mM cumene hydroperoxide. Enzyme activity was determined from the loss of absorbance at 340 nm as NADPH was oxidised after accounting for control reactions. The controls used were; NADPH alone, NADPH + GSH, NADPH + GSH + glutathione reductase, NADPH + GSH +

enzyme extract, and reaction mixtures containing all the reagents except peroxide or enzyme extract. H_2O_2 was used in the determination of selenium dependent glutathione peroxidase, and cumene hydroperoxide for the determination of selenium independent enzyme activity.

4.6.6 NADPH glyceraldehyde 3-phosphate dehydrogenase

Enzyme activity was assayed according to the method described by Jackson *et al.* (1978). The 3.0 ml reaction volume contained 67 mM Tris-HCl, pH 7.2, 4 mM EDTA, 10 mM $MgCl_2$, 3.3 mM ATP, 1 mM dithiothreitol, 130 µM NADPH, 3.3 µg ml^{-1} phosphoglycerate kinase and an aliquot of enzyme extract. After incubating at 25°C for 5 min, the reaction was initiated by addition of 5 mM 3-phosphoglycerate and followed by the fall in absorbance at 340 nm as NADPH was oxidised.

4.6.7 Peroxidase

Peroxidase activity was assayed by the method of Braber (1980), using guiacol as a substrate. The 3.0 ml reaction volume contained 40 mM potassium phosphate, pH 5.9, 33 mM guiacol and an aliquot of enzyme extract. The peroxidative reaction was initiated by addition of 0.2 ml of 1% H_2O_2, and the reaction followed from the change in absorbance at 420 nm.

4.6.8 Cytochrome C oxidase

The assay for this enzyme was based on the method described by Tolbert (1974) which measures the loss of absorbance at 550 nm as cytochrome C was oxidised. The 3.0 ml reaction volume contained enzyme extract, 0.1 M potassium phosphate, pH 7.2, 1 mM EDTA and 0.0033% Triton X-100. The reaction was initiated by the addition of

0.2 ml of reduced cytochrome C (5 mg ml^{-1}). Reduced cytochrome C was prepared by adding sodium dithionite to the cytochrome solution until the E_{550}/E_{565} ratio was greater than 6. Excess dithionite was removed by bubbling nitrogen through the solution.

4.6.9 Catalase

Catalase activity was determined polargraphically in a Hansatech oxygen electrode according to the method of Jablonski and Anderson (1978). The 2.0 ml reaction volume contained enzyme extract and 50 mM potassium phosphate buffer, pH 7.0. The reaction was initiated by adding H_2O_2 to give a concentration of 10 mM, and catalase activity monitored from the rate of oxygen evolution.

4.6.10 Glycollate oxidase

Enzyme activity was assayed by following formation of glycollate phenylhydrazone from glycollate (Feierabend and Beevers, 1972). The 3.0 ml reaction volume contained 33 mM triethanolamine, pH 7.8, 2.7 mM EDTA, 5 mM glycollic acid, 3.3 mM phenylhydrazine-HCl, pH 6.8, 0.67 mM GSSG, 0.2 mM flavin mononucleotide, 0.0083% Triton X-100 and an aliquot of enzyme extract. The reaction was monitored by following the increase in absorbance at 342 nm.

4.7 Peroxidation of illuminated chloroplast membranes

Peroxidation of illuminated chloroplast membranes was followed by thiobarbituric acid determination as described by Takahama and Nishimura (1975). Chloroplast membranes (50 µg chlorophyll per ml) were incubated in screw top flasks in 50 mM potassium phosphate buffer, pH 8.0. After incubation in a water bath at 20°C, illuminated from

below (500 μmol $m^{-2}s^{-1}$ photon flux density), aliquots were analysed for thiobarbituric acid reactive material. To a 2.0 ml aliquot in a test tube, 0.5 ml 40% trichloroacetic acid, 0.5 ml 2% thiobarbituric acid and 0.25 ml 5 N HCl were added, mixed and heated in a boiling water bath for 10 min. After cooling and centrifuging at 2 500 g for 5 min, the absorbance of the supernatant was determined at 532 nm, and corrected for non-specific turbidity by subtracting the absorbance at 600 nm.

RESULTS

RESULTS

1. Distribution and activity of chloroplast superoxide and hydrogen peroxide scavenging systems in pea leaves

Chloroplasts from higher plants contain superoxide dismutase, ascorbate peroxidase, dehydroascorbate reductase, and glutathione reductase and millimolar concentrations of ascorbate and glutathione which protects them against $O_2^{\cdot -}$ and H_2O_2 (Foyer and Halliwell, 1976; Halliwell, 1981). These enzymes have been shown to be released from the chloroplast on rupturing the envelope, and are therefore stromal and not bound to the chloroplast envelope (Jackson *et al.*, 1978; Jablonski and Anderson, 1978, 1981, 1982; Nakano and Asada, 1981). The proportion of these enzymes and of ascorbate and glutathione in chloroplasts however remains unclear. The aim of this investigation was to determine the chloroplast activity and leaf distribution of $O_2^{\cdot -}$ and H_2O_2 scavenger mechanisms in pea leaves.

1.1 Characterisation of enzyme activities

Extracts from pea leaves were prepared by grinding approximately 1.0 g of leaf material into 10.0 ml buffer, followed by centrifugation at 5000 g for 10 minutes. Such leaf homogenates catalysed ascorbate dependent reduction of H_2O_2, reduction of DHA to ascorbate by GSH, and NADPH dependent regeneration of GSH from GSSG. The rates of these reactions was proportional to the amount of enzyme added at protein concentrations up to 100 µg ml^{-1} of reaction volume. The activity of these enzymes in leaf extracts was 0.325 µmol H_2O_2 reduced mg $protein^{-1}min^{-1}$ for ascorbate peroxidase, 0.075 µmol DHA reduced mg $protein^{-1}min^{-1}$ for dehydroascorbate reductase and 0.115 µmol NADPH oxidised mg $protein^{-1}min^{-1}$ for glutathione reductase. Leaf homogenates

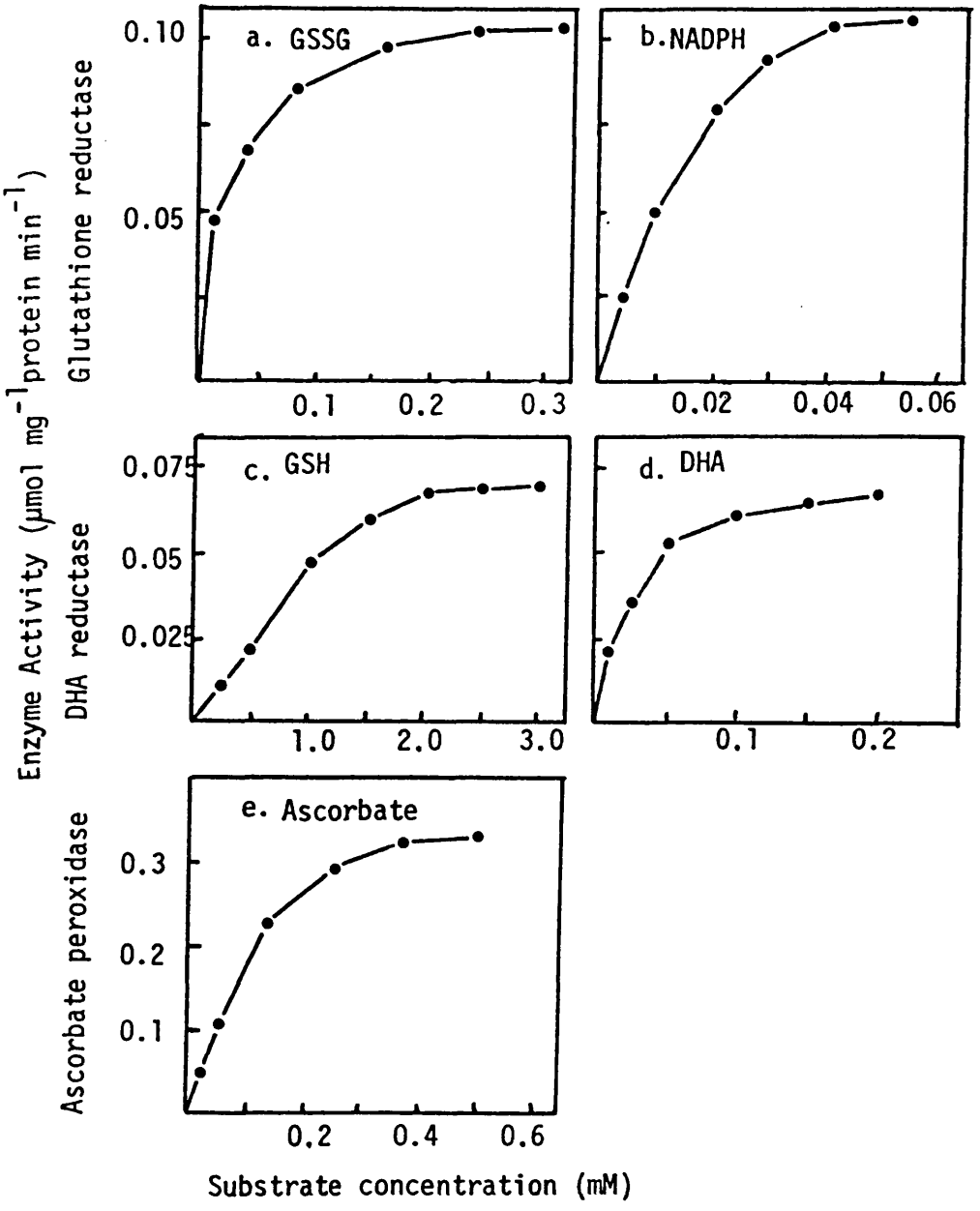


Fig 6. The effect of substrate concentration on the activity of ascorbate peroxidase, DHA reductase and glutathione reductase from pea leaves. Enzyme substrates were: GSSG (a), NADPH (b), GSH (c), DHA (d) and ąscorbate (e).

also showed SOD activity, assessed by inhibition of nitrite formation from hydroxylamine or by nitro blue tetrazolium oxidation to formazan, equivalent to 25 units SOD mg protein^{-1}.

The effect of substrate concentrations on the activity of glutathione reductase, dehydroascorbate reductase and ascorbate peroxidase from pea leaf homogenates (using 75 µg protein ml^{-1}) is shown in Figure 6. The apparent Km values for these enzymes under saturating levels of other substrates, estimated using Lineweaver-Burk plots from this data are shown below:

Glutathione reductase	
Km (NADPH)	26 µM
Km (GSSG)	12 µM
Dehydroascorbate reductase	
Km (DHA)	27 µM
Km (GSH)	4 mM
Ascorbate peroxidase	
Km (ascorbate)	0.5 mM

These values are comparable with those of spinach or pea enzymes reported elsewhere (Jablonski and Anderson, 1978, 1981, 1982; Nakano and Asada, 1981; Gerbling *et al.*, 1984; Hossain and Asada, 1984).

The pH optima of these enzymes assessed in phosphate or Tris-HCl buffer is shown in Figure 7. Optimum enzyme activity was shown at pH 8.0 for glutathione reductase, pH 7.0 for dehydroascorbate reductase and pH 7.5 for ascorbate peroxidase. The rate of H_2O_2 reduction by ascorbate or NADPH oxidation by GSSG was low in the absence of enzyme extracts. At alkaline pH values non enzymic reduction of DHA by GSH exceeded the enzymic rate, as reported previously (Foyer and Halliwell, 1977).

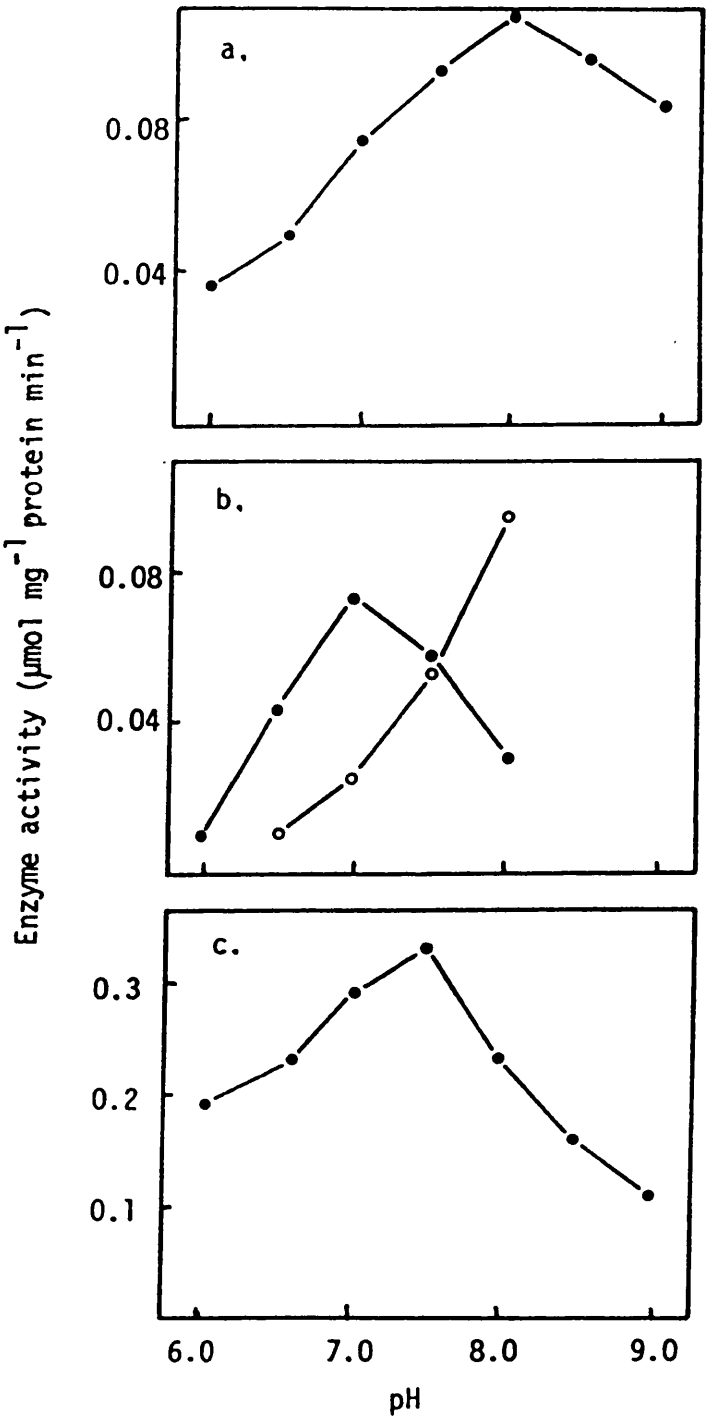


Fig 7. The effect of pH on the activity of glutathione reductase (a), DHA reductase (b) or ascorbate peroxidase (c) from pea leaves. For DHA reductase the enzymic reaction (●–●) is compared with the non-enzymic rate (o–o).

1.2 Subcellular fractionation of pea leaves

To assess the distribution of organelle marker, and active oxygen scavenging enzymes in pea leaves, leaf homogenates were subjected to a fractionation procedure as outlined in Tables 1 and 2. Centrifugation of leaf homogenates at 2500 g for 1-2 minutes left a pellet rich in intact and broken chloroplasts. Further centrifugation at 6000 g for 15 minutes removed mitochondria. Results presented in Table 1 show the distribution of chlorophyll and organelle marker enzymes between these fractions. The chloroplast fraction (chlorophyll marker) also contained mitochondria (cytochrome oxidase marker) and peroxisomes (glycollate oxidase marker). The mitochondrial 6000 g pellet also contained peroxisomes and chloroplast membranes (chlorophyll marker). The 6000 g supernatant contained a high proportion of glycollate oxidase activity indicating that this fraction was rich in peroxisomes. The high activity of NADPH-GPD recovered in this fraction shows that many chloroplasts had lost their envelopes during fractionation.

The distribution of $O_2^{\cdot -}$ and H_2O_2 scavenging enzymes is shown in Table 2. Distribution of SOD, ascorbate peroxidase and glutathione reductase was found to be similar to that of NADPH-GPD. This indicates that a high proportion of their activity was chloroplastic and released from chloroplasts during fractionation.

SOD activity in plants has often been assessed in parallel to catalase and peroxidase levels (Simon *et al.*, 1974; Harper and Harvey, 1978; Dhindsa *et al.*, 1981; McRae and Thompson, 1984). Results presented in Table 2 confirm that chloroplasts contain little or no peroxidase activity, as demonstrated elsewhere (Parrish, 1972), and indicate that much of the activity of this enzyme was in the leaf soluble fraction. Distribution of catalase was similar to that of glycollate

Table 1. Subcellular fractionation of pea leaves: distribution of chlorophyll and organelle marker enzymes between 2500g (chloroplast) pellet, 6000g (mitochondrial) pellet and the 6000g (leaf soluble) supernatant. The recovery of enzyme from the three fractions is also shown.

	Chlorophyll	NADPH-GPD	Cytochrome oxidase	Glycollate oxidase
Homogenate	100.00	100.00	100.00	100.00
2500g pellet	62.10	20.30	21.70	5.95
6000g pellet	3.50	74.10	19.20	79.80
6000g supernatant	26.98	0.51	61.90	19.10
(% recovery)	(97.58)	(91.90)	(103.50)	(104.80)

Table 2. Subcellular fractionation of pea leaves: distribution of enzymes that scavenge $O_2^{\cdot -}$ and H_2O_2.

	superoxide dismutase	ascorbate peroxidase	glutathione reductase	catalase	peroxidase
Homogenate	100.00	100.00	100.00	100.00	100.00
2500g pellet	19.60	19.60	18.90	4.50	0.33
6000g pellet	76.60	76.60	78.30	87.10	92.30
6000g supernatant	4.20	4.20	1.15	14.70	1.10
(% recovery)	(103.60)	(100.40)	(94.40)	(106.30)	(93.70)

oxidase, confirming that this enzyme originates from peroxisomes (Halliwell, 1981). As SOD is predominantly a chloroplast enzyme (Table 2, Jackson *et al.*, 1978), it would be unlikely that peroxidase or catalase could scavenge H_2O_2 generated as a consequence of SOD activity.

Glutathione peroxidase is an important enzyme that scavenges H_2O_2 in animal cells (Halliwell, 1981). This enzyme has also been detected in *Euglena* and other microalgae (Overbaugh and Fall, 1985), and in cultured cells of several higher plant species (Drotar *et al.*, 1985; Overbaugh and Fall, 1985). By contrast, Smith and Shrift (1975) suggested that this enzyme was absent from higher plants. The extracts of pea leaves used in this study did not show glutathione peroxidase activity, using either H_2O_2 or cumene hydroperoxide as substrates to assess selenium independent or selenium dependent forms of the enzyme.

1.3 Chloroplast activity

To further examine chloroplast levels of NADPH-GPD, SOD, ascorbate peroxidase, glutathione reductase and ascorbate, pea chloroplasts of varying degrees of intactness were prepared. The activity present in chloroplast preparations was compared with the activity in leaf homogenates. The proportion of activity was then compared with the intactness of the chloroplast preparation, and these results are shown in Figures 8 and 9. Extrapolation from these figures to the level present in intact chloroplasts shows that 95% of NADPH-GPD would be present (Figure 8a), demonstrating that this enzyme is a valid chloroplast stromal fraction marker. In addition intact chloroplasts would contain 30% of leaf ascorbate (8b), 96% of SOD (9a), 82% of glutathione reducase (9b) and all the ascorbate peroxidase activity (9c).

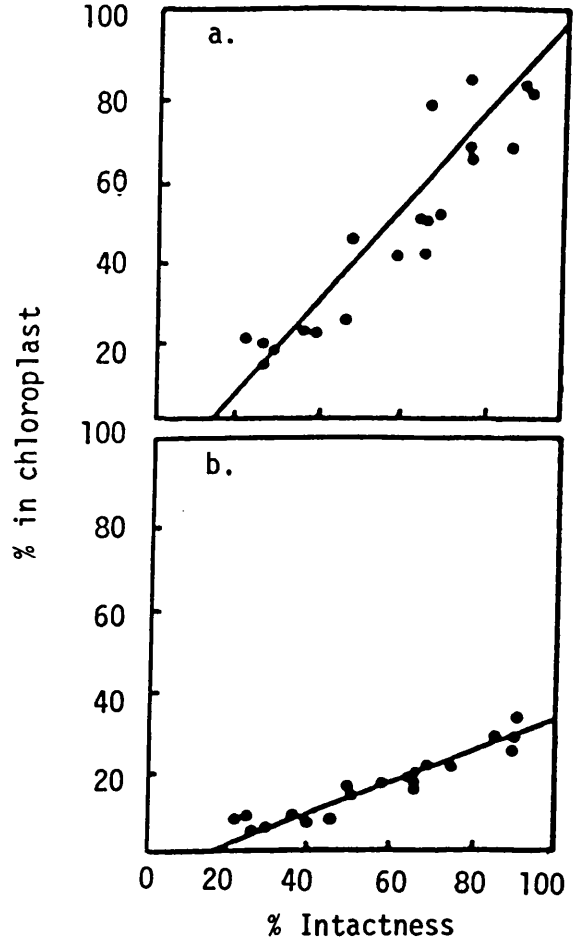


Fig 8. The proportion of leaf NADPH-GPD (a) and ascorbate (b) in pea chloroplasts of varying intactness.

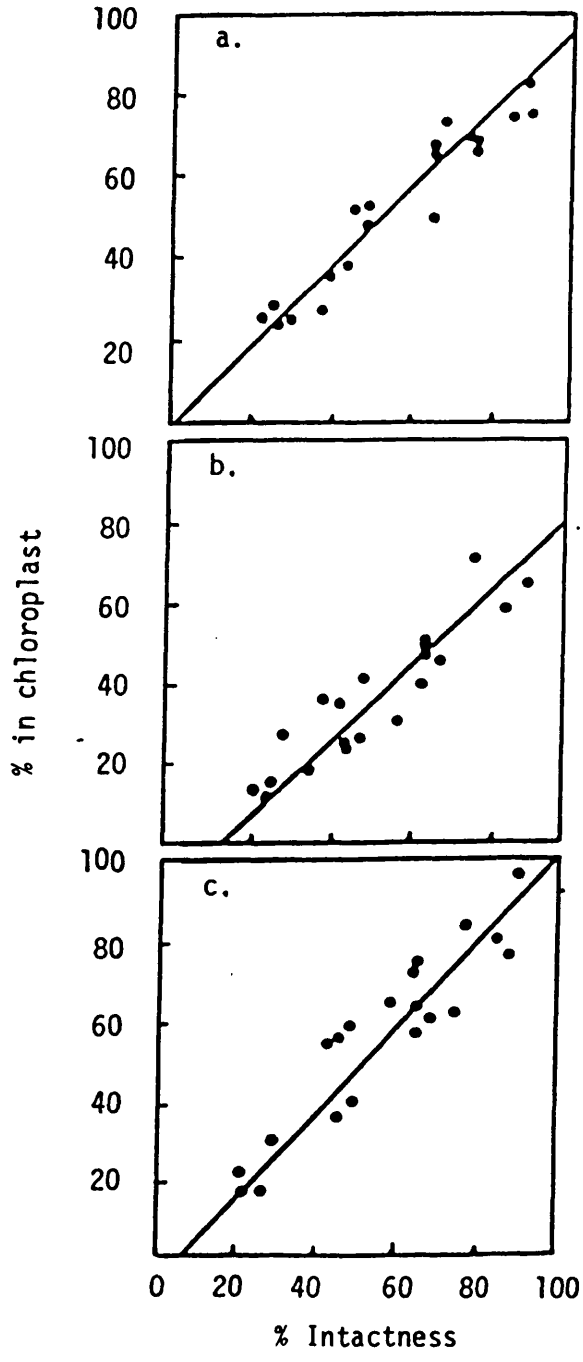


Fig 9. The proportion of leaf SOD (a), glutathione reductase (b) and ascorbate peroxidase (c) in pea chloroplasts of varying intactness.

Table 3. Activity of enzymes scavenging $O_2^{\cdot -}$ and H_2O_2 (a) and the levels of antioxidants (b) in pea leaf homogenates and isolated chloroplasts (85% intact). The proportion of activity present in the chloroplast fraction is also shown.

a.	Activity (μmol mg^{-1}chl h^{-1})		% in chloroplast
	leaf homogenate	chloroplast	
Superoxide dismutase (1)	200.6	151.2	77.8
Ascorbate peroxidase	1198.7	935.5	78.9
Glutathione reductase	45.9	34.7	76.8
Dehydroascorbate reductase	110.2	70.2	64.7

(1) Activity of SOD expressed as units mg^{-1}chl

b.	Level (μmol mg^{-1}chl)		% in chloroplast
	leaf homogenate	chloroplast	
Glutathione	1.00	0.091	9.1
Ascorbate	1.68	0.54	32.3

In addition to these data, chloroplasts(85% intact) were isolated from pea leaves, and levels of ascorbate, glutathione, and $O_2^{\cdot -}$ and H_2O_2 scavenging enzymes were compared to the levels present in pea leaf homogenates. These data are presented in Table 3. These results confirm that SOD, ascorbate peroxidase and glutathione reductase are present mainly in the chloroplast fraction of pea leaves. Additionally a high proportion of leaf dehydroascorbate reductase activity was chloroplastic. Pea chloroplasts contained a low proportion of leaf glutathione (< 10%) and ascorbate (< 35%), which equated to 0.1 and 0.6 µmol mg^{-1} chlorophyll. Assuming a chloroplast volume of 26 µl mg^{-1} chlorophyll (Heldt *et al.*, 1973) this would give a chloroplast ascorbate concentration of 20-25 mM and a glutathione concentration of 3.5-4.0 mM. These values are similar to results published for spinach chloroplasts (Foyer and Halliwell, 1976; Foyer *et al.*, 1983).

1.4 Discussion

Previous investigations have demonstrated that SOD, ascorbate peroxidase, dehydroascorbate reductase and glutathione reductase are present in chloroplasts with activities similar to those shown in Table 3 (Foyer and Halliwell, 1976; Jackson *et al.*, 1978; Jablonski and Anderson, 1978, 1981, 1982; Nakano and Asada, 1981). Furthermore, these studies demonstrated that these enzymes were present in the chloroplast stroma, and not bound to the chloroplast envelope.

Comparisons of the distribution of SOD and glutathione reductase activity with that of NADPH-GPD in spinach leaves (Foyer and Halliwell, 1976; Jackson *et al.*, 1978) produced results similar to those reported in this study. Jackson *et al.* (1978) concluded that much of the total leaf SOD was present in chloroplasts. Foster and Edwards (1980) showed

that 50-80% of leaf SOD was chloroplastic. Chloroplast SOD may be bound to the thylakoid membranes (Lumsden and Hall, 1974; Hayakawa *et al.*, 1985), in the intrathylakoid space (Hayakawa *et al.*, 1984) or in the stroma (Lumsden and Hall, 1974). Intact spinach chloroplasts have also been reported to contain 67% of leaf glutathione reductase activity but only 28% of dehydroascorbate reductase (Anderson *et al.*, 1983). Ascorbate peroxidase has previously been shown to be a chloroplast stromal enzyme (Nakano and Asada, 1981) and bound to the thylakoid membranes (Groden and Beck, 1979). SOD and glutathione reductase have also been detected in a variety of non-photosynthetic tissues, including wheat germ (Conn and Vennesland, 1951; Beauchamp and Fridovich, 1973), etiolated leaves (Giannopolitis and Ries, 1977; Gamble and Burke, 1983) and mitochondria (Young and Conn, 1956; Foster and Edwards, 1980). The activity and subcellular distribution of these enzymes may depend on the stage of plant development or growth conditions. Chloroplasts isolated from pea leaves in this study contained a high proportion of leaf SOD, ascorbate peroxidase, dehydroascorbate reductase, glutathione reductase and millimolar concentrations of ascorbate and glutathione. This indicates their importance in scavenging $O_2^{\cdot -}$ and H_2O_2 in that organelle. Catalase, glutathione peroxidase and non-specific peroxidase appear to have no significant role in scavenging H_2O_2 in pea chloroplasts. These enzymes may however be important in scavenging H_2O_2 in other organelles or plant parts.

2. Chloroplast protection in greening leaves

The transfer of etiolated plants to light induces many changes in chloroplasts that leads to full photosynthetic competence. Illumination promotes the reorganisation of the prolamellar body to form thylakoids and induces the synthesis of chlorophyll. Photosystem I activity can be detected after a few minutes illumination, although whole chain electron transport and photosynthetic CO_2 exchange do not commence until several hours after transfer to light (Bradbeer *et al.*, 1977; Bradbeer, 1981). The development of the photosynthetic apparatus increases the potential for the generation of damaging oxygen species in chloroplasts. Greening must therefore be carefully controlled, both to restrict the formation of these radicals, and to ensure that a full range of anti-oxidant protective mechanisms are present early in development. Many studies have shown changes in the photosynthetic apparatus during greening (Bradbeer *et al.*, 1977; Bradbeer, 1981). It is not clear whether enzymes and antioxidants that protect chloroplasts against photo-oxidative damage are present in etiolated leaves, or how their levels change during greening.

2.1 Pigments

The accumulation of chlorophyll and carotenoids that occurred in the leaves of eight day old etiolated pea seedlings when transferred to continuous illumination is shown in Figure 10a. In agreement with previous investigations (Goodwin, 1958; Lichtenthaler, 1969) etiolated leaves were shown to contain appreciable levels of carotenoid pigments. No chlorophyll was formed until seedlings were illuminated, reflecting the light requirement for the accumulation of protochlorophyll, and for its conversion to chlorophyll (Castelfranco and Beale, 1981). The

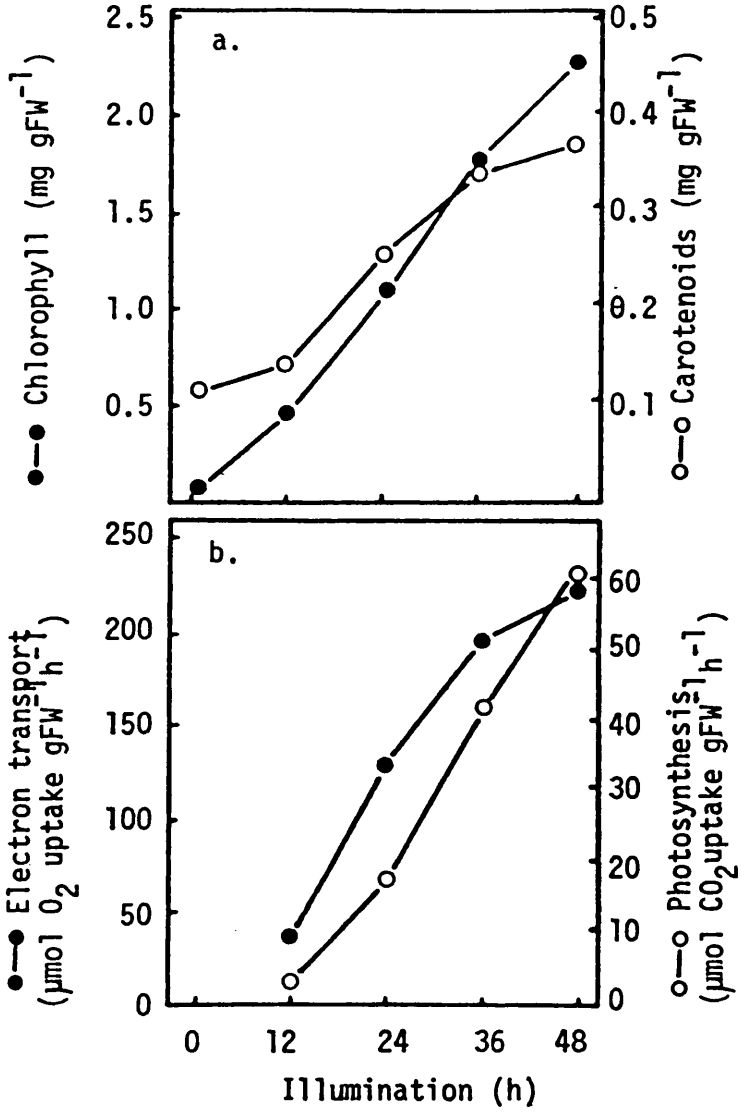


Fig 10. The effect of illumination on chlorophyll and carotenoid levels (a) and photosynthetic activity (b) in etiolated pea leaves.

Table 4. The ratio of chlorophyll to carotenoid during the greening of etiolated pea leaves.

Hours illumination	Chl: Car ratio
0.1	0.336
12	3.36
24	4.56
36	5.07
48	6.28

Table 5. Changes in the photosynthetic activity of thylakoid membranes isolated from etiolated and greening pea leaves.

Hours illumination.	P/S electron flow ($H_2O \rightarrow PQ$) $\mu mol\ O_2 mg^{-1} chl\ h^{-1}$	$\mu mol\ O_2\ gFW^{-1} h^{-1}$
0.1	0	0
12	78.3	36.7
24	110.8	129.6
36	109.3	192.4
48	108.0	222.8

relationship between chlorophyll and carotenoid levels during greening is shown in Table 4. During the first 12 hours of illumination there was a ten-fold increase in the ratio of chlorophyll to carotenoid. Subsequently the ratio of chlorophyll to carotenoid increased to approach that of mature pea leaves (5-6 : 1).

2.2 Photosynthetic activity

The development of photosynthetic electron flow and CO_2 exchange is shown in Figure 10b. Both photosynthetic processes were active after 12 hours illumination and subsequently increased in parallel with chlorophyll accumulation. When the rate of photosynthetic electron flow in greening leaves was calculated per unit of chlorophyll, no marked change in activity was detected after 24 hours illumination (Table 5). This indicates that the increase in photosynthetic electron flow shown in Figure 10b was due to the accumulation of light harvesting chlorophyll.

2.3 Superoxide and hydrogen peroxide scavenging systems

The results presented in Figure 11 show the activity of SOD, ascorbate peroxidase and glutathione reductase, and the level of ascorbate in leaf homogenates from etiolated and greening pea leaves. Etiolated leaves contained these enzymes and ascorbate. Transfer to light promoted a marked increase in ascorbate peroxidase activity and ascorbate levels during the first 12 hours illumination (Figure 11a). The activity of glutathione reductase and SOD did not change throughout the greening period (Figure 11b).

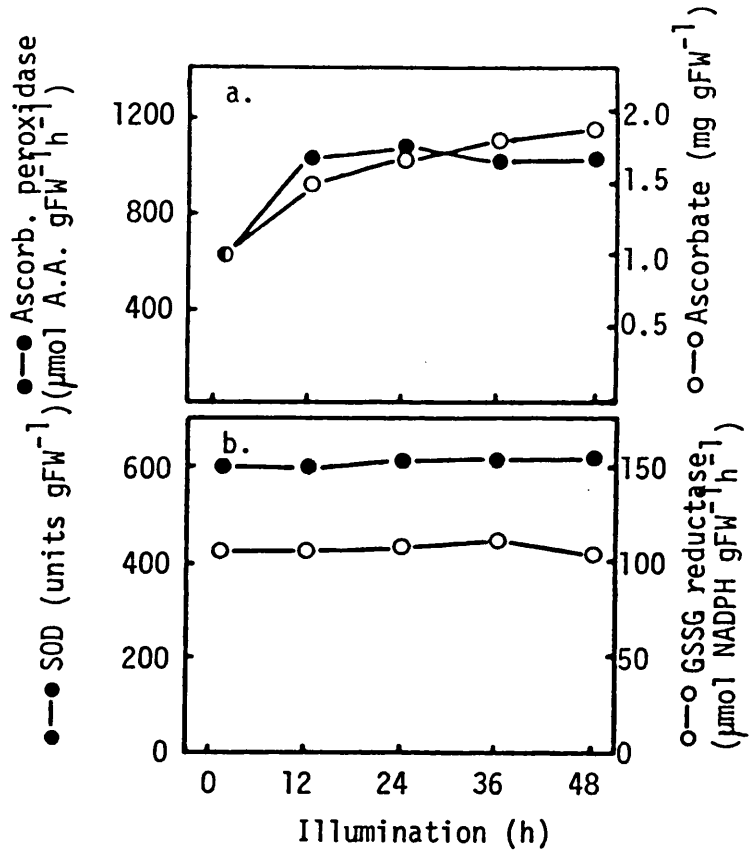


Fig 11. The effect of illumination on ascorbate peroxidase and ascorbate (a) and SOD and glutathione reductase levels (b) in etiolated leaves

2.4 Discussion

Although the greening of etiolated seedlings is a somewhat artificial experimental system, it does provide a convenient and commonly used method for studying chloroplast development. Seedlings probably encounter a certain degree of etiolation during their early growth through soil prior to their emergence at the surface. It is important however that the development of the photosynthetic apparatus occurs rapidly on emergence into the light, so as to remove the dependence on stored reserves. Several aspects of the development of etioplasts into chloroplasts are probably significant in restricting photo-oxidative damage during the early stages of greening. During this period several hours may elapse between the appearance of chlorophyll and the detection of functional electron transport activity (Bradbeer, 1981). In this period carotenoid pigments, which are present in etiolated leaves, would have an important role in protecting chloroplasts against photo-oxidative damage. The importance of carotenoids in protecting seedlings against photosensitized reactions during greening has been clearly demonstrated elsewhere. Anderson and Robertson (1960) demonstrated that in maize mutants lacking carotenoids, chlorophyll was rapidly bleached on exposure to high light intensities. Such mutants retained pigments if they were maintained in darkness or illuminated in an atmosphere containing no oxygen. Similar results have been observed with plants treated with carotenoid synthesis inhibitor herbicides (Bartels and Watson, 1978; Ridley and Ridley, 1979). The results presented in Figure 12 demonstrate the effect of fluridone, a carotenoid synthesis inhibitor herbicide, on the accumulation of chlorophyll in etiolated peas during their subsequent illumination at low light intensity (50 μmol $m^{-2}s^{-1}$ photon flux density). No chlorophyll accumulation occurred

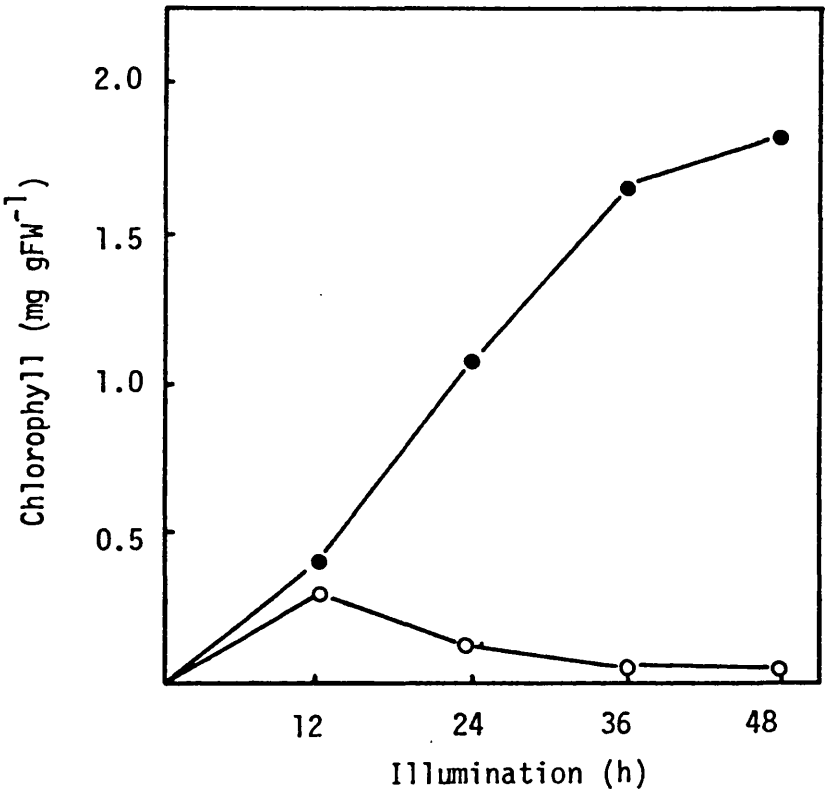


Fig 12. Chlorophyll accumulation in etiolated pea leaves sprayed with H_2O (●–●) or 10μM fluridone (o–o) and illuminated for up to 48hours at low light intensity.

in plants sprayed with fluridone (10 μM, 50 ml per 100 x 210 mm tray) immediately prior to the transfer of etiolated plants to light (Figure 12). This observation, and the high ratio of carotenoid to chlorophyll observed during the early hours of greening (Table 4), indicates that carotenoids are important in preventing photo-oxidation of chlorophyll before photosynthetic electron flow is fully functional. The data in Figure 12 also illustrates that carotenoid synthesis must be maintained, following the transfer of etiolated plants to light, for greening to occur.

Much of the light harvesting chlorophyll was not synthesised until photosynthetic activity had developed (Table 5, Bradbeer, 1981). While this may reflect a role for photosynthesis in supplying substrates necessary for chlorophyll synthesis (Dodge *et al.*, 1971), it would also restrict the potential for damaging photosensitized reactions. If chlorophyll were present in developing chloroplasts in excess of the level required to saturate photosynthesis, potentially lethal energy spillover from chlorophyll to singlet oxygen would occur.

Photosynthetic electron transport activity, and therefore the potential to reduce oxygen to $O_2^{\cdot -}$ by photosystem I, was detected after 12 hours greening (Table 5). In mature chloroplasts electron flow to oxygen may be an important reaction of photosynthesis to generate ATP in the absence of $NADP^+$ reduction (Halliwell, 1981; Furbank, 1984). Electron flow to oxygen during photosynthesis may also be important to dissipate photochemical energy under conditions when the rate of CO_2 assimilation is low (Furbank, 1984). A similar mechanism of energy dissipation may occur during the early stages of greening, when the rate of photosynthetic electron flow per unit of chlorophyll is high, but the dark reactions of photosynthesis are not fully functional

(Popovic *et al.*, 1984). Such a mechanism of energy dissipation requires that chloroplast $O_2^{\cdot -}$ and H_2O_2 scavengers are present very early in chloroplast development. SOD and chloroplast enzymes that scavenge H_2O_2 were present in etiolated pea leaves and throughout greening. The chloroplast would therefore be well protected against damaging $O_2^{\cdot -}$ and H_2O_2 during its early development.

3. Active oxygen species and leaf senescence

The senescence of leaves proceeds in a specific, genetically controlled pattern, characterised by a decline in photosynthetic activity, a fall in chlorophyll, protein and nucleic acid levels, and a loss of membrane integrity (Thomas and Stoddart, 1980; Woolhouse, 1982). However the mechanism of senescence induction is not well understood. Theories accounting for the initiation of senescence include changing hormonal control, competition for nutrient or environmental change (Thomas and Stoddart, 1980). Increased accumulation of free radicals in ageing leaves has also attracted recent attention (Leshem, 1981). Woolhouse (1984) postulated that deterioration of thylakoid membrane proteins in senescent leaves led to increased formation of $O_2^{\cdot -}$, which contributed to the degradation of chlorophyll and membrane lipids. If $O_2^{\cdot -}$ or H_2O_2 derived from $O_2^{\cdot -}$ dismutation are important in controlling the degradation that occurs in ageing leaves, chloroplasts as potential sites of oxygen radical formation may have an important role in the initiation of senescence. The activity of enzymes that scavenge $O_2^{\cdot -}$ and H_2O_2 in chloroplasts would also be important in the control of senescence. In this study photosynthetic activity and the level of chloroplast $O_2^{\cdot -}$ and H_2O_2 scavengers have been monitored during the ageing of attached flax cotyledons and isolated pea leaf discs.

3.1 Senescence and regreening in flax cotyledons

Cotyledons excised from 5, 10, 15 and 20 day old flax seedlings were analysed for pigment levels, photosynthetic activity and enzyme levels, and the results presented in Figures 13 and 14. For regreening experiments young shoots above the senescent cotyledons were removed and cotyledon activities monitored at 2 and 4 days regreening.

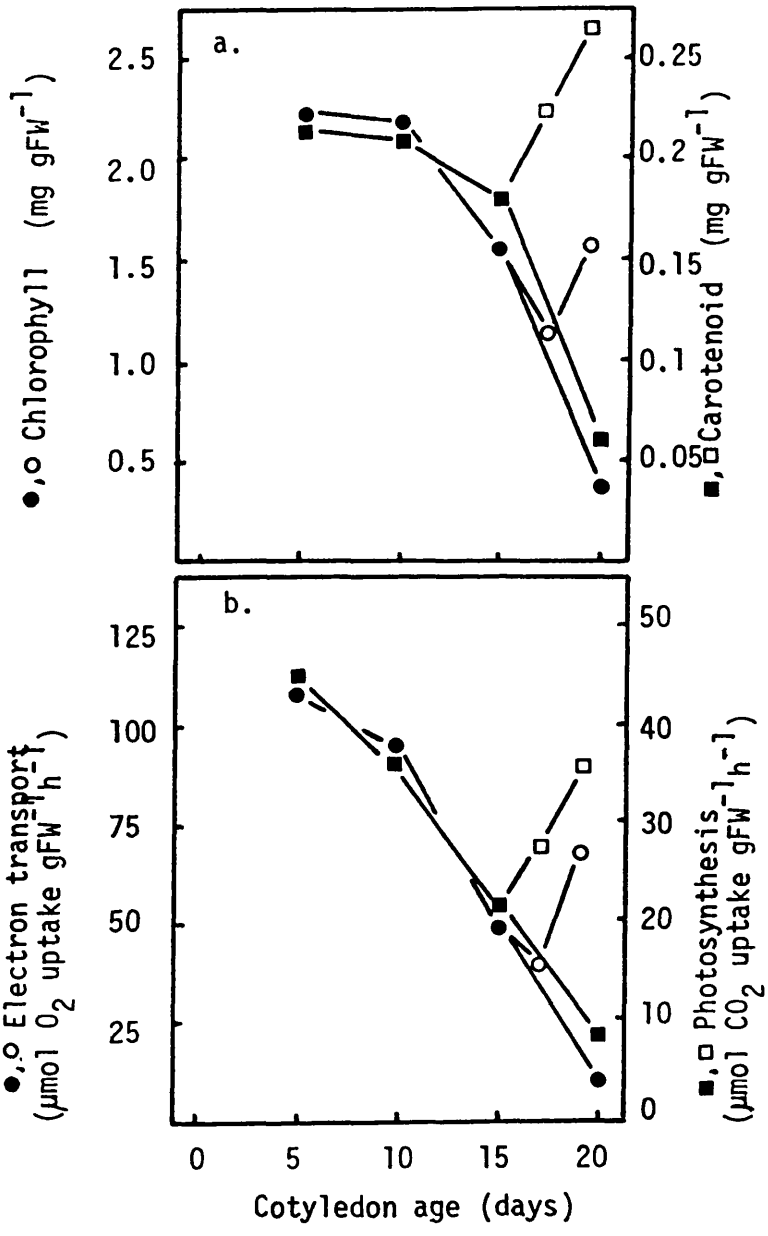


Fig 13. Pigment levels (a) and photosynthetic activity (b) during the senescence (●,■) and regreening (○,□) of flax cotyledons.

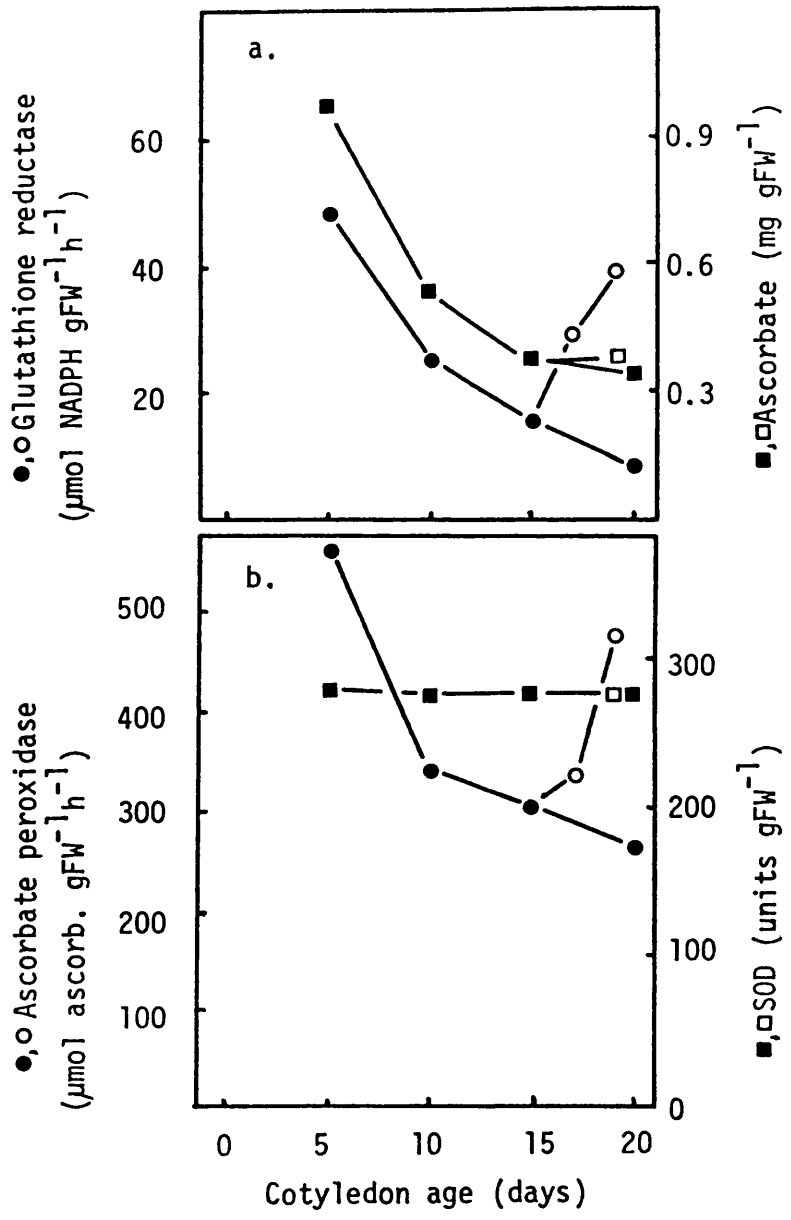


Fig 14. Ascorbate and glutathione reductase (a) and ascorbate peroxidase and SOD (b) during the senescence (●,■) and regreening (○,□) of flax cotyledons.

3.1.1 Pigments and photosynthetic activity

Chlorophyll and carotenoid levels in flax cotyledons declined after 10 days growth(Figure 13a). Photosynthetic activity of senescent flax cotyledons is shown in Figure 13b. Photosynthetic electron flow and CO_2 exchange declined progressively from 5 days. Removal of shoots above senescent cotyledons after 15 days stimulated accumulation of chlorophyll and carotenoid and restoration of photosynthetic activity.

3.1.2 Superoxide and hydrogen peroxide scavengers

Changes in the activity of SOD, ascorbate peroxidase and glutathione reductase and levels of ascorbate in ageing flax cotyledons are shown in Figure 14. The levels of ascorbate peroxidase, glutathione reductase and ascorbate declined between 5 and 10 days. This preceeded the loss of pigments or photosynthetic activity. No loss of SOD activity was detected during flax cotyledon senescence. Regreening of flax cotyledons was accompanied by increased ascorbate peroxidase and glutathione reductase activity. No change in SOD or ascorbate levels occurred during regreening.

3.2 Senescence in pea leaf discs

Discs cut from the sub-apical leaves of bean plants were floated on distilled water in glass petri dishes under constant illumination for up to nine days. Discs were analysed at 24 hour intervals for pigments, photosynthetic activity and levels of chloroplast $O_2^{\cdot -}$ and H_2O_2 scavengers.

3.2.1 Pigment levels and photosynthetic activity

The results presented in Figure 15 show the changes in chlorophyll

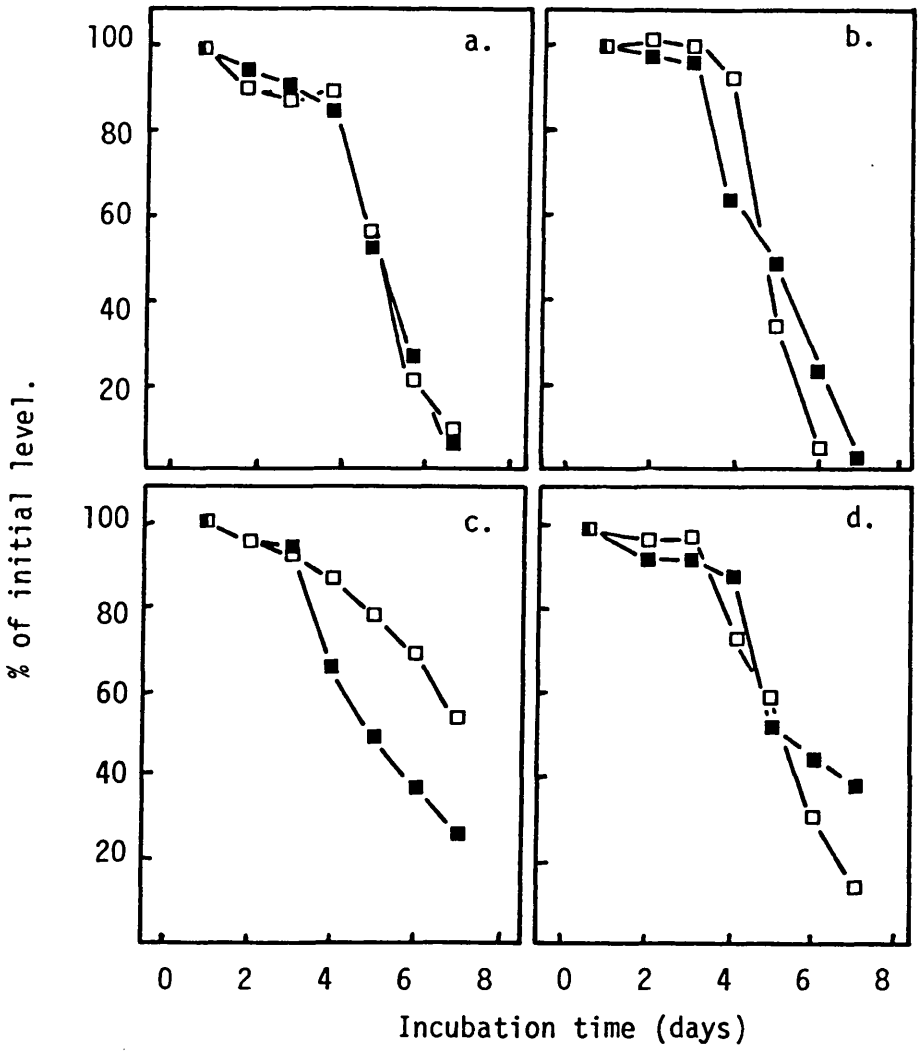


Fig 15. Cellular disruption in ageing pea leaf discs. Leaf discs were incubated on H_2O for up to 8 days, and cellular breakdown was assessed as loss of:

a. chlorophyll (■–■) and carotenoid (□–□).

b. Photosynthetic electron flow (■–■) and CO_2 exchange (□–□).

c. Ascorbate peroxidase (■–■) and SOD (□–□).

d. Ascorbate (■–■) and GSSG reductase (□–□).

and carotenoid levels (Figure 15a) and photosynthetic electron flow and CO_2 exchange (Figure 15b) during the ageing of pea leaf discs. Chlorophyll and carotenoid levels showed no marked change during the first four days incubation, but then declined rapidly. Photosynthetic electron flow in thylakoids isolated from ageing leaf discs declined in parallel to chlorophyll. Photosynthetic CO_2 exchange of pea leaf discs showed a marked loss of activity between three and four days incubation, prior to the decline in leaf pigments or photosynthetic electron flow. This may reflect the loss of Calvin cycle enzymes that occurs during leaf senescence (Woolhouse, 1982).

3.2.2 Superoxide and hydrogen peroxide scavengers

The levels of SOD, ascorbate peroxidase, glutathione reductase and ascorbate during the ageing of pea leaf discs are shown in Figure 15 (c and d). Ascorbate peroxidase (Figure 15c) and ascorbate (Figure 15d) declined after three days incubation. Marked loss of glutathione reductase (Figure 15d) was not detected until after four days incubation. SOD activity (Figure 15c) declined progressively throughout the incubation period.

3.3 The role of light and oxygen in pigment and protein breakdown

The effect of incubating pea leaf discs for up to nine days under different light intensities (100, 200 or 400 µmol $m^{-2}s^{-1}$ photon flux density) on their chlorophyll and leaf soluble protein content is shown in Figure 16 (a and b). The loss of chlorophyll and protein was dependent on light intensity.Stronger light intensities promoted their degradation, while incubation at lower light intensities retarded the loss of chlorophyll and protein.

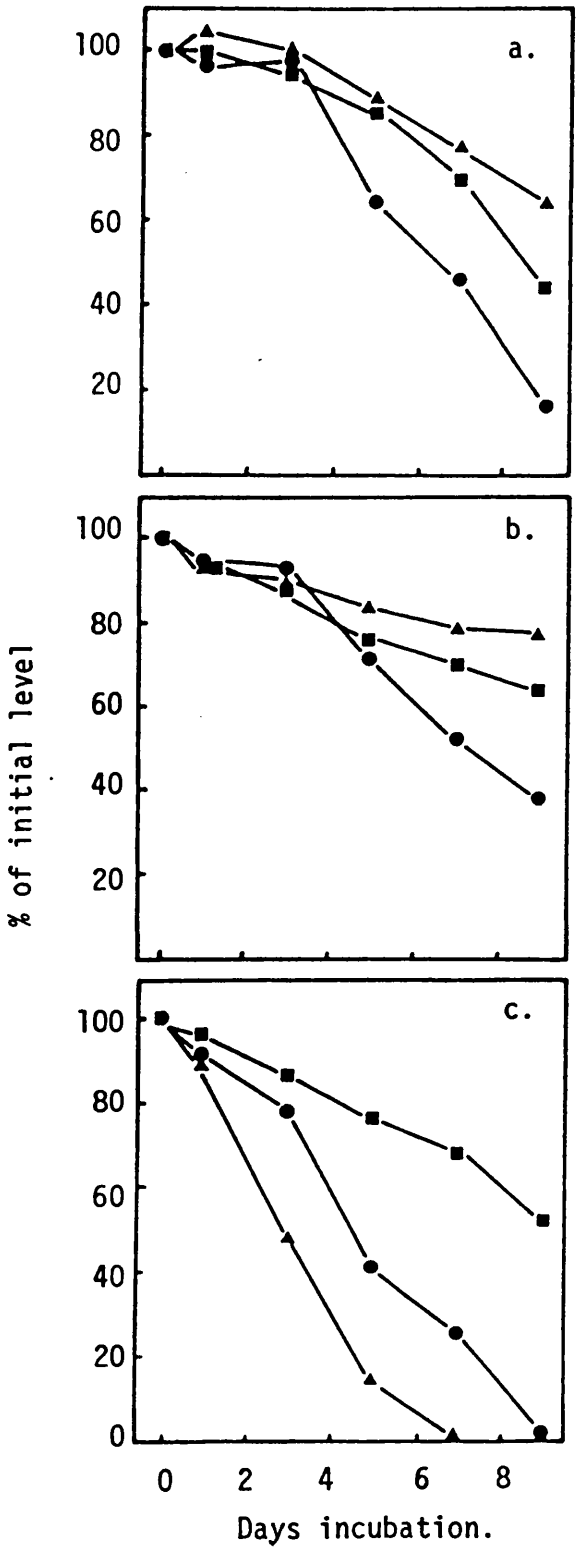


Fig 16. The role of light and oxygen in pea leaf disc senescence. Chlorophyll bleaching (a) or protein loss (b) at 100 (▲), 200 (■) or 400 (●) μmol $m^{-2}s^{-1}$ PFD, or chlorophyll bleaching (c) of discs incubated under air (●), N_2 (■) or O_2 (▲).

Chlorophyll bleaching in leaf discs incubated in sealed vials flushed with air, N_2 or O_2 at the start of the experiment, and subsequently incubated under constant illumination is shown in Figure 16c. Chlorophyll breakdown was enhanced by incubation under an oxygen enriched atmosphere, but delayed by incubation under nitrogen.

3.4 Discussion

Chloroplasts from higher plants may form $^1O_2$, $O_2^{\cdot -}$ and H_2O_2 as a consequence of photosynthetic electron flow. If leaf senescence is initiated or promoted by these active oxygen species (Dhindsa *et al.*, 1981; McRae and Thompson, 1983), chloroplasts as a major potential site of their formation, might be expected to show the first signs of cellular disruption. Ultrastructural studies have shown that the loss of chloroplast thylakoid structure in senescent leaves preceeded the degradation of other organelles (Butler, 1967). Furthermore, protein synthesis in chloroplasts of senescent *Perilla* leaves declined prior to cytoplasmic protein synthesis (Callow *et al.*, 1972). The activity of chloroplast stromal enzymes in wheat leaves declined from full leaf expansion, although cytoplasmic enzyme activities remained high until the latter stages of senescence (Camp *et al.*, 1984). These results are consistent with leaf senescence being first manifest in chloroplasts.

An indication that photo-oxidative mechanisms contributed to the cellular disruption occurring in ageing leaves was shown in Figure 16. Light and oxygen, either alone or as a component of air, promoted the loss of chlorophyll and protein from pea leaf discs. Previous studies have shown that protein degradation in ageing oat leaf segments was promoted by oxygen and retarded by nitrogen (Salter and Thimann, 1983). Chlorophyll bleaching of sycamore leaves was promoted by light

(Maunders and Brown, 1983). Senescence in maize and rice leaves was enhanced by H_2O_2 but delayed by antioxidants (Mondal and Choudhuri, 1981, 1982). These studies indicate that photo-oxidative degradation contributes to cellular disruption in ageing leaves.

One mechanism which may account for the increased photo-oxidative damage in senescent leaves is for a reduction in the capacity of leaves to scavenge active oxygen species during senescence. In ageing pea leaf discs loss of $O_2^{\cdot -}$ and H_2O_2 scavengers occurred slightly in advance of, or at the same time as the decline in photosynthetic activity and leaf pigments. In flax cotyledons however there was a marked loss of ascorbate peroxidase, ascorbate and glutathione reductase early in senescence. A decline in SOD and catalase activity has also been correlated with the onset of leaf senescence in tobacco (Dhindsa *et al.*, 1981). Reduced capacity to scavenge $O_2^{\cdot -}$ and H_2O_2 in ageing leaves may contribute to an accumulation of free radicals, and therefore photo-oxidative cellular disruption. A decline in enzyme synthesis, coupled with increased protein hydrolysis has been identified by some workers as an important event in the initiation of leaf senesence (Martin and Thimann, 1972; Thomas, 1976). The loss of activity of enzymes that scavenge $O_2^{\cdot -}$ and H_2O_2 in chloroplasts observed in this study was probably one consequence of a redirection of protein metabolism in senescent leaves. The accumulation of free radicals and active oxygen species in senescent leaves would therefore be a consequence of the onset of leaf senescence rather than the initiating step.

Alternatively photo-oxidative damage to chlorophyll, protein and membrane lipids may occur as a consequence of increased formation

of active oxygen species in senescent leaves. Disruption of the photosynthetic apparatus leading to inhibition of CO_2 exchange or photosynthetic electron flow may promote the formation of $O_2^{\cdot -}$ or $^1O_2$. McRae and Thompson (1983) observed that the production of $O_2^{\cdot -}$ by illuminated chloroplasts isolated from bean leaves increased four fold during the early stages of leaf senescence. This coincided with the initiation of membrane disruption. Increased $O_2^{\cdot -}$ formation in chloroplasts together with the observed decline in the activity of enzymes that scavenge $O_2^{\cdot -}$ and H_2O_2 in pea and flax leaves in this study would contribute to an increased rate of photo-oxidative degradation.

While photo-oxidative reactions occurring through increased formation of active oxygen species, or a decline in scavenger enzyme activities may contribute to cellular disruption in ageing leaves, it is difficult to assess their significance. Leaf senescence is clearly a very organised process, as indicated by the controlled reversal that occurs when shoots are removed above senescent leaves, or by the application of cytokinins (Dhindsa *et al*., 1982; Venkatarayappa *et al*., 1984). Such treatments, as shown in this study, promoted pigment and protein synthesis and restoration of photosynthetic activity. These events are in marked contrast to the damage that occurs when leaves are treated with certain herbicides that promote the formation of $^1O_2$, $O_2^{\cdot -}$ and H_2O_2 (Harris and Dodge, 1972; Pallett and Dodge, 1979, 1980; Chia *et al*., 1982). Such herbicides induced extensive cellular disruption that was essentially irreversible. If photosynthetically derived activated oxygen species are important in cellular degradation in senescent leaves, their formation and accumulation must be carefully controlled.

4. Seasonal variations and control by light of chloroplast superoxide and hydrogen peroxide scavengers from pea leaves

Changes in the levels of SOD, ascorbate peroxidase, glutathione reductase, ascorbate and glutathione have been observed in higher plants in response to a variety of environmental stresses or air pollutants (Tanaka and Sugahara, 1980; Tanaka *et al.*, 1982a, 1985; de Kok and Oosterhuis, 1983; Gamble and Burke, 1984). In other sections of this thesis, changes in the levels of chloroplast $O_2^{\cdot -}$ and H_2O_2 scavengers have been observed in pea leaves during greening and leaf senescence or in response to the herbicide paraquat (Section 8). As a consequence of the routine analysis of pea plants grown under glasshouse conditions at different times of the year, a seasonal variation in the levels of ascorbate peroxidase, glutathione reductase and ascorbate has been observed, which is reported here.

4.1 Seasonal variations in activity

The results of analysis of the youngest fully expanded (sub-apical leaves of pea plants grown under glasshouse conditions between April 1984 and May 1985 are shown in Figures 17 and 18. All data points are the means of three separate analyses from different batches of leaves. Levels of ascorbate peroxidase, ascorbate and glutathione reductase (Figure 17) showed marked seasonal variation, with a peak in early summer (May - June) and a sharp decline in winter (Dec - Jan). The sampling procedure entailed using the youngest fully expanded leaves to prepare homogenates for analysis. Changes in ascorbate, ascorbate peroxidase and glutathione reductase levels between summer and winter were therefore not due to leaf samples being of different ages. The activity of SOD and chlorophyll and carotenoid levels between

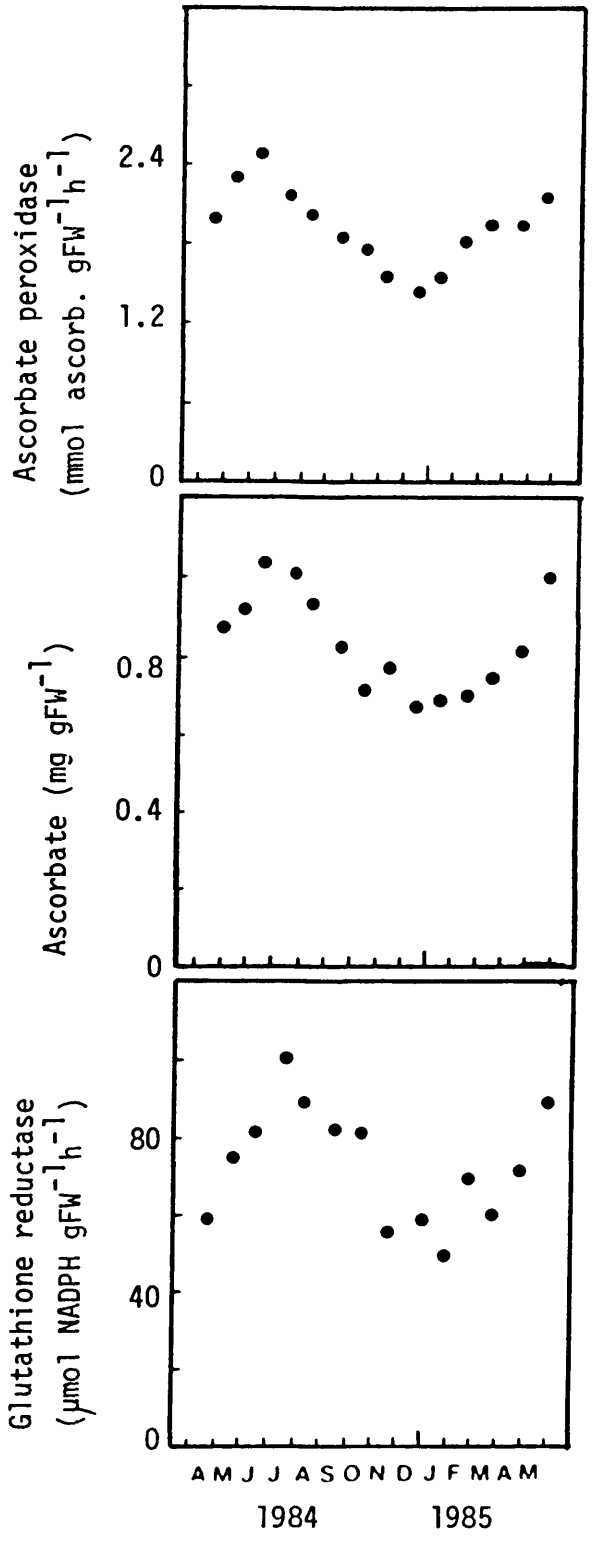


Fig 17. Ascorbate peroxidase, ascorbate and glutathione reductase levels in pea leaves grown under glasshouse conditions between April 1984 and May 1985.

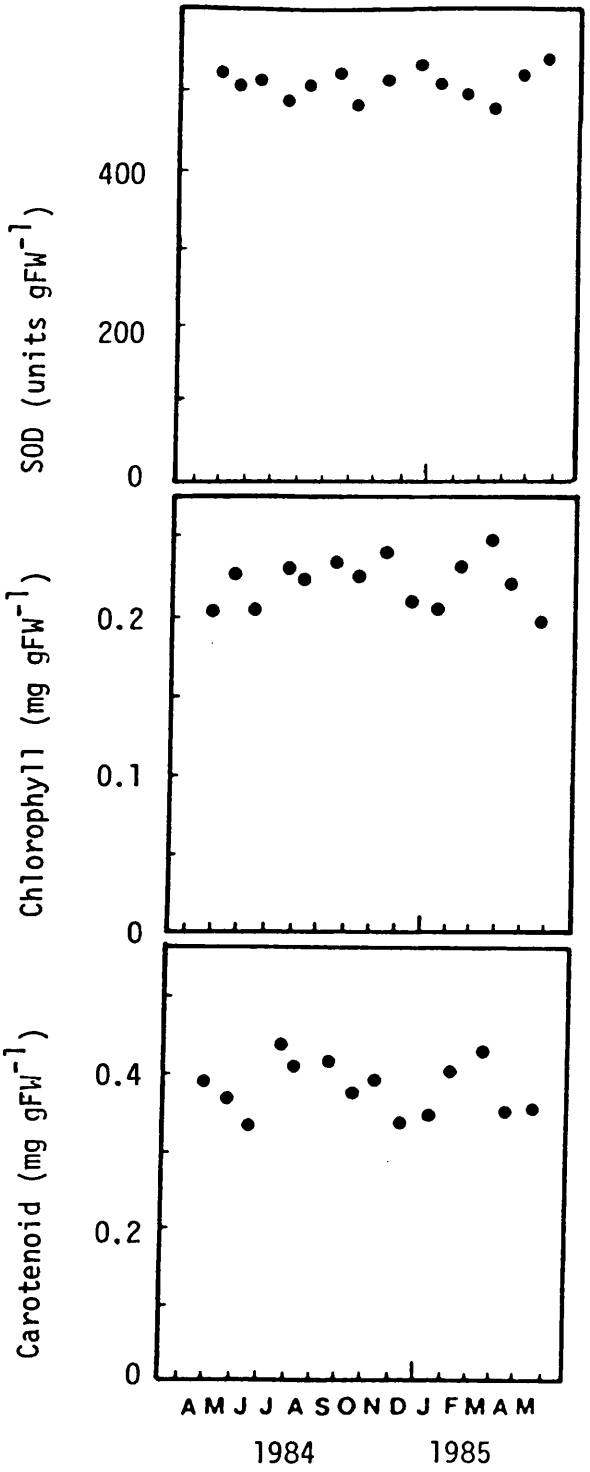


Fig 18. SOD, chlorophyll and carotenoid levels in the leaves of pea plants grown under glasshouse conditions between April 1984 and May 1985.

April 1984 and May 1985 are shown in Figure 18. Although there was some variation in their levels throughout the year, there was no marked fluctuation between summer and winter.

A high proportion of leaf SOD, ascorbate peroxidase and glutathione reductase is present in chloroplasts. To demonstrate that seasonal variations in the leaf activity of these enzymes occurred in chloroplasts, these organelles (75% intact) were isolated from pea plants grown under glasshouse conditions in Feb. 1985 and July 1985. Levels of SOD, ascorbate peroxidase, glutathione reductase and ascorbate were then assessed (Table 6). No difference in SOD activity was detected between the two samples of chloroplasts. Chloroplasts isolated from plants grown in July contained increased levels of ascorbate, ascorbate peroxidase and glutathione reductase. These results show a similar trend to those presented in Figure 17.

One environmental factor likely to have influenced chloroplast ascorbate and enzyme levels was light. The data in Figure 19 shows the mean daily irradiance for each month between April 1984 and May 1985 falling at Long Ashton Research Station (15 miles WNW of Bath University). Although the variation in total irradiation between summer and winter was greater than the variations in enzyme and ascorbate levels shown in Figure 17, the pattern of change appears similar. Growth in early summer, the period of highest mean daily irradiance, corresponded with the highest levels of ascorbate peroxidase, ascorbate and gluathione reductase. By contrast the low levels of these enzymes and of ascorbate as a consequence of growth in winter matched the period of lowest mean daily irradiance.

Table 6. Activity of enzymes that scavenge $O_2^{\cdot -}$ and H_2O_2, and ascorbate levels, in pea chloroplast (75% intact) isolated from plants grown under glasshouse conditions in February or July 1985.

	Activity. (μmol mg^{-1}chl h^{-1})	
	Feb 1985	July 1985
Ascorbate peroxidase	792	1185
Glutathione reductase	24.9	34.6
SOD[1]	142.5	148.5

1 Activity expressed as units mg^{-1}chl.

	Level (μmol mg^{-1}chl)	
Ascorbate	0.35	0.55

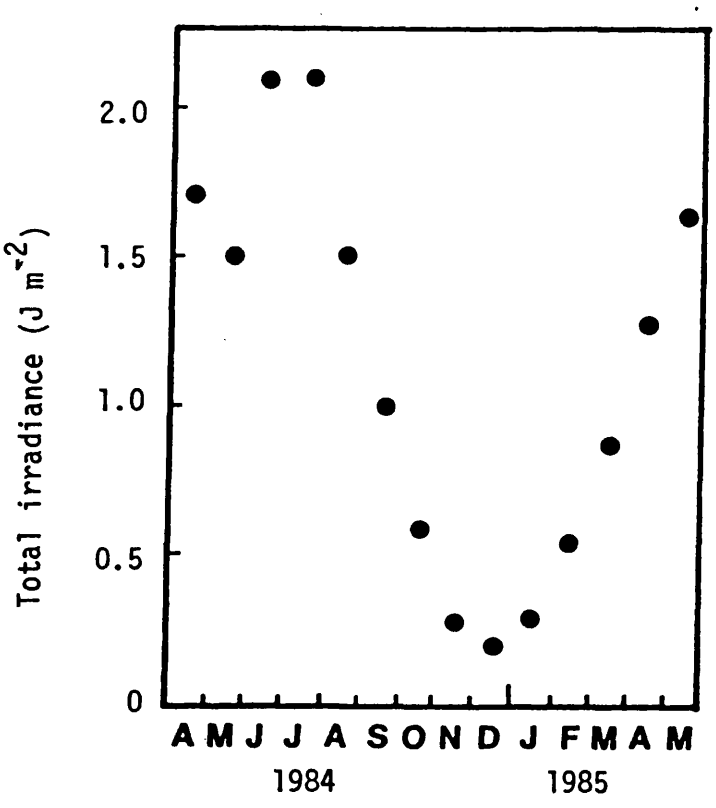


Fig 19. Mean total daily irradiance falling at Long Ashton Research Station for each month between April 1984 and May 1985.

4.2 Control by light

The effect of light intensity on the activity of chloroplast $O_2^{\cdot -}$ and H_2O_2 scavenging systems was investigated in pea plants grown in a constant environment chamber for 14-21 days under continuous illumination of 100 or 400 μmol $m^{-2}s^{-1}$ photon flux density. Chloroplasts were isolated and analysed for SOD, ascorbate peroxidase, dehydroascorbate reductase and glutathione reductase activity and levels of ascorbate and glutathione (Table 7). No difference was detected in chloroplast SOD activity or glutathione levels between the two batches of plants. Plants grown at the higher light intensity contained enhanced levels of ascorbate peroxidase, glutathione reductase, dehydroascorbate reductase and ascorbate. These results indicate that light intensity during growth may have a significant role in regulating the chloroplast activity of $O_2^{\cdot -}$ and H_2O_2 scavengers.

4.3 Discussion

The results presented here indicate that seasonal variations in ascorbate, ascorbate peroxidase and glutathione reductase levels in pea leaves and chloroplasts may have been caused by changes in monthly mean daily irradiance between summer and winter. Control of chloroplast antioxidant protective mechanism levels by light was confirmed by studies of plants grown in a controlled environment chamber. Light intensity has a strong influence on chloroplast structure and function. Adaptations that favour growth at high or low light intensity include changes in chloroplast orientation, the degree of thylakoid stacking, levels of chlorophyll and light harvesting pigment protein complexes and the level of electron transport intermediates (Boardman, 1977; Lichtenthaler, 1983). Light intensity can also influence the level

Table 7. The effect of growth at 100 or 400μmol $m^{-2}s^{-1}$ photon flux density on the activity of enzymes that scavenge $O_2^{\cdot-}$ and H_2O_2 (a) and level of antioxidants (b) in isolated pea chloroplasts (80-85% intact).

	Activity (μmol mg^{-1}chl h^{-1})	
	100	400
Superoxide dismutase (1)	160.7	170.8
Ascorbate peroxidase	542.2	925.4
Glutathione reductase	28.1	44.5
Dehydroascorbate reductase	37.2	58.5

(1) Activity of SOD expressed as units mg^{-1}chl

	Level (μmol mg^{-1}chl)	
	100	400
Ascorbate	0.29	0.48
Glutathione	0.085	0.86

and activity of chloroplast carboxylative enzymes (Huffaker *et al.*, 1966; Hatch *et al.*, 1969). One consequence of these adaptations is that plants grown under strong light show high maximum photosynthetic rates, whereas low light adapted or shade plants with a well developed light harvesting apparatus show greater photosynthetic efficiency at low light intensities. The increased chloroplast levels of ascorbate, ascorbate peroxidase and glutathione observed in this study in plants grown at a moderate compared with low light intensity, or in summer, may permit high photosynthetic rates whilst ensuring that chloroplasts are well protected against damaging $O_2^{\cdot -}$ and H_2O_2.

The effect of daylength on levels of chloroplast $O_2^{\cdot -}$ and H_2O_2 scavenger enzymes was not investigated. Daylength, through photoperiodic control by phytochrome, affects many aspects of plant growth and development, including germination, flowering and leaf fall (Hillman, 1969). Phytochrome may also control the activity of many leaf enzymes (Schopfer, 1977). It remains to be determined whether seasonal variations in chloroplast ascorbate, ascorbate peroxidase and glutathione reductase observed in pea leaves in this study were in response to light intensity, daylength or total light received. While light may have contributed to changes in levels of chloroplast $O_2^{\cdot -}$ and H_2O_2 scavengers in pea leaves between summer and winter, it is possible that annual fluctuations in temperature, water status or air pollutants may also exert some control. Reports in the literature indicate that low temperature, drought, O_3, SO_2 or increased oxygen tensions affect ascorbate, glutathione, ascorbate peroxidase, glutathione reductase or SOD levels in leaves (Grill *et al.*, 1979; Tanaka and Sugarhara, 1980; Foster and Hess, 1980; McKersie *et al.*, 1982; Guy and Carter, 1984; Gamble and Burke, 1984; Tanaka *et al.*, 1985). Clearly a wide variety of

environmental factors can influence the levels of chloroplast $O_2^{\cdot -}$ and H_2O_2 scavenging enzymes and antioxidants. Great care must therefore be taken in the growth of plant material for such investigations.

5. Photo-oxidative damage in chloroplasts: The action of light activated herbicides

Many herbicides are known to interact with photosynthesis inducing photo-oxidative damage to plants. These include electron transport inhibitors such as triazines, ureas or uracil compounds, bipyridyl compounds such as paraquat and diquat, which promote oxygen reduction by photosystem I, and diphenyl ether (DPE) compounds that may inhibit electron flow or promote photosystem I oxygen reduction (Dodge, 1983; Ridley, 1983; Böger, 1984). The mode of action of DPE herbicides however remains unclear. In addition photosensitizers such as rose bengal and xanthene dyes induce light and oxygen dependent damage to leaves and chloroplasts (Knox and Dodge, 1984, 1985). Damage induced by these compounds probably occurs because increased formation of $O_2^{\cdot -}$, H_2O_2 and $^1O_2$ exceeds the capacity of chloroplast protective mechanisms to scavenge them (Dodge, 1983). Damage induced to pea leaves and chloroplasts by monuron (electron flow inhibitor), paraquat (bipyridyl) and rose bengal (photosensitizer) has been compared here to that induced by the DPE herbicides acifluorfen and oxyfluorfen.

5.1 Assessment of herbicide injury

Damage to plant tissues induced by paraquat, monuron, rose bengal and acifluorfen was assessed in leaf discs, cut from sub-apical leaves of 14-21 day old pea plants, incubated on 25 μM herbicide solutions in glass petri dishes or sealed vials under constant illumination (300 μmol $m^{-2}s^{-1}$ photon flux density). Damage was monitored by following photosynthetic CO_2 exchange, chlorophyll bleaching, electrolyte leakage and ethane evolution from leaf discs. These results are presented in Figures 20 and 21. Paraquat and monuron inhibited photosynthesis

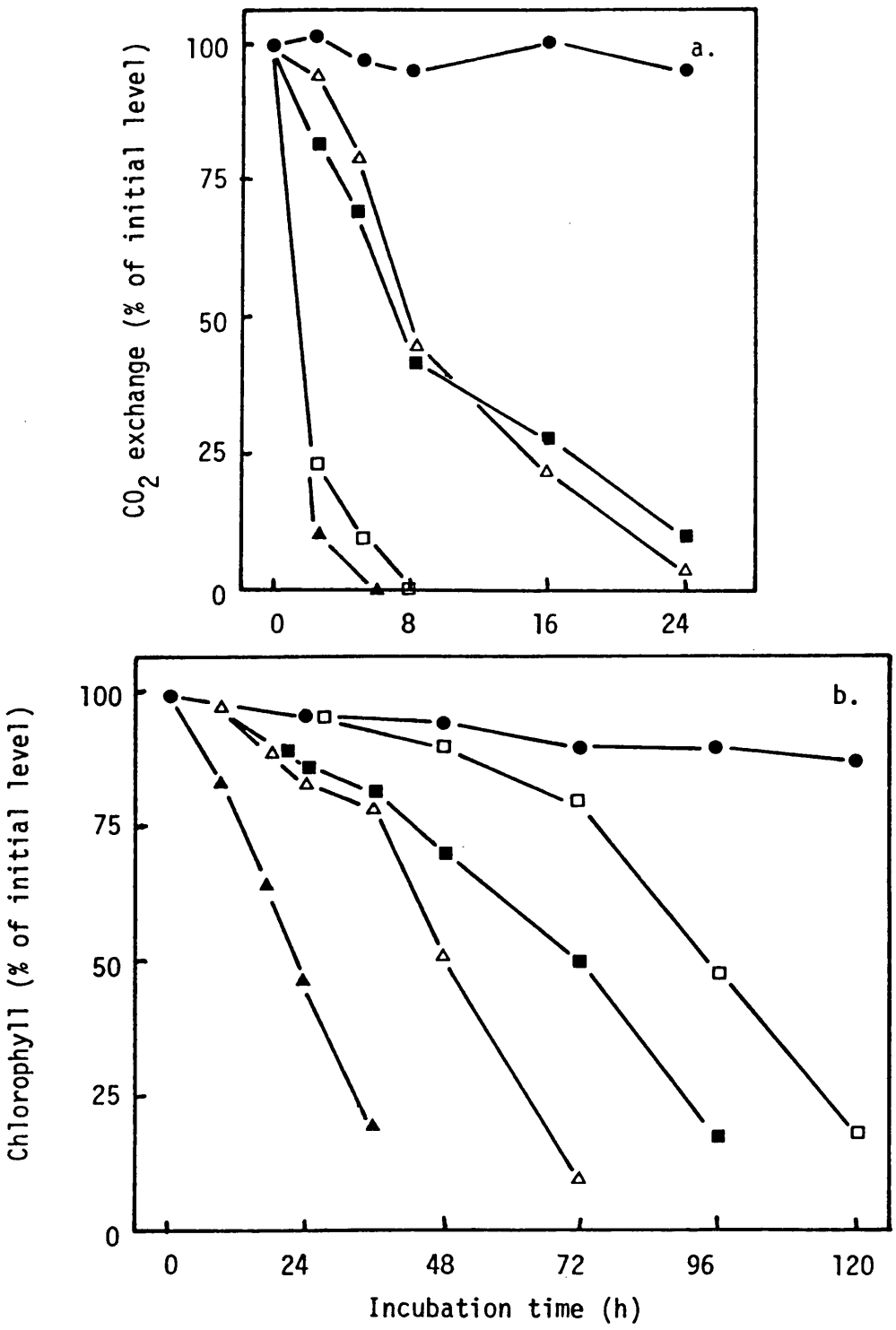


Fig 20. The effect of 25μM paraquat (▲), monuron (□), acifluorfen (△) and rose bengal (■) on photosynthetic CO_2 exchange (a) and chlorophyll bleaching (b) in pea leaf discs. (control treatment: ●—●)

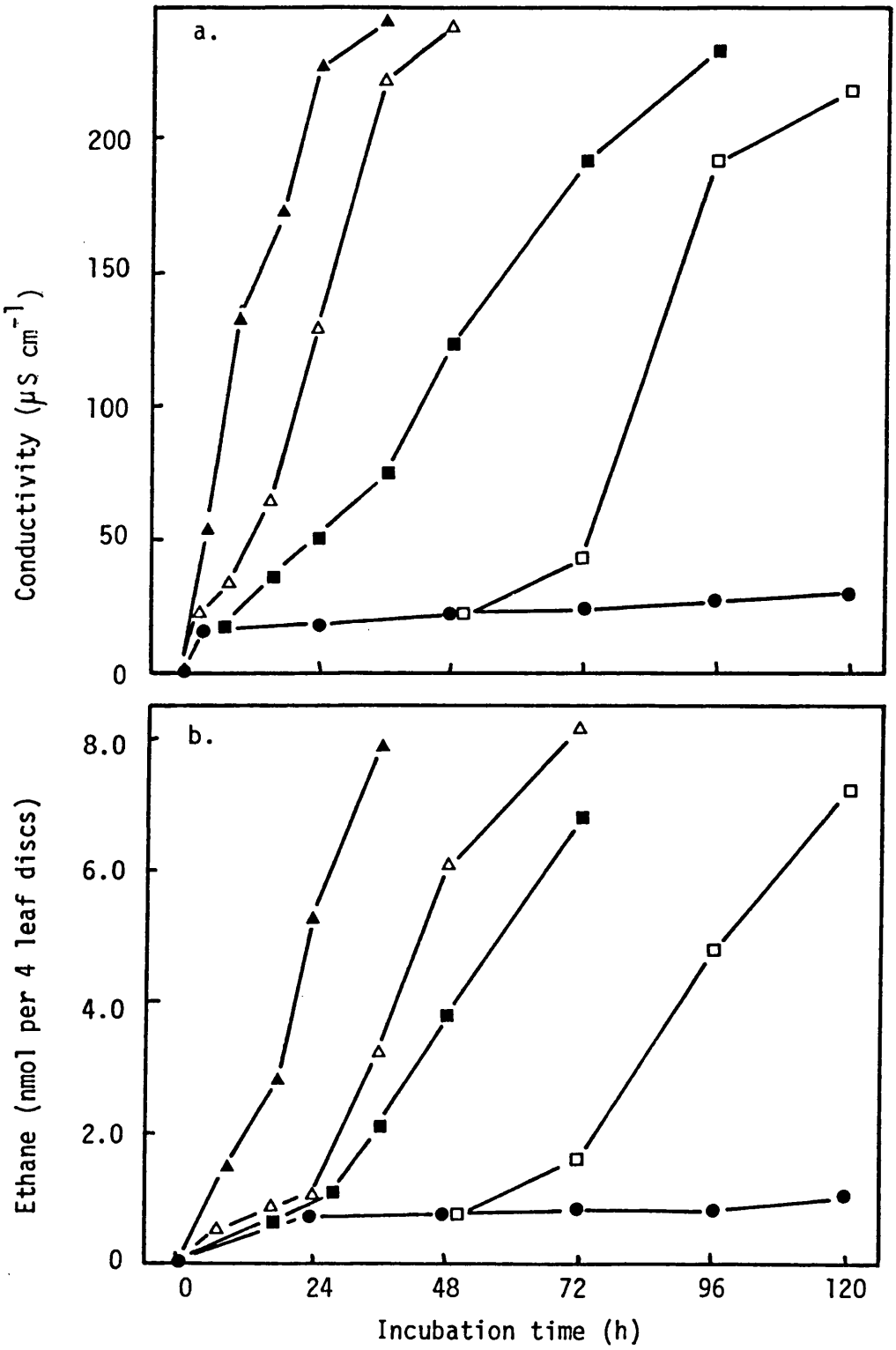


Fig 21. The effect of 25μM paraquat (▲), monuron (□), acifluorfen (△) and rose bengal (■) on electrolyte leakage (a) or ethane evolution (b) from pea leaf discs. (control treatment: ●—●)

in illuminated leaf discs within eight hours treatment (Figure 20a). Acifluorfen and rose bengal inhibited photosynthesis by 50% after eight hours, although 24 hours incubation was required to abolish photosynthetic activity. Chlorophyll bleaching (Figure 20b) was detected in paraquat treated leaf discs after eight hours, and 50% loss of chlorophyll was observed after 24 hours. Leaf discs incubated on acifluorfen, rose bengal and monuron showed 50% loss of chlorophyll after 48, 72 and 96 hours respectively. Thus although photosynthetic inhibition was detected after a few hours herbicide treatment, there was a considerable delay before chlorophyll bleaching was observed. Damage to membranes in herbicide treated leaf discs was assessed by monitoring electrolyte leakage into the bathing medium (Figure 21a) or by lipid peroxidation (ethane evolution, Figure 21b). Paraquat induced increased electrolyte leakage from leaf discs after four hours, and was followed by lipid peroxidation (8-16 hours incubation). Electrical conductivity changes in the bathing medium surrounding acifluorfen or rose bengal treated leaf discs were detected after 16 hours illumination, although ethane evolution was not detected until 36 hours incubation. Membrane damage induced by monuron was observed after 72 hours.

Damage induced by electron transport inhibitor herbicides, bipyridyls and photosensitizers is dependent on light and oxygen (Dodge, 1982). The role of light and oxygen in DPE herbicide injury to pea leaf discs is shown in Figures 22 and 23. Chlorophyll bleaching and ethane evolution was assessed in leaf discs incubated on 10 μM acifluorfen or oxyfluorofen in sealed vials flushed with air, oxygen or nitrogen for five minutes prior to illumination (Figure 22). These results demonstrate that chlorophyll bleaching and membrane lipid peroxidation was dependent

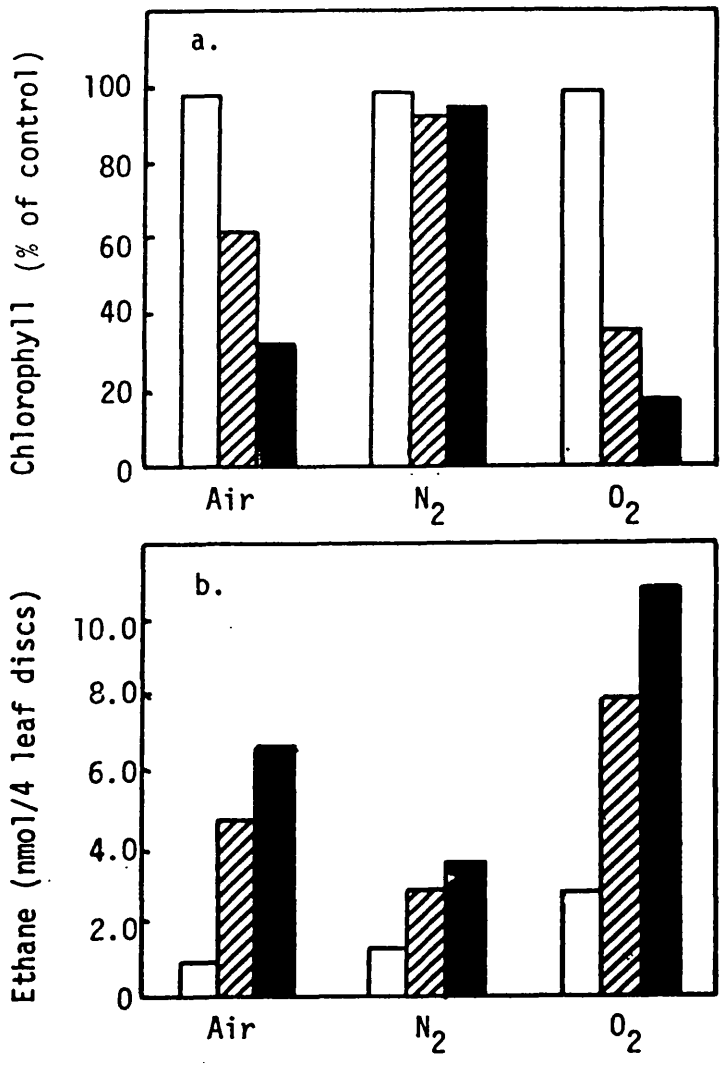
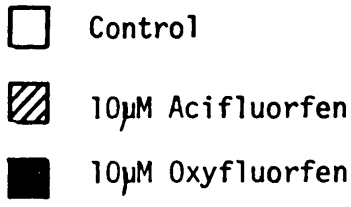


Fig 22. The effect of incubating pea leaf discs under air, nitrogen or oxygen on acifluorfen and oxyfluorfen induced chlorophyll bleaching (a) or ethane evolution (b).



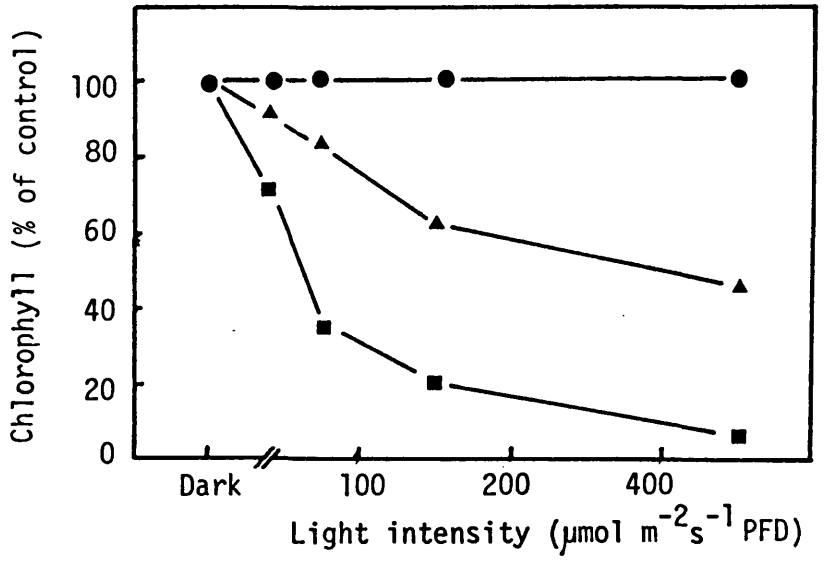


Fig 23. The effect of light intensity on chlorophyll bleaching in pea leaf discs. Discs were incubated on H_2O (●), 10μM acifluorfen (▲) or 10μM oxyfluorfen (■).

on oxygen, either alone or as a component of air. Damage was retarded by incubation under nitrogen and enhanced by oxygen. The role of light intensity in acifluorfen and oxyfluorfen induced chlorophyll bleaching of pea leaf discs is shown in Figure 23. No chlorophyll loss was detected in leaf discs incubated for 48 hours in darkness. Bleaching occurred only in light and was greater after 48 hours incubation at highest light intensities. Ethane evolution from acifluorfen or oxyfluorfen treated pea leaf discs was also light dependent. No lipid peroxidation was detected after 48 hours dark incubation (results not shown).

Herbicide damage induced by acifluorfen, paraquat, monuron and rose bengal was also assessed in illuminated chloroplast membranes. Thylakoids isolated from pea leaves were incubated in a 20 ml reaction volume containing phosphate buffer (50 mM, pH 7.6) and 10 μM herbicides. Damage was assessed following periods of illumination, by monitoring lipid peroxidation (malondialdehyde formation) or chlorophyll bleaching. The results, presented in Figure 24, show that rose bengal induced rapid membrane peroxidation and chlorophyll bleaching of thylakoids. Acifluorfen, paraquat and monuron induced damage to thylakoids at similar rates, although no marked lipid peroxidation or chlorophyll bleaching was detected before 180 minutes illumination.

5.2 Discrimination between herbicidal modes of action

The results presented in Figures 20 - 24 demonstrated that paraquat, monuron, rose bengal and acifluorfen induced similar symptoms of injury to pea leaf discs and chloroplasts. However, such studies indicate little about the site of activation or mode of action of these herbicides. The effect of these chemicals against photosynthetic reactions of

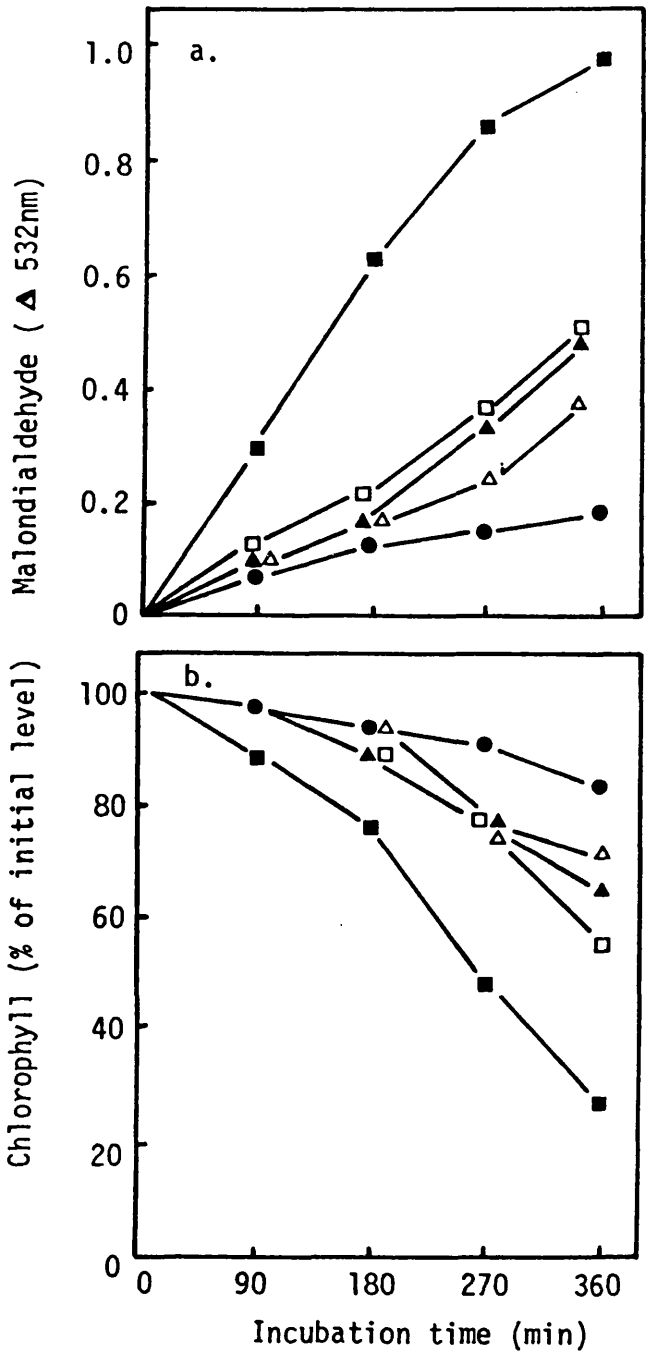


Fig 24. The effect of 10μM paraquat (▲), monuron (□), acifluorfen (△) or rose bengal (■) on lipid peroxidation (a) or chlorophyll bleaching (b) of illuminated thylakoids. (control treatment: ●—●)

leaf discs or isolated thylakoids, and the role of photosynthetic electron flow in herbicide activation are compared here in an attempt to discriminate between their distinct modes of action.

All four classes of herbicide inhibited photosynthesis in pea leaf discs after 24 hours illumination (Figure 20). The results in Table 8 show the effects of these herbicides on photosynthetic activity of pea leaf discs after 24 hours dark incubation. Both paraquat and monuron abolished photosynthesis within 24 hours incubation. Rose bengal had no effect on photosynthetic CO_2 exchange, although both acifluorfen and oxyfluorfen retarded photosynthesis by 20-30% after 24 hours dark incubation.

The results in Table 9 and Figure 25 demonstrate the effect of herbicides on photosynthetic electron flow reactions of illuminated thylakoids. Monuron abolished photosystem II ferricyanide reduction at low concentrations. As a consequence electron flow through photosystem I and II using paraquat as an electron acceptor (Table 9), and $NADP^+$ reduction by illuminated thylakoids (Figure 25) was also inhibited by monuron. Paraquat had no effect on photosystem II activity, but promoted oxygen uptake by photosystem I. Paraquat promotion of electron flow from photosystem I to oxygen caused the inhibition of $NADP^+$ reduction (Figure 25) and photosynthetic CO_2 exchange (Table 8). The DPE herbicides acifluorfen and oxyfluorfen had no marked effect on electron flow through photosystem I or II at concentrations up to 50 μM. These herbicides did however inhibit $NADP^+$ reduction by illuminated thylakoids (Figure 25). This indicates that the inhibition of CO_2 exchange induced by these herbicides in pea leaf discs (Table 8) was a consequence of DPE herbicide interaction with $NADP^+$ reduction. Rose bengal did not inhibit electron flow reactions through photosystem I or II.

Table 8. The effect of herbicides on photosynthetic CO_2 exchange in pea leaf discs after 24 hours dark incubation.

	CO_2 uptake (μmol CO_2 $gFW^{-1}h^{-1}$)
Control	71.88 (100%)
25μM paraquat	0
25μM monuron	0
25μM rose bengal	71.55 (99.5%)
25μM acifluorfen	55.3 (76.8%)
25μM oxyfluorfen	51.6 (71.8%)

Table 9. The effect of paraquat, monuron, rose bengal, acifluorfen and oxyfluorfen on electron transport reactions of isolated pea thylakoids.

	$H_2O \rightarrow FeCN$ (PS II)	$H_2O \rightarrow PQ$ (PS I+II)	Ascorb/DCPIP $\rightarrow O_2$ (PS I)		$H_2O \rightarrow O_2$ (PS I+II)	
Control rate	125.1[a] (100%)	134.5[b] (100%)	20.13[b] (100%)		29.5[b] (100%)	
	Inhibitor concentration (μM)					
	10	50	50	10	50	50
Paraquat	100%	98.0%	—[c]	977%	992%	837%
Monuron	1.6%	0%	0%	—[d]	—	0%
Rose bengal	95.3%	90.0%	93.0%	—[e]	—	107%
Acifluorfen	101.6%	100%	91.6%	106%	100%	108%
Oxyfluorfen	100%	94.7%	95.5%	103%	100%	89%

a. Results as μmol O_2 evolution mg^{-1}chl h^{-1}
b. Results as μmol O_2 uptake mg^{-1}chl h^{-1}
c. Reaction mix contained paraquat
d. Reaction mix contained monuron
e. No result- Rose bengal sensitised the oxidation of ascorbate.

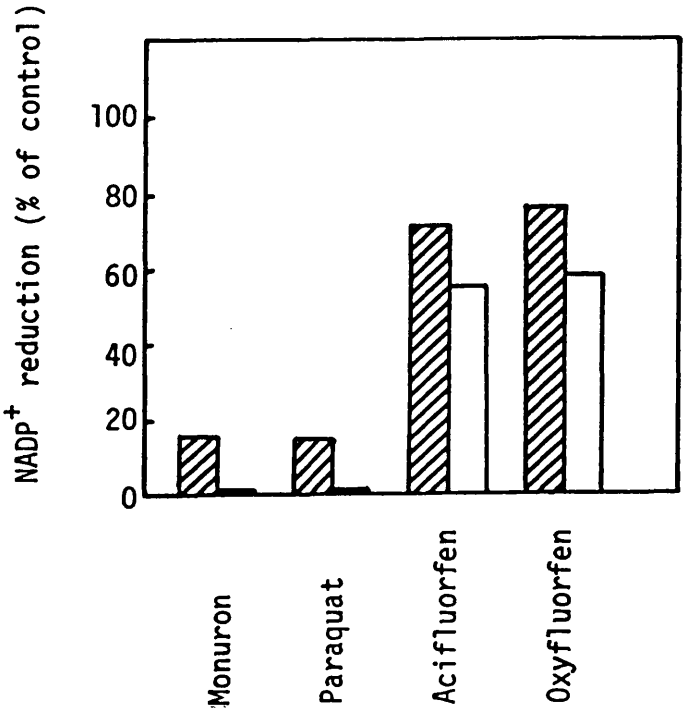


Fig 25. The effect of paraquat, monuron, acifluorfen and oxyfluorfen on $NADP^+$ reduction by illuminated thylakoid membranes.

Results presented here indicate that inhibition of photosynthetic electron flow is unlikely to account for the herbicidal action of DPE compounds. Functional photosynthetic electron flow was however necessary for acifluorfen and oxyfluorfen activity in pea leaf discs. Leaf discs were incubated for 24 hours in darkness on water or monuron (0.1 mM) and transferred to 10 μM DPE herbicide solutions under constant illumination. Herbicide damage was assessed by chlorophyll bleaching and lipid peroxidation (Figure 26). Leaf discs preincubated on water and then transferred to acifluorfen or oxyfluorfen showed chlorophyll bleaching and lipid peroxidation after 24 to 48 hours illumination. Pre incubation on monuron retarded damage induced by these DPE herbicides. Similar experiments on the role of photosynthetic electron flow in chlorophyll bleaching induced by paraquat or rose bengal are shown in Table 10. Inhibition of electron flow by monuron retarded paraquat induced bleaching of pea leaf discs. By contrast chlorophyll bleaching induced by rose bengal was unaffected by monuron. This shows that although symptoms of injury induced by rose bengal were similar to those induced by paraquat, monuron and DPE herbicides, this occurred independently of photosynthetic electron flow.

5.3 The role of antioxidants in preventing injury

Chloroplasts are normally protected to some degree against photoxidative damage *in vivo* by antioxidants and enzymes in the stroma and thylakoid membranes. Peroxidation of illuminated thylakoids can however be promoted by herbicides such as monuron, paraquat, rose bengal and acifluorfen. Herbicide promoted chloroplast membrane peroxidation was investigated further in the experiments shown in Figures 27 - 30. The aim of this study was to determine the effect of various antioxidants on herbicide induced chloroplast damage, and to investigate whether anti-

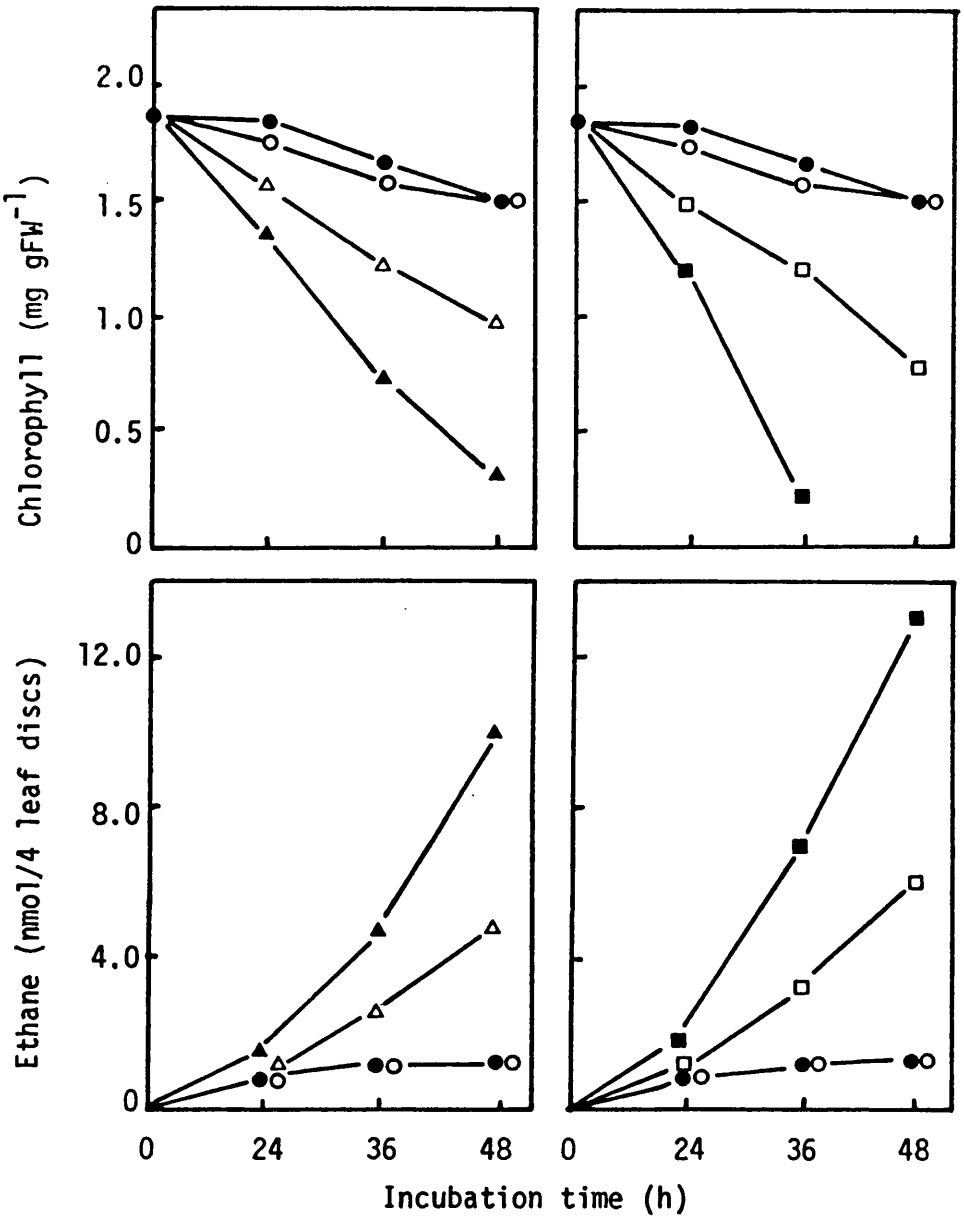


Fig 26. Chlorophyll bleaching and ethane evolution from pea leaf discs incubated on H_2O (●,○), acifluorfen (▲,△) or oxyfluorfen (■,□). Leaf discs were preincubated for 24 hours in darkness on H_2O (●,▲,■) or 0.1mM monuron (○,△,□).

Table 10. The role of photosynthetic electron transport in paraquat and rose bengal induced chlorophyll bleaching of pea leaf discs. Discs were preincubated on herbicide solutions for 24 hours in darkness prior to illumination.

	Chlorophyll (as % of control)
25μM paraquat	24.1%[a]
25μM paraquat + 0.1mM monuron	59.3%[a]
25μM rose bengal	30.8%[b]
25μM rose bengal + 0.1mM monuron	29.6%[b]

a, after 24 hours illumination

b. after 72 hours illumination

oxidants may discriminate between the damaging action of different herbicide classes. Peroxidation of illuminated thylakoids was promoted by paraquat (Figure 27), monuron (Figure 28), acifluorfen (Figure 29) and rose bengal (Figure 30). The other results in these figures demonstrate the effect of adding 10 mM ascorbate, 1 mM α-tocopherol, 1 mM DABCO (diazobicyclooctane, an $^1O_2$ quencher), 1 mM crocetin (a carotenoid isolated from saffron), or 2000 units of SOD plus catalase on lipid peroxidation induced by these herbicides.

Chloroplast membrane peroxidation induced by all four herbicides was strongly retarded by 10 mM ascorbate, an antioxidant normally present in the chloroplast stroma at concentrations up to 25 mM (Section 1). Carotenoids and α-tocopherol, which are present in thylakoid membranes, strongly retarded herbicide induced thylakoid membrane peroxidation. The $^1O_2$ quencher DABCO gave no protection against paraquat and acifluorfen induced lipid peroxidation. DABCO did however restrict peroxidation induced by monuron and rose bengal. SOD plus catalase had no significant effect in reducing thylakoid peroxidation induced by any of the herbicides tested. In addition 1.0 mM or 5.0 mM copper penacillamine, a copper complex with SOD activity, did not protect thylakoids against herbicide induced peroxidation (results not shown).

Discrimination between herbicidal modes of action by antioxidants was restricted to the protection by DABCO against the herbicides monuron and rose bengal. Both compounds promote $^1O_2$ formation, either directly or as a consequence of the inhibition of photosynthetic electron flow (Dodge, 1982). Other antioxidants tested were either ineffective at quenching damage (SOD plus catalase or PaCu), or retarded lipid peroxidation induced by all four herbicides (ascorbate, α-tocopherol and crocetin). The effectiveness of ascorbate, α-tocopherol and crocetin in preventing injury may reflect an ability to quench lipid peroxidation

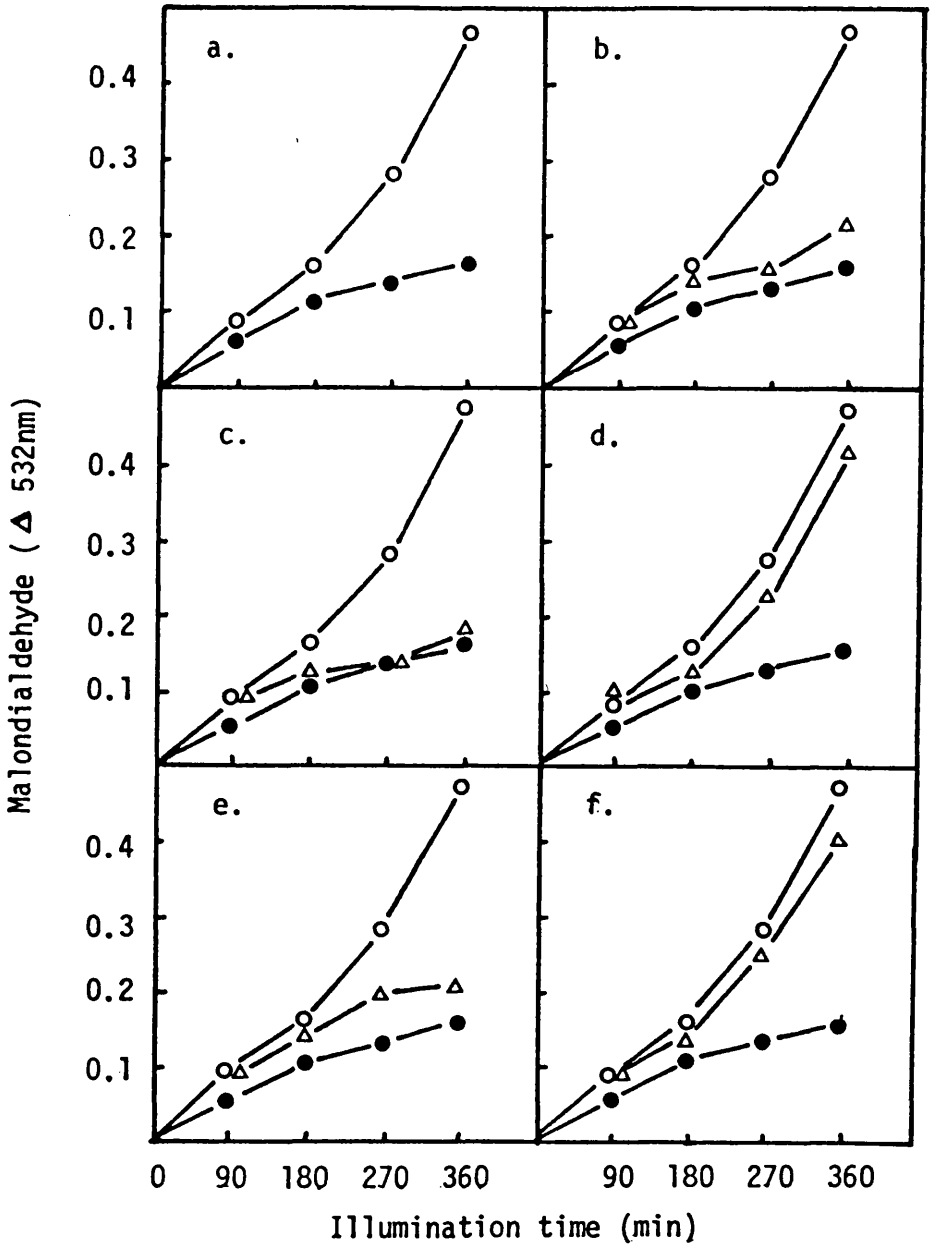


Fig 27. The effect of 10μM paraquat (o) on the peroxidation of illuminated thylakoids. Control (●). Other additions (△) were:
(b) 10mM ascorbate + paraquat, (c) 1mM α-tocopherol + paraquat, (d) 1mM DABCO + paraquat, (e) 1mM crocetin + paraquat, (f) SOD / catalase + paraquat.

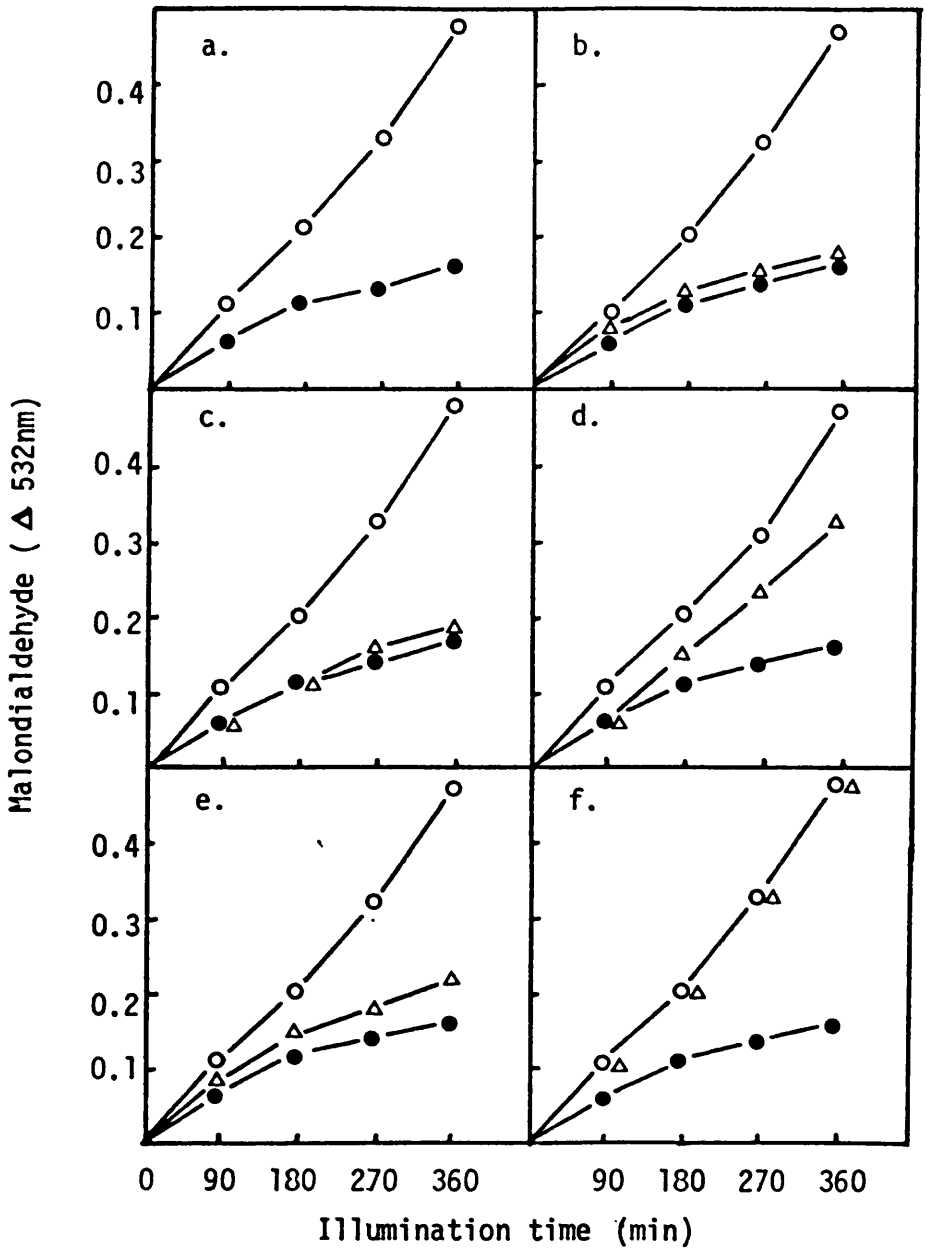


Fig 28. The effect of 10μM monuron (o) on the peroxidation of illuminated thylakoids. Control (●). Other additions (△) were:
(b) 10mM ascorbate + monuron. (c) 1mM α-tocopherol + monuron. (d) 1mM DABCO + monuron. (e) 1mM crocetin + monuron. (f) SOD / catalase + monuron.

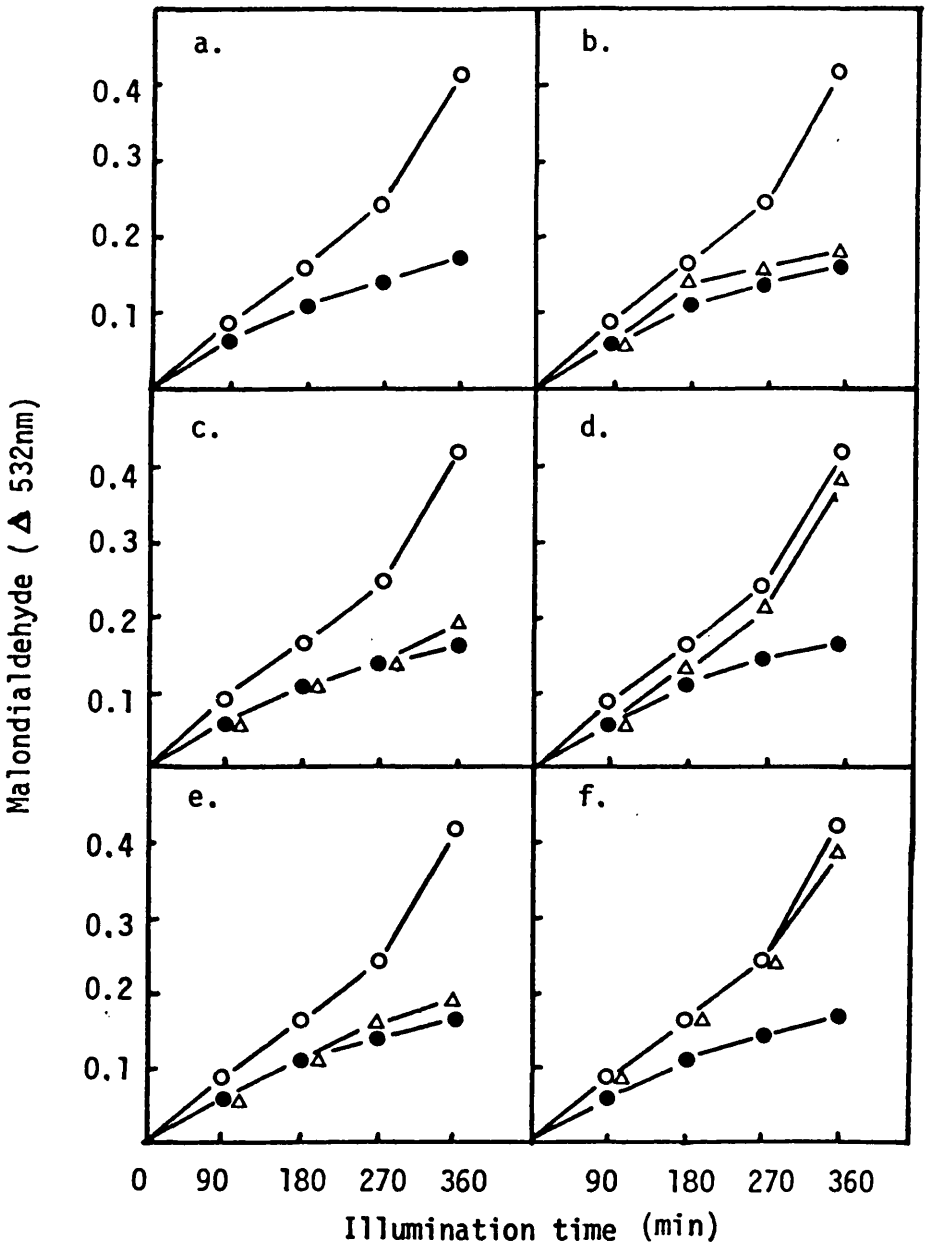


Fig 29. The effect of 10μM acifluorfen (o) on the peroxidation of illuminated thylakoids. Control (●). Other additions (△) were:
(b) 10mM ascorbate + acifluorfen. (c) 1mM α-tocopherol + acifluorfen. (d) 1mM DABCO + acifluorfen. (e) 1mM crocetin + acifluorfen. (f) SOD / catalase + acifluorfen.

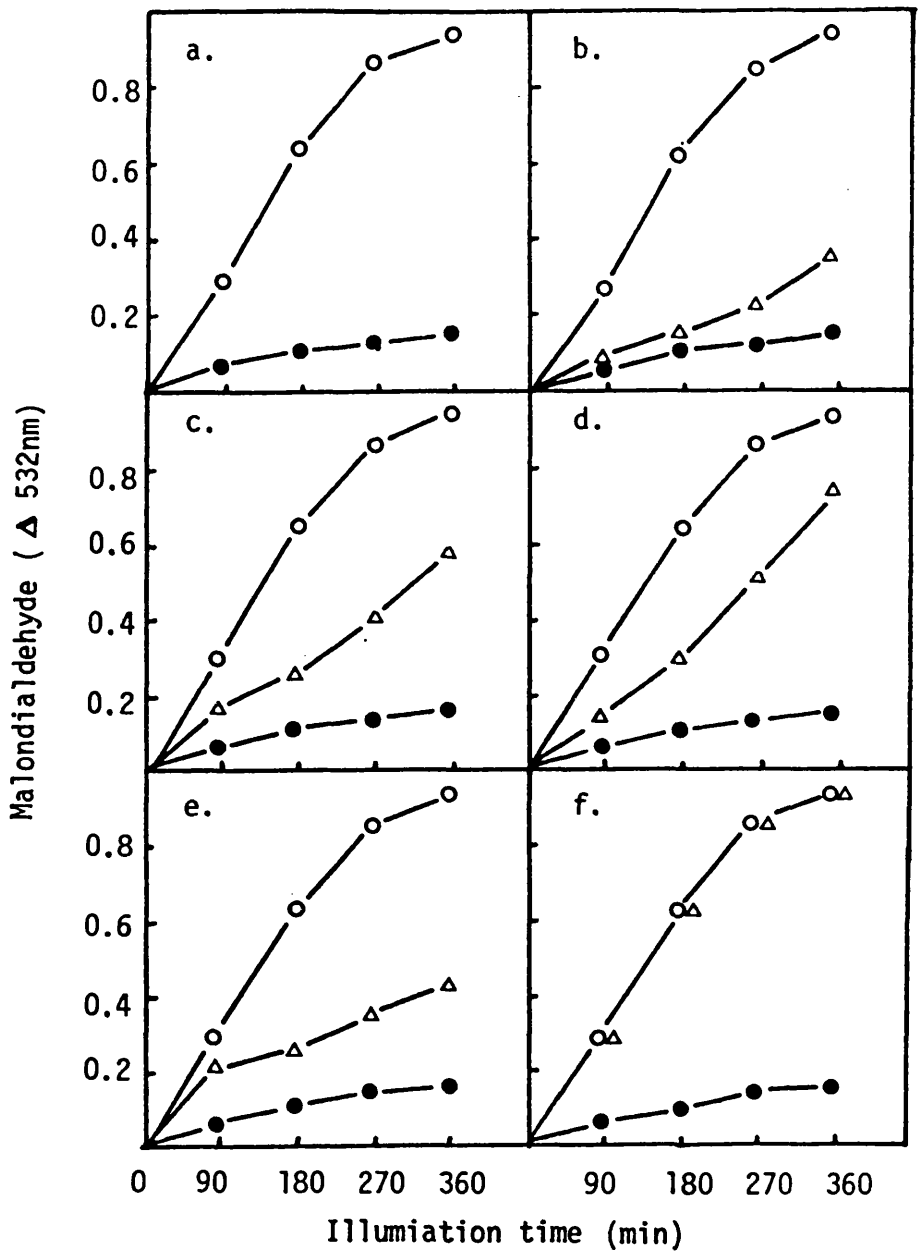


Fig 30. The effect of 10μM rose bengal (o) on the peroxidation of illuminated thylakoids. Control (●). Other additions (△) were:
(b) 10mM ascorbate + rose bengal. (c) 1mM α-tocoperol + rose bengal. (d) 1mM DABCO + rose bengal. (e) 1mM crocetin + rose bengal. (f) SOD / catalase + rose bengal.

directly as well as scavenge $O_2^{\cdot -}$, H_2O_2 and $^1O_2$ generated as a consequence of herbicide action.

Several recent studies of herbicide action, particularly with DPE herbicides, have attempted to use antioxidants to evaluate the role of $O_2^{\cdot -}$ and $^1O_2$ in herbicide action. Experiments by Kunert and Böger (1981) and Ensminger and Hess (1985) showed that PaCu, DABCO and SOD did not prevent acifluorfen or oxyfluorfen induced injury. These workers concluded from these results that generation of $O_2^{\cdot -}$ and $^1O_2$ was not the primary mechanism of action of these compounds. Results presented in Figures 27 - 30 show that the use of antioxidants to scavenge these radicals and thus prevent tissue injury is not a specific method for evaluating the role of such radicals in herbicide action.

5.4 Discussion

The herbicides paraquat, monuron, acifluorfen and rose bengal induced lipid peroxidation and chlorophyll bleaching of illuminated thylakoids, and loss of photosynthetic activity, membrane disruption and chlorophyll bleaching of pea leaf discs. Although the symptoms of injury induced by these chemicals were similar, the effects against photosynthetic reactions and requirement for electron flow in herbicide activity indicated distinct modes of action.

Bipyridyl herbicides such as paraquat are readily reduced by photosystem I electron flow. Their subsequent reoxidation by oxygen promotes $O_2^{\cdot -}$ accumulation in chloroplasts (Dodge, 1983). This is illustrated in this study by paraquat induced oxygen uptake by illuminated thylakoids (Table 9), thus inhibiting $NADP^+$ reduction and photosynthetic CO_2 exchange.

Accumulation of $O_2^{\cdot -}$ and H_2O_2, and the formation of $OH^{\cdot}$ by interaction between $O_2^{\cdot -}$ and H_2O_2 initiates damage. Damage symptoms can be observed rapidly in paraquat treated leaf material, illustrated by chlorophyll bleaching and membrane disruption observed after 8 hours paraquat treatment. This may reflect the positive generation of damaging oxygen species that occurs in chloroplasts as a consequence of herbicide action.

Electron flow inhibitor herbicides induce damage to leaf material more slowly. Although monuron abolished photosynthesis in pea leaf discs after 4-8 hours incubation, chlorophyll bleaching and membrane disruption was not detected until after 72 hours treatment. Electron flow inhibitors such as monuron inhibit the Hill reaction at concentrations below 1 μM (Corbett, 1974). Thus photosystem II ferricyanide reduction and $NADP^+$ reduction by isolated thylakoids, and CO_2 exchange in leaf discs were strongly inhibited by monuron. Inhibition of electron flow prevents the normal dissipation of light energy absorbed by chlorophyll through photosynthetic carbon metabolism. Excitation energy absorbed by chlorophyll in monuron inhibited chloroplasts promotes the formation of ^3Chl and $^1O_2$ (Dodge, 1983). Damage induced by monuron is therefore a consequence of prolonged illumination of photosynthetically incompetent chloroplasts, and may be compared to the positive generation of active oxygen species that occurs when photosynthesis is inhibited by paraquat.

The photosensitizer rose bengal is a well known generator of $^1O_2$ (Ito, 1978). Light energy absorbed by the dye is transferred directly to a biological substrate in a type I mechanism, or to oxygen forming $^1O_2$ in a type II mechanism (Foote, 1976). Compounds such as rose bengal do not inhibit electron flow reactions of isolated thylakoids, or require electron flow for their phytotoxic action, even though loss of photo-

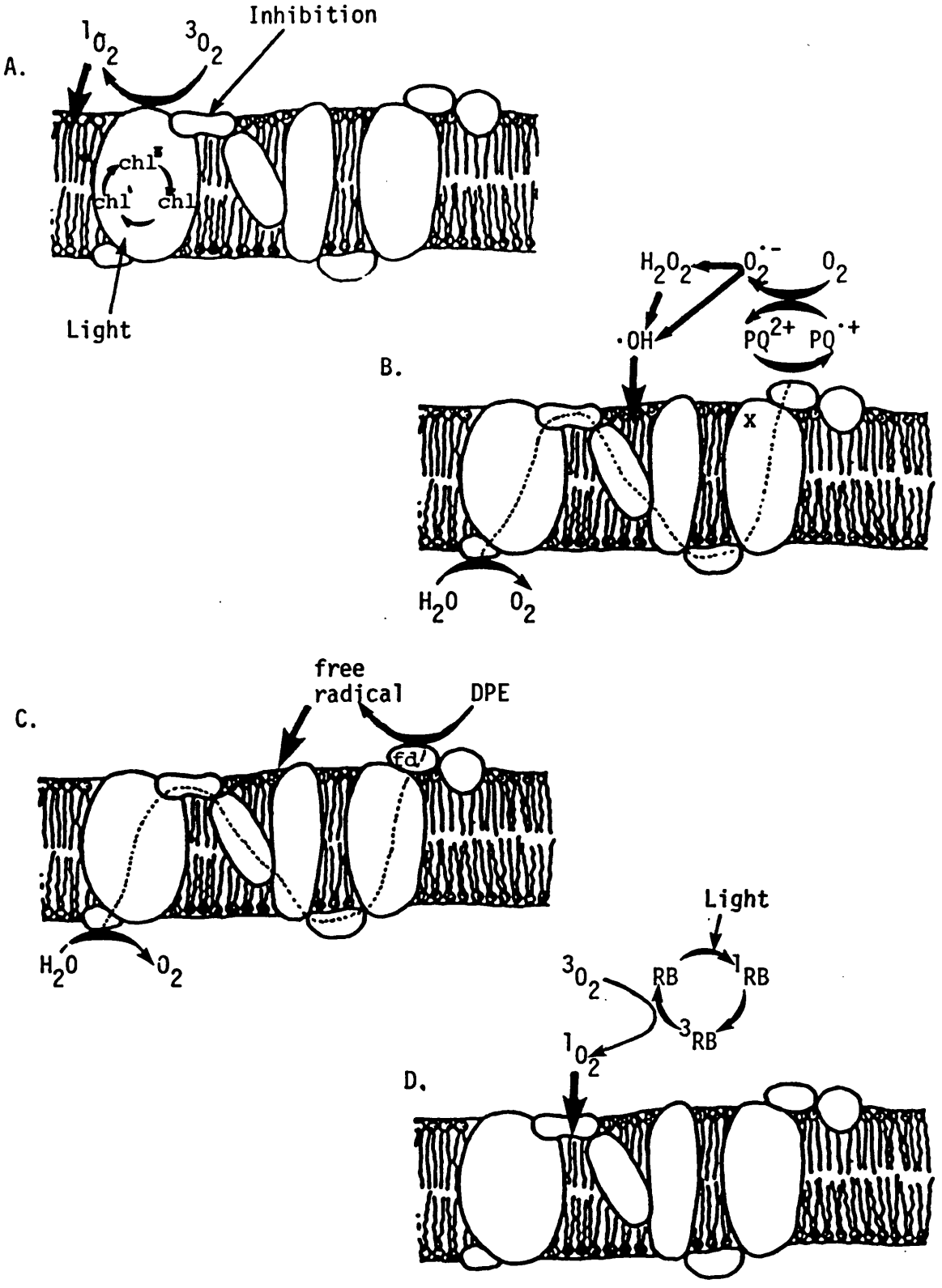


Fig 31. Proposed sites of interaction of monuron (a), paraquat (b) aciluorfen (c) and rose bengal (d) with photosynthetic electron flow.

synthetic activity was one of the first symptoms of injury in rose bengal treated leaf discs (Figure 20). This may reflect the sensitivity of photosynthetic reactions to photo-oxidative disruption. A scheme outlining the damaging action of paraquat, monuron, acifluorfen and rose bengal, and their interaction with photosynthetic electron flow is shown in Figure 31.

The DPE herbicides acifluorfen and oxyfluorfen initiated membrane damage to pea leaf discs after 8-16 hours illumination, followed by chlorophyll bleaching. Several studies have indicated that the primary mode of action of this group of herbicides is to initiate membrane lipid peroxidation. This has been demonstrated in studies of electrolyte leakage or lipid peroxidation in DPE herbicide treated material, or by ultrastructural investigations (Vanstone and Stobbe, 1979; Kunert and Böger, 1981; Orr and Hess, 1982; Kenyon *et al.*, 1985). Although these herbicides are dependent on light for activity the mechanism of light activation remains unclear. Results from this study support observations by Kunert and Böger (1981) that functional photosynthetic electron flow is necessary for DPE herbicide activity. Some DPE herbicides have been shown to inhibit electron flow through photosystem II, while others promote oxygen uptake and $O_2^{\cdot -}$ formation by photosystem I electron flow (van den Burg and Tipker, 1983; Ridley, 1983). Results presented here indicate that such mechanisms do not account for the herbicidal action of acifluorfen or oxyfluorfen. These herbicides had no effect on electron flow through photosystem I and II, although ferredoxin dependent $NADP^+$ reduction was retarded. Thus activation by electron flow may occur at a site on the thylakoid membrane close to ferredoxin or ferredoxin $NADP^+$ reductase. The mechanism of activation and mode of action of DPE herbicides will be considered further in

a later section of this thesis.

The role of light and oxygen in DPE herbicide damage to pea leaf discs may reflect a photo-oxidative damage mechanism involving the generation of activated oxygen species ($O_2^{\cdot -}$, $^1O_2$ or H_2O_2) in chloroplasts similar to the damaging action of paraquat, monuron and rose bengal. The increased formation of free radicals or toxic oxygen species in herbicide treated chloroplasts probably exceeds the capacity of endogenous antioxidant mechanisms to scavenge them. Thus the antioxidants ascorbate, α-tocopherol and crocetin restricted the peroxidation of illuminated thylakoids induced by these herbicides. While such antioxidant treatments could not be used to discriminate between the damaging action of these herbicides *in vitro*, their presence *in vivo* may be a significant factor accounting for the delay between the rapid loss of photosynthetic activity and the development of chlorophyll bleaching or lipid peroxidation in herbicide treated leaf discs. Alterations in the endogenous levels of these antioxidants in chloroplasts may be one mechanism whereby increased or decreased tolerance to these herbicides is achieved.

6. Modes of action of nitrodiphenyl ether herbicides

Diphenyl ether herbicides induce light and oxygen dependent inhibition of photosynthesis, chlorophyll bleaching and membrane damage to leaf material similar to that induced by photosynthetic inhibitor herbicides. Activation by photosynthetic electron flow induces the peroxidation of chloroplast membranes, although the site of activation and damaging action remains unclear. The action of DPE herbicides are considered further in this section by comparing the damaging action of acifluorfen with a range of other DPE chemicals.

6.1 The role of ferredoxin in herbicide activation

Acifluorfen and oxyfluorfen were shown previously to retard ferredoxin-dependent $NADP^+$ reduction by illuminated thylakoids, although no inhibition of electron flow through photosystem I and II was detected. Activation of these herbicides by photosynthetic electron flow may therefore occur at a site on the thylakoid membrane in the region of ferredoxin. Electron flow through ferredoxin and ferredoxin $NADP^+$ reductase is investigated here using the diaphorase properties of this enzyme to catalyse NADPH oxidation.

To a basic reaction mixture of Tris-HCl buffer containing NADPH was added ferredoxin and ferredoxin $NADP^+$ reductase extracted from spinach leaves. Other additions are shown in Table 11. In the presence of this enzyme, ferredoxin promoted the oxidation of NADPH according to the pathway shown. The reaction proceeded at a slow rate if either ferredoxin or enzyme were omitted from the reaction mixture. This experimental system has been used in several investigations to assess the role of ferredoxin in reducing a variety of compounds (Wessels, 1965; Forti and Grubas, 1985; Camilleri *et al.*, 1985). Comparisons

Pathway of electron flow from NADPH to ferredoxin via ferredoxin $NADP^+$ reductase:

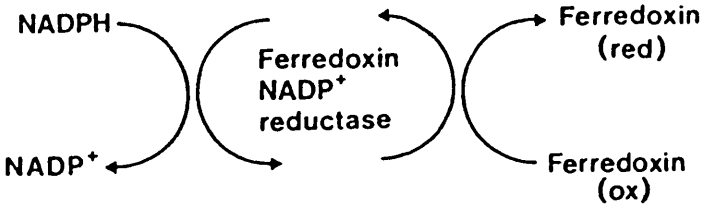


Table 11. The effect of acifluorfen, oxyfluorfen, cytochrome C and myoglobin on the rate of oxidation of NADPH by ferredoxin and ferredoxin $NADP^+$ reductase. The basic 3.0ml reaction mix contained 0.33M phosphate buffer, pH 8.0, and 80µg NADPH.

	Rate of NADPH oxidation (nmol min^{-1})				
Additions to basic reaction:	Control	25µM acifluorfen	25µM oxyfluorfen	25µM cytochrome	25µM myoglobin
Fd-$NADP^+$ reductase	3.78	4.4	4.6	6.29	5.17
Ferredoxin	1.79	1.62	1.62	1.83	1.97
Ferredoxin + reductase	13.32	16.01 (+20.2%)	15.86 (+19.1%)	23.67 (+78.4%)	22.32 (+67.7%)
Ferredoxin + reductase + thylakoids	13.25	18.4 (+38.9%)	19.8 (+49.4%)	23.72 (+72.0%)	21.96 (+65.7%)

of the effect of the DPE herbicides acifluorfen and oxyfluorfen with cytochrome C and myoglobin on NADPH oxidation by ferredoxin and ferredoxin $NADP^+$ reductase are shown in Table 11. All four compounds promoted the oxidation of NADPH provided that both ferredoxin and ferredoxin $NADP^+$ reductase were present in the reaction mixture. This indicates that all four compounds can be reduced by ferredoxin but not ferredoxin $NADP^+$ reductase. Reduction of myoglobin and cytochrome C by ferredoxin has been confirmed elsewhere by following the change in absorbance of these compounds as they were reduced (Davenport and Hill, 1960). Experiments shown here indicate that acifluorfen and oxyfluorfen behave in a similar manner to cytochrome C and myoglobin in this experimental system. The rate of NADPH oxidation catalysed by ferredoxin and ferredoxin $NADP^+$ reductase and promoted by DPE herbicides was however significantly enhanced if chloroplast membranes (30 µg chlorophyll) were added to the reaction mixture. No such stimulation by chloroplasts was observed in experiments using cytochrome C and myoglobin. The promotion by chloroplasts of NADPH oxidation in experiments using DPE herbicides may have been by providing a herbicide binding site as a prerequisite of herbicide activity in this system. Binding of DPE herbicides to chloroplast thylakoids and their subsequent reduction by ferredoxin may account for the observed role of photosynthetic electron flow in herbicide activation.

Ferredoxin has an important role in a number of biological electron transfer reactions, which include $NADP^+$ reduction, fatty acid desaturation reactions, oxygen reduction to $O_2^{\cdot -}$ and the activation of certain chloroplast stromal enzymes by ferredoxin-ferredoxin thioreductase (Hall and Rao, 1977; Halliwell, 1981). Ferredoxin has also been demonstrated to reduce a variety of compounds including cytochrome C and

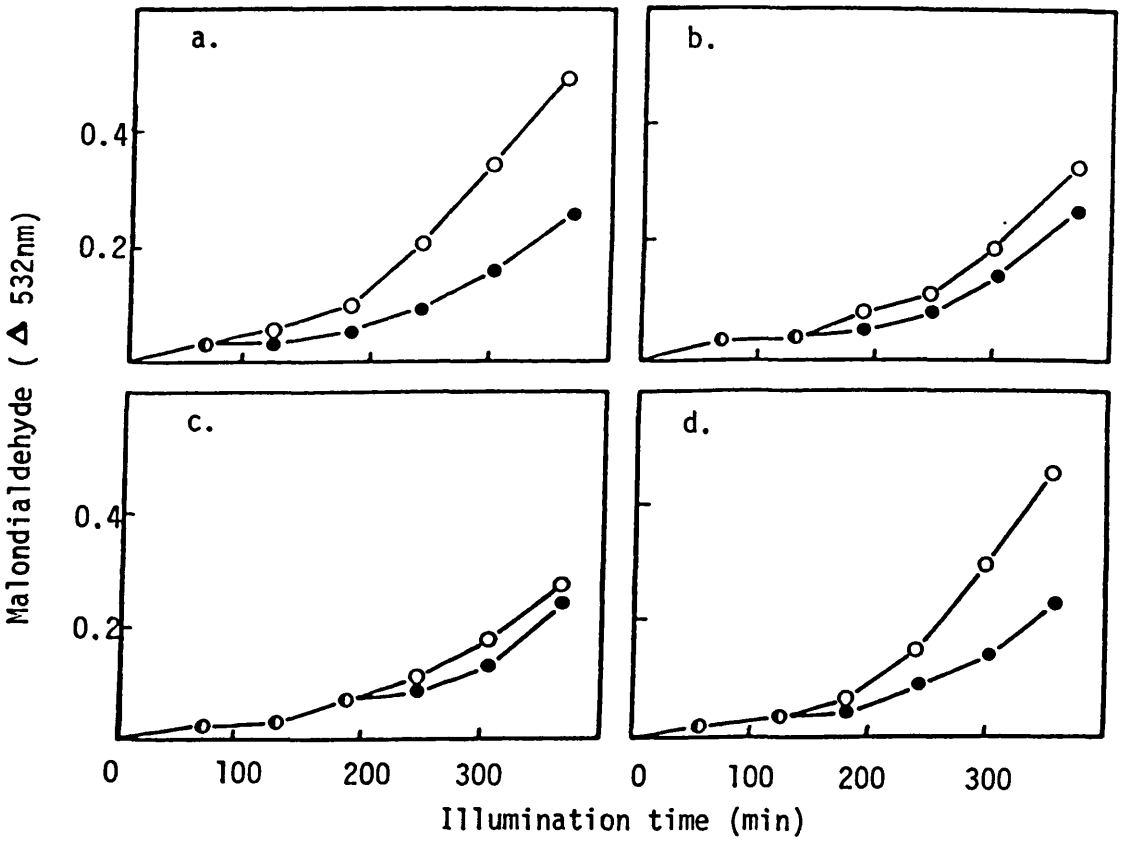


Fig 32. Membrane lipid peroxidation of illuminated thylakoids. Control (●) and the effect of 10μM acifluorfen (o). Thylakoids were unwashed (a), washed once (b) or twice (c) prior to the experiment. The effect of adding ferredoxin to the washed thylakoids is shown in figure d.

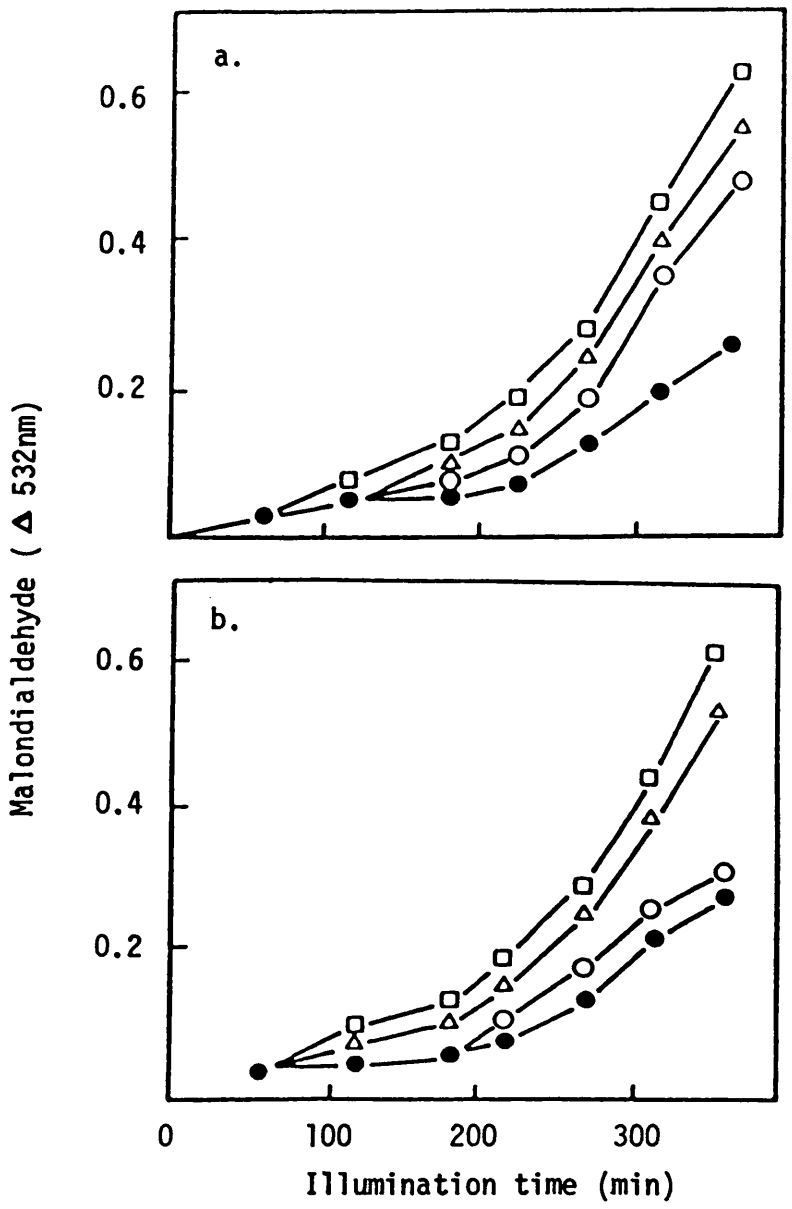
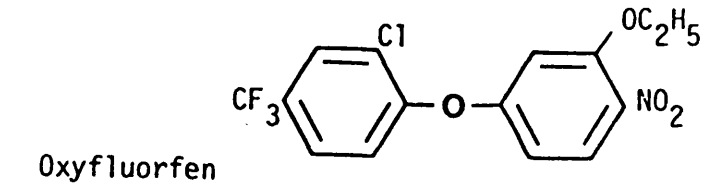


Fig 33. The effect of paraquat (△), monuron (□) and acifluorfen (○) compared with the control (●) on lipid peroxidation of illuminated thylakoids. Thylakoids were unwashed (a) or washed twice (b) prior to the experiment.

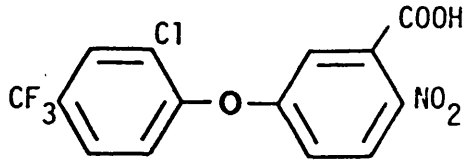
myoglobin, dinitrophenols and heteropentalenes (Davenport and Hill, 1960; Wessels, 1965; Camilleri *et al.*, 1985). Studies of the redox chemistry of acifluorfen-methyl and several other nitro DPE herbicides showed that their one electron reduction potentials did not preclude reduction by the acceptor site of photosystem I (Orr *et al.*, 1983 a,b, Bowyer *et al.*, 1986). Ferredoxin reduction of DPE herbicides in illuminated chloroplasts may therefore be a feasible reaction.

The role of ferredoxin in DPE herbicide activation was examined further by following the effect of acifluorfen on the peroxidation of illuminated thylakoids (Figure 32), using the experimental system described previously (Section 5.1). Membrane lipid peroxidation was promoted by acifluorfen (Figure 32a). This promotion of lipid peroxidation was strongly retarded if thylakoids were washed with 50 mM Hepes-NaOH buffer either once or twice prior to use (Figure 32 b, c). Membrane lipid peroxidation was therefore dependent on a chloroplast component loosely attached to the thylakoids and easily removed by washing. Ferredoxin is known to be readily lost from chloroplasts by washing (Barber, 1983). Results presented in Figure 32d demonstrate the effect of adding ferredoxin to washed thylakoid preparations on acifluorfen induced peroxidation. Ferredoxin almost completely restored the acifluorfen promotion of membrane peroxidation.

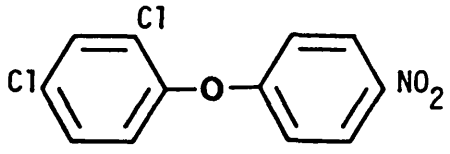
Lipid peroxidation of illuminated thylakoids can be promoted by acifluorfen, paraquat and monuron to a similar extent (Section 5.1). Peroxidation induced by these herbicides on unwashed thylakoids was compared with thylakoids washed twice prior to use (Figure 33). Washing thylakoid membranes twice prior to use did not affect the peroxidation promoted by paraquat and monuron, although thylakoid membrane lipid peroxidation induced by acifluorfen was retarded (Figure 33b).



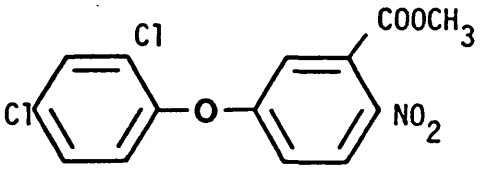
Oxyfluorfen



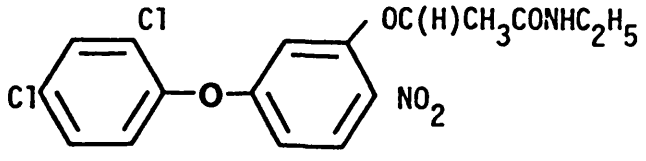
Acifluorfen



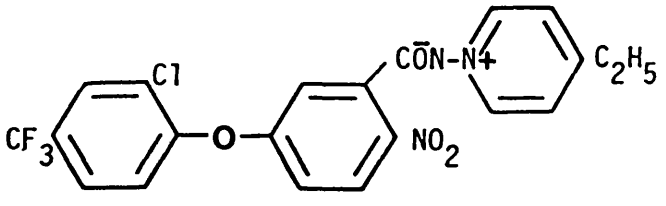
Nitrofen



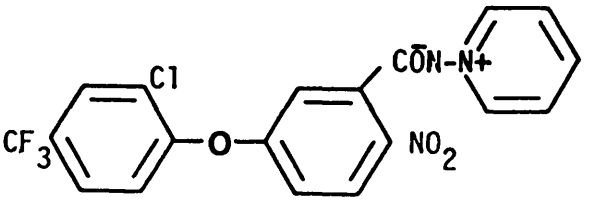
Bifenox



Etnipromid



92 611



38 968

Fig 34. Diphenyl ether herbicide structures.

These results show that the interaction of acifluorfen with chloroplast membrane and the subsequent induction of membrane peroxidation is fundamentally different than that induced by paraquat or monuron.

6.2 Comparisons with other DPE herbicides

Acifluorfen induced light dependent peroxidation of leaf discs or isolated thylakoids. Activation was dependent on photosynthetic electron flow via ferredoxin, although the nature of the radical species formed by DPE herbicide activation is unclear. To determine if other DPE herbicides are dependent on a similar mechanism for herbicide activation, the action of acifluorfen in pea leaf discs and chloroplasts has been compared with a range of other experimental DPE compounds shown in Figure 34.

Visible symptoms of injury to pea leaf discs were assessed following incubation on DPE herbicide solutions (25 μM) for up to 48 hours under constant illumination (300 μmol $m^{-2}s^{-1}$ photon flux density). The effect of DPE herbicides on membrane permeability, assessed by increased electrolyte leakage into the bathing medium, is shown in Figure 35. All six DPE herbicides induced electrolyte leakage from pea leaf discs after 8-16 hours illumination, indicating membrane damage. The effect of herbicide treatments on membrane lipid peroxidation and chlorophyll bleaching from leaf discs, and the requirement for photosynthetic electron flow in DPE herbicide activity is shown in Figure 36. Pea leaf discs were preincubated for 24 hours in darkness on water or 0.1 mM monuron prior to transfer to DPE herbicide solutions and illumination for 48 hours. All DPE herbicides tested induced some chlorophyll bleaching and membrane lipid peroxidation in leaf discs. No chlorophyll bleaching or lipid peroxidation was detected in leaf discs incubated on DPE

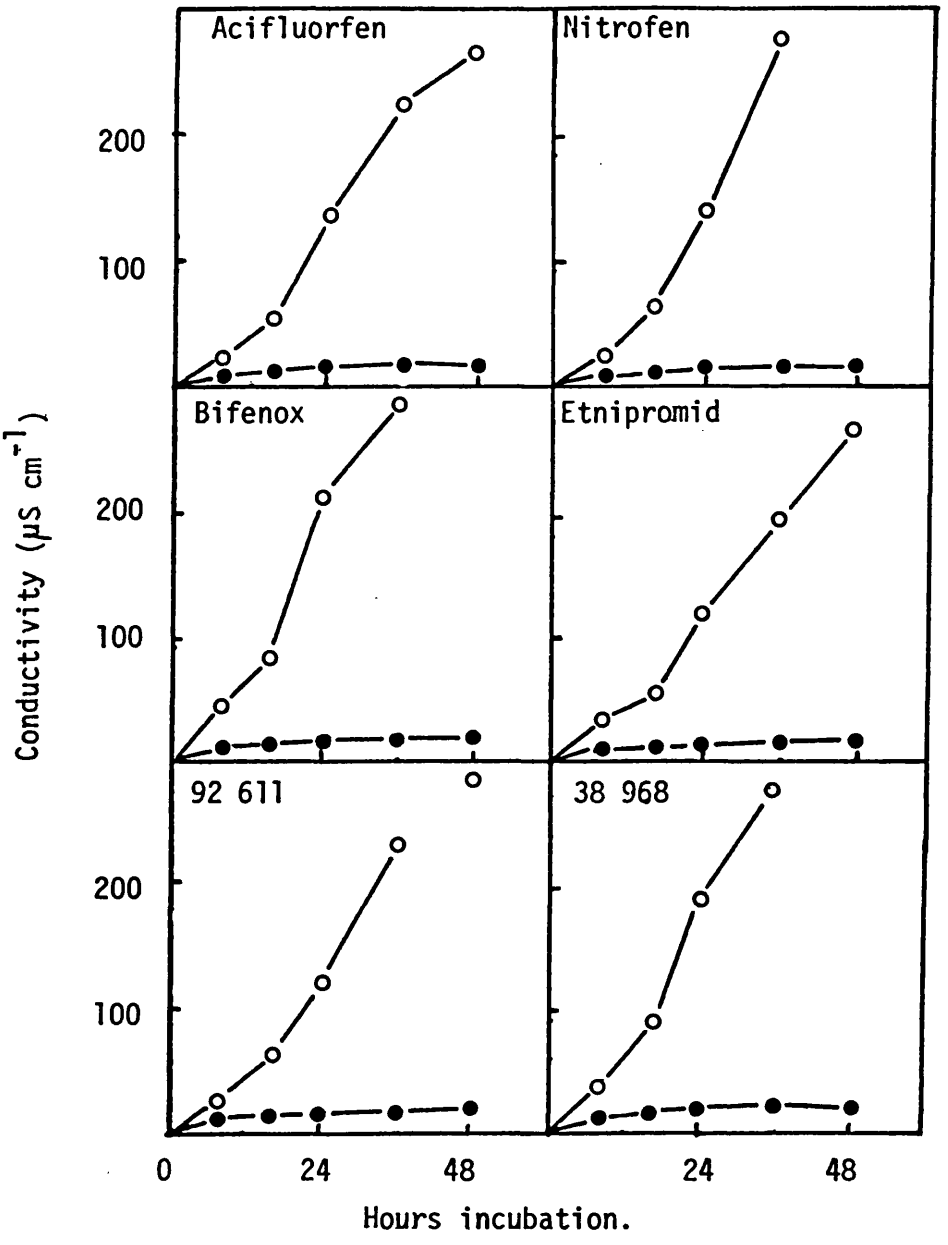


Fig 35. The effect of 25μM DPE herbicides (o) compared with the control treatment (●) on electrolyte leakage from illuminated pea leaf discs.

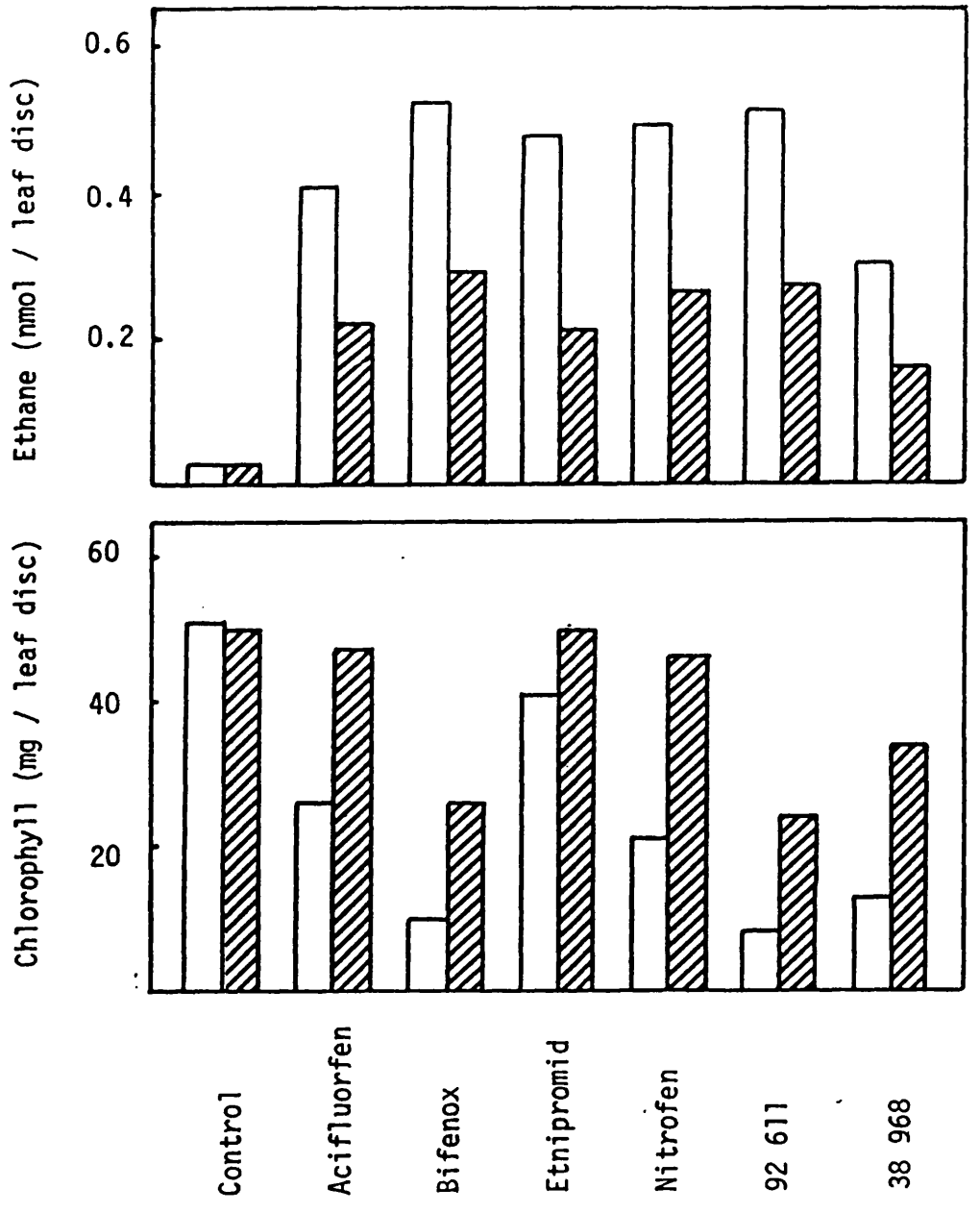


Fig 36. Ethane evolution and chlorophyll bleaching from pea leaf discs incubated on 25µM DPE herbicides for 48h. Leaf discs were preincubated for 24h in darkness on H_2O (□) or 0.1mM monuron (▨).

herbicides for 48 hours in darkness (data not shown). Pea leaf discs pre-incubated on monuron prior to DPE herbicide treatments showed less chlorophyll bleaching and membrane lipid peroxidation than leaf discs pre-incubated on water. DPE herbicide activity therefore required photosynthetic electron flow.

The results presented in Table 12 show that DPE herbicides (25 µM) retarded photosynthetic CO_2 exchange in pea leaf discs after 24 hours dark incubation. Inhibition of photosynthesis was not complete, although CO_2 exchange was totally abolished by these herbicides after a similar period of light incubation (data not shown). DPE herbicides have been reported to promote oxygen uptake by photosystem I or inhibit photosynthetic electron flow (Ridley, 1983; van den Burg and Tipker, 1983; Böger, 1984). To examine the interaction of the compounds shown in Figure 34 with photosynthetic electron flow reactions, thylakoids were isolated from pea leaves and electron transport through photosystem I and II assessed (Table 13). Although acifluorfen showed no inhibition of photosystem II ferricyanide reduction at concentrations up to 50 µM, this was retarded by other DPE herbicides. The inhibitory effect of DPE herbicides such as nitrofen was however poor compared with urea herbicides which inhibit photosystem II function at concentrations below 1 µM (Corbett, 1974). Acifluorfen can induce the peroxidation of chloroplast thylakoids at a concentration of 0.5 µM (Figure 37), and other DPE herbicides induce damage symptoms to leaves at concentrations below 0.1 µM (Orr and Hess, 1981; Böger, 1984). A 50% inhibition of photosystem II electron flow is induced by many DPE herbicides at concentrations above 10 µM (van den Burg and Tipker, 1983). Inhibition of electron flow is therefore unlikely to be the primary mode of action of this group of chemicals. Several DPE herbicides

Table 12. The effect of diphenyl ether herbicides on photosynthetic CO_2 exchange in pea leaf discs after 24 hours dark incubation

	CO_2 uptake (μmol CO_2 $gFW^{-1}h^{-1}$)
Control	71.88 (100%)
25μM Acifluorfen	55.3 (76.8%)
25μM Bifenox	31.2 (43.4%)
25μM Etnipromid	54.6 (75.9%)
25μM Nitrofen	40.7 (56.6%)
25μM 92611	44.0 (61.2%)
25μM 38968	55.4 (77.1%)

Table 13. The effect of diphenyl ether herbicides on electron transport reactions of isolated pea thylakoids.

	$H_2O \rightarrow FeCN$ (PS II)		$H_2O \rightarrow PQ$ (PS I+II)	Ascorb/DCPIP $\rightarrow O_2$ (PS I)	
Control rate	130.5[a] (100%)		146.9[b] (100%)	18.66[b] (100%)	
	Inhibitor concentration (μM)				
	10	50	50	10	50
Acifluorfen	101.6%	100%	91.6%	106%	100%
Bifenox	96.9%	68.7%	73.2%	159%	271%
Etnipromid	96.9%	57.8%	71.8%	153%	297%
Nitrofen	65.1%	23.3%	21.4%	100%	93.2%
92611	100%	79.6%	95.8%	147%	247%
38968	102%	86.5%	95.5%	153%	199%

a. Results as μmol O_2 evolution mg^{-1}chl h^{-1}
b. Results as μmol O_2 uptake mg^{-1}chl h^{-1}

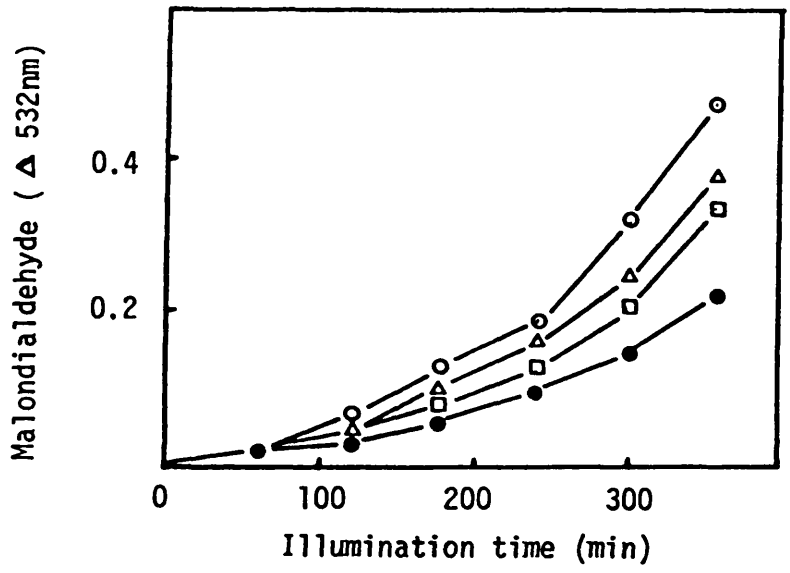


Fig 37. The effect of acifluorfen at 0.5μM (△), 1.0μM (□) or 10μM (○) compared with the control treatment (●) on the peroxidation of illuminated thylakoids.

can promote oxygen uptake by photosystem I (Table 13; bifenox, etnipromid, 38 968, 92 611), although the effect is poor compared with that induced by paraquat (Table 9). These results indicate that depending on their chemical structure, some DPE herbicides can inhibit photosystem II electron flow and promote oxygen uptake by photosystem I. While these effects are unlikely to be the primary damaging action of these compounds, they may contribute to their herbicidal action.

Studies with acifluorfen and oxyfluorfen indicated that herbicide activity was dependent on photosynthetic electron flow through ferredoxin. The role of ferredoxin in the activity of other DPE herbicides was assessed by following the peroxidation of illuminated thylakoids induced by these compounds (Figure 38). All DPE herbicides tested promoted the peroxidation of illuminated thylakoids. Membrane lipid peroxidation that was promoted by acifluorfen and 38 968 was abolished if thylakoids were washed twice prior to use. Peroxidative activity of these two herbicides was restored if exogenous ferredoxin (300 µg) was added to washed thylakoid preparations. The peroxidation of illuminated thylakoids that was promoted by etnipromid or bifenox was unaffected by washing. Herbicide activity, although dependent on functional electron flow (Figure 36) was independent of ferredoxin. Lipid peroxidation induced by nitrofen or 92 611 was partially reduced by thylakoid washing, and this was restored by the addition of exogenous ferredoxin. Damage induced by these two herbicides was probably enhanced by electron flow through ferredoxin although herbicide activation could occur in the absence of ferredoxin.

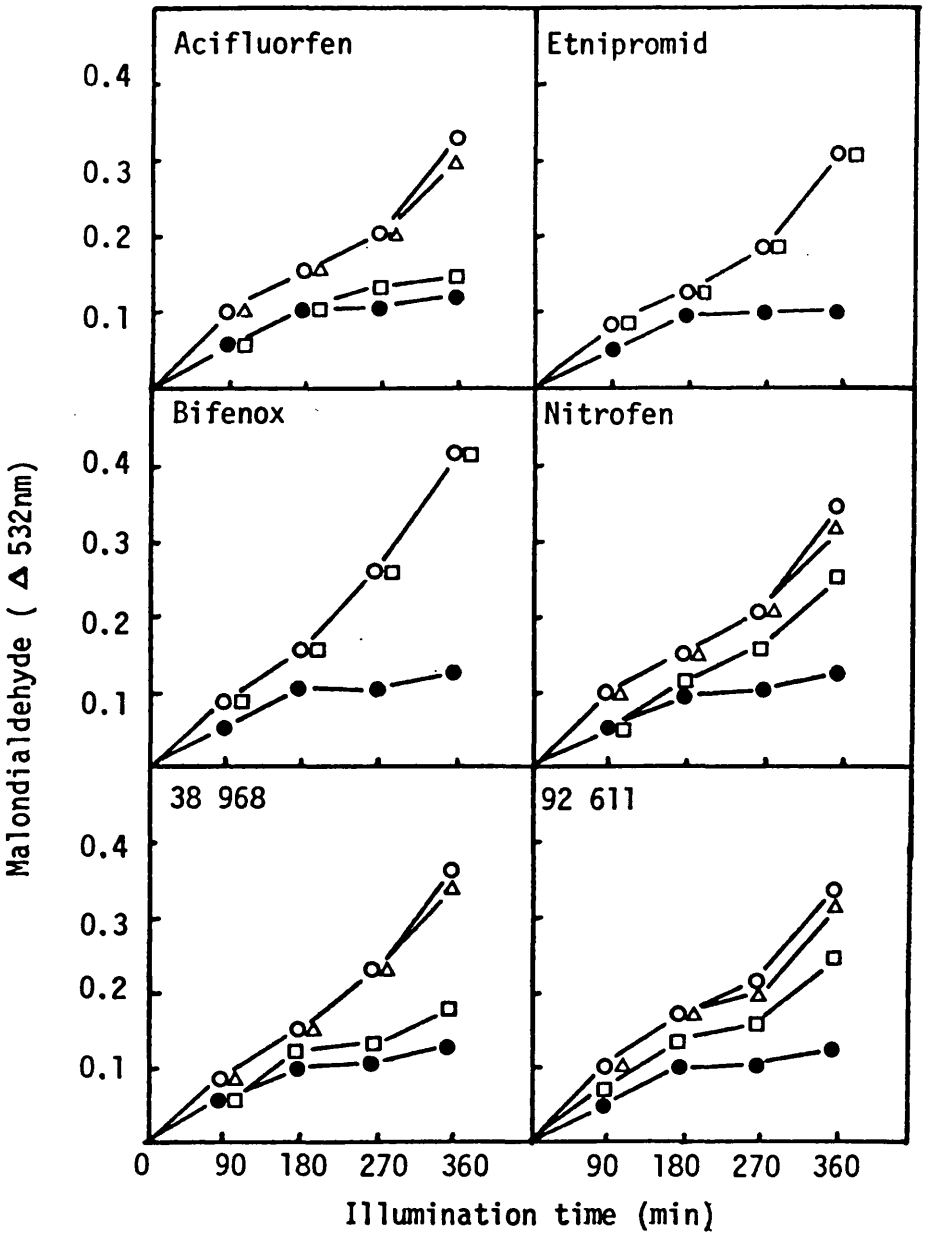


Fig 38. The effect of 25μM DPE herbicides on the peroxidation of illuminated thylakoids. DPE treatments (o,□,△) are compared with the control(●). Thylakoids were unwashed (●,o) or washed twice (□) prior to the experiments, or washed twice and exogenous ferredoxin added (△).

6.3 Discussion

Several studies have indicated that the primary effect of DPE herbicides to plant tissues is to induce light dependent membrane damage. These herbicides caused increased membrane permeability, membrane lipid peroxidation, loss of membrane structure shown by ultrastructural studies, and wilting and desiccation of whole plants (Vanstone and Stobbe, 1979; Kunert and Böger, 1981; Orr and Hess, 1981, 1982; Kenyon *et al.*, 1985). The mechanism of light activation remains unclear, although results from this study support the observations by Kunert and Böger (1981) that photosynthetic electron flow is necessary for the activity of DPE herbicides.

Elucidating the mechanism of DPE herbicide activation by light, and the nature of the radical species formed by light activation that initiates membrane lipid peroxidation may indicate the primary mode of action of this group of chemicals. Draper and Casida (1985) demonstrated the reduction of the nitro DPE herbicide nitrofen to p-nitroso derivatives, and the binding of these derivatives to unsaturated lipids. This promoted the formation of nitroxide radicals that were sufficiently reactive to initiate membrane lipid peroxidation. The initial reduction step was proposed to have been coupled to photosynthetic electron flow. Studies of the redox chemistry of nitrofen, acifluorfen methyl and a range of other nitro-DPE herbicides have shown that their reduction potentials do not preclude reduction by the acceptor site of photosystem I (Orr *et al.*, 1983 a, b; Bowyer *et al.*, 1986). Reduction and reoxidation by oxygen forming $O_2^{\cdot -}$ was not a feasible reaction, thus precluding a paraquat type mode of action (Orr *et al.*, 1983 a, b). Reduction of DPE herbicides by photosystem I or ferredoxin as indicated in this study may therefore be an important

initial step in herbicide activation. Recent studies of other DPE compounds where the p-nitro group was replaced by p-H or p-Cl have shown that although these compounds were herbicidally active, their reduction potentials precluded direct reduction by photosynthetic electron flow (Orr *et al.*, 1983 b; Ensminger *et al.*, 1985). Reduction of the nitro group to a p-nitroso derivative as proposed by Draper and Casida (1985) could not occur in these compounds. It does remain possible that herbicidally active non-nitro DPE compounds are metabolised to nitro DPE compounds *in vivo*. Herbicide activation could then occur by a similar mechanism to the nitro DPE compounds used in this study.

Identification of the radical species formed by DPE herbicide activation that initiates membrane lipid peroxidation may indicate the mechanism of light activation of these herbicides. Electron spin trapping (ESR) techniques have demonstrated the formation of $O_2^{\cdot -}$ in chloroplasts following paraquat treatment (Harbour and Bolton, 1975; Miller and MacDowall 1975; Chia *et al.*, 1982). Similar ESR techniques with illuminated chloroplasts treated with DPE herbicides have shown the formation of radicals, although they were not identified (Lambert *et al.*, 1985). Radical formation induced by DPE herbicides in illuminated chloroplasts was inhibited by diuron, confirming the role of photosynthetic electron flow in herbicide activation (Lambert *et al.*, 1985). Radicals induced by nitro and p-Cl DPE herbicides in these studies were similar, indicating a common mechanism of herbicide activation. Studies by Draper and Casida (1985) using ESR techniques showed the generation of nitroxide radicals in illuminated beet leaves that had been treated with nitrofen and illuminated. Further such studies using other DPE herbicides may indicate whether nitroxide radicals are important

in the activation and herbicidal mode of action of nitro and non-nitro DPE compounds.

Structure activity studies with DPE herbicides have shown that the most active compounds are derivatives of nitrofen and nitrofluorfen with 2,4-Cl or 2,4-CF_3 substitution and a 4'-NO_2 group (see Figure 34). Appropriate substitutions at the 3' position, as in acifluorfen or bifenox, enhance herbicide activity (Lambert *et al.*, 1983). Compounds with 3,5 substitution or substituents at the 2', 5' or 6' positions were inactive (Matsunaka, 1969; Swithenbank, 1982; Lambert *et al.*, 1983). The specific requirement for substituents in certain positions on the ring structures may reflect a specific receptor or binding site for DPE molecules prior to light activation (Swithenbank, 1982). Identification of the nature and location of that binding site may indicate the mechanism of DPE herbicide activation.

Several reports of the mode of action of DPE herbicides have indicated that these herbicides are active in etiolated, non photosynthetic leaves, or in non-chlorophyll containing mutants (Matsunaka, 1969; Orr and Hess, 1982). Furthermore, several workers have shown that inhibition of photosynthetic electron flow prior to DPE herbicide treatments did not affect the development of herbicide injury (Orr and Hess, 1982; Kenyon *et al.*, 1985; Ensminger and Hess, 1985). DPE herbicide activity was observed in mutants that contained carotenoids but not chlorophyll, but not in mutants lacking both chlorophyll and carotenoids (Matsunaka, 1969). Herbicide activity was also observed in etiolated leaves that contained carotenoids but not chlorophyll (Orr and Hess, 1982). These workers suggested that DPE herbicide activation occurred through carotenoid pigments, although no mechanism was proposed.

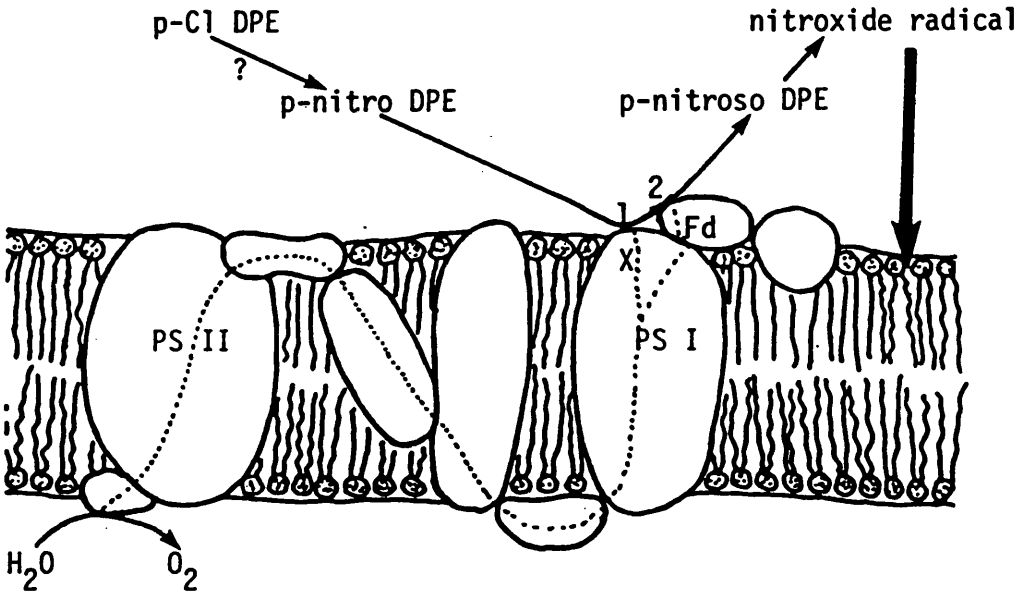


Fig 39. Scheme outlining possible site of activation of DPE herbicides by photosynthetic electron flow. Reduction of nitro DPE's by PS I (1) or ferredoxin (2) forms p-nitroso derivatives and nitroxide radicals that initiate membrane damage. Non-nitro DPE's may be metabolised in vivo to nitro derivatives.

Although damage induced by DPE herbicides has been well documented, their mechanism of activation by light remains unclear. Whether activation occurs by carotenoid pigments or photosynthetic electron flow is unresolved. Results from this study indicate that in photosynthetically active tissues, chloroplast electron flow is important in DPE herbicide activity, activation occurring via photosystem I or ferredoxin. Interaction with photosynthetic electron flow at a site on the thylakoid close to ferredoxin may promote the formation of nitroxide radicals that initiate lipid peroxidation, as proposed by Draper and Casida (1985). Activation of non-nitro DPE herbicides may occur through their conversion to nitro compounds *in vivo*, followed by herbicide activation. This proposed mechanism of DPE herbicide activation is shown in Figure 39. The identification of the site of DPE herbicide binding to membranes, and the nature of radicals formed after light activation that initiate subsequent membrane damage are central to elucidating the mode of action of this group of herbicides.

7. Environmental stress and photoinhibition of chloroplast reactions

Light energy absorbed by chloroplasts that is not utilised in photosynthetic carbon metabolism or other light driven metabolic reactions may damage the photosynthetic apparatus. Such damage has been observed if photosynthesis is impaired during chilling or drought stress, or if shade adapted plants are transferred to strong light (Osmond, 1981; Powles, 1984). Damage,termed photoinhibition, induces the loss of photosystem II function and has been extensively studied in leaves, leaf cells, isolated chloroplasts and thylakoids (Krause *et al.*, 1978, 1985; Powles *et al.*, 1979, 1983; Critchley, 1981; Cornic *et al.*, 1982; Powles, 1984). Photoinhibition is promoted by light, although the mechanism of damage is unclear (Powles, 1984). Several reports have indicated that reduced photosynthetic activity during stress may promote the formation of toxic oxygen species that induce damage to the photosynthetic apparatus (van Hasselt, 1972, 1974; Osmond, 1981; Barényi and Krause, 1985; Krause *et al.*, 1985). Damage would probably occur analogous to the action of certain herbicides that inhibit photosynthesis. In this study the photoinhibition of photosynthesis has been assessed in leaves incubated at chilling temperatures, and in isolated chloroplasts incubated in the absence of electron acceptors.

7.1 Photoinhibition of photosynthesis induced by chilling temperatures

The effect of chilling on the photosynthetic activity of pea leaf discs or maize leaf segments was investigated. Leaf material was incubated on distilled water and maintained at 20°C or 5°C, using an ice bath, in darkness or under illumination. Leaves were returned to room temperature and photosynthetic activity assessed. The results presented in Figure 40 show the effect of 5 hours incubation at 20°C

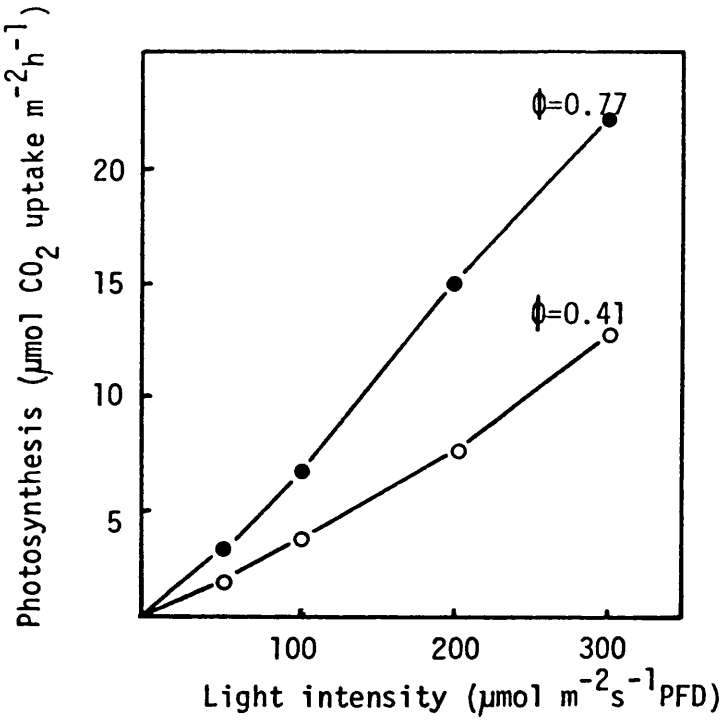


Fig 40. The effect of 5h illumination at 20°C (●) or 5°C (o) on the rate of subsequent CO_2 exchange in pea leaf discs. Photosynthesis was assessed over a range of light intensities, and the slope of the plot used to estimate the apparent quantum yield of the reaction (ϕ).

or 5°C on the subsequent rate of photosynthetic CO_2 exchange measured at room temperature. Photoinhibition characteristically reduces the rate of light saturated photosynthesis and the quantum yield (Osmond, 1981; Long *et al.*, 1983). The results presented in Figure 40 demonstrate that chilling pea leaf discs for 5 hours reduced the quantum yield of photosynthesis by nearly 50%. In subsequent experiments photosynthetic CO_2 exchange was assessed at 300 μmol $m^{-2}s^{-1}$ photon flux density as an indicator of photoinhibition. The results presented in Figure 41 demonstrate the effect of the chilling period on the development of photoinhibition, assessed by photosynthetic CO_2 exchange (Figure 41a) or chlorophyll fluorescence (Figure 41b).

Chlorophyll fluorescence kinetics have been increasingly used in recent years to assess the inhibition of photosynthetic reactions *in vivo* (Papageorgiou, 1975; Baker and Bradbury, 1981). The induction of chlorophyll a fluorescence in photosynthetic cells has been resolved into fast and slow phases. The fast phase, of a few seconds, consists of a biphasic rise from the initial level, through O, to a maximum P followed by a slow decline in fluorescence yield (Figure 42a). These changes are considered to reflect photochemical events associated with photosystem II (Baker and Bradbury, 1981). The initial rise to O represents the fluorescence of constant yield from photosystem II, occurring prior to excitation energy inducing photochemical electron flow through the photosystem II reaction centre. The rise above O, the variable component of fluorescence, is correlated with photosystem II mediated electron flow that reduces the primary electron acceptor Q. Following the peak, P, fluorescence slowly declines as electron flow mediated by photosystem I and CO_2 reduction initiates the reoxidation of Q. The transient F_I through P and the subsequent fluorescence decline

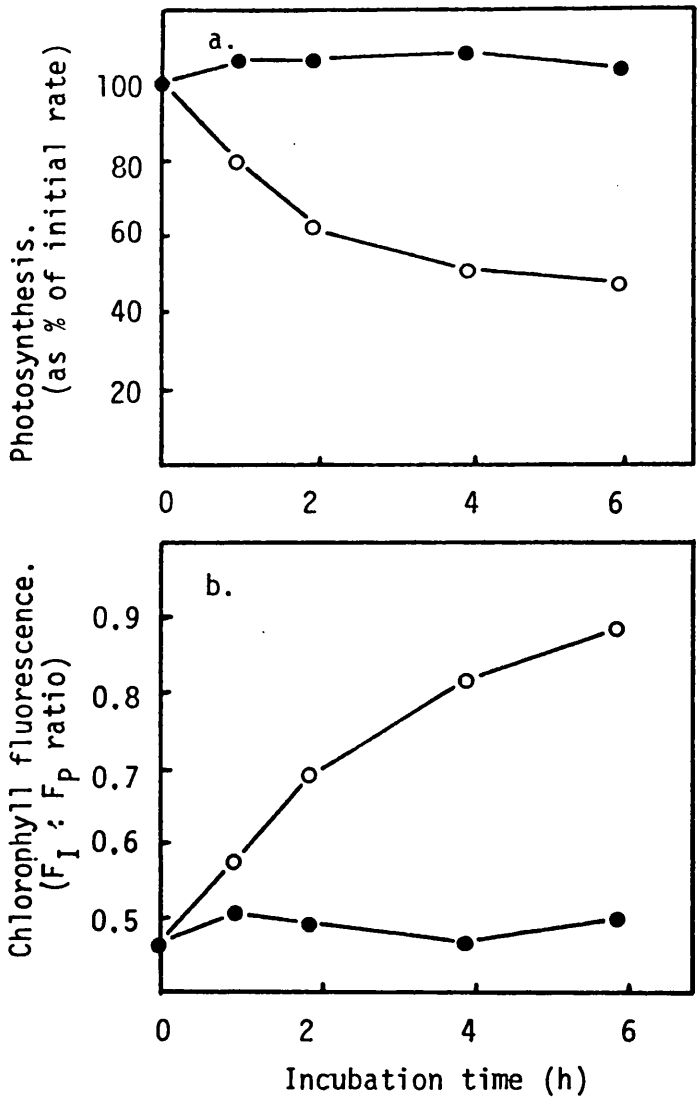


Fig 41. The development of photoinhibition of photosynthesis in pea leaf discs incubated in light at 20°C (●) or 5°C (○). Photoinhibition was subsequently assessed at room temperature by CO_2 exchange (a) or chlorophyll fluorescence kinetics (b).

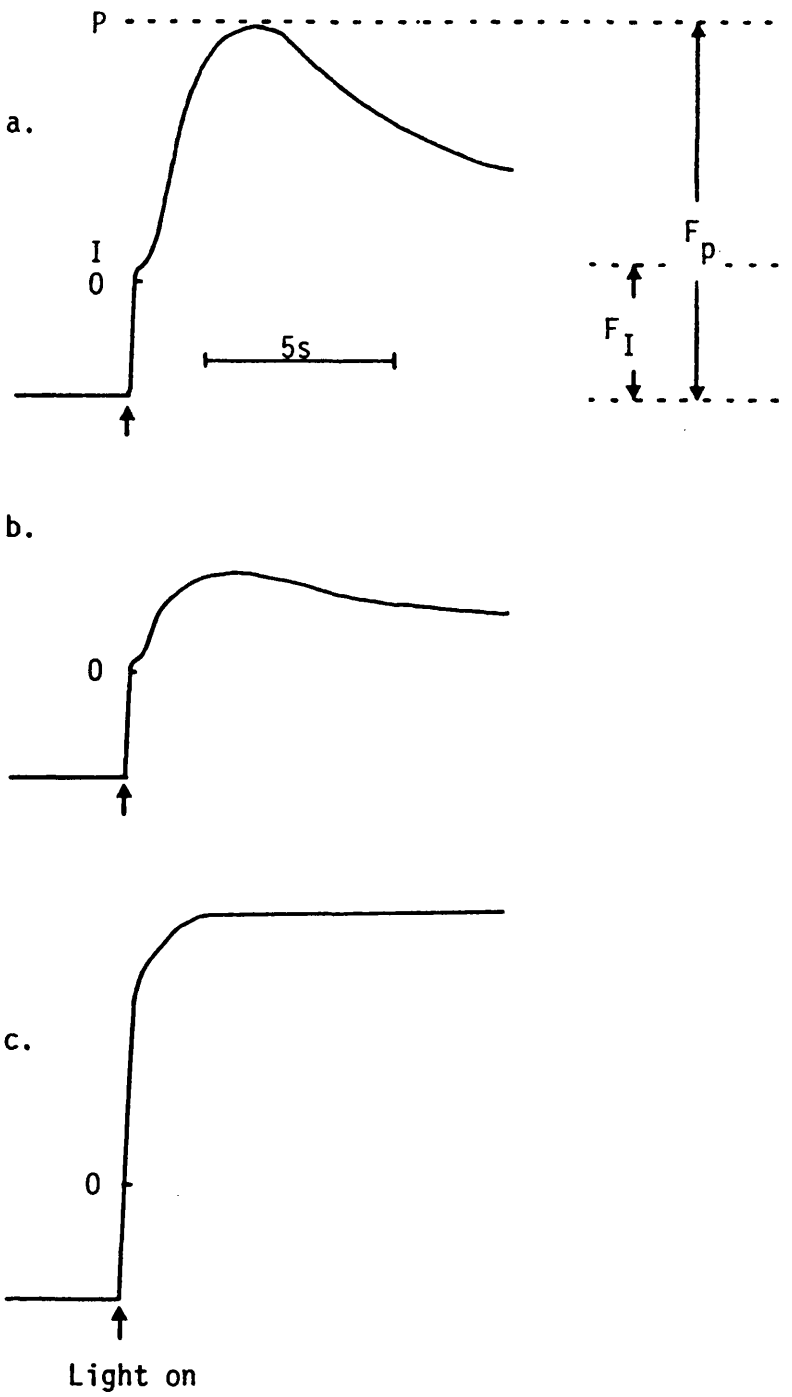


Fig 42. Chlorophyll fluorescence induction curves from pea leaves, showing untreated control (a), chlorophyll fluorescence following 5 hours illumination at 5°C (b) and the effect of monuron (c).

is considered to reflect the redox state of Q (Papageorgiou, 1975). The arbitrary ratio $F_I:F_P$ has been used in this study to quantify the variable fluorescence yield of leaf discs following chilling treatments. Treatments that prevent photosystem II activity reduce the fluorescence peak, giving an increased $F_I:F_P$ ratio (Figure 42b). Inhibition of electron flow beyond Q, as occurs when electron flow is inhibited by monuron (Figure 42c), or if photosystem I is damaged, prevents the reoxidation of Q. Fluorescence yield is therefore at maximum value and does not decline beyond P. Incubating pea leaf discs at chilling temperatures increased the $F_I:F_P$ ratio (Figure 41b), indicating damage to photosystem II function.

The role of light intensity in the photoinhibition of pea and maize leaves incubated at 20°C or 5°C for 5 hours is shown in Figure 43. No photoinhibition was detected after 5 hours dark incubation. Damage was promoted by increased light intensities.

Photoinhibition is commonly characterised by reduced photosystem II function although loss of photosystem I activity may also occur (Powles, 1984). To assess the site of photoinhibition of photosynthesis induced by chilling, pea and maize leaves were incubated for 5 hours at 5°C or 20°C under constant illumination (400 μmol $m^{-2}s^{-1}$ photon flux density). Chloroplast thylakoids were isolated from leaf material and photosynthetic electron flow reactions assessed (Figure 44). Photoinhibition of photosynthesis in pea and maize resulted in loss of photosystem II function, using either FeCN or SiMo as photosystem II electron acceptors, or paraquat to assess electron flow through photosystem I and II. Diphenyl carbazide (DPC), which can donate electrons to photosystem II (Izawa, 1980) was unable to restore electron flow. This indicated that damage had occurred to the photosystem II reaction

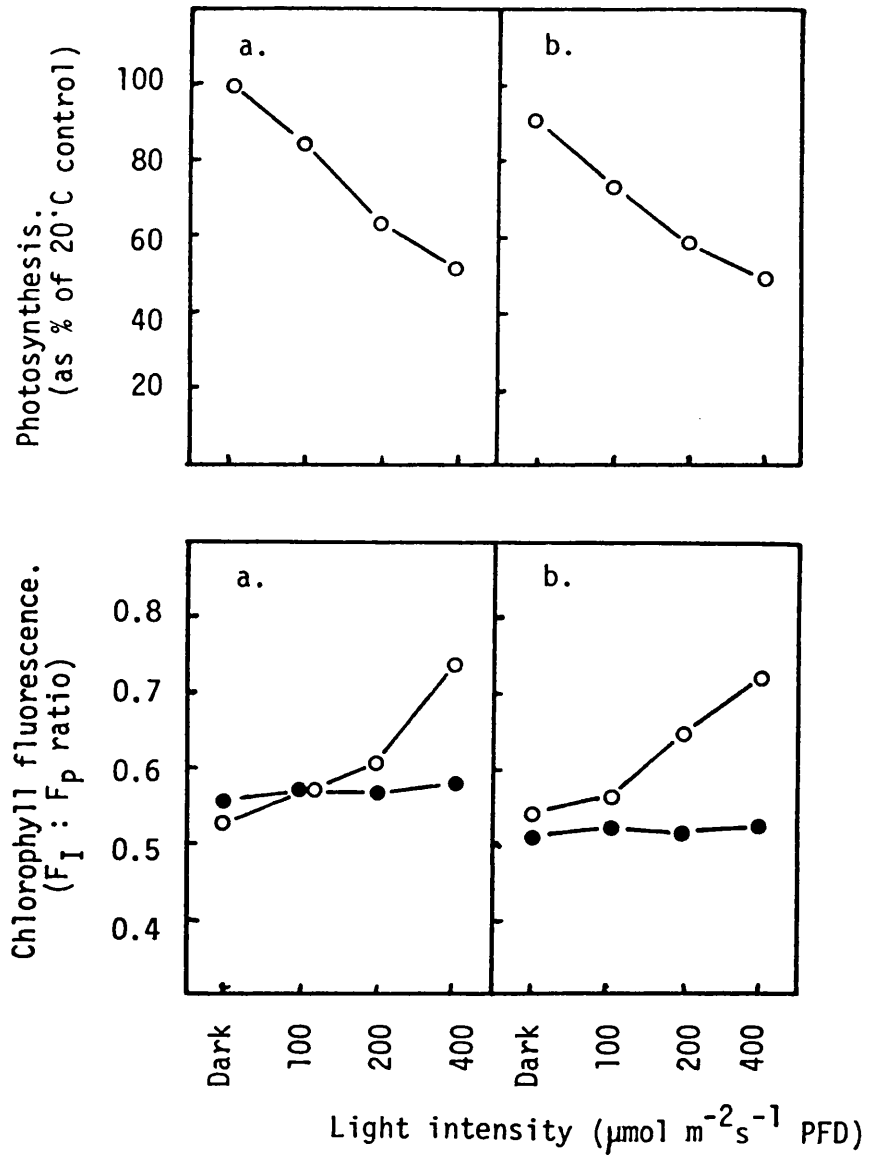


Fig 43. The effect of light intensity during incubation on the development of photoinhibition in pea (a) or maize (b) leaves incubated at 5°C (o) or 20°C (●). Photosynthetic activities were assessed at room temperature after 5h preincubation at 100, 200 or 400 μmol $m^{-2}s^{-1}$PFD or in darkness.

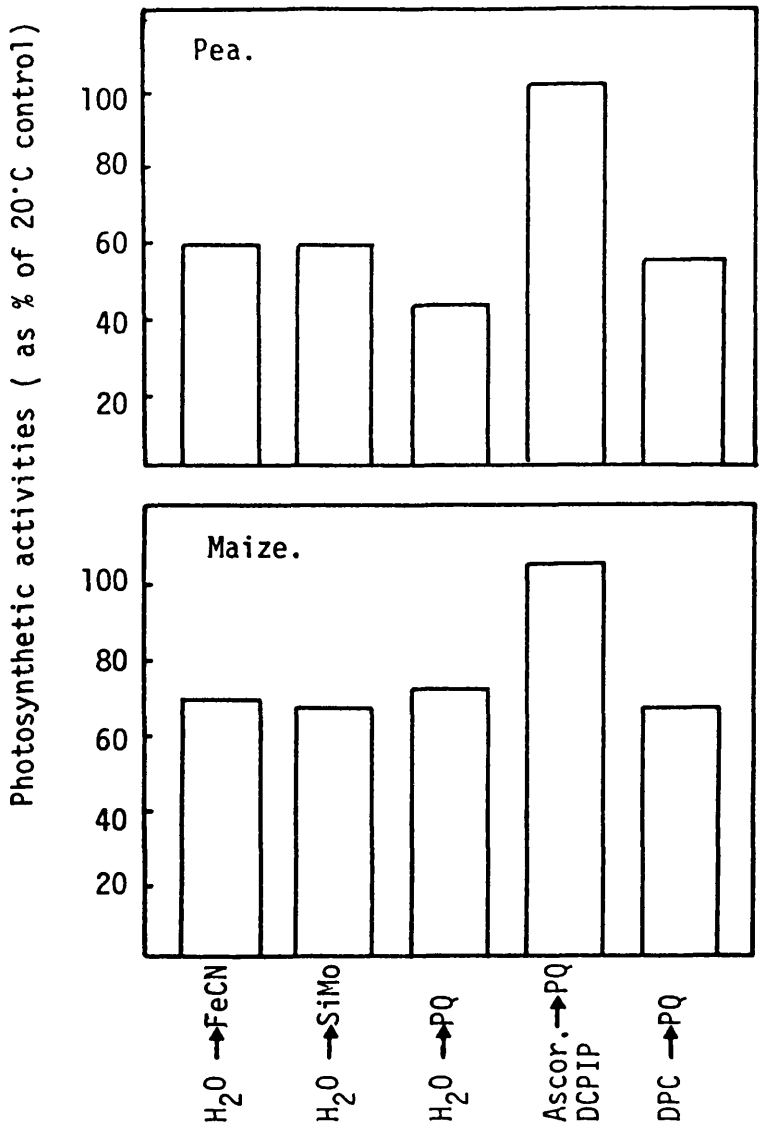


Fig 44. Inhibition of photosynthetic electron flow reactions in thylakoids isolated from pea or maize leaves incubated at 20°C or 5°C for 5h. The results are expressed as electron flow rates at 5°C as a percentage of the 20°C control.

centre, and not to the water splitting reaction. Electron flow through photosystem I using ascorbate/DCPIP as an electron donor coupled to paraquat as an acceptor was unaffected by chilling pretreatments.

Inhibition of photosynthetic electron flow by urea herbicides has been reported to reduce chilling induced photoinhibition of photosynthesis (Mustardy *et al.*, 1984). To assess the role of photosynthetic electron flow in photoinhibition of pea and maize leaves during chilling, leaves were incubated on water or 10 μM monuron for 5 hours at 5°C or 20°C and illuminated. Chloroplasts were isolated from chilled or unchilled leaves and photosynthetic electron flow assessed. If photoinhibition was induced by a promotion of $O_2^{\cdot -}$ formation during chilling, it may be expected that impaired rates of electron flow during chilling by monuron would provide some protection against damage. Protection would be similar to that provided by monuron against the herbicical action of paraquat (Section 5.2). The results presented in Figures 44 and 45 show the rates of photosynthetic electron flow in thylakoids isolated from leaves incubated on water (Figure 44) or monuron (Figure 45). The results show the rate of electron flow in leaves incubated at 5°C as a percentage of the rates from leaves incubated at 20°C. The photosystem II electron acceptor SiMo is particularly useful in such studies. SiMo accepts electrons prior to the site of action of monuron (Izawa, 1980), and can therefore be used to assess photosystem II activity in leaf material incubated on monuron solutions. Leaf material incubated on water for 5 hours at 5°C showed 40% loss of photosystem II (SiMo reduction) activity in pea and 35-40% inhibition in maize compared to controls incubated at 20°C (Figure 44). Leaf material incubated on 10 μM monuron for 5 hours at 5°C showed similar photosystem II inhibition (Figure 45). Monuron thus provided no protection against

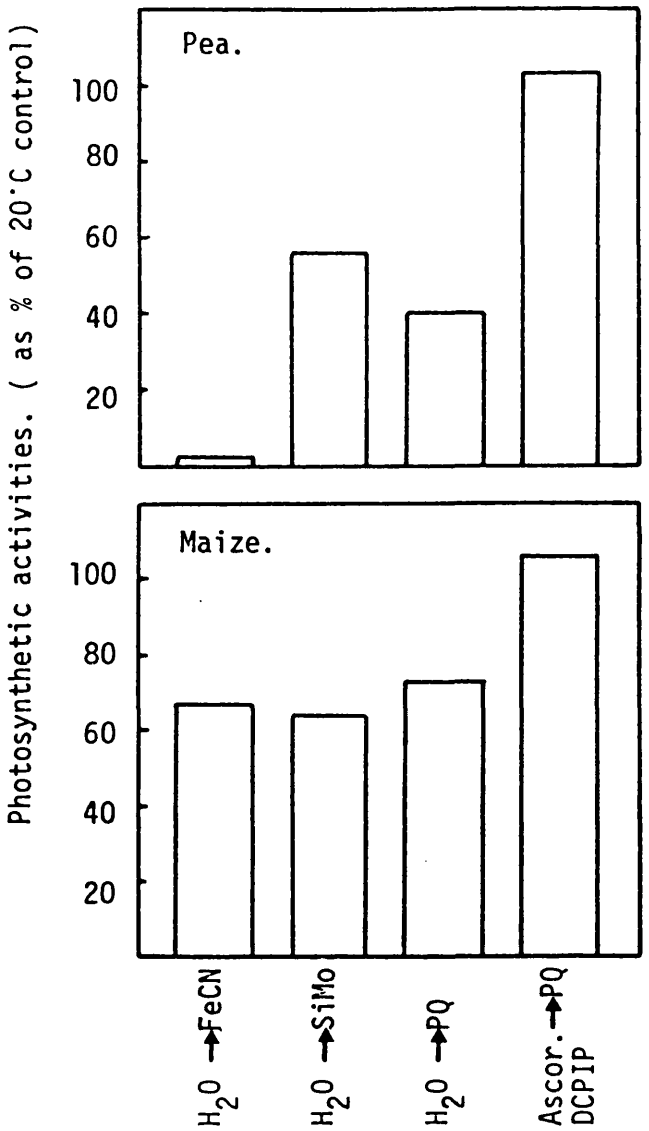


Fig 45. Inhibition of photosynthetic electron flow reactions in thylakoids isolated from pea or maize leaves incubated on 10μM monuron at 20°C or 5°C for 5h. The results are expressed as the electron flow rate in thylakoids from leaves at 5°C as a percentage of those incubated at 20°C.

chilling induced loss of photosystem II activity. Electron flow through photosystem I and II, using paraquat as an electron acceptor in thylakoids isolated from leaves incubated on water (Figure 44) or monuron (Figure 45) at 5°C or 20°C showed similar photoinhibition.

These results can be compared with the effect of monuron on the loss of photosystem II activity that occurred when leaves were incubated on paraquat and illuminated (Figure 46). Paraquat promotes oxygen reduction to $O_2^{\cdot -}$ by photosystem I, and thus its damaging action is dependent on photosynthetic electron flow. Thylakoids isolated from pea leaf discs incubated on 10 μM paraquat for 5 hours under continuous illumination showed 35-40% loss of photosystem II activity using either FeCN or SiMo as electron acceptors. Electron flow through photosystem I and II was retarded to a similar extent, although photosystem I electron flow was unaffected (Figure 46a). The effect of incubating leaf discs on monuron (10 μM) or monuron plus paraquat on electron transport activity is shown in Figure 46b. The activity of electron flow reactions in thylakoids isolated from leaf discs incubated on paraquat plus monuron is shown as a percentage of the activity of discs incubated on monuron alone. Monuron almost completely protected photosystem II activity against paraquat induced damage. This indicates that reduced rates of electron flow in monuron treatments restricted paraquat induced formation of $O_2^{\cdot -}$ by photosystem I. Protection was therefore similar to monuron protection against paraquat induced chlorophyll bleaching of pea leaf discs demonstrated previously (Table 10). These results demonstrate that the mechanism of chilling induced damage to photosystem II occurred by a different mechanism from damaged induced by paraquat.

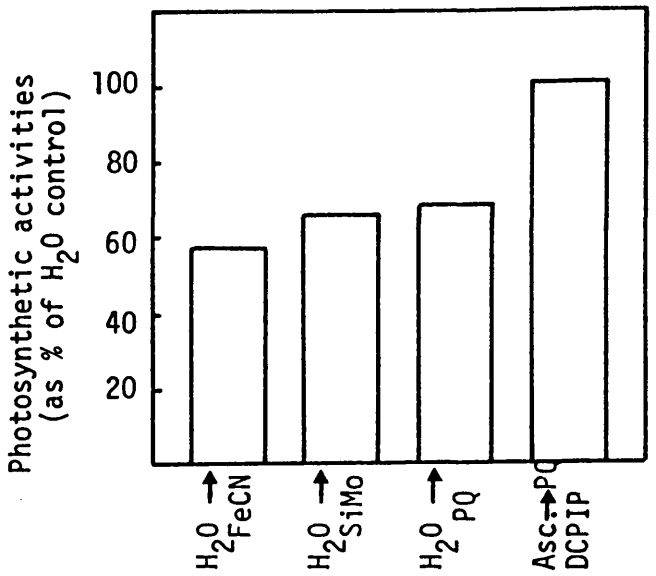


Fig 46a. Inhibition of photosynthetic electron flow in thylakoids isolated from pea leaf discs incubated for 5h on H_2O or 10μM paraquat. The results shown are the activity of thylakoids from leaves incubated on paraquat as a percentage of the H_2O control treatment.

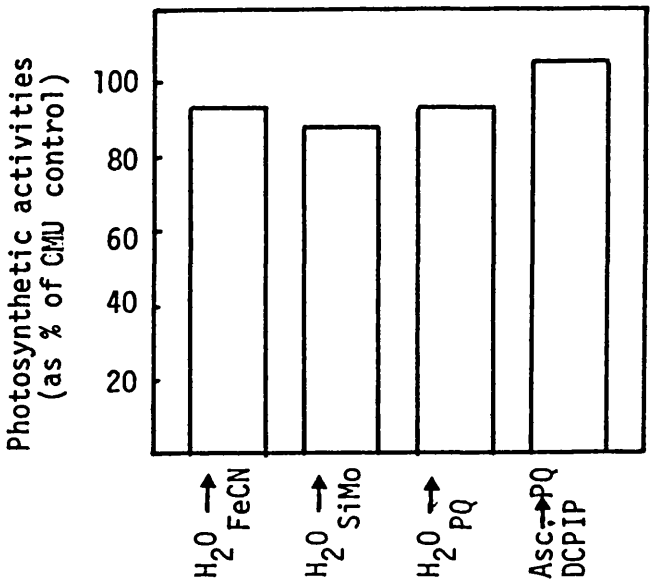


Fig 46b. Inhibition of photosynthetic electron flow in thylakoids isolated from pea leaf discs incubated for 5h on 10μM monuron or 10μM paraquat + 10μM monuron. The results shown are the activity of thylakoids from leaves incubated on paraquat + monuron as a percentage of the monuron treatment.

Studies by Michalski and Kanuiga (1981) have indicated that one consequence of chilling was a loss of SOD activity from leaves. Long (1983) suggested that chilling-induced photoinhibition in maize may occur if the activities of chloroplast $O_2^{\cdot -}$ and H_2O_2 scavenger enzymes were inhibited by chilling treatments. Homogenates of pea or maize leaves that had been incubated at 5°C for 5 hours showed no loss of SOD, ascorbate peroxidase, glutathione reductase or ascorbate compared to leaves incubated at 20°C (data not shown). Reduced enzyme activity at low temperatures may however impair their ability to scavenge $O_2^{\cdot -}$ and H_2O_2 in chloroplasts. This may contribute to the photo-oxidative damage that occurs if chilling treatments are prolonged (van Hasselt, 1972, 1974).

7.2 Photoinhibition of isolated chloroplasts

Photoinhibition of electron transport can be induced in isolated chloroplasts or chloroplast fragments incubated in the absence of electron acceptors (Krause *et al.*, 1978; Barényi and Krause, 1985). Such studies may assist in the elucidation of the mechanism of photo-inhibition, and the role of activated oxygen species in the induction of damage.

Chloroplast membranes (100 µg chlorophyll) isolated from pea plants were incubated in vials containing 30 mM Tricine-NaOH buffer (2.5 ml) at varying light intensities for up to 15 minutes. The contents were transferred to the reaction chamber of an oxygen electrode at three minute intervals and the rate of photosynthetic electron flow assessed using paraquat as an electron acceptor. Electron flow in chloroplasts that had been illuminated was compared with that of chloroplasts incubated in darkness. These results are presented in Figure 47a. A

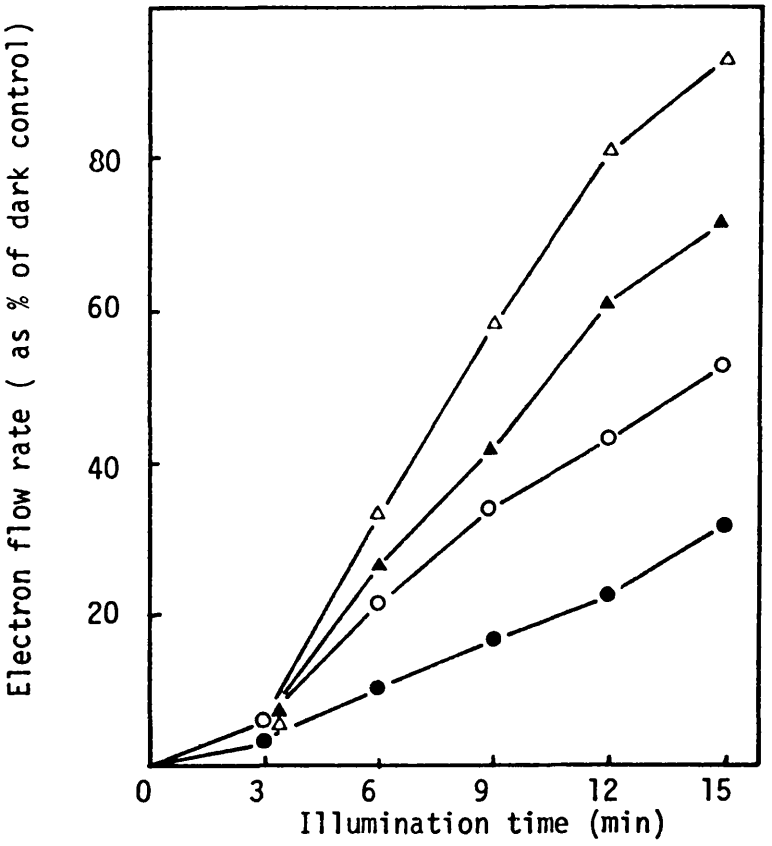


Fig 47a. Inhibition of photosynthetic electron flow induced by incubation of thylakoids in the absence of electron acceptors at 250 (●), 500 (○) 750 (▲) or 1000 (■) μmol $m^{-2}s^{-1}$PFD for up to 15 minutes.

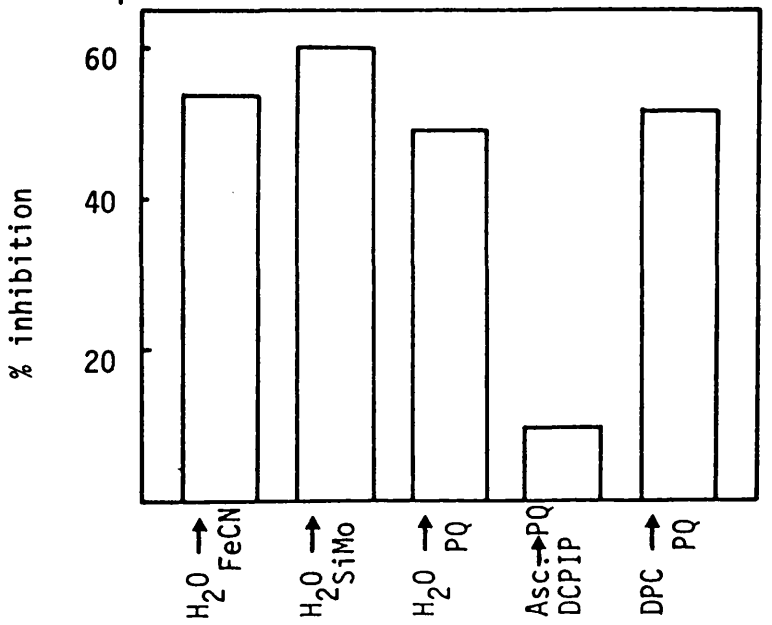


Fig 47b. Inhibition of electron flow in pea thylakoids atfer 10 minutes preincubation at 750 μmol $m^{-2}s^{-1}$ PFD.

reduction in photosynthetic electron flow in illuminated chloroplast thylakoids compared with those incubated in darkness was observed after six minutes incubation. Photoinhibition was more marked in thylakoids incubated at higher light intensities.

To investigate the site of photoinhibition, chloroplast membranes were incubated for 10 minutes at 750 μmol $m^{-2}s^{-1}$ photon flux density prior to the assessment of electron transport (Figure 47b). Photoinhibition of photosynthetic electron flow was expressed as inhibition of photosystem II activity. Photosystem I activity was retarded by 10% as a consequence of preillumination. The inability of DPC to restore electron flow through photosystem II indicated that damage had occurred to the photosystem II reaction centre.

The results presented in Figure 48 show the effect of various additions during the chloroplast preincubation period on the rate of subsequent electron flow. They are expressed as the percentage inhibition of ferricyanide reduction that occurred in illuminated chloroplasts compared with controls maintained in darkness. Preincubation of chloroplasts induced a 40-50% loss of photosystem II activity. Addition of FeCN (1.0 mM) during the preincubation period reduced the degree of photoinhibition. Protection provided by FeCN was reduced if monuron concentrations (1.0 μM) that partially inhibit electron flow were also added during the preincubation period. These results indicate that photoinhibition of chloroplast reactions achieved by incubation in the absence of electron acceptors could be mimicked by incubating thylakoids with an electron acceptor in the presence of monuron. In the absence of electron acceptors light energy absorbed by chlorophyll cannot be dissipated by photosynthetic electron flow. Thus damage induced to the photosystem II reaction centre would be

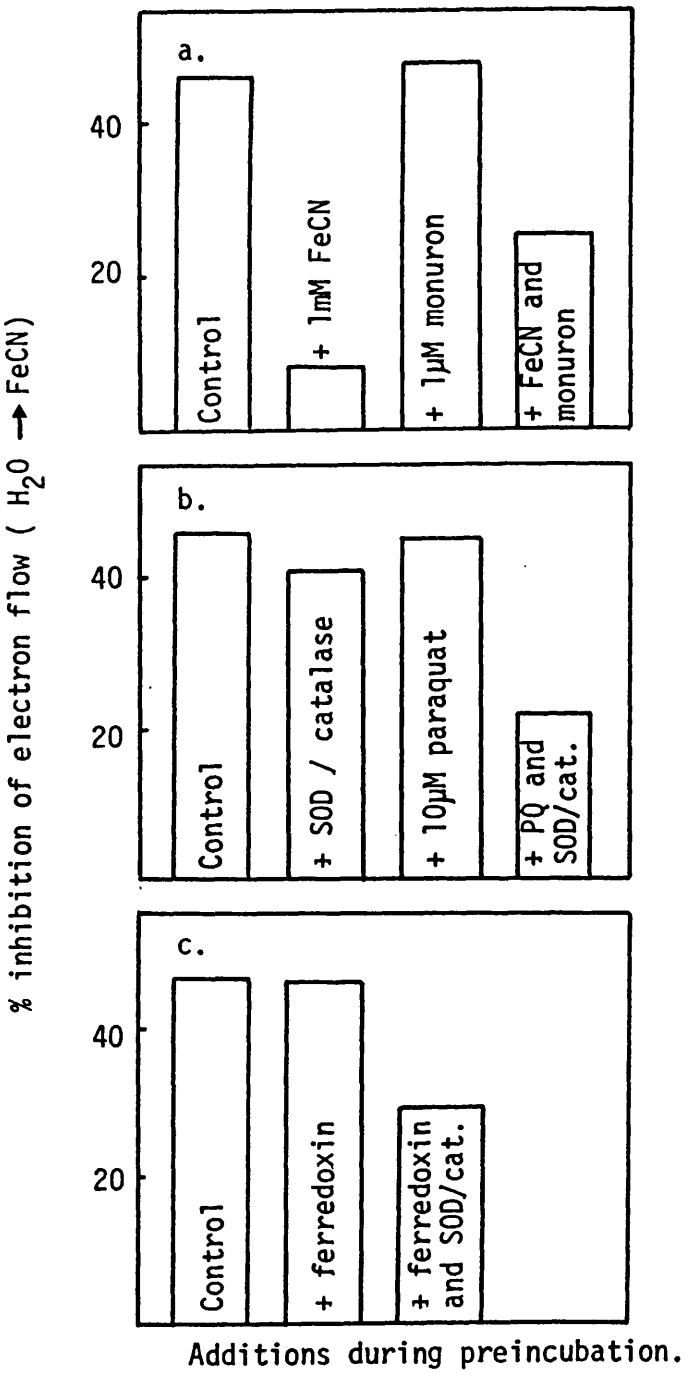


Fig 48. Photoinhibition of photosynthetic electron flow induced by 10 minutes incubation in light. The effect of various additions during the preincubation period on the inhibition of electron flow.

similar to damage induced by inhibition of photosynthetic electron flow with monuron. Addition of FeCN to thylakoids, or bicarbonate to intact chloroplasts (Krause *et al.*, 1978; Barényi and Krause, 1985) permits maintained electron flow through photosystem II during pre-incubation, thus preventing over-reduction of the electron carriers and hence, photoinhibition.

The results in Figure 48b show the effect of paraquat (10 μM) and SOD plus catalase (250 units) added during the preincubation period on the inhibition of photosystem II ferricyanide reduction. Neither paraquat or SOD plus catalase alone protected photosystem II against photoinhibition. Thus even though paraquat promotes electron flow to oxygen, thereby maintaining electron flow through photosystem I and II, this did not prevent photoinhibition. Results presented previously (Figure 46) demonstrated that paraquat can induce damage to photosystem II. Superoxide formed as a consequence of paraquat action probably initiates damage. Incubation of chloroplasts with paraquat and SOD plus catalase significantly retarded damage to photosystem II (Figure 48b). These results indicate that photoinhibition of isolated thylakoids can be prevented by electron flow to oxygen provided that a catalyst of oxygen reduction is present (paraquat), and $O_2^{\cdot -}$ formed is scavenged by SOD and catalase. SOD and catalase did not protect photosystem II against photoinhibition unless paraquat was also present. This indicates that the endogenous rate of oxygen reduction by photosystem I was too low to maintain sufficient electron flow through photosystem II to prevent photoinhibition. In chloroplasts *in vivo* oxygen reduction by photosystem I is catalysed by ferredoxin. Addition of ferredoxin to illuminated thylakoids promotes oxygen uptake by photosystem I (Halliwell, 1981).

The results presented in Figure 48c show the effect of ferredoxin and SOD plus catalase on photoinhibition of illuminated thylakoids. Addition of ferredoxin (150 μg) alone did not prevent loss of photosystem II function that occurred as a consequence of preincubation in light. However ferredoxin together with SOD plus catalase did reduce the degree of photoinhibition. This was similar to that shown in Figure 48b with paraquat and SOD plus catalase. These results indicate that either ferredoxin or paraquat can promote electron flow to oxygen. Provided that the products of oxygen reduction are scavenged, photoinhibition can be restricted.

7.3 Discussion

Although the inhibition of photosynthesis induced by chilling leaves in light has been extensively studied (Taylor and Rowley, 1971; Rowley and Taylor, 1972; Lindenman, 1979; Powles *et al.*, 1983), the mechanism of damage remains unclear. Photosynthesis is one of the first processes to be adversely affected by low temperatures (Berry and Björkman, 1980). Chilling temperatures at high light intensities in the presence of oxygen cause damage to the photosystem II reaction centre (Rowley and Taylor, 1972; Powles *et al.*, 1983), as also demonstrated in this study. Reduced photosynthetic function at low temperature may occur as a conseqeunce of reduced activity of carboxylative enzymes, or because phase changes in thylakoid membrane lipids impair electron flow (Berry and Björkman, 1980; Oquist, 1984). One consequence of reduced photosynthetic function at chilling temperatures is that less light is required to saturate photosynthesis than at higher tempertures (Berry and Björkman, 1980). Incubating leaves at low temperature and at light intensities in excess of that required to saturate

photosynthesis induces photoinhibition. Photosynthetic reactions are unable to dissipate light energy trapped by chloroplast pigments under such conditions. The mechanism of damage is probably similar to that occurring when shade adapted plants are transferred to strong light (Powles and Critchley, 1980; Critchley, 1981), or if photosynthesis is restricted during drought stress (Newton *et al.*, 1981; Björkman and Powles, 1984), or by incubating leaves in the absence of both CO_2 and O_2 (Powles and Osmond, 1978; Krause *et al.*, 1985). Impaired photosynthetic carbon metabolism reduces the availability of $NADP^+$ as a photosystem I electron acceptor. Illumination may promote electron flow to oxygen with the formation of $O_2^{\cdot -}$. Alternatively the reduced availability of electron acceptors, or disruption of the electron transport pathway itself as a consequence of membrane damage during stress, may promote energy spillover from chlorophyll to form ^3Chl or $^1O_2$. Damage by these mechanisms would therefore occur in a manner analogous to the mode of action of paraquat or photosynthetic electron flow inhibitor herbicides. Results from this study indicate that $O_2^{\cdot -}$ did not initiate photoinhibition of photosynthesis in pea or maize leaves during chilling. While monuron prevented damage to photosystem II induced by paraquat, no such protection was provided against chilling induced damage. Chilling-induced photoinhibition probably occurred because photosynthetic electron flow was impaired by low temperature, promoting energy spillover from chlorophyll to form ^3Chl and $^1O_2$ as proposed by van Hasselt (1974). Photoinhibition and subsequent membrane damage during chilling would therefore be similar to the damage occurring when photosynthetic energy dissipation from chlorophyll is inhibited by certain herbicides that prevent photosynthetic electron flow (Dodge, 1983).

The mechanism of photoinhibition was examined further in experiments with isolated thylakoids. Photoinhibition was induced by incubating thylakoids in light in the absence of electron acceptors. Addition of ferricyanide reduced damage to photosystem II. Similar experiments (Barényi and Krause, 1985; Krause *et al.*, 1985) showed that photoinhibition of intact chloroplasts or thylakoids induced by illumination in the absence of electron acceptors, could be prevented by the addition of bicarbonate to intact chloroplasts, or ferricyanide to thylakoids. These experiments indicate that in the absence of photosynthetic electron flow acceptors, over-reduction of the electron transport chain induces damage to photosystem II. Damage probably occurs because over-reduction of the electron transport chain promotes oxygen reduction to $O_2^{\cdot -}$ by photosystem I, or because energy spillover from chlorophyll increases the formation of ^3Chl and $^1O_2$. The experiments in this study indicate that both mechanisms of damage may occur. Photoinhibition of photosystem II activity in illuminated thylakoids was relieved by addition of ferricyanide during the preincubation period. If however electron flow was impaired by monuron, inhibition of photosystem II activity was observed. This indicates that photoinhibition occurred because electron flow beyond photosystem II was impaired, either as a consequence of illuminating chloroplasts without electron acceptors, or because electron flow was inhibited by herbicides. Reduction of oxygen by photosystem I did not protect thylakoids against photoinhibition unless a catalyst of oxygen reduction (paraquat or ferredoxin), and a scavenger of the products of oxygen reduction (SOD plus catalase) were added. In the absence of SOD and catalase damage occurred presumably because $O_2^{\cdot -}$ formed by photosystem I oxygen reduction initiates damage to photosystem II.

Studies of the role of oxygen in photoinhibition of thylakoids, intact chloroplasts, leaf cells or whole leaves have shown that oxygen can promote or protect against photoinhibition. Damage induced by incubating thylakoids or leaves in light under anaerobic conditions was reduced if oxygen was present (Krause *et al.*, 1985). Similar experiments have shown that photoinhibition induced by illuminating leaves under low partial pressures of oxygen in the absence of CO_2 was prevented if the oxygen concentration was increased (Powles and Osmond, 1978; Powles *et al.*, 1979, 1983). Photoinhibition during chilling was by contrast dependent on oxygen (Lindenman, 1979; Powles *et al.*, 1983). Studies of photoinhibition in intact chloroplasts or isolated leaf mesophyll cells illuminated in CO_2 free buffer showed that damage occurred only if oxygen was present (Krause *et al.*, 1978). Photoinhibition in leaves induced by high light intensities was similar whether the oxygen tension was that of normal air or close to zero (Powles, 1984). A scheme to account for possible mechanisms of photoinhibitory damage to photosystem II, and the role of oxygen in promoting or preventing damage is shown in Figure 49.

Light energy absorbed by chlorophyll is normally dissipated through photosynthetic electron flow to $NADP^+$. A pool of chloroplast $NADP^+$ is maintained by photosynthetic carbon metabolism that utilises NADPH (Figure 49a). During normal photosynthesis a small proportion of electron flow may be lost to oxygen with the formation of $O_2^{\cdot -}$. Superoxide formed is efficiently removed by stromal SOD enzymes (Halliwell, 1981).

Photoinhibition induced by the transfer of shade adapted plants to full sunlight probably occurs because the well developed light harvesting apparatus in such plants absorbs more light than can be dissipated by electron flow reactions (Osmond, 1981). Photoinhibitory

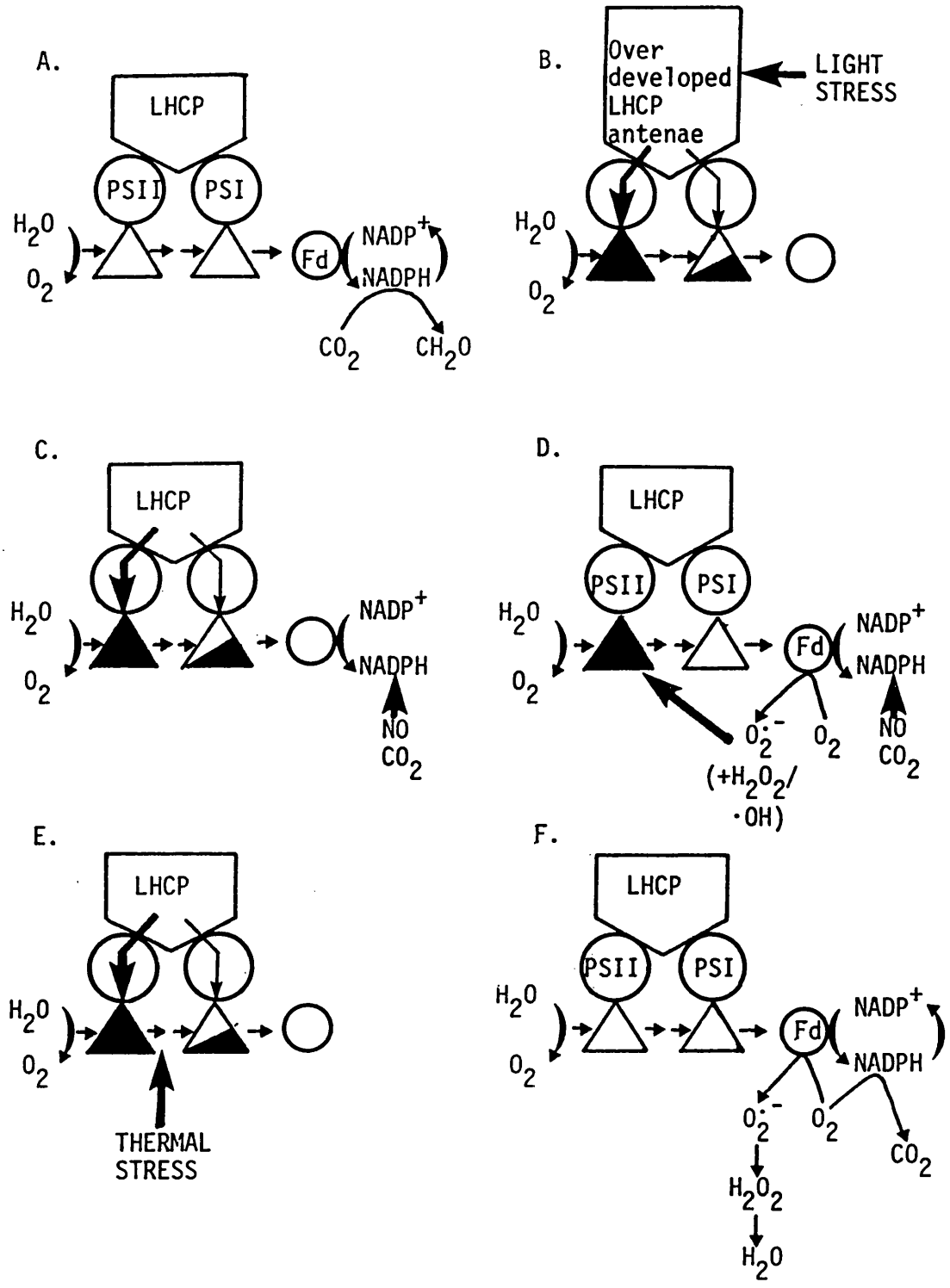


Fig 49. Photoinhibition of photosynthetic electron flow, and the role of oxygen in restricting photo-inhibition. See text for explanations.

damage to photosystem II is caused by direct photochemical damage (Figure 49b) and is dependent on light but not oxygen (Powles, 1984).

Environmental conditions that prevent normal photosynthetic CO_2 metabolism (incubation of leaves in the absence of CO_2, drought or chilling induced loss of Calvin cycle enzyme activity) would prevent the oxidation of NADPH, thus leading to the reduced availability of photosystem I electron acceptors. Over-reduction of the electron transport chain may promote energy spillover from light harvesting pigments to ^3Chl and $^1O_2$ initiating damage to the photosystem reaction centres (Figure 49c).

Reduced rates of photosynthetic CO_2 metabolism may alternatively promote ferredoxin catalysed reduction of oxygen to $O_2^{\cdot -}$. The subsequent generation of the H_2O_2 and $OH^{\cdot}$ probably induces damage to photosystem II (Figure 49d). Damage by this mechanism may occur in isolated thylakoids provided that a catalyst of oxygen reduction is present (ferredoxin or paraquat). In intact chloroplasts, cells or leaves, the increased formation of $O_2^{\cdot -}$ probably exceeds the capacity of chloroplast enzymes and antioxidants to scavenge it. Damage would therefore occur in a manner analogous to the herbicidal action of paraquat. This mechanism was unlikely to have accounted for chilling induced photoinhibition of photosynthesis in pea or maize leaves observed in this study as the mechanism of damage appeared to be different from damage induced by paraquat.

An alternative mechanism to account for chilling induced photoinhibition is shown in Figure 49e. Disruption of electron flow may occur because thylakoid lipids change from fluid to gel phase at low temperature (Oquist, 1983). Inhibition of electron flow would be analogous to that induced by electron transport inhibitor herbicides.

Damage presumably occurred because energy spillover from light harvesting chlorophyll promoted the formation of ^3Chl and $^1O_2$. Enhanced formation of $^1O_2$ may account for the oxygen dependence of chilling induced photoinhibition and would be similar to the requirement for oxygen in damage to leaves or chloroplasts induced by electron transport inhibitor herbicides (Dodge, 1982).

The role of oxygen in preventing photoinhibition is outlined in Figure 49f. Under conditions where low rates of CO_2 assimilation limit photosynthesis, oxygen can promote energy dissipation in chloroplasts via photorespiration (Heber and Krause, 1978). Such a mechanism would be operative only in leaves or intact cells containing functional chloroplasts, mitochondria and peroxisomes. Alternatively oxygen reduction to $O_2^{\cdot -}$ would maintain electron flow through photosystem I and II. Damage would be prevented provided that the products of oxygen reduction were efficiently scavenged. Such a mechanism may operate in intact chloroplasts catalysed by ferredoxin, or in isolated thylakoids provided exogenous ferredoxin or paraquat and SOD plus catalase were added, as demonstrated in this study.

The mechanism of photoinhibition induced by a variety of stresses, and the role of oxygen, may therefore depend on the nature of that stress and the type of experimental material (intact or broken chloroplasts, leaf cell or whole leaf). Photoinhibitory damage to photosystem II although induced by a variety of stresses (Powles, 1984) probably occurs in a manner analogous to the mode of action of photosynthetic electron flow inhibitor or bipyridyl herbicides.

8. Aspects of paraquat action

Several studies have indicated that SOD activity is correlated with paraquat tolerance in photosynthetic organisms (Harper and Harvey, 1978; Rabinowitch *et al.*, 1983; Rabinowitch and Fridovich, 1985). In addition bacteria or green algae grown in the presence of low concentrations of paraquat contained enhanced SOD activity (Hassan and Fridovich, 1977a; Rabinowitch *et al.*, 1983). In this study the relationship between paraquat toxicity and chloroplast $O_2^{\cdot -}$ and H_2O_2 scavenger enzyme levels in higher plants has been investigated.

8.1 Herbicidal symptoms of paraquat injury

Pea leaf discs were incubated on a range of paraquat concentrations in glass petri dishes and illuminated (250 µmol $m^{-2}s^{-1}$ photon flux density) for up to 72 hours. The effect of these treatments on chlorophyll and carotenoid levels, photosynthetic activity and membrane lipid peroxidation is shown in Figures 50 and 51. Incubation of pea leaf discs on 10 µM paraquat abolished photosynthetic CO_2 exchange within 24 hours treatment (Figure 50c). This was followed by pigment bleaching, loss of photosynthetic electron transport activity in thylakoids isolated from paraquat treated leaves (Figure 50d), and enhanced membrane lipid peroxidation (Figure 51). Leaf discs incubated on 1 µM paraquat showed similar symptoms of injury, although damage developed more slowly. Compared with the control treatment, 0.1 µM paraquat induced no loss of chlorophyll or carotenoid and did not promote membrane lipid peroxidation in leaf discs. Photosynthetic CO_2 exchange in pea leaf discs was inhibited by 30% after 24 hours, indicating paraquat uptake and herbicide activity.

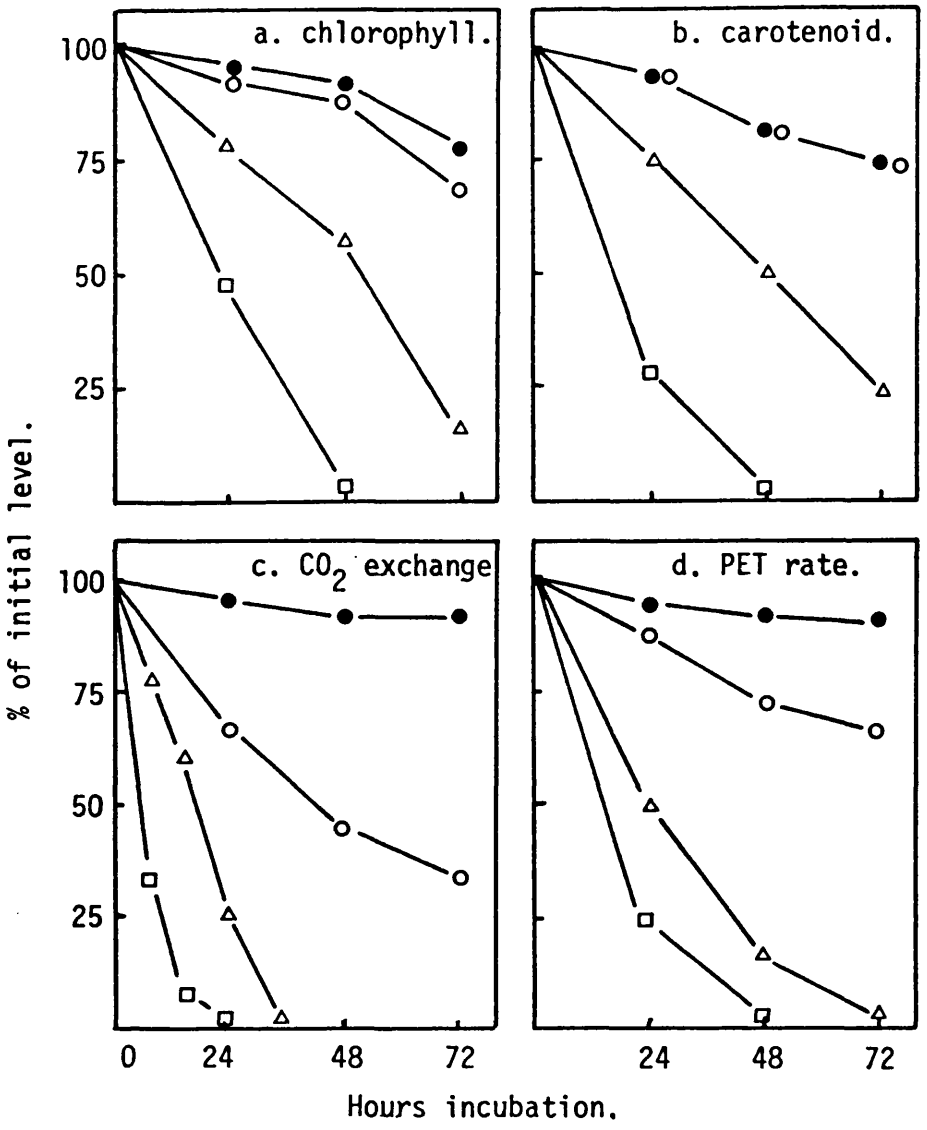


Fig 50. The effect of paraquat at 0.1 (○), 1.0 (△) or 10μM (□) compared to the water control (●) on chlorophyll (a) and carotenoid (b) levels and photosynthetic CO_2 exchange (c) or electron transport activity (d) of pea leaf discs.

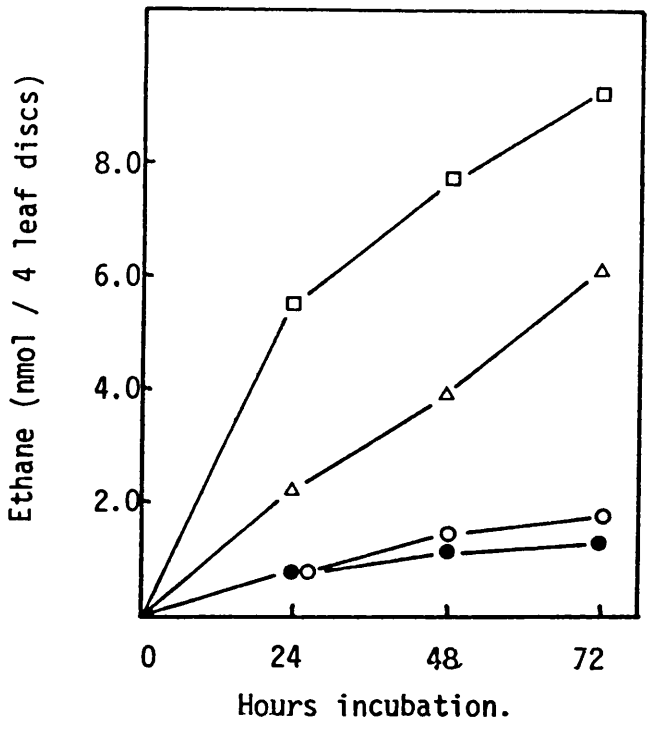


Fig 51. The effect of paraquat at 0.1 (o), 1.0 (△) or 10μM (□) compared with the water control (●) on ethane evolution from pea leaf discs.

8.2 The effect of paraquat on chloroplast superoxide and hydrogen peroxide scavengers

Levels of ascorbate, and the activity of SOD, ascorbate peroxidase and glutathione reductase in homogenates from pea leaf discs incubated on paraquat solutions are shown in Figure 52. Leaf discs incubated on 10 μM paraquat showed a marked loss of ascorbate and enzyme activity. This presumably occurred as a consequence of increased photo-oxidative damage to chloroplasts. Leaf discs incubated on 0.1 μM paraquat showed no marked change in ascorbate (Figure 52a) or SOD (Figure 52b) levels. The activity of ascorbate peroxidase (Figure 52c) and glutathione reductase (Figure 52d) were however enhanced after 24 hours treatment. The activity of these two enzymes remained higher than the control level after 72 hours. These results indicate that although 0.1 μM paraquat showed herbicide activity against photosynthesis, and therefore increased $O_2^{\cdot -}$ formation in chloroplasts, damage symptoms were not expressed. The increased activity of ascorbate peroxidase and glutathione reductase observed in paraquat treated leaf discs may have prevented the accumulation of H_2O_2 in chloroplasts. Thus photo-oxidative damage was prevented. Pea leaf discs incubated on 1 μM paraquat showed initial increased activity of ascorbate peroxidase and glutathione reductase, although as herbicidal damage developed the activity of these enzymes also declined. These results indicate that in higher plants stimulation of $O_2^{\cdot -}$ generation in chloroplasts can enhance the activities of ascorbate peroxidase and glutathione reductase but not SOD. If the generation of $O_2^{\cdot -}$ is excessive or prolonged as occurs with herbicidal concentrations of paraquat, damage symptoms prevail. Loss of activity of protective enzymes, together with pigment bleaching and membrane lipid peroxidation then occurs.

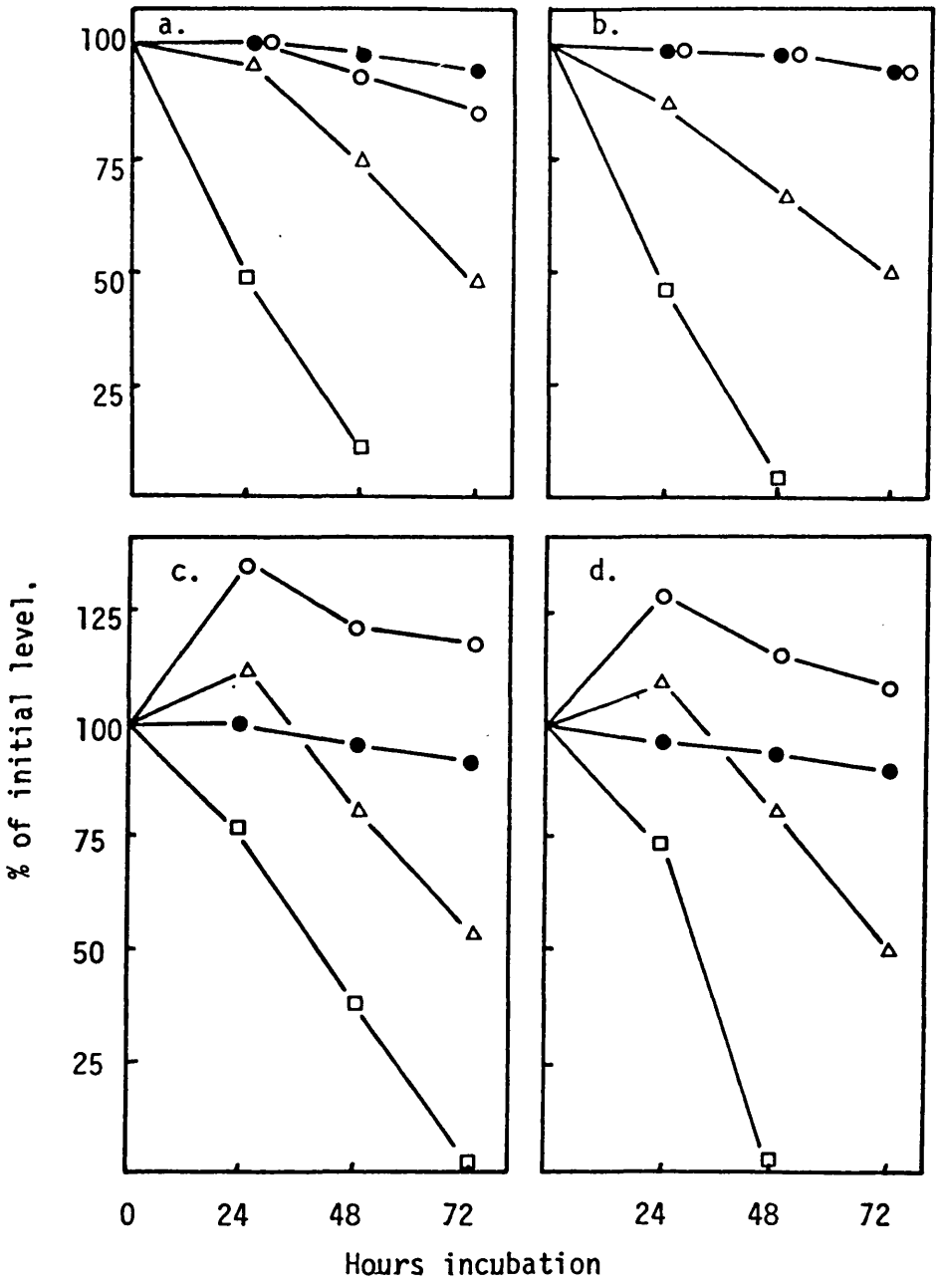


Fig 52. The effect of paraquat at 0.1 (o), 1.0 (Δ) or 10μM (□) compared with the water control (●) on ascorbate (a) and the activity of SOD (b), ascorbate peroxidase (c) and glutathione reductase (d) in pea leaf discs.

8.3 The role of chloroplast protective enzymes in paraquat tolerance

Paraquat promotes the formation of $O_2^{\cdot -}$ in chloroplasts. Accumulation of H_2O_2 and the formation of $OH^{\cdot}$ initiates photo-oxidative damage (Dodge, 1983). Increased levels of enzymes that scavenge $O_2^{\cdot -}$ and H_2O_2 in chloroplasts may prevent the accumulation of toxic oxygen species, and thus restrict herbicidal damage symptoms. Chloroplasts isolated from pea plants grown at a moderate compared with a low light intensity were shown previously to contain elevated levels of ascorbate, ascorbate peroxidase, dehydroascorbate reductase and glutathione reductase. To assess whether the increased chloroplast protective enzyme levels affected paraquat tolerance, leaf discs were cut from pea plants grown at 100 or 400μmol $m^{-2}s^{-1}$ photon flux density and incubated for up to 48 hours on 10 μM paraquat. Injury was assessed by following chlorophyll bleaching (Figure 53). Leaf discs cut from plants grown at the lower light intensity and subsequently incubated on paraquat showed rapid chlorophyll bleaching (50% loss after 12 hours). Chlorophyll bleaching in leaf discs excised from plants grown at the higher light intensity was, by comparison, considerably delayed (50% loss of chlorophyll after 36 hours). This difference was not due to different rates of paraquat uptake, as photosynthetic CO_2 exchange in both sets of leaf discs was inhibited by paraquat after a similar time period (results not shown). These results indicate that the increased tolerance to paraquat in plants grown at the higher light intensity may have been caused by enhanced chloroplast levels of ascorbate, ascorbate peroxidase, dehydroascorbate reductase and glutathione reductase.

The role of SOD in protecting plants against paraquat toxicity was investigated using copper penacillamine (PaCu), a copper complex with SOD activity (Lengfelder *et al.*, 1979). Leaf material incubated on PaCu readily take it up into chloroplasts (Youngman, 1980). Pea leaf discs were preincubated on water or 1 mM PaCu in darkness prior

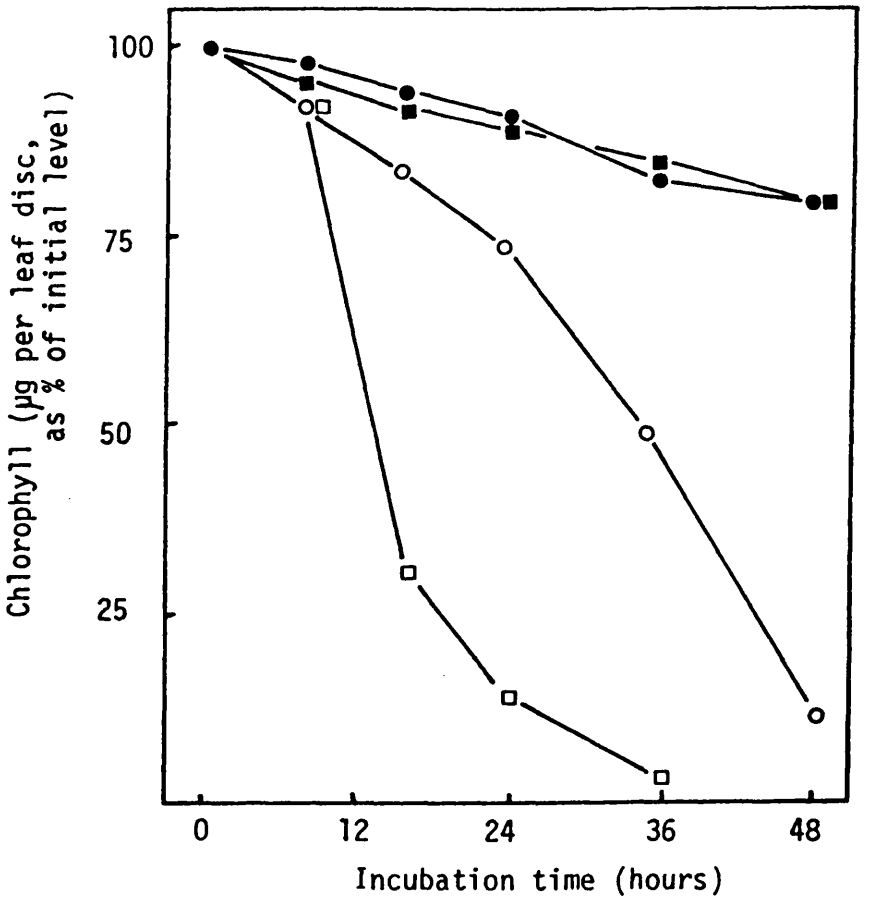


Fig 53. Chlorophyll bleaching in pea leaf discs cut from plants grown at 100 (■,□) or 400 (●,○) µmol $m^{-2}s^{-1}$ PFD and incubated on H_2O (●,■) or 10µM paraquat (○,□).

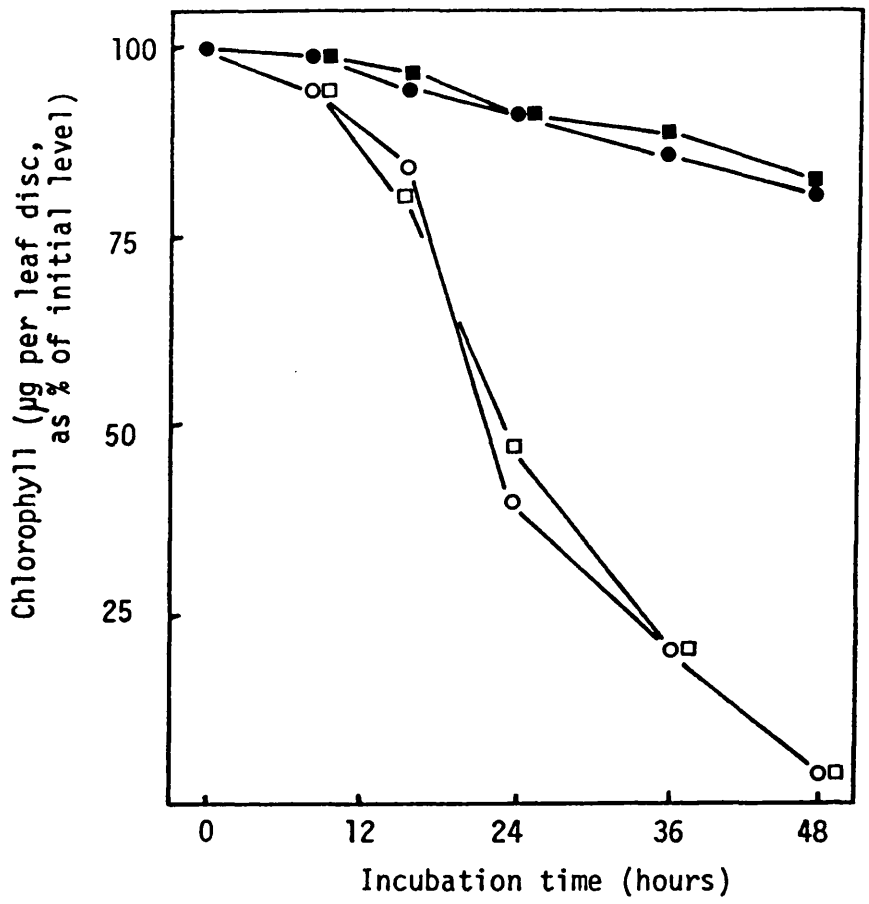


Fig 54. Chlorophyll bleaching in pea leaf discs incubated on H_2O (●), 10µM paraquat (○), 1mM PaCu (■) or paraquat + PaCu (□).

to transfer to 10 μM paraquat and illumination. Damage was assessed by loss of chlorophyll from leaf discs (Figure 54). Preincubation on PaCu had no effect on paraquat induced chlorophyll bleaching from pea leaf discs compared with leaf discs preincubated on water. These results indicate that elevated levels of $O_2^{\cdot -}$ scavengers did not enhance the tolerance of leaf discs to paraquat.

8.4 The potential of chloroplast antioxidant mechanisms as a target for herbicide action

Several recent investigations have shown that the copper chelating compound diethyl dithiocarbamate (DDTC) inhibits SOD function. This chemical has been used to investigate the protective role of SOD in plants (Asada *et al.*, 1974b; Tanaka and Sugarhara, 1980; Rabinowitch and Fridovich, 1983). The results presented in Figure 55 demonstrate the effect of DDTC (1 and 10 mM) on the activity of SOD extracted from spinach leaves. Enzyme activity was assessed by measuring the inhibition of nitro blue tetrazolium oxidation to formazan, a reaction initiated by $O_2^{\cdot -}$. This reaction was inhibited by 50% with 23 μl of spinach SOD. The activity of the spinach SOD preparation therefore equated to 44 units ml^{-1}. Addition of 1 mM or 10 mM DDTC to the SOD assay reduced the activity of spinach SOD by 39% and 66% respectively (calculated assuming that 1 unit of SOD represents the amount of enzyme that inhibited NBT oxidation to formazan by 50%). The activity of SOD was also reduced in pea leaf discs incubated on a range of DDTC concentrations for up to 72 hours in light (Figure 56) or darkness (Table 14). These results demonstrate that DDTC is an effective inhibitor of SOD, although millimolar concentrations are required to abolish enzyme activity.

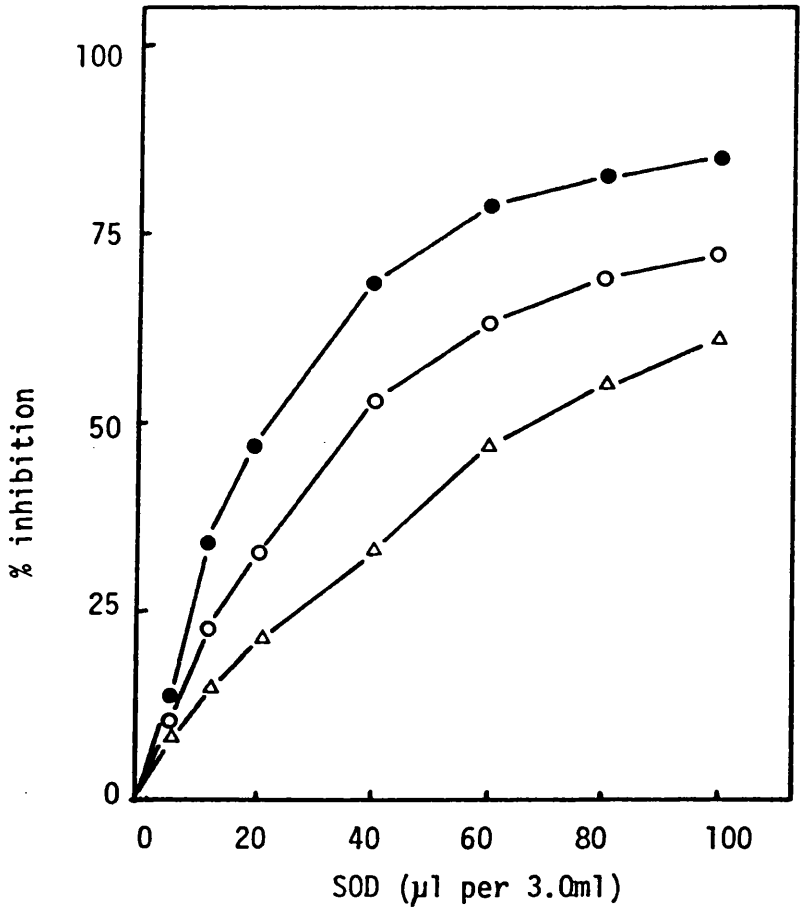


Fig 55. The effect of diethyl dithiocarbamate at 1.0mM (o), or 10mM (△) compared with the control (●) on the activity of SOD purified from spinach leaves. SOD activity was assessed by the inhibition of formazan production from nitro blue tetrazolium.

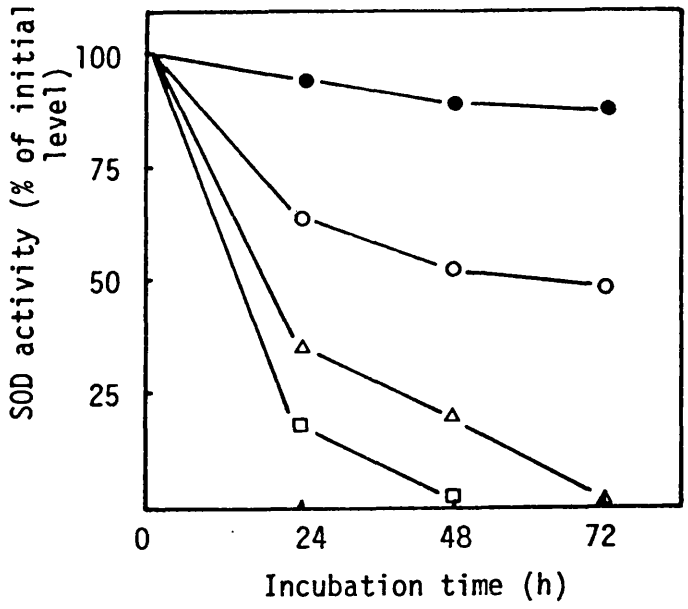


Fig 56. The effect of 1.0 (o), 10 (△) and 20mM (□) DDTC compared with the water control (●) on the SOD activity of pea leaf discs incubated on test solutions for up to 72 hours in light.

Table 14. The effect of incubating pea leaf discs on 10mM DDTC compared to a water control on SOD activity after 24 or 48 hours darkness.

	DDTC treatment.	
	24h	48h
SOD (% of control level)	36.3%	21.4%

The effect of DDTC on chlorophyll bleaching in pea leaf discs is shown in Figure 57. Leaf discs were incubated on a range of DDTC concentrations in light for up to 72 hours. Those discs incubated on 10 mM or 20 mM DDTC showed extensive chlorophyll loss compared with the control treatment. Lower concentrations of DDTC induced limited chlorophyll bleaching. Thus although 1 mM DDTC induced a 50% loss of SOD activity in pea leaf discs after 24 hours illumination (Figure 56), this did not induce significant chlorophyll bleaching. Chlorophyll loss induced by 10 mM DDTC from leaf discs was light dependent (Figure 57b), being enhanced at higher light intensities.

The effect of DDTC induced loss of SOD activity on the toxicity of paraquat to pea leaf discs is shown in Figure 58. Leaf discs were incubated on DDTC (1 mM) or paraquat (1 μM), alone or in combination, for up to 48 hours under constant illumination. Damage was assessed by chlorophyll bleaching in leaf discs. Both DDTC or paraquat alone induced some chlorophyll loss from leaf discs (20 and 40% loss of chlorophyll after 48 hours respectively). Incubation on DDTC and paraquat in combination induced 80% loss of chlorophyll after 48 hours. This result indicates that inhibited SOD activity led to enhanced paraquat toxicity.

8.5 Discussion

Low concentrations of paraquat have been reported to enhance the SOD activity of some bacteria and green algae (Hassan and Fridovich, 1977a; Rabinowitch *et al.*, 1983), although this has not been shown previously with higher plants. Results from this study showed that incubating pea leaf discs on sub-lethal concentrations of paraquat enhanced the activity of ascorbate peroxidase and glutathione reductase,

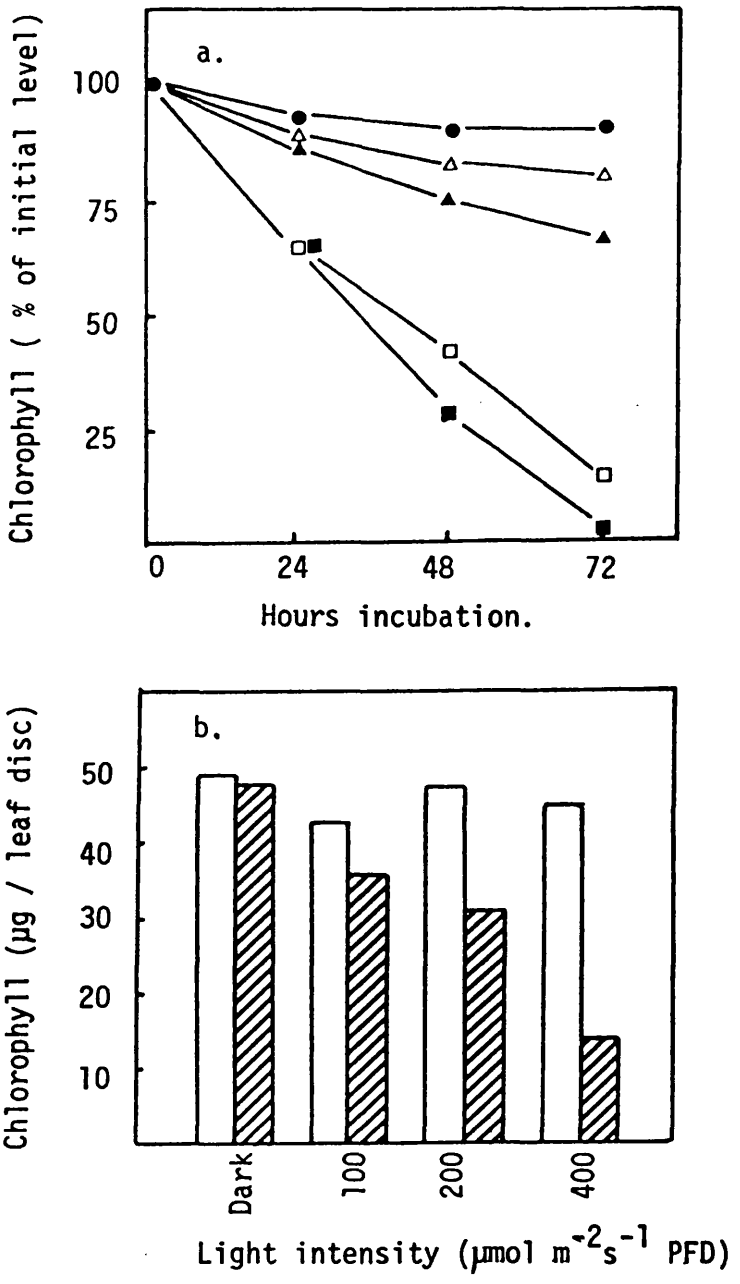


Fig 57. Chlorophyll bleaching of pea leaf discs induced by incubation on DDTC. Leaf discs were incubated for up to 72h on H_2O (●) or 0.1 (△), 1.0 (▲), 10 (□) and 20mM(■) DDTC (a), or were incubated for 48h on H_2O (□) or 10mM (▨) DDTC at different light intensities (b).

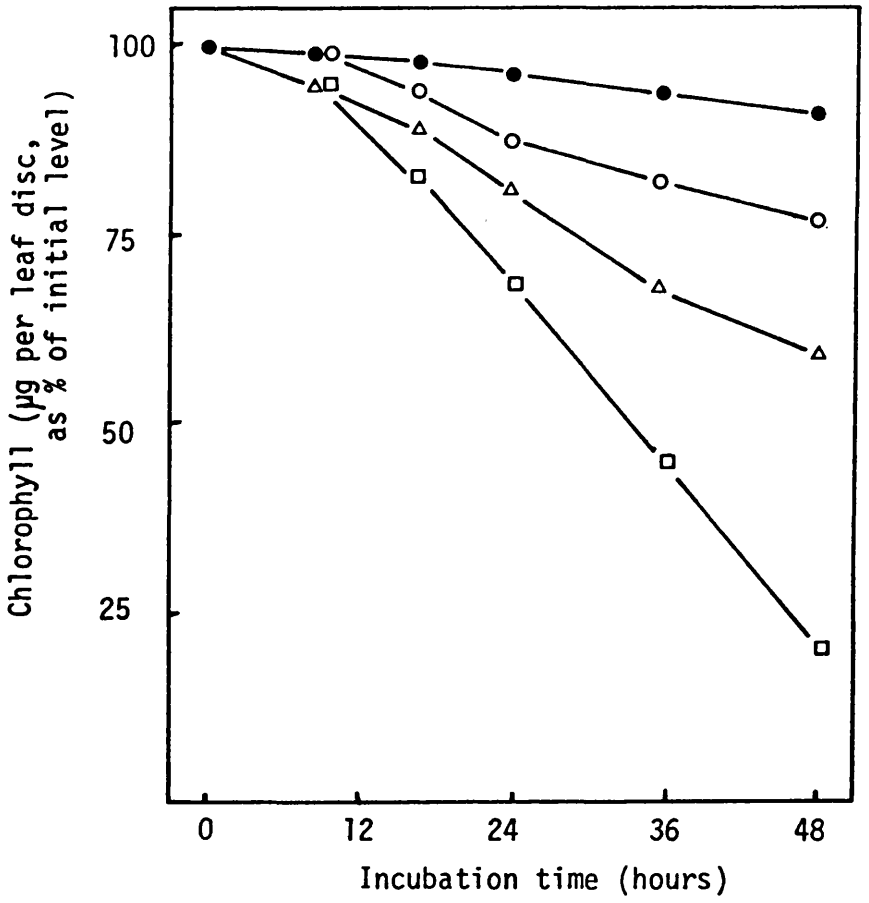


Fig 58. Chlorophyll bleaching in pea leaf discs incubated on H_2O (●), 1mM DDTC (○), 1µM paraquat (△) or DDTC + paraquat (□).

although there was no effect on SOD. Higher paraquat concentrations reduced the levels of chloroplast $O_2^{\cdot -}$ and H_2O_2 scavenger enzymes from pea leaf discs, presumably as a consequence of herbicide damage. This indicates that there is a close relationship between $O_2^{\cdot -}$ formation in chloroplasts and the activity of enzymes that scavenge $O_2^{\cdot -}$ and H_2O_2. Low paraquat concentrations enhanced $O_2^{\cdot -}$ formation and stimulated the levels of ascorbate peroxidase and glutathione reductase. Increased paraquat concentrations generate $O_2^{\cdot -}$ levels that exceed the capacity of these enzymes, and hence damage symptoms occur. Accumulation of H_2O_2 in chloroplasts inhibits Calvin cycle enzymes and also chloroplast CuZn SOD (Asada *et al.*, 1975; Kaiser, 1979). Interactions between $O_2^{\cdot -}$ or reduced paraquat and H_2O_2 may generate $OH^{\cdot}$ that can react with most biological molecules (Halliwell, 1981; Winterbourn, 1981).

The role of SOD in paraquat tolerance has been widely investigated. Resistance to paraquat has been observed in several weed species, and paraquat resistant plants have also been obtained by *in vitro* selection procedures from cell cultures (Harper and Harvey, 1978; Miller and Hughes, 1980; Youngman and Dodge, 1981). One feature of paraquat resistant plants is that they contain higher levels of SOD than susceptible plants (Harper and Harvey, 1978; Youngman and Dodge, 1981; Hughes *et al.*, 1984). Increased capacity to scavenge $O_2^{\cdot -}$ may therefore be an important part of the resistance mechanism, although herbicide exclusion from chloroplasts may also contribute to resistance (Fuerst *et al.*, 1985). Increased tolerance to paraquat has also been observed in plants in which SOD levels had been enhanced by benzyl viologen (Lewinsohn and Gressel, 1984) or with PaCu (Youngman and Dodge, 1979). Paraquat protection by PaCu was not demonstrated in this

study. It would be interesting to determine whether EDU (N-[2-(2-oxy-1-imadazolidinyl)ethyl]-N'-phenyl urea) which promotes SOD levels in plants, and has been used as an antiozonant (Lee and Bennett, 1982), provides any significant protection against paraquat toxicity.

The role of chloroplast H_2O_2 scavenger mechanisms in the tolerance of plants to paraquat has not previously been investigated. If elevated levels of SOD are to provide significant protection against this herbicide, this must presumably be coupled to enhanced activities of enzymes that scavenge H_2O_2. Results presented in this study indicated that plants with varying levels of ascorbate peroxidase, dehydroascorbate reductase and glutathione reductase achieved by growth at two different light intensities do show differences in their tolerance to paraquat. The role of $O_2^{\cdot -}$ and H_2O_2 scavenger enzymes in paraquat tolerance is therefore worthy of further investigation.

Many herbicides are known to inhibit photosynthetic reactions, thus promoting the formation of free radicals and toxic oxygen species in chloroplasts (Section 5). Damage probably occurs because the formation of damaging radicals exceeds the capacity of endogenous antioxidant mechanisms to remove them (Dodge, 1983). Chemicals that reduce the capacity of chloroplasts to scavenge these radicals may enhance herbicide activity, or be herbicidally toxic themselves. Studies here indicate that the activity of chloroplast ascorbate peroxidase and glutathione reductase may influence paraquat toxicity, while reduced levels of SOD, achieved by DDTC, enhance paraquat toxicity. Higher DDTC concentrations induced chlorophyll bleaching, indicating that inhibition of chloroplast protective enzyme function could be a potential herbicide target.

Studies shown previously on the peroxidation of illuminated thylakoids showed that ascorbate, carotenoids and α-tocopherol were effective

quenchers of herbicide induced damage (Section 5.3). Chemicals that enhance or reduce the levels of these antioxidants may have potential as antagonists or safeners against herbicides that promote the formation of active oxygen species in chloroplasts. As the search for new herbicides becomes more expensive, an increased understanding of the mechanisms of herbicide action becomes more important. Compounds that enhance or reduce the activity of these herbicides may have increased commercial significance. Results presented here indicate that control of chloroplast antioxidant and protective enzyme levels may be one mechanism by which enhanced or reduced activity of some herbicides is achieved.

CONCLUDING DISCUSSION

CONCLUDING DISCUSSION

Chloroplasts from higher plants contain the enzymes SOD, ascorbate peroxidase, dehydroascorbate reductase and glutathione reductase that scavenge $O_2^{\cdot -}$ and H_2O_2 formed by electron leakage to oxygen during photosynthesis. In addition millimolar concentrations of ascorbate and glutathione in the chloroplast stroma plus carotenoids and α-tocopherol in the thylakoid membranes quench $O_2^{\cdot -}$, H_2O_2 and $^1O_2$.

Several workers have considered the role of chloroplast protective mechanisms, particularly SOD, in preventing photo-oxidative injury during leaf development and senescence, and in the tolerance of plants to certain air pollutants, environmental stresses and herbicides such as paraquat (Rabinowitch and Fridovich, 1983; Guy and Carter, 1984; Gamble and Burke, 1984; Tanaka *et al.*, 1985). In many such studies enzyme levels have been assessed in leaf homogenates from whole plants or isolated leaf discs that have been subjected to stress. The demonstration in this study that a high proportion of leaf SOD, ascorbate peroxidase, dehydroascorbate reductase and glutathione reductase were present in chloroplasts of pea leaves is therefore significant. Alterations in activity of these enzymes in leaf homogenates during ageing, changes in plant growth conditions or in response to environmental stress are probably a good indication of increased or decreased chloroplast protection against $O_2^{\cdot -}$ and H_2O_2.

In studies of the physiological role of enzymes that protect plants against $O_2^{\cdot -}$ and H_2O_2, SOD activity has commonly been assessed in parallel with changes in catalase and peroxidase (Simon *et al.*, 1974; Harper and Harvey, 1978; Dhindsa *et al.*, 1981; Lee and

Bennett, 1982; McRae and Thompson, 1983; Kar and Feierabend, 1984). Results from this study confirm that while SOD is predominantly a chloroplast stromal enzyme, chloroplasts contain little or no catalase or peroxidase (Parrish, 1972; Halliwell, 1981). Catalase is found mainly in peroxisomes (Halliwell, 1981) and probably scavenges H_2O_2 formed during photorespiration. Peroxidase activity is commonly assessed using a wide range of artificial substrates such as guiacol or catechol, and its role in scavenging H_2O_2 *in vivo* is therefore difficult to assess (Halliwell, 1981). The physiological role of this enzyme may involve biological oxidations that require H_2O_2 such as lignification of cell walls (Elstner, 1982). Chloroplasts contain the enzymes ascorbate peroxidase, dehydroascorbate reductase and glutathione reductase, and the physiological properties of these enzymes are consistent with the scavenging of H_2O_2 in that organelle (Jablonski and Anderson, 1978, 1981, 1982; Nakano and Asada, 1981; Gerbling *et al.*, 1984).

Results presented in this study indicate that the activity of ascorbate peroxidase in pea chloroplasts was 10-20 times higher than the capacity of dehydroascorbate reductase and glutathione reductase to regenerate ascorbate. Similar observations with spinach chloroplasts indicated the presence of an additional enzyme in ascorbate metabolism, monodehydroascorbate free radical reductase (Hossain *et al.*, 1984). Oxidation of ascorbate by H_2O_2 probably forms both dehydroascorbate and monodehydroascorbate free radicals (Arrigoni *et al.*, 1981). Monodehydroascorbate radicals break down spontaneously (Bielski *et al.*, 1981) or catalysed by the reductase enzyme (Arrigoni *et al.*, 1981; Hossain *et al.*, 1984) to form ascorbate and dehydroascorbate. Dehydroascorbate would then be

reduced to ascorbate via the dehydroascorbate reductase and glutathione reductase enzymes. The activity of monodehydroascorbate reductase in spinach chloroplast was probably sufficient to account for the difference in activity between ascorbate peroxidase and dehydroascorbate reductase (Hossain *et al.*, 1984).

Monodehydroascorbate reductase has been identified in chloroplasts, mitochondria, peroxisomes and the leaf soluble fraction of leaves, and in etiolated shoots, roots, buds and tubers from a variety of plants (Arrigoni *et al.*, 1981). Ascorbate, found at high concentrations in plant cells, can be oxidised by $O_2^{\cdot -}$ and H_2O_2 at significant rates (Halliwell, 1981), probably forming monodehydroascorbate radicals (Arrigoni *et al.*, 1981). Oxidation of ascorbate by $O_2^{\cdot -}$ and H_2O_2, and its regeneration by monodehydroascorbate reductase, may therefore represent an additional mechanism for scavenging these active oxygen species. Results from this study showed that a high proportion of leaf SOD and ascorbate peroxidase were present in chloroplasts, and are therefore unlikely to scavenge $O_2^{\cdot -}$ and H_2O_2 at significant rates outside that organelle. The role of ascorbate and monodehydroascorbate reductase in scavenging $O_2^{\cdot -}$ and H_2O_2 may therefore be significant in non-photosynthetic organelles or plant cells.

Results presented in this study showed that the transfer of etiolated plants to light stimulated increased levels of ascorbate and ascorbate peroxidase in pea leaves. Levels of ascorbate, ascorbate peroxidase and glutathione reductase were also enhanced in leaf homogenates and intact chloroplasts from peas grown at moderate compared with low light intensity, or in summer compared with

winter. Leaf discs incubated on low concentrations of paraquat also showed enhanced ascorbate peroxidase and glutathione reductase activity. The activity of SOD was by contrast unaffected by these changes in growth conditions or paraquat treatment. Hyperbaric oxygen tensions were shown elsewhere to promote glutathione reductase activity in leaves, although SOD activity was not affected (Foster and Hess, 1980, 1982). Fumigation of leaves with low levels of O_3 enhanced the activity of ascorbate peroxidase and monodehydroascorbate reductase, again without effect on SOD levels (Tanaka *et al.*, 1985). Glutathione reductase levels in leaves have also been observed to increase in response to low temperature hardening (de Kok and Oosterhuis, 1983; Guy and Carter, 1984), or drought stress (Gamble and Burke, 1984; Burke *et al.*, 1985).

Hydrogen peroxide is extremely toxic to chloroplasts through inhibition of CuZn SOD (Asada *et al.*, 1975; Rabinowitch and Fridovich, 1983) and Calvin cycle enzymes (Kaiser, 1979; Charles and Halliwell, 1980; Tanaka *et al.*, 1982b). One important adaptation of the photosynthetic apparatus to stress would therefore appear to be increased activity of enzymic mechanisms that scavenge H_2O_2. SOD activity was unaffected by conditions promoting the levels of ascorbate, ascorbate peroxidase or glutathione reductase. This indicates that the chloroplast capacity to scavenge $O_2^{\cdot -}$ is higher than the capacity to remove H_2O_2. This is supported by observations of the effect of SO_2 on spinach chloroplasts (Tanaka *et al.*, 1982a). This air pollutant promoted oxygen uptake and consequently $O_2^{\cdot -}$ formation in chloroplasts. Spinach leaves fumigated with SO_2 accumulated H_2O_2 in chloroplasts thus although SO_2 promoted $O_2^{\cdot -}$ formation in chloroplasts, this was reduced to H_2O_2 by SOD. The activity of enzymes that scavenge H_2O_2 were however insufficient to prevent its accumulation.

Studies with bacteria, yeast and green algae have by contrast demonstrated that one facet of adaptation to increased oxygen levels, paraquat or SO_2 fumigation was an enhanced level of SOD (Gregory and Fridovich, 1973; Gregory *et al.*, 1974; Pullich, 1974; Hassan and Fridovich, 1977a, 1978; Rabinowitch *et al.*, 1983; Clare *et al.*, 1984; Rabinowitch and Fridovich, 1985). SOD levels in *Escherichia coli* were also enhanced by compounds that promoted the intracellular formation of $O_2^{\cdot -}$ (Hassan and Fridovich, 1979). Metal chelators, absence of glucose or accumulation of organic acids in the growth media had a similar effect (Hassan and Fridovich, 1977b; Pugh and Fridovich, 1985). The Mn SOD in these organisms would therefore appear to be inducible in response to stress. Levels of CuZn SOD in higher plant chloroplasts by contrast did not change in response to similar stresses. Thus in bacteria, yeast and algae, increased SOD activity was a feature of adaptation to oxidative stress such as high light levels, increased oxygen tensions, SO_2 or paraquat. Similar stresses to plants promoted the activity of enzymes that scavenge H_2O_2 in chloroplasts. The different mechanisms of adaptation to stress between these organism compared with higher plants may reflect differences in inducibility between Mn SOD and CuZn SOD, and the susceptibility of chloroplasts to H_2O_2 toxicity.

Incubation of pea leaf discs on paraquat solutions showed that while low herbicide concentrations promoted the activity of ascorbate peroxidase and glutathione reductase, higher herbicide concentrations induced loss of enzyme activity, chlorophyll bleaching and membrane lipid peroxidation. Photo-oxidative damage to plants induced by herbicides, air pollutants and environmental stresses has been well documented. A large number of herbicides inhibit photosynthetic electron flow, thus preventing CO_2 exchange and initiating oxygen and

light dependent damage (Dodge, 1983). Other herbicides such as the bipyridyls divert photosynthetic electron flow to oxygen, stimulating $O_2^{\cdot -}$ formation in chloroplasts and initiating damage reactions. Similar mechanisms may cause the photoinhibition of photosynthesis that is observed if plants are subjected to certain environmental stresses at high light intensities. Results from this study showed that photoinhibition of chloroplast reactions was initiated by incubating leaves at chilling temperatures in light, or by illuminating thylakoids in the absence of electron flow acceptors. Damage probably occurred because light energy absorbed by the chloroplasts could not be dissipated by photosynthetic reactions. Energy spillover from light harvesting chlorophyll to form ^3Chl or $^1O_2$ may then occur in a similar way to damage induced by photosynthetic inhibitor herbicides. Alternatively, over-reduction of the photosynthetic electron carriers may promote electron flow to oxygen, forming $O_2^{\cdot -}$, and this would initiate damage that is analogous to the herbicidal action of paraquat. Short periods of stress (chilling, drought, heat, high light intensities or incubation of leaves in the absence of CO_2 and O_2) cause damage to the photosystem II reaction centre (Krause *et al.*, 1978; 1985; Santarius and Muller, 1979; Critchley, 1981; Powles *et al.*, 1983; Powles, 1984; Björkman and Powles, 1984). Prolonged stress probably initiates chlorophyll bleaching and membrane lipid peroxidation similar to that observed when leaves were incubated for several days in strong light at chilling temperatures (van Hasselt, 1972; 1974). Damage symptoms would therefore be similar to those induced by herbicides in this study.

Photo-oxidative damage to leaves and chloroplasts can also be initiated by air pollutants such as O_3 and SO_2 which cause inhibition of photosynthesis, pigment bleaching and membrane lipid peroxidation (Shimazaki *et al.*, 1980; 1984; Sakaki *et al.*, 1983; Tanaka *et al*, 1985). The similarity between damage induced by these air pollutants

to that induced by photosynthetic inhibitor herbicides observed in this study indicates that damage may be initiated by similar mechanisms. The toxicity of SO_2 and O_3 may involve the enhanced formation of $O_2^{\cdot -}$ in chloroplasts, although $^1O_2$, H_2O_2 and $OH^{\cdot}$ may also cause damaged induced by these air pollutants (Shimazaki *et al.*, 1980; Tanaka and Sugahara, 1980; Tanaka *et al.*, 1982b; Sakaki *et al.*, 1983; de Kok *et al.*, 1983).

Photo-oxidative damage to plants may also occur if the activity of chloroplast antioxidant mechanisms is impaired. Leaves incubated at chilling temperatures in darkness showed a loss of SOD activity. This was followed by a marked increase in membrane lipid peroxidation when the chilled leaves were subsequently illuminated (Michalski and Kaniuga, 1981). Results from this study showed that reduction of leaf SOD levels by incubation with DDTC induced light dependent chlorophyll bleaching. Photo-oxidative destruction of chloroplast pigments has also been observed in plants that do not contain carotenoids (Anderson and Robertson, 1960; Bartels and Watson, 1978). Reduced carotenoid levels increased the susceptibility of chloroplast membranes to damage induced by ^3Chl and $^1O_2$. During leaf senescence reduced activity of chloroplast antioxidant mechanisms, as observed in this study, may promote photo-oxidative damage reactions which contribute to cellular breakdown.

The photosynthetic apparatus is particularly susceptible to photo-oxidative damage. Injury probably occurs because the formation of free radicals, $O_2^{\cdot -}$, H_2O_2 or $^1O_2$ exceeds the capacity of endogenous antioxidants to scavenge them. This may occur either because the formation of toxic species is enhanced, or the activity of antioxidant protective mechanisms are lowered. This hypothesis is supported by several lines of evidence from this study and elsewhere. Results in this thesis demonstrated that the peroxidation of illuminated thylakoid membranes

by herbicides *in vitro* was retarded by ascorbate, α-tocopherol and carotenoids. These antioxidants are present in chloroplasts *in vivo*, and this observation indicates their potential role in preventing photo-oxidative damage to membranes. Herbicidal injury induced by paraquat was greater in leaf discs cut from plants grown at a low light intensity compared with discs cut from plants grown at a moderate light intensity. This was probably related to the increased activity of H_2O_2 scavenger enzymes and a higher ascorbate concentration in chloroplasts from plants grown at the higher light intensity. Incubation of pea leaf discs on DDTC, which impaired SOD activity, enhanced the toxicity of paraquat. Cotton plants subjected to drought stress were shown to contain enhanced glutathione reductase activity. The increased activity of this enzyme may have contributed to the increased tolerance of these plants to paraquat (Burke *et al.*, 1985). Some plant varieties resistant to paraquat have been reported to contain elevated SOD levels compared with paraquat susceptible varieties (Harper and Harvey, 1978; Youngman and Dodge, 1981; Furusawa *et al.*, 1984). These reports indicate that an increased capacity to scavenge $O_2^{\cdot -}$ and H_2O_2 in chloroplasts could contribute to herbicide tolerance. Damage induced by the air pollutants SO_2 and O_3 may also be related to the level of enzymes and antioxidants that scavenge $O_2^{\cdot -}$ and H_2O_2 in chloroplasts. Spinach cultivars tolerant to O_3 contained higher levels of ascorbate and glutathione than O_3 susceptible plants (Tanaka *et al.*, 1985). Plants treated with EDU, a chemical that promotes SOD levels, also showed enhanced tolerance to O_3 (Lee and Bennett, 1982; Lee and Chen, 1982). Young leaves of poplar, that contained higher levels of SOD than older leaves, showed increased SO_2 tolerance (Tanaka and Sugahara, 1980). Leaves treated with DDTC, which reduced SOD levels,

also showed increased sensitivity to SO_2 (Tanaka and Sugahara, 1980). These studies indicate that damage induced by SO_2 and O_3 is related to endogenous $O_2^{\cdot -}$ and H_2O_2 scavenger levels.

Increased levels of antioxidants, SOD or enzymes that scavenge H_2O_2 in chloroplasts may confer tolerance to O_3, SO_2 and paraquat. A correlation between drought stress and paraquat tolerance, mediated by enhanced glutathione reductase activity has been proposed (Burke *et al.*, 1985). In studies with the green algae *Chlorella* SOD activity has been correlated with paraquat tolerance and susceptibility to chilling injury. *Chlorella* strains resistant to chilling contained higher levels of SOD than chilling sensitive strains. Growth of the chilling sensitive strain with paraquat induced an elevated SOD level and an increased tolerance to chilling (Clare *et al.*, 1984). Other studies with *Chlorella* have demonstrated that SOD levels in this organism were enhanced by growth in the presence of low concentrations of paraquat or with sulphite (Rabinowitch *et al.*, 1983; Rabinowitch and Fridovich, 1985). Elevated SOD levels conferred increased tolerance to paraquat toxicity. These studies indicated that there was a correlation between tolerance to paraquat, chilling injury and sulphite toxicity in *Chlorella*. Comparative plant studies may show similar co-tolerance to such stresses, and are worthy of further investigation.

Control of the activity and level of chloroplast antioxidants and enzymes may, as indicated in this study, be one important mechanism by which increased or decreased tolerance to herbicides, environmental stresses or air pollutants that promote the formation of free radicals, $O_2^{\cdot -}$, H_2O_2 or $^1O_2$ in chloroplasts is achieved.

REFERENCES

REFERENCES

Allen, J.F. (1976) Oxygen reduction and optimum production of ATP in photosynthesis. Nature 256; 599-600.

Allen, J.F. (1977a) Oxygen: A physiological electron acceptor in photosynthesis. Curr. Adv. Plant Sci. 9; 459-469.

Allen, J.F. (1977b) Effect of washing and osmotic shock on catalase activity of intact chloroplast preparations. FEBS Lett. 84; 221-224.

Allen, J.F., Hall, D.O. (1973) Superoxide reduction as a mechanism of ascorbate stimulated oxygen uptake by chloroplasts. Biochem. Biophys. Res. Comm. 52; 856-862.

Anbar, M., Neta, P. (1967) A compilation of specific bimolecular rate constants for the reactions of hydrated electrons, hydrogen atoms and hydroxyl radicals with inorganic and organic compounds in aqueous solution. Int. J. App. Rad. Isotopes 18; 493-523.

Anderson, I.C., Robertson, D.S. (1960) The role of carotenoids in protecting chlorophyll from photodestruction. Plant Physiol. 35; 531-534.

Anderson, J.W., Foyer, C.H., Walker, D.A. (1983) Light-dependent reduction of dehydroascorbate and uptake of exogenous ascorbate by spinach chloroplasts. Planta 158; 442-450.

Anderson, S.M., Krinsky, N.I. (1973) Protective action of carotenoid pigments against photodynamic damage to liposomes. Photochem. Photobiol. 18; 403-408.

Anderson, S.M., Krinsky, N.I., Stone, M.J., Clagett, D.C. (1974) Effect of singlet oxygen quenchers on oxidative damage to liposomes initiated by photosensitisation or by radiofrequency discharge. Photochem. Photobiol. 20; 65-69.

Archibald, F.S., Fridovich, I. (1981) Manganese, superoxide dismutase and oxygen tolerance in some lactic acid bacteria. J. Bacteriol. 146; 928-936.

Arnon, D.I. (1949) Copper enzymes in isolated chloroplasts. Polyphenol oxidase in *Beta vulgaris* L. Plant Physiol. 24; 1-15.

Arrigoni, O., Dipierro, S., Borraccino, G. (1981). Ascorbate free radical reductase: A key enzyme of the ascorbic acid system. FEBS Lett. 125; 242-244.

Asada, K., Badger, M.R. (1984) Photoreduction of $^{18}O_2$ and $H_2{}^{18}O_2$ with concominant evolution of $^{16}O_2$ in intact spinach chloroplasts: Evidence for scavenging of hydrogen peroxide by peroxidase. Plant Cell Physiol. 25; 1169-1179.

Asada, K., Kiso, K. (1973) Initiation of aerobic oxidation of sulphite by illuminated spinach chloroplasts. Eur. J. Biochem. 33; 253-257.

Asada, K., Kiso, K., Yoshikawa, K. (1974a) Univalent reduction of molecular oxygen by spinach chloroplasts on illumination. J. Biol. Chem. 249; 2175-2181.

Asada, K., Takahashi, M., Nagete, M. (1974b) Assay and inhibitors of spinach superoxide dismutase. Agr. Biol. Chem. 38; 471-473.

Asada, K., Takahashi, M., Tanaka, K., Nakano, Y. (1977) Formation of active oxygen and its fate in chloroplasts. In Biochemical and Medical Aspects of Active Oxygen.(O. Hayaishi & K. Asada, eds.), pp. 45-63. University of Tokyo Press.

Asada, K., Urano, M., Takahashi, M. (1973) Subcellular location of superoxide dismutase in spinach leaves and preparation and properties of crystalline spinach superoxide dismutase. Eur. J. Biochem. 36; 257-266.

Asada, K., Yoshikawa, K., Takahashi, M., Maeda, Y., Enmanji, K. (1975). Superoxide dismutase from a blue green algae *Plectonema boryanum*. J. Biol. Chem. 250; 2801-2807.

Avron, M., Jagendorf, A.J. (1956). A TPNH diaphorase from chloroplasts. Arch. Biochem. Biophys. 65; 475-490.

Baker, N.R., Bradbury, M. (1981) Possible applications of chlorophyll fluorescence techniques for studying photosynthesis *in vivo*. In: Plants and the daylight spectrum: Proceedings of the First International Symposium of the British Photobiology Society. (H. Smith, ed.) pp. 355-373.

Barber, J. (1983). Photosynthetic electron transport in relationship to thylakoid membrane composition and organisation. Plant Cell Environ. 6; 311-322.

Barényi, B., Krause, G.H. (1985). Inhibition of photosynthetic reactions by light. A study with isolated spinach chloroplasts. Planta 163; 218-226.

Bartels, P.G.. Watson, C.W. (1978) Inhibition of carotenoid synthesis by fluridone and norflurazon. Weed Sci. 26; 198-203.

Bassham, J.A. (1977) The reductive pentose phosphate cycle and its regulation. In Encyclopedia of Plant Physiology New Series, Vol. 6. (A. Trebst & M. Avron, eds.) pp. 9-30. Springer-Verlag.

Beauchamp, C.O.,Fridovich, I. (1971). Superoxide dismutase; Improved assays and an assay applicable to acrylamide gels. Anal. Biochem. 44; 276-287.

Beauchamp, C.O., Fridovich, I. (1973) Isozymes of superoxide dismutase from wheat germ. Biochim. Biophys. Acta 317; 50-64.

Berry, J., Björkman, O. (1980) Photosynthetic response and adaptation to temperature in higher plants. Ann. Rev. Plant Physiol. 31; 491-543.

Bielski, B.H.J. (1978) Re-evaluation of the spectral and kinetic properties of H_2O_2 and $O_2^{\cdot -}$ free radicals. Photochem. Photobiol. 28; 645-649.

Bielski, B.H.J., Allen, A.O., Schwarz, H.A. (1981). Mechanism of disproportionation of ascorbate radicals. J. Am. Chem. Soc. 103; 3516-3518.

Birker, P.J.M.W.L., Freeman, H.C. (1977) Structure, properties and function of a copper (I) copper (II) complex of D-penicillamine: pentathallium (I), μ_8-chlorododeca (D-penicillaminato)-octacuprate (I) hexacuprate (II) n-hydrate. J. Am. Chem. Soc. 99; 6890-6899.

Björkman, O., Powles, S.B. (1984) Inhibition of photosynthetic reactions under water stress: Interaction with light level. Planta 161; 490-504.

Boardman, N.K. (1977) Photosynthesis in sun and shade plants. Ann. Rev. Plant Physiol. 26; 355-377.

Bodannes, R.S., Chan, P.C. (1979) Ascorbic acid as a scavenger of singlet oxygen. FEBS Lett. 105; 195-196.

Böger, P. (1984) Multiple modes of action of diphenyl ethers. Z. Naturforsch. 39c; 468-475.

Bowyer, J.R., Camilleri, P., O'Neil, P. (1986) Physical-chemical and biochemical techniques in the study of photosystem I herbicides and related molecules. Aspects of App. Biol. 11; 85-96.

Braber, J.M. (1980) Catalase and peroxidase in primary bean leaves during development and senescence. Z. Pflanzenphysiol. 97; 135-144.

Bradbeer, J.W. (1981) Development of photosynthetic function during chloroplast biogenesis. In: The Biochemistry of Plants, Vol. 8 (P.K. Stumpf & E.E. Conn, eds.) pp. 432-472. Academic Press.

Bradbeer, J.W., Arron, G.P., Herra, A., Kemble, R.J., Montes, G., Sherratt, D., Wara-Asswapati, O. (1977) The greening of leaves. Symp. Soc. Expt. Biol. 31; 195-219.

Bridges, S.M., Salin, M.L. (1981) Distribution of iron containing superoxide dismutases in vascular plants. Plant Physiol. 68; 275-278.

Buchanan, B.B., Arnon, D.I. (1969) Ferredoxins from photosynthetic bacteria, algae and higher plants. Methods in Enzymol. 23; 413-440.

Bucke, C. (1968) The distribution and stability of α-tocopherol in subcellular fractions of broad bean leaves. Phytochem. 7; 693-700.

Burg, G. van den, Tipker, J. (1983) Quantitative structure-activity relationships of the inhibition of photosynthetic electron transport by substituted diphenyl ethers. Pestic. Sci. 13; 29-38.

Burke, J.J., Gamble, P.E., Hatfield, J.L., Quisenberry, J.E. (1985) Plant morpholgocial and biochemical responses to field water deficits. I. Responses of glutathione reductase activity and paraquat sensitivity. Plant Physiol. 79; 415-419.

Butler, R.D. (1967) The fine structure of senescing cotyledons of cucumber. J. Expt. Bot. 18; 535-543.

Callow, J.A., Callow, M.E., Woolhouse, H.W. (1972) *In vitro* protein synthesis, RNA synthesis and polyribosomes in senescing leaves of *Perilla*. Cell Differ. 1; 79-90.

Camilleri, P., Bowyer, J.R., Weaver, R.C. (1985) Catalysis by heteropentalenes of electron transfer to oxygen from ferredoxin-$NADP^+$ oxidoreductase reduced by NADPH. Biochim. Biophys. Acta 810; 385-387.

Camp, P.J., Huber, S.C., Moreland, D.E. (1984) Changes in enzymes of sucrose metabolism and the activation of certain chloroplast enzymes during wheat leaf senescence. J. Expt. Bot. 35; 659-668.

Castelfranco, P.A., Beale, S.I. (1981) Chlorophyll biosynthesis. In Biochemistry of Plants, vol. 8 (P.K. Stumpf & E.E. Conn, eds.) pp. 375-421. Academic Press.

Chance, B., Williams, G.R. (1956). The respiratory chain and oxidative phosphorylation. Adv. Enzymol. 17; 65-134.

Charles, S.A., Halliwell, B. (1980) Effect of hydrogen peroxide on spinach (*Spinacea oleracea*) chloroplast fructose bisphosphatase. Biochem. J. 189; 373-376.

Chia, L.S., McRae, D.G., Thompson, J.E. (1982) Light dependence of paraquat-initiated membrane deterioration in bean plants. Evidence for the involvement of superoxide. Physiol. Plant. 56; 492-499.

Chou, Pi.T., Khan, A.U. (1983) L ascorbic acid quenching of singlet delta molecular oxygen in aqueous media: Generalised antioxidant properties of vitamin C. Biochem. Biophys. Res. Comm. 115; 932-937.

Clare, D.A., Rabinowitch, H.D., Fridovich, I. (1984) Superoxide dismutase and chilling injury in *Chlorella ellipsoidea*. Arch. Biochem. Biophys. 231; 158-163.

Conn, E.E., Vennesland, B. (1951) Glutathione reductase in wheat germ. J. Biol. Chem. 192; 17-28.

Corbett, J.R. (1974) The biochemical mode of action of pesticides. Academic Press.

Cornic, G., Woo, K.C., Osmond, B. (1982) Photoinhibition of CO_2 dependent O_2 evolution by intact chloroplasts isolated from spinach leaves. Plant Physiol. 70; 1310-1315.

Critchley, C. (1981) Studies on the mechanism of photoinhibition in higher plants: (1) Effects of high light intensity on chloroplast activities in cucumber adapted to low light. Plant Physiol. 67; 1161-1165.

Davenport, H.E., Hill, R. (1960) A protein from leaves catalysing the reduction of Haem-protein compounds by illuminated chloroplasts. Biochem. J. 74; 493-501.

Dhindsa, R.S., Plumb-Dhindsa, P., Thorpe, T.A. (1981) Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. J. Exp. Bot. 32; 93-101.

Dhindsa, R.S., Plumb-Dhindsa, R.L., Reid, D.M. (1982) Leaf senescence and lipid peroxidation: Effects of some phytohormones and scavengers of free radicals and singlet oxygen. Physiol. Plant. 56; 453-457.

Dodge, A.D. (1982) The role of light and oxygen in the action of photosynthetic inhibitor herbicides. In: Biochemical responses induced by herbicides (D.E. Moreland, J.F. St. John & F.D. Hess, eds.) pp. 57-77. ACS Symposium Series No. 181.

Dodge, A.D. (1983) Toxic oxygen species and herbicide action. In: Human Welfare and the Environment (J. Miyamoto, ed.) pp. 59-66. IUPAC Pesticide Chemistry. Pergamon Press, Oxford.

Dodge, A.D., Alexander, D.J., Blackwood, G.C. (1971) The contribution of photosynthesis to chlorophyll formation in etiolated mung bean leaves. Physiol. Plant.25; 71-74.

Draper, W.M., Casida, J.E. (1985). Nitroxide radical adducts of nitrodiphenyl ether herbicides and other nitroaryl pesticides with unsaturated cellular lipids. J. Agric. Food Chem. 33; 103-108.

Drotar, A., Phelps, P., Fall, R. (1985) Evidence for glutathione peroxidase activities in cultured plant cells. Plant Science 42; 35-40.

Edwards, G., Walker, D.A. (1983) C_3, C_4: Mechanisms and cellular and environmental regulation of photosynthesis. pp. 496-511. Blackwell Scientific Publications, Oxford.

Egneus, H., Heber, U., Matthiesen, U., Kirk, M. (1975) Reduction of oxygen by the electron transport chain of chloroplasts during assimilation of carbon dioxide. Biochim. Biophys. Acta 408; 252-268.

Ellyard, P.W., Gibbs, M. (1969) Inhibition of photosynthesis by oxygen in isolated spinach chloroplasts. Plant Physiol. 44; 1115-1121.

Elstner, E.F. (1982) Oxygen activation and oxygen toxicity. Ann. Rev. Plant Physiol. 33; 73-96.

Elstner, E.F., Heupel, A. (1974) On the mechanism of photosynthetic oxygen reduction by isolated chloroplast lamellae. Z. Naturforsch. 29c; 564-571.

Elstner, E.F., Heupel, A. (1976) Inhibition of nitrite formation from hydroxylamine ammonium chloride: A simple assay for SOD. Anal. Biochem. 70; 616-620.

Elstner, E.F., Stoffer, C., Heupel, A. (1975) Determination of superoxide free radical and hydrogen peroxide as products of

photosynthetic oxygen reduction. Z. Naturforsch. 30c: 53-56.

Ensminger, M.P., Hess, F.D. (1985) Photosynthetic involvement in the mechanism of action of diphenyl ether herbicides. Plant Physiol. 78; 46-50.

Ensminger, M.P., Hess, F.D., Bahr, J.T. (1985) Nitro free radical formation of diphenyl ether herbicides is not necessary for their toxic action. Pestic. Biochem. Physiol. 23; 163-170.

Epel, B.L., Neumann, J. (1973) The mechanism of the oxidation of ascorbate and Mn^{2+} by chloroplasts. The role of the radical superoxide. Biochim. Biophys. Acta 325; 520-529.

Fahrenholtz, S.R., Doleiden, F.H., Trozzolo, A.M., Lamola, A.A. (1974) On the quenching of singlet oxygen by α-tocopherol. Photochem. Photobiol. 20; 505-509.

Feierabend, J., Beevers, H. (1972) Developmental studies on microbodies in wheat leaves. I. Conditions influencing enzyme development. Plant Physiol. 49; 28-32.

Foote, C.S. (1968) Mechanisms of photosensitized oxidations. Science 162; 963-970.

Foote, C.S., Denny, R.W. (1968) Chemistry of singlet oxygen XII. Quenching by β carotene. J. Am. Chem. Soc. 90; 6233-6235.

Foote, C.S. (1976) Photosensitised oxidation and singlet oxygen: consequences in biological systems. In Free Radicals in Biology Vol. 11 (W.A. Pryor, ed.) pp. 85-133. Academic Press.

Forti, G., Grubas, P.M.G. (1985) Two sites of interaction of ferredoxin with thylakoids. FEBS Lett. 186; 149-152.

Foster, J.G., Edwards, G.E. (1980) Localisation of superoxide dismutase in leaves of C_3 and C_4 plants. Plant Cell Physiol. 21; 895-906.

Foster, J.G., Hess, J.L. (1980) Responses of superoxide dismutase and glutathione reductase activities in cotton leaf tissue exposed to an atmosphere of enriched oxygen. Plant Physiol. 66; 482-487.

Foster, J.G., Hess, J.L. (1982) Oxygen effects on maize leaf superoxide dismutase and glutathione reductase. Phytochem. 21; 1527-1532.

Foyer, C.H. (1984) Photosynthesis p. 28. Wiley, New York.

Foyer, C.H., Hall, D.O. (1980a) Superoxide dismutase in the functioning chloroplast. In: Chemical and Biochemical aspects of superoxide and superoxide dismutase (J.V.S. Banister & H.A.O. Hill, eds.) pp 380-389. Elsevier/North Holland.

Foyer, C.H., Hall, D.O. (1980b) Oxygen metabolism in the active chloroplast. TIBS. 188; 91-94.

Foyer, C.H., Halliwell, B. (1976) The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. Planta 133; 21-25.

Foyer, C.H., Halliwell, B. (1977) Purification and properties of dehydroascorbate reductase from spinach leaves. Phytochem. 16; 1347-1350.

Foyer, C.H., Rowell, J., Walker,D.A. (1983) Measurement of the ascorbate content of spinach leaf protoplasts and chloroplasts during illumination. Planta 157; 239-244.

Fridovich, I. (1975) Superoxide dismutases. Ann. Rev. Biochem. 44; 147-159.

Fridovich, I. (1976) Oxygen radicals, hydrogen peroxide and oxygen toxicity. In:Free Radicals in Biology Vol. I. (W.A. Pryor, ed.) pp. 239-277. Academic Press.

Fridovich, I. (1978) The biology of oxygen radicals. Science 201; 875-880.

Friend, J. and Mayer, A.M. (1960) The enzymic destruction of carotenoids by isolated chloroplasts. Biochim. Biophys. Acta 41; 422-429.

Fuerst, E.P., Nakatini, H.Y., Dodge, A.D., Penner, D.S., Arntzen, C.J. (1985) Paraquat resistance in *Conyza*. Plant Physiol. 77; 984-989.

Furbank, R.T. (1984) Photoreduction of oxygen in higher plants: mechanisms and physiological functions. Whats new in Plant Physiol. 15; 33-36.

Furusawa, I., Tanaka, K., Thanutong, P., Mizuguchi, A., Yazaki, M., Asada, K. (1984) Paraquat resistant tobacco calluses with enhanced superoxide dismutase activity. Plant Cell Physiol. 25; 1247-1254.

Gamble, P.E., Burke, J.J. (1983) Glutathione reductase distribution in etiolated and green wheat leaves. Plant Physiol. 72; S-42.

Gamble, P.E., Burke, J.J. (1984) Effect of water stress on the chloroplast antioxidant system. I. Alterations in glutathione reductase activity. Plant Physiol. 76; 615-621.

Gerbling, K.P., Kelly, G.J., Fischer, K.H., Latzko, E. (1984) Partial purification and properties of soluble ascorbate peroxidase from pea leaves. J. Plant Physiol. 115; 59-67.

Giannopolitis, C.N., Ries, S.K. (1977) Superoxide dismutases. I. occurrence in higher plants. Plant Physiol. 59; 315-318.

Ginkel, G. van, Brown, J.S. (1978) Endogenous catalase and SOD activities in photosynthetic membranes. FEBS Lett. 94; 284-296.

Glidewell, S.M., Raven, J.A. (1975) Measurement of simultaneous oxygen evolution and uptake in *Hydrodictoyon africanum*. J. Expt. Bot. 26; 479-488.

Good, N.E., Hill, R. (1955) Photochemical reduction of oxygen in chloroplast preparations. II. Mechanisms of the reaction with oxygen. Arch. Biochem. Biophys. 57; 355-366.

Goodwin, T.W. (1958) Studies in carotenogenesis. 25. The incorporation of ^{14}C (2-^{14}C) acetic acid and (2-^{14}C) mevalonate into β carotene by illuminated etiolated maize seedlings. Biochem. J. 70; 612-617.

Gregory, E.M., Fridovich, I. (1973) Induction of superoxide dismutase by molecular oxygen. J. Bacteriol. 114; 543-548.

Gregory, E.M., Goscin, S.A., Fridovich, I. (1974) Superoxide dismutase and oxygen toxicity in a eucaryote. J. Bacteriol. 117; 456-460.

Grill, D., Esterbaur, H., Klosch, U. (1979) Effect of sulphur dioxide on glutathione in leaves of plants. Environ. Pollut. 19; 187-194.

Groden, D., Beck, E. (1979) H_2O_2 destruction by ascorbate-dependent systems from chloroplasts. Biochim. Biophys. Acta 546; 426-435.

Guy, C.L., Carter, J.V. (1984) Characterisation of partially purified glutathione reductase from cold hardened and non-hardened spinach leaf tissue. Cryobiology 21; 454-464.

Haber, F., Weiss, J. (1934) The catalytic decomposition of hydrogen peroxide by iron salts. Proc. Roy. Soc. Lond. Ser. A.147; 332-351.

Hall, D.O. (1972) Nomenclature for isolated chloroplasts. Nature 235; 125-126.

Hall, D.O., Rao, K.K. (1977) Ferredoxin. In: Encyclopedia of plant Physiology New Series. Vol. 6 (A. Trebst and M. Avron, eds.) pp. 206-216. Springer-Verlag.

Halliwell, B. (1976) An attempt to demonstrate a reaction between superoxide and hydrogen peroxide. FEBS Lett. 72; 8-10.

Halliwell, B. (1981) Chloroplast metabolism: the structure and function of chloroplasts in green leaf cells. Clarendon Press, Oxford.

Halliwell, B., Foyer, C.H., Charles, S.A. (1981) The fate of hydrogen peroxide in illuminated chloroplasts. In: Photosynthesis II. electron transport and photophosphorylation. (G. Akoyonaglou, ed.) pp. 279-283. Babalan Int. Sci. Series, Philadelphia.

Halliwell, B., Gutteridge, J.M.C. (1985) Free radicals in biology and medicine. Clarendon Press, Oxford.

Harbour, J.R., Bolton, J.R. (1975) Superoxide formation in spinach chloroplasts: electron spin resonance detection by spin trapping. Biochem. Biophys. Res. Comm. 64; 803-807.

Harper, D.B., Harvey, B.M.R. (1978) Mechanisms of paraquat tolerance in perennial ryegrass II. Role of superoxide dismutase, catalase and peroxidase. Plant Cell Environ. 1; 211-215.

Harris, N., Dodge, A.D. (1972) The effect of paraquat on flax cotyledon leaves: changes in fine structure. Planta 104; 201-209.

Harris, T.J., Auffret, A.D., Northrop, F.D., Walker, J.E. (1980) Structural comparisons of superoxide dismutases. Eur. J. Biochem. 106; 297-303.

Hartree, E.F. (1972) Determination of protein: a modification of the Lowry method that gives a linear photometric response. Anal. Biochem. 48; 422-427.

Hassan, H.M., Fridovich, I. (1977a) Regulation of the synthesis of superoxide dismutase in *Escherichia coli*: Induction by methyl viologen. J. Bacteriol. 252; 7667-7672.

Hassan, H.M., Fridovich, I. (1977b) Regulation of superoxide dismutase in *Escherichia coli*: Glucose effect. J. Bacteriol. 132; 505-510.

Hassan, H.M., Fridovich, I. (1978) Superoxide radical and the oxygen enhancement of the toxicity of paraquat in *E.coli*. J. Biol. Chem. 253; 8143-8148.

Hassan, H.M., Fridovich, I. (1979) Intracellular production of superoxide radical and of hydrogen peroxide by redox active compounds. Arch. Biochem. Biophys. 196; 385-395.

Hasselt, Ph.R. van (1972) Photo-oxidation of leaf pigments in *Cucumis* leaf discs during chilling. Acta Bot. Neerl. 21; 539-543.

Hasselt, Ph.R. van (1974) Photo-oxidation of unsaturated lipids in *Cucumis* leaf discs during chilling. Acta Bot. Neerl. 23; 159-169.

Hatch, M.D., Slack, C.R., Bull, J.A. (1969) Light induced changes in the control of some enzymes of the C_4-dicarboxylic acid pathway of photosynthesis and its effect on other characteristics of photosynthesis. Phytochem. 8; 697-706.

Haugaard, N. (1968) Cellular mechanisms of oxygen toxicity. Physiol. Rev. 48; 311-373.

Hayakawa, T., Kanematsu, S., Asada, K. (1984) Occurrence of Cu, Zn-superoxide dismutase in the intrathylakoid space of spinach chloroplasts. Plant Cell Physiol. 25; 883-889.

Hayakawa, T., Kanematsu, S., Asada, K. (1985) Purification and characterisation of thylakoid-bound Mn-superoxide dismutase in spinach chloroplasts. Planta 166; 111-116.

Heber, U., Krause, G.H. (1980) What is the physiological role of photorespiration? TIBS. 5; 32-34.

Heldt, H.W., Chon, C.J., Lilley, R.McC., Portis, A. (1978) The role of fructose- and sedoheptulose bisphosphatase in the control of CO_2 fixation. Evidence from the effects of Mg^{2+}, concentration, pH and H_2O_2. In: Photosynthesis 1977 (D.O. Hall, J. Coombs & T.W. Goodwin, eds.) pp. 469-478. Biochemical Society, London.

Heldt, H.W., Werdan, K., Milovaner, M., Geller, G. (1973) Alkalisation of the chloroplast stroma caused by light dependent proton flux into the thylakoid space. Biochim. Biophys. Acta 314; 224-241.

Hill, R. (1937) Oxygen evolution by isolated chloroplasts. Nature 139; 881-882.

Hill, R. (1939) Oxygen produced by isolated chloroplasts. Proc. Roy. Soc. Lond. Ser. B. 127; 192-210.

Hill, R., Bendall, F. (1960) Function of the two cytochrome components in chloroplasts: a working hypothesis. Nature 186; 136-137.

Hillman, W.S. (1969) Photoperiodism and Vernalisation. In: Physiology of plant growth and development (M.B. Wilkins, ed.) pp. 556-601. McGraw-Hill.

Hossain, M.A., Asada, K. (1984) Purification of dehydroascorbate reductase from spinach and its characterization as a thiol enzyme. Plant Cell Physiol. 25; 85-92.

Hossain, M.A., Nakano, Y., Asada, K. (1984) Monodehydroascorbate reductase in spinach chloroplasts and its participation in regeneration of ascorbate for scavenging hydrogen peroxide. Plant Cell Physiol. 25; 385-395.

Huffaker, R.C., Obendorf, R.L., Keller, C.J., Kleinkopf, G.E. (1966) Effects of light intensity on photosynthetic carboxylative phase enzymes and chlorophyll synthesis in greening leaves of *Hordeum vulgare* L. Plant Physiol. 41; 913-918.

Hughes, C.T., Gaunt, J.K., Laidman, D.I. (1971) The subcellular distribution of tocopherols in the green leaves of *Pisum sativum*. Biochem. J. 124; 9P.

Hughes, K.W., Negrotto, D., Daub, M.E., Meeusen, R.L. (1984) Free-radical stress response in paraquat sensitive and resistant tobacco plants. Environ. Expt. Bot. 24; 151-157.

Ito, T. (1978) Cellular and subcellular mechanisms of photodynamic action: the $^1O_2$ hypothesis as a driving force in recent research. Photochem. Photobiol. 28; 493-508.

Izawa, S. (1980) Acceptors and donors for chloroplast electron transport. Methods in Enzymol. 69; 413-434.

Jablonski, P.P., Anderson, J.W. (1978) Light dependent reduction of oxidised glutathione by ruptured chloroplasts. Plant Physiol. 61; 221-225.

Jablonski, P.P., Anderson, J.W. (1981) Light dependent reduction of dehydroascorbate by ruptured pea chloroplasts. Plant Physiol. 67; 1239-1244.

Jablonski, P.P., Anderson, J.W. (1982) Light dependent reduction of hydrogen peroxide by ruptured pea chloroplasts. Plant Physiol. 69; 1407-1413.

Jackson, C., Dench, J., Moore, A.L., Halliwell, B., Foyer, C., Hall, D.O. (1978) Subcellular localisation and identification of superoxide dismutase in the leaves of higher plants. Eur. J. Biochem. 91; 339-344.

Joliot, P., Kok, B. (1975) Oxygen evolution in photosynthesis. In: Bioenergetics of Photosynthesis. (Govindjee, ed.) pp. 387-412. Academic Press.

Jones, P., Suggett, A. (1968) The catalase H_2O_2 system: Kinetics of catalytic action at high substrate concentrations. Biochem. J. 110; 617-629.

Kaiser, W.M. (1976) The effect of hydrogen peroxide on CO_2 fixation of isolated intact chloroplasts. Biochim. Biophys. Acta 440; 476-482.

Kaiser, W.M. (1979) Reversible inhibition of the Calvin cycle and the activation of the pentose phosphate pathway in isolated chloroplasts by hydrogen peroxide. Planta 145; 377-382.

Kar, M., Feierabend, J. (1984) Metabolism of activated oxygen in detached wheat and rye leaves and its relevance to the initiation of senescence. Planta 160; 385-391.

Kenyon, W.H., Duke, S.O., Vaughn, K.C. (1985) Sequence of effects of acifluorfen on physiological and ultrastructural parameters in cucumber cotyledon discs. Pestic. Biochem. Physiol. 24; 240-250.

Khan, A.U. (1970) Singlet molecular oxygen from superoxide anion and sensitized fluorescence of organic molecules. Science 168; 476-477.

Kirk, J.T.O., Allen, R.L. (1965) Dependence of chloroplast pigment synthesis on protein synthesis: Effect of actidione. Biochem. Biophys. Res. Comm. 21; 523-530.

Knox, J.P., Dodge, A.D. (1984) Photodynamic damage to plant leaf tissue by rose bengal. Plant Sci. Letts. 37; 3-7.

Knox, J.P., Dodge, A.D. (1985) The photodynamic action of eosin, a singlet oxygen generator. The inhibition of photosynthetic electron flow. Planta 164; 30-34.

Kok, L.J.de, Oosterhuis, F.A. (1983) Effect of frost hardening and salinity on glutathione and sulphydryl levels and on glutathione reductase activity in spinach leaves. Physiol. Plant. 58; 47-51.

Kok, L.J.de, Thompson, C.R., Kuiper, P.J.C. (1983) Sulphite induced oxygen uptake by isolated spinach chloroplasts catalysed by photosynthetic electron transport. Physiol. Plant. 59; 19-32.

Kono, Y., Takahashi, M., Asada, K. (1976) Oxidation of manganous pyrophosphate by superoxide radicals and illuminated spinach chloroplasts. Arch. Biochem. Biophys. 174; 454-462.

Krause, G.H., Kirk, M., Heber, U., Osmond, C.B. (1978) O_2 dependent inhibition of photosynthetic capacity in intact isolated chloroplasts and isolated cells from spinach leaves illuminated in the absence of CO_2. Planta 142; 229-233.

Krause, G.H., Köster, S., Wong, S.C. (1985) Photoinhibition of photosynthesis under anaerobic conditions studied with leaves and chloroplasts of *Spinacea oleracea* L. Planta 165; 430-438.

Krinsky, N.I. (1977) Singlet oxygen in biological systems. TIBS. 2; 35-38.

Krinsky, N.I. (1979) Carotenoid protection against oxidation. Pure Appl. Chem. 51; 649-660.

Kunert, K.J., Böger, P. (1981) The bleaching effect of the diphenyl ether herbicide oxyfluorfen. Weed Sci. 29; 169-171.

Lai, E.K., Fong, K.L., McCay, P.B. (1978) Studies on the properties of the singlet oxygen like factor produced during lipid peroxidation. Biochim. Biophys. Acta 528; 497-506.

Lambert, R., Kroneck, P.M., Böger, P. (1985) Radical formation and peroxidative action of phytotoxic diphenyl ethers. Z. Naturforsch. 39c; 486-491.

Lambert, R., Sandmann, G., Böger, P. (1983) Correlation between structure and phytotoxic activities of nitro diphenyl ethers. Pestic. Biochem. Physiol. 19; 309-320.

Law., M.Y., Charles, S.A., Halliwell, B. (1983) Glutathione and ascorbic acid in spinach (*Spinacia oleracea*) chloroplasts. The effect of hydrogen peroxide and paraquat. Biochem. J. 210; 899-903.

Lee, E.H., Bennett, J.H. (1982) Superoxide dismutase. A possible protective enzyme against ozone injury in snap beans (*Phaseolus vulgaris* L.). Plant Physiol. 69; 1444-1449.

Lee, E.H., Chen, C.M. (1982) Studies on the mechanism of ozone tolerance: Cytokinin like activity of N-[2-(2-oxo-1-imidazolidinyl)-ethyl]-N'-phenyl urea, a compound protecting against ozone injury. Physiol. Plant. 56; 486-491.

Lengfelder, E., Fuchs, C., Younes, M., Weser, U. (1979) Functional aspects of the superoxide dismutative action of Cu-penicillamine. Biochim. Biophys. Acta 567; 492-502.

Leshem, Y.Y. (1981) Oxy free radicals in plant senescence. Whats new in Plant Physiol. 12; 1-4.

Lewinsohn,E., Gressel, J. (1984) Benzyl viologen-mediated counteraction of diquat and paraquat phytotoxicities. Plant Physiol. 76; 125-130.

Lichtenthaler, H.K. (1969) Light stimulated synthesis of plastid quinones and pigments in etiolated barley seedlings. Biochim. Biophys. Acta 184; 164-172.

Lichtenthaler, H.K., Gurystahler, R., Buschmann, C., Meier, D., Prenzel, U., Schonthal, A. (1983) Effect of high light and high

light stress on composition, function and structure of the photosynthetic apparatus. In: Effects of stress on photosysnthesis. (R. Marcelle, H. Clijsters and M. van Puch, eds.), pp. 353-370. Martinus Nijhoff.

Lichtenthaler, H.K., Wellburn, A.R. (1983) Determinations of total carotenoids and chlorophyll a and b of leaf extracts in different solvents. Biochem. Soc. Trans. 11; 591-592.

Lindenman, W. (1979) Inhibition of photosynthesis in *Lemna minor* by illumination during chilling in the presence of oxygen. Photosynthetica 13; 175-185.

Long, S.P. (1983) C_4 photosynthesis at low temperature. Plant Environ. 6; 345-363.

Long, S.P., East, T., Baker, N. (1983) Chilling damage to photosynthesis in young *Zea mays*. I. Effects of light and temperature variations on photosynthetic CO_2 assimilation. J. Expt. Bot. 34; 177-188.

Lumsden, J., Hall, D.O. (1974) Soluble and membrane bound superoxide dismutases in a blue-green alga (*Spirulina*) and spinach. Biochem. Biophys. Res. Comm. 58; 35-41.

Lumsden, J., Hall, D.O. (1975) Chloroplast manganese and superoxide. Biochem. Biophys. Res. Comm. 64; 595-602.

Martin, C., Thimann, K.V. (1972) The role of protein synthesis in the senescence of leaves. I. The formation of protease. Plant Physiol. 49; 64-71.

Matsunaka, S. (1969) Acceptor of light energy in photoactivation of diphenyl ether herbicides. J. Agric. Food Chem. 17; 171-175.

Maunders, M.J., Brown, S.B. (1983) The effect of light on chlorophyll loss in senescing leaves of sycamore (*Acer pseudoplatanus* L.). Planta 158; 309-311.

McClune, G.J., Fee, J.A. (1976) Stopped flow spectrophotometric observation of superoxide dismutation in aqueous solution. FEBS Lett. 67; 394-398.

McCord, J.M., Fridovich, I. (1969) Superoxide dismutase: An enzymic function for erythrocuperin (haemocuperin). J. Biol. Chem. 244; 6049-6055.

McKersie, B.D., Beversdorf, W.D., Hucl, P. (1983) The relationship between ozone insensitivity, lipid soluble antioxidants and superoxide dismutase in *Phaseolus vulgaris*. Can. J. Bot. 60; 2686-2691.

McRae, D.G., Thompson, J.E. (1983) Senescence-dependent changes in superoxide anion production by illuminated chloroplasts from bean leaves. Planta 158; 185-193.

Mehler, A.H. (1951a) Studies on reactions of illuminated chloroplasts. I. Mechanisms of the reduction of oxygen and other Hill reagents. Arch. Biochim. Biophys. 33; 65-77.

Mehler, A.H. (1951b) Studies on reactions of illuminated chloroplasts. II. Stimulation and inhibition of the reaction with molecular oxygen. Arch. Biochem. Biophys. 34; 339-351.

Mehler, A.H., Brown, A.H. (1952) Studies on reactions of illuminated chloroplasts. III. Simultaneous photoproduction and consumption of oxygen studied with oxygen isotopes. Arch. Biochem. Biophys. 38; 365-370.

Michalski, W.S., Kaniuga, Z. (1981) Photosynthetic apparatus of chilling resistant plants. X. Relationship between superoxide dismutase activity and photoperoxidation of chloroplast lipids. Biochem. Biophys. Acta 637; 159-167.

Miller, O.K., Hughes, K.W. (1980) Selection of paraquat resistant varieties of tobacco from cell cultures. In Vitro. 16; 1085-1095.

Miller, R.W., MacDowall, F.D.H. (1975) The tiron free radical as a sensitive indicator of chloroplastic photoautoxidation. Biochim. Biophys. Acta 387; 176-187.

Mitchell, P. (1966) Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. Biol. Rev. 41; 445-502.

Mondal, R., Choudhuri, M.A. (1981) Role of hydrogen peroxide in senescence of excised leaves of rice and maize. Biochem. Physiol. Pflanzen. 176; 700-709.

Mondal, R., Choudhuri, M.A. (1982) Regulation of senescence of excised leaves of C_3 and C_4 species by endogenous H_2O_2. Biochem. Physiol. Pflanzen.177; 403-417.

Mukherjee, S.P., Choudhuri, M.A. (1983) Implications of water stress induced changes in the levels of endogenous ascorbic acid and hydrogen peroxide in *Vigna* seedlings. Plant Physiol. 58; 166-170.

Mustardy, L.A., Rozsa, Z.S., Faludi Daniel, A. (1984) Chilling syndrome in light exposed maize leaves and its easing by low doses of DCMU. Physiol. Plant. 60; 572-576.

Nakano, Y., Asada, K. (1980) Spinach chloroplasts scavenge hydrogen peroxide on illumination. Plant Cell Physiol. 21; 1295-1307.

Nakano, Y., Asada, K. (1981) Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 22; 867-880.

Newton, B.A., Baker, N.R., Long, S.P., Lawlor, D.A. (1983) *In vivo* photochemical function in water stressed leaves of *Zea mays*. In: Photosynthesis VI. Photosynthesis and productivity, photosynthesis and environment. (G. Akoyonaglou, ed.). pp. 209-218. Babalan Int. Sci. Series, Philadelphia.

Nilsson, R., Kearns, D.R. (1973) A remarkable deuterium effect on the rate of photosensitized oxidation of alcohol dehydrogenase and trypsin. Photochem. Photobiol. 17; 65-68.

Nishikimi, M. (1975) Oxidation of ascorbic acid with superoxide anion generated by xanthine-xanthine oxidase. Biochem. Biophys. Res. Comm. 63; 463-468.

Oquist, G. (1983) Effects of low temperature on photosynthesis. Plant Cell Environ. 6; 281-300.

Orr, G.L., Elliott, M.C., Hogan, M.E. (1983a) Determination of redox behaviour *in vitro* of nitrodiphenyl ether herbicides using cyclic voltametry. J. Agric. Food Chem. 31; 1192-1195.

Orr, G.L., Elliot, C.M., Hogan, M.E. (1983b) Activity *in vivo* and redox states *in vitro* of nitro- and chlorodiphenyl ether herbicide analogs. Plant Physiol. 73; 939-944.

Orr, G.L., Hess, F.D. (1981) Characterisation of herbicidal injury by acifluorfen-methyl in excised cucumber (*Cucumis sativus* L.) cotyledons. Pestic. Biochem. Physiol. 16; 171-178.

Orr, G.L., Hess, F.D. (1982) Mechanism of action of the diphenyl ether herbicide acifluorfen-methyl in excised cucumber (*Cucumis sativus* L.) cotyledons. Plant Physiol. 69; 502-507.

Oser, B.L. (1979) Hawks Physiological Chemistry. pp 702-705. McGraw Hill, New York.

Osmond, C.B. (1981) Photorespiration and photoinhibition. Some implications for photosynthesis. Biochim. Biophys. Acta 639; 77-98.

Overbaugh, J.M., Fall, R. (1982) Detection of glutathione peroxidases in some microalgae. FEMS Microbiol. Lett. 13; 371-375.

Overbaugh, M.J., Fall, R. (1985) Characterization of selenium independent glutathione peroxidase from *Euglena gracilis*. Plant Physiol. 77; 437-442.

Packer, J.E., Slater, T.F., Wilson, R.L. (1979) Direct observations of a free radical reaction between vitamin E and vitamin C. Nature 278; 737-738.

Pallett, K.E., Dodge, A.D. (1979) Role of light and oxygen in the action of the photosynthetic inhibitor herbicide monuron. Z. Naturforsch. 34; 1058-1061.

Pallett, K.E., Dodge, A.D. (1980) Studies into the action of some photosynthetic inhibitor herbicides. J. Expt. Bot. 31; 1051-1066.

Papageorgiou, G. (1975) Chlorophyll fluorescence: an intrinsic probe of photosynthesis. In: Bioenergetics of photosynthesis (Gorindjee, ed.) pp. 320-366. Academic Press, New York.

Parrish, R.W. (1972) The intracellular location of phenol oxidases, peroxidase and phosphatase in the leaves of spinach beet (*Beta vulgaris* L. subspecies *vulgaris*). Eur. J. Biochem. 31; 446-455.

Popovic, R., Fraser, D., Vidaver, W., Colbow, K. (1984) Oxygen quenched chlorophyll a fluorescence and electron transport in barley during greening. Physiol. Plant 62; 344-348.

Powles, S.B. (1984) Photoinhibition of photosynthesis induced by visible light. Ann. Rev. Plant Physiol. 35; 15-44.

Powles, S.B., Critchley, C. (1980) Effect of light intensity during growth on photoinhibition of intact attached bean leaflets. Plant Physiol. 65; 1181-1187.

Powles, S.B., Berry, J.A., Bjorkman, O. (1983) Interaction between light and chilling temperatures on the inhibition of photosynthesis in chilling sensitive plants. Plant Cell Environ. 6; 117-123.

Powles, S.B., Osmond, B. (1978) Inhibition of the capacity and efficiency of photosynthesis in bean leaflets illuminated in a CO_2 free atmosphere at low oxygen. A possible role for photorespiration. Aust. J. Plant Physiol. 5; 619-629.

Powles, S.B., Osmond, B., Thorne, S.W. (1979) Photoinhibition in intact attached leaves of C_3 plants illuminated in the absence of both carbon dioxide and of photorespiration. Plant Physiol. 64; 982-988.

Pryor, W.A. (1978) The formation of free radicals and the consequences of their reactions *in vivo*. Photochem. Photobiol. 28; 787-801.

Pugh, S.Y.R., Fridovich, I. (1985) Induction of superoxide dismutase in *Escherichia coli* B by metal chelators. J. Bacteriol. 162; 196-202.

Rich, P.R., Bonner, W.D. (1978) The sites of superoxide anion generation in higher plant mitochondria. Arch. Biochem. Biophys. 188; 206-213.

Ridley, S.M. (1983) Interaction of chloroplasts with inhibitors. Effects of two diphenyl ether herbicides, fomesafen and nitrofluorfen on electron transport, and some comparisons with dibromothymoquinone, diuron and paraquat. Plant Physiol. 72; 461-468.

Ridley, S.M., Ridley,J. (1979) Interaction of chloroplasts with inhibitors. Location of carotenoid synthesis and inhibition during chloroplast development. Plant Physiol. 63; 392-398.

Rowley, J.A., Taylor,A.O. (1978) Plants under climatic stress (IV). Effects of CO_2 and O_2 on photosynthesis under high light low temperature stress. New Phytol. 71; 477-481.

Rowley, D.A., Halliwell, B. (1983) Superoxide dependent and ascorbate dependent formation of hydroxyl radicals in the presence of copper salts. A physiologically significant reaction. Arch. Biochem. Biophys. 225; 279-284.

Sakaki, T., Kondo, N., Sugahara, K. (1983) Breakdown of photosynthetic pigments and lipids in spinach leaves with ozone fumigation. Role of active oxygen. Physiol. Plant. 59; 28-34.

Salter, S.O., Thimann, K.V. (1983) Metabolism of oat leaves during senescence VIII. The Interaction of $CO_2$and other atmospheric gases with lght in controlling chlorophyll loss and senescence. Plant Physiol. 71; 67-70.

Sandemann, G., Böger, P. (1982) Volatile hydrocarbons from photosynthetic membranes containing different fatty acids. Lipids. 17; 35-41.

Santarius, K.A., Muller, M. (1979) Investigations on heat resistance of spinach leaves. Planta 46; 529-538.

Scandalios, J.G., Liu, E..H., Campeau, M.A. (1972) The effect of intragenic and intergenic complementation on catalase structure and function in maize ; a molecular approach to heterosis. Arch. Biochem. Biophys. 153; 695-705.

Schaedle, M., Bassham, J.A. (1977) Chloroplast glutathione reductase, Plant Physiol. 59; 1011-1012.

Schopfer, P. (1977) Phytochrome control of enzymes. Ann. Rev. Plant Physiol. 28; 223-252.

Shimazaki, K., Nakamachi, K., Kondo, N., Sugahara, K. (1984) Sulfite inhibition of photosystem II in illuminated spinach leaves. Plant Cell Physiol. 25; 337-341.

Shimazaki, K., Sakaki, T., Kondo, T., Sugahara, K. (1980) Active oxygen participation in chlorophyll destruction and lipid peroxidation in SO_2 fumigated leaves. Plant Cell Physiol. 21; 1193-1204.

Shin, J. (1969) Ferredoxin $NADP^+$ reductase from spinach.Methods in Enzymol. 23; 440-447.

Simon, L.M., Fatrai, Z., Jonas, D.E., Matovics, B. (1974) Study of peroxide metabolism enzymes during the development of *Phaseolus vulgaris*. Biochem. Physiol. Pflanzen 166; 387-392.

Smith, J., Shrift, A. (1979) Phylogenic distribution of glutathione peroxidase. Comp. Biochem. Physiol. 63; 39-44.

Steinitz, Y., Mazor, Z., Shilo, M.(1979) A mutant of the cyanobacterium *Plectonema boryanum* resistant to photo-oxidation. Plant Sci. Lett. 16; 327-335.

Swithenbank, C. (1982) The discovery of aci- and oxy-fluorofen: Structure/Activity studies of herbicidal diphenyl ethers using an enzyme inhibition model. In Human Welfare and the Environment. (ed. J. Miyomoto), IUPAC Pesticide Chemistry. Pergamon Press, Oxford.

Takahama, U. , Nishimura, M. (1975) Formation of singlet molecular oxygen in illuminated chloroplasts. Effects on photoinactivation and lipid peroxidation. Plant Cell Physiol. 16; 737-748.

Tanaka, K., Kondo, N., Sugahara, K. (1982a) Accumulation of hydrogen peroxide in chloroplasts of SO_2 fumigated spinach leaves. Plant Cell Physiol. 23; 999-1007.

Tanaka, K., Otsuko, T., Kondo, N. (1982b) Particpation of hydrogen peroxide in the inactivation of Calvin cycle - SH enzynes in SO_2 fumigated spinach leaves. Plant Cell Physiol. 23; 1009-1018.

Tanaka, K., Suda, Y., Kondo, N., Sugahara, K. (1985) O_3 tolerance and the ascorbate-dependent H_2O_2 decomposing system in chloroplasts. Plant Cell Environ. 26; 1425-1431.

Tanaka, K., Sugahara, K. (1980) Role of superoxide dismutase in defence against SO_2 toxicity and an increase in superoxide dismutase activity with SO_2 fumigation. Plant Cell Physiol. 21; 601-611.

Taube, H. (1965) Mechanisms of oxidation with oxygen. J. Gen. Physiol. 49; 29-50.

Taylor, A.O., Rowley, J.A. (1971) Plants under climatic stress: I. Low temperature, high light stress on photosynthesis. Plant Physiol. 47; 713-718.

Thomas, H. (1976) Delayed leaf senescence in leaves treated with the protein synthesis inhibitor MDMP. Plant Sci. Lett. 6; 369-377.

Thomas, H., Stoddart, J.L. (1980) Leaf Senescence. Ann. Rev. Plant Physiol. 31; 83-111.

Tolbert, N.E. (1974) Isolation of subcellular organelles of metabolism on isopycnic sucrose gradients. Methods in Enzymol. 31A; 734-746.

Tolbert, N.E., Oeser, A., Kisaki, T., Hageman, R.H., Yamazaki, R.K. (1968) Peroxisomes from spinach leaves containing enzymes related to glycollate metabolism. J. Biol. Chem. 243; 5179-5184.

Trebst, A., Avron, M. (1977) In Photosynthesis II, Encyclopedia of Plant Physiology. New Series, Vol. 6 (A. Trebst & M. Avron, eds.) pp. 1-4. Springer-Verlag.

Vanstone, D.E., Stobbe, E.H. (1977) Electrical conductivity - a rapid measure of herbicidal injury. Weed Sci. 25; 352-354.

Vanstone, D.E., Stobbe,E.H. (1979) Light requirement of the diphenyl ether herbicide oxyfluorfen. Weed Sci. 29; 88-91.

Vaughan, D., Kok, P.C.de, Ord, B.G. (1982) The nature and localisation of superoxide dismutase in fronds of *Lemna gibba* L. and the effect of copper and zinc deficiency on its activity. Physiol. Plant. 54; 253-257.

Venkatrayappa, J., Fletcher, R.A., Thompson, J.E. (1984) Retardation and reversal of senescence in bean leaves by benzyl adenine and decapitation. Plant Cell Physiol. 25; 407-418.

Walker, D.A. (1980) Preparation of higher plant chloroplasts. Methods in Enzymol. 69; 94-104.

Wessels, J.C.S. (1965) Mechanism of the reduction of organic nitro compounds by chloroplasts. Biochim. Biophys. Acta 109; 357-371.

Winterbourn, C.C. (1981) Production of hydroxyl radicals from paraquat radicals and hydrogen peroxide. FEBS Lett. 128; 339-342.

Woolhouse, H.W. (1982) Leaf Senescence, In The Molecular Biology of Plant Development (H. Smith & D. Grierson, eds.) pp. 256-281. Blackwell.

Woolhouse, H.W. (1984) The biochemistry and regulation of senescence in chloroplasts. Can. J. Bot. 62; 2934-2942.

Yamauchi, R., Matsushita, S. (1979) Light induced lipid peroxidation in isolated chloroplasts and the role of α-tocopherol. Agric. Biol. Chem. 43; 2157-2161.

Young, L.C.T., Conn, E.E. (1956) The reduction and oxidation of glutathione by plant mitochondria. Plant Physiol. 31; 205-211.

Youngman, R.J. (1980) The role of oxygen in the herbicidal action of paraquat. Ph.D. Thesis, University of Bath.

Youngman, R.J., Dodge, A.D. (1979). Mechanism of paraquat action: Inhibition of herbicidal effect by a copper chelate with superoxide dismutase activity. Z. Naturforsch. 34c; 1032-1035.

Youngman, R.J., Dodge, A.D. (1981) On the mechanism of paraquat resistance in *Conyza* sp. Photosynthesis VI. Photosynthesis and productivity, photosynthesis and environment (G. Akoyunoglou ed.) pp. 537-543. Babalan Int. Sci. Services, Philadelphia.

Planta (1986) 167:246–251

Planta

Hydrogen-peroxide-scavenging systems within pea chloroplasts

A quantitative study

D.J. Gillham and A.D. Dodge
School of Biological Sciences, University of Bath, Claverton Down, Bath BA2 7AY, UK

Abstract. The subcellular distribution of ascorbate peroxidase and glutathione reductase (EC 1.6.4.2) in pea leaves was compared with that of organelle markers. Enzyme distribution was found to be similar to that of the chloroplast enzyme NADPH-glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13). Isolated chloroplasts showed a close correlation between intactness and the percentage of enzyme activity recovered. Chloroplasts of 85% intactness were found to contain a high proportion of leaf dehydroascorbate reductase activity (EC 1.8.5.1), 10% of leaf glutathione and 30% of leaf ascorbate. These results are discussed in relation to the potential role of chloroplast antioxidant systems in plant resistance to environmental and other stress conditions.

Key words: Ascorbate – Chloroplast – Glutathione – Hydrogen peroxide – *Pisum* (superoxide) – Superoxide.

Introduction

Superoxide formation within the chloroplast may occur as an accidental result of electron leakage to oxygen (Allen 1977). Chloroplasts contain, however, high activities of the enzyme superoxide dismutase (SOD) and this catalyses the dismutation of superoxide (O_2^-) to hydrogen peroxide (H_2O_2). Although H_2O_2 is strongly inhibitory towards some of the Calvin-cycle enzymes (Kaiser 1976), chloroplasts do possess high activities of an ascorbate-specific peroxidase that can scavenge H_2O_2 efficiently (Nakano and Asada 1981; Jablonski and Anderson 1982). Ascorbate peroxidase catalyses the peroxidation of ascorbate to dehydroascorbate, and dehydroascorbate reductase utilises reduced glutathione to maintain the ascorbate pool in a reduced form (Jablonski and Anderson 1981). Glutathione is in turn reduced by NADPH via the enzyme glutathione reductase (Foyer and Halliwell 1976).

High activities of SOD, ascorbate peroxidase, dehydroascorbate reductase and glutathione reductase, and high levels of ascorbate and glutathione have been detected in chloroplasts. Furthermore, these enzymes have been shown to be released from the chloroplast stroma on rupturing the envelope. They are therefore presumably stromal in origin and not bound to the chloroplast envelope (Jackson et al. 1978; Jablonski and Anderson 1978, 1981, 1982; Nakano and Asada 1981). The proportion of these enzymes and of ascorbate and glutathione in chloroplasts however remains unclear. Changes in activity of some of these enzymes have been recorded in plants in reponse to water stress (Gamble and Burke 1984), chilling stress (de Kok and Oosterhuis 1983; Guy and Carter 1984), treatment with low levels of paraquat (Gillham and Dodge 1984), SO_2 fumigation (Tanaka and Sugahara 1980) or growth in hyperbaric oxygen levels (Foster and Hess 1981, 1982). Superoxide dismutase activity has also been correlated with the onset of leaf senescence (Dhindsa et al. 1981) resistance to paraquat (Harper and Harvey 1978), drought tolerance (Dhindsa and Matowe 1981), and resistance to ozone (Lee and Bennett 1982) and SO_2 (Tanaka and Sugahara 1980). In all of these experiments, enzyme activities were determined in crude leaf extracts. In order to assess the importance of these results, and the relevance of changes in activity of these enzymes to the chloroplast antioxidant system, it is essential

Abbreviations: GSH = reduced glutathione; GSSG = oxidized glutathione; NADPH-GPD = glyceraldehyde-3-phosphate dehydrogenase; SOD = superoxide dismutase

to determine the proportion of activity present in the chloroplast. In this study, we have examined the subcellular distribution and activity of ascorbate peroxidase, glutathione reductase, dehydroascorbate reductase, ascorbate and glutathione.

Material and methods

Plant Material. Pea (*Pisum sativum* L. cv. Meteor) seedlings were grown for 14–21 days in Levington universal compost in a glasshouse with a 14-h photoperiod (natural daylength extended by Thorn (London, UK) 400-W mercury-vapour lamps) and a mean air temperature of 22° C.

Subcellular fractionation. For isolated chloroplasts, 25 g of leaf material was homogenised (three 2-s bursts) with domestic blendor in 100 ml ice slush of grinding buffer according to the method described by Walker (1980). The extract was squeezed through two layers of muslin and filtered through eight layers of muslin, plus one layer of cotton wool. Chloroplasts were recovered by centrifugation at 4500 rpm for 20 s in an MSE (Crawley, Sussex, UK) chillspin centrifuge (maximum radius 16.8 cm). The pellet was superficially washed with 50 ml of washing buffer and was resuspended in 1–2 ml incubating buffer. The respective composition of buffers were: grinding buffer: 0.33 M sorbitol, 50 mM Na_2HPO_4, 50 mM KH_2PO_4, 5 mM $MgCl_2$, 0.1% (w/v) NaCl, and 0.1% bovine serum albumin (BSA) type V adjusted to pH 6.5 with KOH; washing buffer: 0.33 M sorbitol, 5 mM $MgCl_2$, 0.1% (w/v) BSA and 4 ml incubating buffer per 100 ml of washing buffer; incubating buffer: 0.33 M sorbitol, 50 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes), 2 mM disodium ethylenediaminetetraacetic acid (Na_2 EDTA), 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 0.1% (w/v) BSA adjusted to pH 7.6 with KOH. This technique routinely yielded chloroplasts that were approx. 60% intact. To vary the intactness of the chloroplast preparation the amount of superficial washing of the crude chloroplast pellet was altered. Increased or decreased washing changed the proportion of broken chloroplasts retained in the pellet. Alternatively, for chloroplasts of 80% intactness, the chloroplast suspension was layered over 10 ml of 40% Percoll containing 0.33 M sorbitol and 50 mM Hepes adjusted to pH 7.6. After centrifugation at 4500 rpm for 1–2 min the pellet was washed to remove Percoll and resuspended in 1–2 ml of incubating buffer (Edwards and Walker 1983). Chloroplast intactness was determined by the ferricyanide method (Lilley et al. 1975).

For enzyme-distribution studies, chloroplasts were prepared as above. The pellet from the centrifugation was left unwashed and contained intact and broken chloroplasts, nuclei and cell debris, and was taken as the crude chloroplast fraction. The supernatant from this stage was further centrifuged at 6000 g for 15 min at 0–4° C. The pellet, containing mitochondria, peroxisomes and broken chloroplasts was resuspended in 1–2 ml of incubating medium, and was taken as the crude mitochondrial fraction. The supernatant from this step is referred to as the "leaf soluble fraction". Enzyme activity recovered in each fraction was compared with the activity in crude leaf homogenates prepared by grinding 0.5–1.0 g leaf material in 10 ml of chloroplast grinding medium containing no osmoticum. This was squeezed through two layers of muslin to remove cellular debris.

Enzyme assays. All enzyme assays were carried out at 25° C. Glyceraldehyde-3-phosphate dehydrogenase (NADPH-GPD; EC 1.2.1.13) was assayed by the loss in absorbance at 340 nm as NADPH was oxidised. A 3.0-ml reaction volume contained 67 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)-HCl pH 7.2, 3.3 mM ATP, 10 mM $MgCl_2$, 4 mM EDTA, 130 μM NADPH, 3.3 $\mu g \cdot ml^{-1}$ phosphoglycerate kinase, 1 mM dithiothreitol (DTT) and 100 μl of extract (Jackson et al. 1978). Glycollate oxidase (EC 1.1.3.1) was assayed by the formation of glycollate phenylhydrazone in a 3.0-ml reaction volume containing 33 mM triethanolamine pH 7.8, 2.7 mM EDTA, 0.0083% Triton X-100, 0.67 mM oxidized glutathione (GSSG), 0.2 mM flavin mononucleotide (FMN), 5 mM glycollic acid and 100–200 μl of extract (Feierabend and Beevers 1972). The assay for cytochrome-*c* oxidase (EC 1.9.3.1) was based on the method of Tolbert (1974), measuring the loss in absorbance at 550 nm as reduced cytochrome-*c* was oxidased. The 3.0-ml reaction volume contained enzyme, 0.1 M phosphate buffer pH 7.2, 1 mM EDTA, and 0.0033% Triton X-100. The reaction was initiated by the addition of 0.2 ml reduced cytochrome-*c* (5 $mg \cdot ml^{-1}$). Glutathione reductase (EC 1.6.4.2) was assayed in a 3.0-ml reaction volume containing 100–200 μl sample, 0.4 M phosphate buffer pH 8.0, 0.4 mM EDTA, 5 mM GSSG and 0.5 mM NADPH (Jablonski and Anderson 1978). Activity was determined by the fall in absorbance at 340 nm as NADPH was oxidised. Correction was made for oxidation of NADPH in the absence of GSSG or enzyme extract, although these were less than 5% of the GSSG, and enzyme-dependent rates. Dehydroascorbate reductase (EC 1.8.5.1) was determined according to the method described by Nakano and Asada (1981) in a 3.0-ml reaction volume containing 50 mM phosphate buffer pH 7.0, 0.1 mM EDTA, 2.5 mM reduced glutathione (GSH) and 0.2 mM dehydroascorbate. The reaction was followed by the increase in absorbance at 265 nm as dehydroascorbate was reduced to ascorbate. Correction was made for reduction of dehydroascorbate in the absence of enzyme, although this was normally less than 1% of the enzyme-dependent rate. Ascorbate peroxidase activity was determined from the fall in absorbance at 290 nm as ascorbate was oxidised, in a 3.0-ml reaction volume containing enzyme, 50 mM Hepes buffer pH 7.6, 0.1 mM EDTA, 0.5 mM ascorbate and 0.1 mM H_2O_2 (Nakano and Asada 1981). Correction was made for the oxidation of ascorbate in the absence of H_2O_2, although non-enzymic rates of oxidation of ascorbate were less than 5% of the enzymic rate. Catalase (EC 1.11.1.6) activity was determined polargraphically in a Hansatech (King's Lynn, Norfolk, UK) oxygen electrode. The 2.0-ml reaction volume contained enzyme and 50 mM phosphate buffer pH 7.0. The reaction was initiated by adding H_2O_2 to give a final concentration of 10 mM (Jablonski and Anderson 1981). Guiacol peroxidase (EC 1.11.1.7) activity was determined according to Braber (1979). The 3.0-ml reaction volume contained enzyme, 40 mM phosphate buffer pH 5.9, and 33 mM guiacol. The peroxidative reaction was initiated by adding 0.2 ml of 1% H_2O_2, and the reaction monitored by the increase in absorbance at 420 nM.

Determinations. Ascorbate was determined according to the method of Mukherjee and Choudhuri (1983) following reaction with 2,4-dinitrophenylhydrazine. Glutathione was determined from the specific reaction of glutathione with glutathione reductase as described by Law et al. (1983). Chlorophyll was extracted from samples with 80% acetone and determined according to Arnon (1949).

Results

The distribution of enzymes from the subcellular fractionation of pea leaves is shown in Tables 1 and 2. One problem of studies of this type is that chloroplast fractions are heavily contaminated

Table 1. Subcellular fractionation of pea leaves: distribution of chlorophyll and organelle marker enzymes between 2500-*g* (chloroplast) pellet, 6000-*g* (mitochondrial) pellet and 6000-*g* (leaf soluble) supernatant. The recovery of enzymes from the three fractions is also shown

	Chlorophyll	NADPH-GPD	Cytochrome oxidase	Glycollate oxidase
Homogenate	100.00	100.00	100.00	100.00
2500-*g*	67.10	20.30	21.70	5.95
6000-*g* supernatant	3.50	74.10	19.90	79.80
6000-*g* pellet	26.98	0.51	61.90	19.10
(% recovery)	(97.58)	(91.90)	(103.50)	(104.80)

Table 2. Subcellular fractionation of pea leaves: distribution of enzymes scavenging H_2O_2

	Ascorbate peroxidase	Glutathione reductase	Catalase	Peroxidase
Homogenate	100.00	100.00	100.00	100.00
2500-*g* pellet	19.60	18.90	4.50	0.33
6000-*g* supernatant	76.60	78.30	87.10	92.30
6000-*g* pellet	4.20	1.15	14.70	1.10
(% recovery)	(100.40)	(94.40)	(106.30)	(93.70)

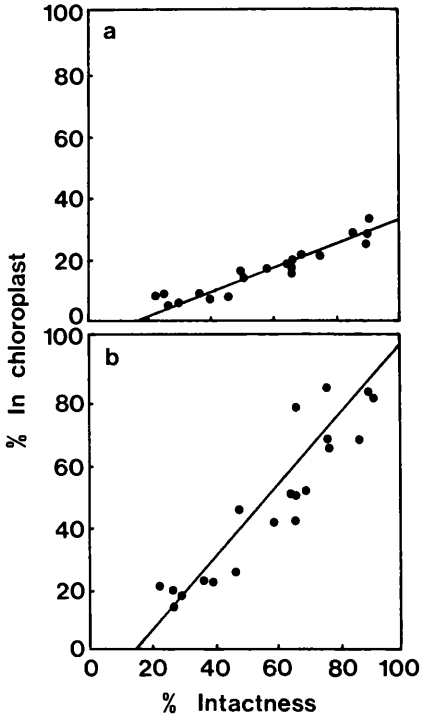


Fig. 1a, b. Determination of the proportion of **a** glutathione reductase and **b** ascorbate peroxidase activity in pea chloroplasts. Chloroplasts of varying degrees of intactness were prepared as described in Material and methods. Intactness is plotted against the proportion of total enzyme activity recovered in the preparation

with mitochondria and peroxisomes (Halliwell 1981). The crude chloroplast pellet (Table 1) was rich in mitochondria (cytochrome-*c*-oxidase marker; Tolbert 1974) and peroxisomes (glycollate-oxidase marker; Tolbert 1969). The mitochondrial 6000-*g* pellet, was also heavily contaminated with peroxisomes. This pellet also contained a high proportion of chloroplast membranes (chlorophyll marker), presumably broken, as indicated by the low activity of NADPH-GPD activity recovered in this fraction. A high proportion of the activity of this enzyme was recovered in the 6000-*g* supernatant, indicating that many chloroplasts had lost their envelopes during fractionation. Distribution of oxygen-scavenging enzymes in pea leaves is shown in Table 2. The distribution of ascorbate peroxidase and glutathione reductase was found to be similar to that of NADPH-GPD. Only a small proportion of these enzymes were recovered in the 6000-*g* mitochondrial fraction which indicates that mitochondria contain little or no activity of these enzymes. It is assumed that much of the enzyme is released from chloroplasts during fractionation. Changes in activity of peroxidase and catalase have often been followed in plants as enzymes important in scavenging H_2O_2 formed by SOD activity (e.g. Harper and Harvey 1978; Dhindsa et al. 1981; McRae and Thompson 1983; Kar and Feierabend 1984). Results from Table 2 confirm that chloroplasts contain little or no non-specific peroxidase (Parrish 1972) and indicate that much of the activity of this enzyme is in the leaf soluble fraction. Distribution of catalase was similar to that of glycollate oxidase confirming that this enzyme was mainly of peroxisomal origin (Halliwell 1981). As SOD is predominantly a chloroplast enzyme (Jackson et al. 1978), it would be unlikely that peroxidase or catalase could scavenge H_2O_2 generated by SOD activity.

To examine further the chloroplast activity of ascorbate peroxidase, glutathione reductase, NADPH-GPD and the level of ascorbate, pea chloroplasts of varying degrees of intactness were prepared and activity in the chloroplast pellet was compared with activity in a total leaf homogenate. The proportion of activity in the chloroplast was then compared with the intactness of the chloroplast preparation. These results are shown in Figs. 1 and 2. Over 95% of the NADPH-GPD was present in the chloroplast (Fig. 2b). In addition,

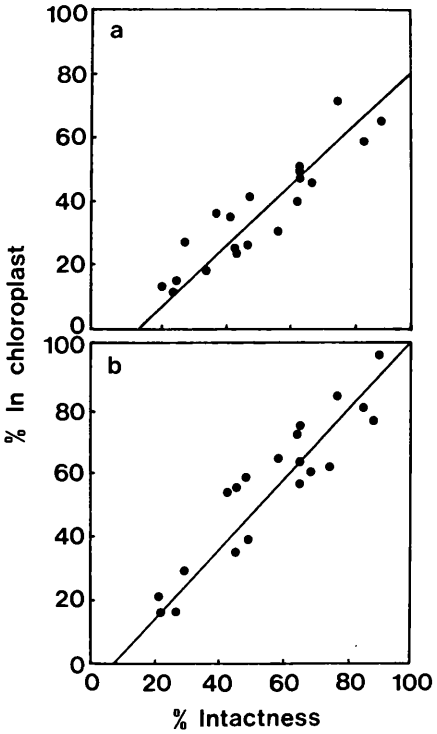


Fig. 2a, b. Determination of the proportion of **a** ascorbate and **b** NADPH-GPD activity recovered in chloroplasts of varying degrees of intactness

Table 3a, b. Activity of enzymes scavening H_2O_2 (**a**) and level of antioxidants (**b**) in pea leaf homogenates and isolated chloroplasts (85% intact). The proportion of activity present in the chloroplast fraction is also shown

a)

	Activity ($\mu mol \cdot mg^{-1}$ Chl$\cdot h^{-1}$)		% in chloroplast
	Leaf homogenate	Chloroplast	
Ascorbate peroxidase	1198.7	935.5	78.9
Glutathione reductase	45.9	34.7	76.8
Dehydroascorbate reductase	110.2	70.2	64.7

b)

	Activity ($\mu mol \cdot mg^{-1}$ Chl$\cdot h^{-1}$)		% in chloroplast
	Leaf homogenate	Chloroplast	
Glutathione	1.00±0.10	0.091±0.01	9.1
Ascorbate	1.68±0.11	0.54 ±0.04	32.3

chloroplasts were found to contain 82% of the glutathione reductase activity (Fig. 1a) and all the ascorbate peroxidase activity (Fig. 1b). Only 30% of the leaf ascorbate was detected in chloroplasts (Fig. 2a), a value which is in close agreement with data published for spinach chloroplasts (Foyer et al. 1983).

In addition to these data, chloroplasts of 85% intactness were isolated from pea leaves and the activity of these enzymes and dehydroascorbate reductase and glutathione levels were compared with the levels in crude leaf extracts. These data are presented in Table 3. While much of the leaf ascorbate peroxidase and glutathione-reductase activity was confirmed to be chloroplastic, high activities of dehydroascorbate reductase were also detected.

The ascorbate and glutathione content of pea leaves grown during March was typically found to be 1.7 and 1.0 $\mu mol \cdot mg^{-1}$ chlorophyll, respectively. Chloroplasts however, were found to contain 0.6 and 0.1 $\mu mol \cdot mg^{-1}$ chlorophyll. Assuming a chloroplast volume of 26 $\mu l \cdot mg^{-1}$ chlorophyll (Heldt et al. 1973), this would give a chloroplast ascorbate concentration of 20–25 mM and a glutathione concentration of 3.5–4 mM. This is in good agreement with the results from spinach chloroplasts (Foyer and Halliwell 1976; Law et al. 1983).

Discussion

The results presented in this paper demonstrate that chloroplasts contain a high proportion of the total leaf ascorbate peroxidase, dehydroascorbate reductase and glutathione reductase activities. However, only 10% of leaf glutathione and 30% of ascorbate was present in chloroplasts.

Previous investigations have confirmed the presence of these enzymes in chloroplasts of pea and spinach leaves at activities similar to those reported in this paper. (Foyer and Halliwell 1976; Jablonski and Anderson 1978, 1981, 1982; Nakano and Asada 1981).

Comparison of glutathione reductase distribution with that of NADPH-GPD (Foyer and Halliwell 1976) produced results similar to those presented in this paper. Jackson et al. (1978) concluded that much of the total leaf SOD was present in the chloroplast, while Foster and Edwards (1980) found 50–80% of leaf SOD to be chloroplastic. Anderson et al. (1983) found spinach chloroplasts to contain 67% of glutathione-reductase activity but only 28% of dehydroascorbate-reductase activity. Ascorbate peroxidase has previously been reported as a chloroplast stromal enzyme (Nakano and Asada 1981) bound to chloroplast

membranes (Groden and Beck 1979) and present in the leaf soluble fraction (Kelly and Latzko 1979; Gerbling et al. 1984). Reports of a cytoplasmic form of this enzyme may reflect release of ascorbate peroxidase from chloroplasts during preparation of crude enzyme extracts. Both SOD and glutathione reductase activity have also been detected in a variety of non-photosynthetic tissues and organelles including wheat germ (Conn and Vennesland 1951; Beauchamp and Fridovich 1973), etiolated leaves (Giannopolitis and Ries 1977; Gamble and Burke 1983) and mitochondria (Young and Conn 1956; Foster and Edwards 1980). Investigations showing differences in the distribution of these enzymes and their proportion in chloroplasts may reflect changes in their activity and location dependent on plant development or growing conditions.

The results presented in this paper are important in view of several recent reports into the effects of environmental and other stresses on the activity of these enzymes. Water stress (Gamble and Burke 1984) low-temperature hardening (de Kok and Oosterhuis 1983; Guy and Carter 1984) and hyperbaric oxygen (Foster and Hess 1981, 1982) were shown to stimulate glutathione reductase activity in leaves. Incubation of leaf discs with low concentrations of paraquat promoted the activity of ascorbate peroxidase (Gillham and Dodge 1984) and gluatathione reductase (Gillham and Dodge unpublished result). In these experiments, enzyme activities were estimated on crude leaf homogenates. The demonstration in this present work that a high proportion of activity of these enzymes is chloroplastic indicates that changes in activity of these enzymes may be an important adaptation of the photosynthetic apparatus in response to stress.

This work was supported by a University of Bath research studentship to D.J.G.

References

Allen, J.F. (1977) Oxygen – A physiological electron acceptor in photosynthesis? Curr. Adv. Plant Sci. **29**, 459–469

Anderson, J.W., Foyer, C.H., Walker, D.A. (1983) Light-dependent reduction of dehydroascorbate and uptake of exogenous ascorbate by spinach chloroplasts. Planta **158**, 442–450

Arnon, D.I. (1949) Copper enzymes in isolated chloroplasts. Polyphenol oxidase in *Beta vulgaris* L. Plant Physiol. **24**, 1–15

Beauchamp, C.O., Fridovich, I. (1973) Isozymes of superoxide dismutase from wheat germ. Biochim. Biophys. Acta. **317**, 50–64

Braber, J.M. (1980) Catalase and peroxidase in primary bean leaves during development and senescence. Z. Pflanzenphysiol. **97**, 135–144

Conn, E.E., Vennesland, B. (1951) Glutathione reductase in wheat germ. J. Biol. Chem. **192**, 17–28

de Kok, L.J., Oosterhuis, F.A. (1983) Effect of frost hardening and salinity on glutathione and sulphydryl levels and on glutathione reductase activity in spinach leaves. Physiol. Plant. **58**, 47–51

Dhindsa, R.S., Matowe, W. (1981) Drought tolerance in two mosses: correlated with enzymic defence against lipid peroxidation. J. Exp. Bot. **32**, 79–91

Dhindsa, R.S., Plumb-Dhindsa, P., Thorpe, T.A. (1981) Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. J. Exp. Bot. **32**, 93–101

Edwards, G., Walker, D.A. (1983) C_3, C_4: Mechanisms and cellular and environmental regulation of photosynthesis, pp. 496–511, Blackwell scientific publications, Oxford

Feierabend, J., Beevers, H. (1972) Developmental studies on microbodies in wheat leaves. I. Conditions influencing enzyme development. Plant Physiol. **49**, 28–32

Foster, J.G., Edwards, G.E. (1980) Localisation of superoxide dismutase in leaves of C_3 and C_4 plants. Plant Cell Physiol. **21**, 895–906

Foster, J.G., Hess, J.L. (1980) Responses of superoxide dismutase and glutathione reductase activities in cotton leaf tissue exposed to an atmosphere of enriched oxygen. Plant Physiol. **66**, 482–487

Foster, J.G., Hess, J.L. (1982) Oxygen effects on maize leaf superoxide dismutase and glutathione reductase. Phytochemistry **21**, 1527–1532

Foyer, C.H., Halliwell, B. (1976) The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. Planta **133**, 21–25

Foyer, C.H., Rowell, J., Walker, D.A. (1983) Measurement of the ascorbate content of spinach leaf protoplasts and chloroplasts during illumination. Planta **157**, 239–244

Gamble, P.E., Burke, J.J. (1983) Glutathione reductase distribution in etiolated and green wheat leaves. (Abstr.) Plant Physiol. **72**, Suppl., 42

Gamble, P.E., Burke, J.J. (1984) Effect of water stress on the chloroplast antioxidant system. 1. Alterations in glutathione reductase activity. Plant Physiol. **76**, 615–621

Gerbling, K.P., Kelly, G.J., Fischer, K.H., Latzko, E. (1984) Partial purification and properties of soluble ascorbate peroxidase from pea leaves. J. Plant Physiol. **115**, 59–67

Giannopolitis, C.N., Ries, S.K. (1977) Superoxide dismutases. 1. Occurrence in higher plants. Plant Physiol. **59**, 315–318

Gillham, D.J., Dodge, A.D. (1984) Some effects of paraquat on plants. (Abstr.) Plant Physiol. **75**, Suppl., 51

Groden, D., Beck, E. (1979) H_2O_2 destruction by ascorbate-dependent systems from chloroplasts. Biochim. Biophys. Acta. **546**, 426–435

Guy, C.L., Carter, J.V. (1984) Characterisation of partially purified glutathione reductase from cold hardened and non-hardened spinach leaf tissue. Cryobiology **21**, 454–464

Halliwell, B. (1981) Chloroplast metabolism: the structure and function of chloroplasts in green leaf cells, pp. 179–205, Clarendon Press, Oxford

Harper, D.B., Harvey, B.M.R. (1978) Mechanism of paraquat tolerance in perennial ryegrass. II. Role of superoxide dismutase, catalase and peroxidase. Plant Cell Environ. **1**, 211–215

Heldt, H.W., Werdan, K., Milovaner, M., Geller, G. (1973) Alkalisation of the chloroplast stroma caused by light dependent proton flux into the thylakoid space. Biochim. Biophys. Acta. **314**, 224–241

Jablonski, P.P., Anderson, J.W. (1978) Light dependent reduc-

tion of oxidised glutathione by ruptured chloroplasts. Plant Physiol. **61**, 221–225

Jablonski, P.P., Anderson, J.W. (1981) Light dependent reduction of dehydroascorbate by ruptured pea chloroplasts. Plant Physiol. **67**, 1239–1244

Jablonski, P.P., Anderson, J.W. (1982) Light dependent reduction of hydrogen peroxide by ruptured pea chloroplasts. Plant Physiol. **69**, 1407–1413

Jackson, C., Dench, J., Moore, A.L., Halliwell, B., Foyer, C., Hall, D.O. (1978) Subcellular localisation and identification of superoxide dismutase in the leaves of higher plants. Eur. J. Biochem. **91**, 339–344

Kaiser, W. (1976) The effect of hydrogen peroxide on CO_2 fixation of isolated intact chloroplasts. Biochim. Biophys. Acta **440**, 476–482

Kar, M., Feierabend, J. (1984) Metabolism of activted oxygen in detached wheat and rye leaves and its relevance to the initiation of senescence. Planta **160**, 385–391

Kelly, G.J., Latzko, E. (1979) Soluble ascorbate peroxidase. Detection in plants and use in vitamin C estimation. Naturwissenschaften **66**, 617–618

Law, M.Y., Charles, S.A., Halliwell, B. (1983) Glutathione and ascorbic acid in spinach (*Spinacia oleracea*) chloroplasts. The effect of hydrogen peroxide and paraquat. Biochem. J. **210**, 899–903

Lee, E.H., Bennett, J.H. (1982) Superoxide dismutase. A possible protective enzyme against ozone injury in snap beans (*Phaseolus vulgaris* L). Plant Physiol. **69**, 1444–1449

Lilley, R.M.C., Fitzgerald, M.P., Rienits, K.G., Walker, D.A. (1975) Criteria of intactness and the photosynthetic activity of spinach chloroplast preparations. New Phytol. **75**, 1–10

McRae, D.G., Thompson, J.E. (1983) Senescence-dependent changes in superoxide anion production by illuminated chloroplasts from bean leaves. Planta **158**, 185–193

Mukherjee, S.P., Choudhuri, M.A. (1983) Implications of water stress induced changes in the levels of endogenous ascorbic acid and hydrogen peroxide in *Vigna* seedlings. Plant Physiol. **58**, 166–170

Nakano, Y., Asada, K. (1981) Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. Plant Cell Physiol. **22**, 867–880

Parrish, R.W. (1972) The intracellular location of phenol oxidases, peroxidase and phosphatases in the leaves of spinach beet (*Beta vulgaris* L. subspecies *vulgaris*). Eur. J. Biochem. **31**, 446–455

Tanaka, K., Sugahara, K. (1980) Role of superoxide dismutase in defence against SO_2 toxicity and an increase in superoxide dismutase activity with SO_2 fumigation. Plant Cell Physiol. **21**, 601–611

Tolbert, N.E. (1969) Isolation of leaf peroxisomes. Methods Enzymol. **23**, 665–682

Tolbert, N.E. (1974) Isolation of subcellular organelles of metabolism on isopycnic sucrose gradients. Methods Enzymol. **31 A**, 734–746

Walker, D.A. (1980) Preparation of higher plant chloroplasts. Methods Enzymol. **69**, 94–104

Young, L.C.T., Conn, E.E. (1956) The reduction and oxidation of glutathione by plant mitochondria. Plant Physiol. **31**, 205–211

Received 11 July; accepted 8 October 1985

PHYSIOL. PLANT. 65: 393–396. Copenhagen 1985

Chloroplast protection in greening leaves

D. J. Gillham and A. D. Dodge

Gillham, D. J. and Dodge, A. D. 1985. Chloroplast protection in greening leaves. – Physiol. Plant. 65: 393–396.

Changes in photosynthetic activity, leaf pigments and the activities of enzymes that scavenge damaging oxygen species in chloroplasts were followed during the greening of 8-day-old etiolated pea (*Pisum sativum* L. cv. Meteor) seedlings.
Accumulation of chlorophyll and carotenoids was accompanied by development of photosynthetic activity. Carotenoids present in etiolated leaves, and the high ratio of carotenoid to chlorophyll detected during the early hours of greening are suggested to provide important protection against singlet oxygen. Superoxide dismutase, ascorbate peroxidase and glutathione reductase, which scavenge superoxide and hydrogen peroxide in chloroplasts, are present at high activities in etiolated leaves and throughout greening. The mechanisms by which developing chloroplasts may generate damaging oxygen species, and the role of these scavengers during greening is discussed.

Additional keywords – Ascorbate peroxidase, carotenoids, chloroplast development, glutathione reductase, *Pisum sativum*, superoxide dismutase.

D. J. Gillham and A. D. Dodge (reprint requests), School of Biological Science, Univ. of Bath, Bath, Avon, BA2 7AY, United Kingdom.

Introduction

Chloroplasts of higher plants possess the potential for generating several forms of damaging oxygen species. Interaction between triplet chlorophyll (^{3}Chl) and oxygen ($^3O_2$) results in the generation of singlet oxygen ($^1O_2$) (Foote 1968). Oxygen can accept electrons from the terminal electron carriers of photosystem I, being reduced to superoxide (O_2^-). Dismutation of O_2^- by superoxide dismutase (SOD) forms hydrogen peroxide (H_2O_2), while reaction between O_2^- and H_2O_2 generates highly reactive hydroxyl radicals ($OH^\cdot$) (Halliwell 1981). Generation of oxygen radicals in chloroplasts can lead to extensive damage to the photosynthetic apparatus. Low concentrations of H_2O_2 inhibit photosynthesis by inactivating the fructose and sedoheptulose bisphosphatase enzymes of the Calvin cycle (Charles and Halliwell 1981). Both $^1O_2$ and $OH^\cdot$ can initiate peroxidation of membrane unsaturated fatty acids, as well as oxidise certain protein amino acids and other cellular components (Halliwell 1981). Damage is normally restricted by a range of protective mechanisms which reduce the toxicity of the damaging species. Generation of $^1O_2$ is restricted by carotenoids which quench both ^{3}Chl and $^1O_2$ (Foote and Denny 1968). SOD catalyses the conversion of O_2^- to H_2O_2, while ascorbate peroxidase and glutathione reductase are efficient scavengers of H_2O_2 in chloroplasts. In addition, ascorbate, present at high concentrations in the chloroplast stroma, can quench both $^1O_2$ and O_2^- (Halliwell et al. 1981).

The transfer of etiolated plants to light induces many changes in the photosynthetic apparatus. Illumination activates the conversion of protochlorophyll to chlorophyll, followed by de novo synthesis of the pigment. Electron transport and CO_2 fixation commence several hours after the transfer to light (see Bradbeer et al. 1974, Bradbeer 1981).

The development of the photosynthetic apparatus increases the potential for the generation of damaging oxygen species. Greening must therefore be carefully controlled, both to restrict formation of these radicals, and to ensure that a full range of protective mechanisms are present early in development.

In this investigation we have monitored the devel-

Received 26 March, 1985; revised 26 July, 1985

opment of the photosynthetic apparatus and levels of oxygen radical scavengers following transfer of etiolated seedlings to light.

Abbreviations – GSSG, oxidized glutathione; SOD, superoxide dismutase.

Materials and methods

Plant material

Peas (*Pisum sativum* L. cv. Meteor) Were grown in moist Levington compost at 23°C for eight days in total darkness. All manipulations, such as watering were carried out under minimal green light. For greening, plants were transferred to a growth cabinet at 23°C under continuous light (400 μmol m^{-2} s^{-1} photon flux density, provided by warm-white fluorescent tubes 65/85W; Thorn, London, UK).

Photosynthesis

An infra-red gas analyser was used to measure photosynthetic CO_2 exchange in pea leaves. Plant material was incubated in a glass chamber maintained at 25°C. Illumination was provided by a photoflood lamp (Thorn, 400 W) giving 250 μmol m^{-2} s^{-1} photon flux density at the sample chamber.

Chloroplast membranes for electron transport studies were prepared by grinding leaves in a cold mortar in 10–20 ml of 50 m*M* Tricine-NaOH buffer, pH 7.6, containing NaCl (0.3 *M*) and $MgCl_2$ (5 m*M*). The homogenate was filtered through 4 layers of muslin and centrifuged at 4°C at 200 g for 1 min. The supernatant was recentrifuged at 2 500 *g* for 10 min. Chloroplast pellets were resuspended in 2.5 ml cold Tricine buffer, pH 7.6, containing NaCl (0.03 *M*) and $MgCl_2$ (5 m*M*).

Non-cyclic electron transport was estimated in a Hansatech oxygen electrode at 20°C under 500 μmol m^{-2} s^{-1} photon flux density. The electrode chamber contained 3.0 ml of 20 m*M* potassium phosphate buffer, pH 8.0, containing NH_4Cl (1.0 m*M*), NaN_3 (1.0 m*M*), methyl viologen (80 μ*M*) and chloroplast membranes containing 50–100 μg chlorophyll.

Preparation of leaf extracts

For enzyme determinations, cell free homogenates were prepared. Approximately 0.5 g leaf material was ground in 10 ml cold 50 m*M* potassium phosphate buffer, pH 7.6, in a cold mortar. The homogenate was strained through 4 layers of muslin and centrifuged at 4 000 *g* for 10 min. The resulting supernatant was used to estimate enzyme activities.

Enzyme Assays

Enzyme activities were determined at 20°C. The assay for SOD (EC 1.15.1.1.) was as described by Elstner and Heupel (1976). The 2.0 ml reaction mixture contained 65 μ*M* potassium phosphate buffer, pH 7.8, 1 μ*M* hydroxylamine, 1.5 μ*M* xanthine and an aliquot of enzyme extract. The reaction was initiated by adding xanthine oxidase (Sigma) containing 100 μg protein. After incubation at 20°C for 25 min, a 1 ml aliquot was analysed for nitrite as described by Elstner and Heupel (1976).

Ascorbate peroxidase was assayed according to the method of Groden and Beck (1979) in a Hansatech oxygen electrode. The 2.0 ml reaction mixture contained 0.1 *M* Tris-HCl buffer, pH 8.0, sodium isoascorbate (0.1 m*M*) and enzyme extract containing 200–250 mg protein. The peroxidative reaction was initiated by addition of 0.05 ml of 8 m*M* H_2O_2. H_2O_2 remaining after 30 s was determined polargraphically following the addition of 2 500 units of catalase (EC 1.11.1.6.). H_2O_2 consumed by ascorbate peroxidase was calculated from the difference between added and unreacted H_2O_2 after accounting for controls lacking ascorbate or enzyme extract.

Glutathione reductase (EC 1.6.4.2.) was assayed by the method of Jablonski and Anderson (1978). The 3.0 ml reaction mixture contained potassium phosphate buffer, pH 8.0 (0.13 *M*), Na_2 EDTA (0.13 m*M*) GSSG (0.33 m*M*) and enzyme extract containing 150–250 μg protein. The reaction was initiated by the addition of 33 μ*M* NADPH, and the reaction followed by monitoring the decline in absorbance at 340 nm as NADPH was oxidised. Enzyme activity was expressed as the difference in rate of NADPH oxidation (as mol h^{-1}) with and without GSSG.

Pigments and ascorbate

Chlorophyll was determined spectrophotometrically on a sample of leaf material extracted with 80% acetone, using the extinction coefficients of Arnon (1949). Total carotenoid in the acetone extract was estimated from the absorbance at 480 nm after correction for chlorophyll interference using the coefficients of Kirk and Allen (1965) as described by Davis (1976).

For determination of ascorbate, a sample of leaf homogenate containing approximately 100 mg ascorbate was extracted with 6% TCA. Ascorbate was oxidised to dehydroascorbate with acid-washed activated charcoal (Oser 1979). Total ascorbate was then determined spectrophotometrically following its reaction with 2% 2,4–dinitrophenylhydrazine (Mukherjee and Choudhuri 1983).

Results

The accumulation of chlorophyll and carotenoid pigments that occurred when 8-day-old dark grown pea seedlings were transferred to light is shown in Fig. 1a. In agreement with other investigators (Goodwin 1958, Lichtenthaler 1969), etiolated leaves were shown to contain appreciable levels of carotenoid pigments, although no chlorophyll was present. The relationship be-

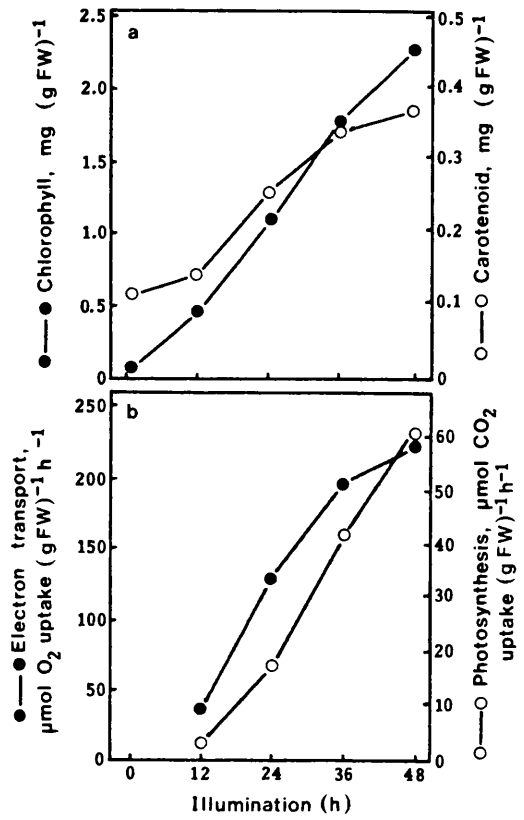


Fig. 1. The effect of illumination on pigment levels and photosynthetic activity of etiolated pea leaves. (a). Chlorophyll and carotenoid levels. (b) Photosynthesis and chloroplast electron transport. Data points represent the mean of three replicate samples.

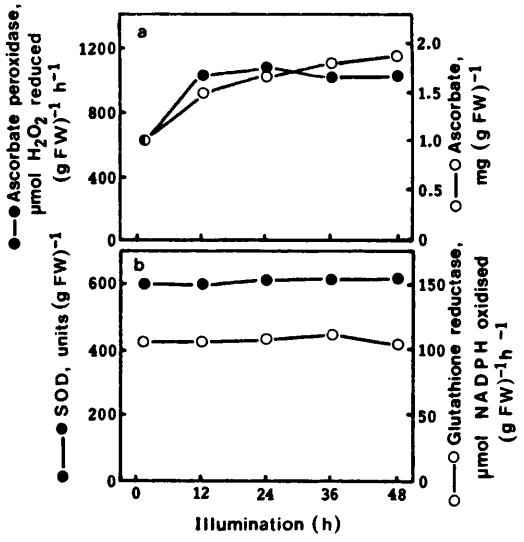


Fig. 2. Levels of oxygen radical scavengers during greening of etiolated pea leaves. (a) Ascorbate and ascorbate peroxidase. (b) SOD and glutathione reductase. Data points represent the mean of three replicate samples.

Tab. 1. Changes in the ratio of chlorophyll to carotenoid during greening in peas.

Hours greening	Chl: Car ratio
0.1	0.33
12	3.36
24	4.56
36	5.07
48	6.28

tween the level of chlorophyll and carotenoid during greening is shown in Tab. 1. Over the first 12 h of illumination there was a ten-fold increase in the ratio of chlorophyll to carotenoid. Subsequently the ratio of chlorophyll to carotenoid progressively increased to approach that of normal green pea leaves of 5.8:1. This has also been described elsewhere (Lichtenthaler 1969).

Development of photosynthetic electron transport activity and CO_2 fixation are shown in Fig. 1b. Both processes were active after 12 h illumination and increased in parallel to chlorophyll accumulation between 12 and 48 h of illumination.

Etiolated leaves also contained high activities of SOD, glutathione reductase and ascorbate peroxidase (Fig. 2). While the activity of ascorbate peroxidase was high in etiolated leaves, enzyme activity was shown to increase from 629 to 917 mol H_2O_2 consumed (g fresh weight)$^{-1}$ during the first 12 h of illumination. The level of ascorbate increased from 1.0 to 1.6 mg (g fresh weight)$^{-1}$ during this period (Fig. 2a). The activity of SOD and glutathione reductase changed little during the greening period (Fig. 2b).

Discussion

Although the greening of etiolated seedlings is a somewhat artificial experimental system, it does provide a convenient and commonly used method for studying chloroplast development. Some seedlings probably encounter a certain degree of etiolation during their early growth through the soil, prior to emergence at the surface. Several aspects of the subsequent development of chloroplasts are probably significant in preventing photo-oxidative damage during greening. Several hours may elapse between the appearance of chlorophyll and the operation of a fully functional electron transport chain (Bradbeer 1981). In this period, carotenoid pigments would have an important role in protecting chloroplasts against photo-oxidative damage. The importance of carotenoids in protecting chlorophyll from photosensitized reactions during greening has been clearly demonstrated elsewhere. Maize mutants which lack carotenoids were shown to be particularly sensitive to chlorophyll bleaching during greening (Anderson and Robertson 1960), as were plants treated with herbicides that inhibit carotenoid synthesis (Ridley and Ri-

dley 1977). Chlorophyll caused considerable damage to the developing chloroplasts of greening wheat seedlings when carotenoid accumulation was prevented (Ryberg et al. 1981). The carotenoids present in etiolated leaves, and the high ratio of carotenoid to chlorophyll found in the early hours of greening in this study, would have an important role in preventing photooxidation and in stabilizing chlorophyll before photosynthetic electron transport is fully functional.

Photosynthetic electron transport activity, and therefore the potential to generate superoxide was detected after 12 h of greening. In mature chloroplasts electron flow to oxygen may be an important reaction in allowing the generation of ATP without $NADP^+$ reduction (Halliwell 1981). Electron flow to oxygen may also be important during the induction of photosynthesis. Electron flow will commence rapidly, but there is a delay before CO_2 fixation is fully functional and here oxygen may act as an electron acceptor (Halliwell 1981). A similar mechanism may operate during the early stages of greening when the rate of electron flow per unit of chlorophyll is high, but the dark reactions of photosynthesis are not fully functional (Popovic et al. 1984). Such mechanisms of energy dissipation require that chloroplast oxygen radical scavengers are present very early in chloroplast development. In the results presented in this paper we have shown that both SOD and the enzymes scavenging H_2O_2 are present at high activities in etiolated leaves and throughout greening and should provide effective protection.

Acknowledgement – This work was supported by a University of Bath research studentship to D. J. G.

References

Anderson, I. C. & Robertson, D. S. 1960. Role of carotenoids in protecting chlorophyll from photodestruction – Plant Physiol. 35: 531–534.

Arnon, D. I. 1949. Copper enzymes in isolated chloroplasts. Polyphenol oxidases in *Beta vulgaris*. – Plant Physiol. 24: 1–15.

Bradbeer, J. W. 1981. Development of photosynthetic function during chloroplast biogenesis – *In* The Biochemistry of Plants (P. K. Stumpf and E. E. Conn, eds), Vol. 8, pp. 432–472. Academic Press. ISBN 0-12-675408-X.

– , Arron, G. P., Herrera, A., Kemble, R. J., Montes, G., Sherratt, D. & Wara-Asswapati, O. 1977. The greening of leaves. – Symp. Soc. Exp. Biol. 31: 195–219.

Charles, S. A. & Halliwell, B. 1981. The effect of hydrogen peroxide on spinach (*Spinacia oleracea*) chloroplast fructose bisphosphatase. – Biochem J. 189: 373–376.

Davies, B. H. 1976. Carotenoids. – *In* Chemistry and Biochemistry of Plant Pigments (T. W. Goodwin, ed.), Vol. 2, pp. 38–165. Academic Press ISBN 0-12.289902-4.

Elstner, E. F. & Heupel, A. 1976. Inhibition of nitrite formation from hydroxylamine ammonium chloride: A simple assay for SOD. – Anal. Biochem. 70: 616–620.

Foote, C. S. 1968. Mechanisms of photosensitized oxidations. – Science 162: 963–970.

– & Denny, R. W. 1968. Chemistry of singlet oxygen XII. Quenching by β carotene. – J. Am. Chem. Soc. 90: 6233–6235.

Goodwin, T. N. 1958. Studies in carotenogenesis 25. – Biochem. J. 70: 612–617.

Groden, D. & Beck, E. 1979. H_2O_2 destruction by ascorbate-dependent systems from chloroplasts. – Biochim. Biophys. Acta 546: 426–435.

Halliwell, B. 1981. Chloroplast metabolism. – Clarendon Press, Oxford. pp. 179–205. ISBN 0-19-854549-5.

– , Foyer, C. H. & Charles, G. A. 1981. The fate of hydrogen peroxide in illuminated chloroplasts. – *In* Photosynthesis II. Electron transport and photophosphorylation (G. Akoyunoglou, ed.), pp. 279–283. Balaban Int. Sci. Series, Philadelphia, PA, U.S.A. ISBN 0-86689-012-2.

Jablonski, P. P. & Anderson, J. W. 1978. Light dependent reduction of oxidised glutathione by ruptured chloroplasts. – Plant Physiol. 61: 221–225.

Kirk, J. T. O. & Allen, R. L. 1965. Dependence of chloroplast pigment synthesis on protein synthesis: Effect of actidione. – Biochem. Biophys. Res. Comm. 21: 523–530.

Lichtenthaler, H. K. 1969. Light-stimulated synthesis of plastid quinones and pigments in etiolated barley seedlings. – Biochim. Biophys. Acta 184: 164–172.

Mukherjee, S. P. & Choudhuri, M. A. 1983. Implications of water stress induced changes in the levels of endogenous ascorbic acid and hydrogen peroxide in *Vigna* seedlings. – Physiol. Plant 58: 166–170.

Oser, B. L. 1979. Hawks Physiological Chemistry. – McGraw Hill N. Y. pp. 702–705. ISBN 0-07-099490-0.

Popovic, R., Fraser, D., Vidaver, W. & Colbow, K. 1984. Oxygen quenched chlorophyll *a* fluorescence and electron transport in barley during greening. – Physiol. Plant 62: 344–348.

Ridley, S. M. & Ridley, J. 1979. Interaction of chloroplasts with inhibitors. Location of carotenoid synthesis and inhibition during chloroplast development. – Plant Physiol. 63: 392–398.

Ryberg, H., Axelson, L., Klockare, B. & Sandelius, A. S. 1981. The function of carotenoids during chloroplast development. III. Protection of the prolamellar body and the enzymes for chlorophyll synthesis from photodestruction, sensitized by early forms of chlorophyll – *In* Photosynthesis V. Chloroplast Development (G. Akoyunoglou, ed.), pp. 295–304. Balaban Int. Sci. Series, Philadelphia. PA, USA, ISBN 0-86689-012-2.

Edited by C. Larsson

Aspects of Applied Biology 11, 1986
Biochemical and physiological techniques in herbicide research

Methods for assessing the action of light activated herbicides. (i). The assessment of lipid peroxidation

A. D. DODGE AND D. J. GILLHAM
School of Biological Sciences, University of Bath, Bath. BA2 7AY

Summary Two methods for assessing lipid peroxidation based on the formation of breakdown products ethane and malondialdehyde are described. These methods are used to compare the effects of monuron, paraquat, rose bengal and acifluorfen on ethane generation from pea leaf tissue, and on malondialdehyde formation from illuminated chloroplasts.

INTRODUCTION

Peroxidation of membrane lipids is an important feature of cellular damage (Mead, 1976). Peroxidation is generally initiated by hydrogen abstraction from an unsaturated fatty acid, forming a fatty acid radical (Fig. 1). Attack by oxygen generates a lipid peroxide, which can abstract a hydrogen atom from an adjacent unsaturated fatty acid, forming a lipid hydroperoxide and thus initiating a chain reaction. Lipid hydroperoxides decompose to give a range of products including malondialdehyde and ethane (Halliwell, 1981).

Chloroplast membranes are particularly susceptible to lipid peroxidation because they contain a high proportion of unsaturated fatty acid (Halliwell, 1981), and are surrounded by a medium containing potentially high oxygen tensions. Lipid peroxidation is also strongly promoted by several classes of herbicides that promote the generation of free radicals, superoxide and singlet oxygen (Dodge, 1983). These herbicides include photosynthetic electron flow inhibitors (e.g. monuron), bipridyls (e.g. paraquat), photosensitisers (e.g. rose bengal) and diphenyl ethers (e.g. acifluorfen). In this paper we describe two methods for assessing lipid peroxidation, of intact leaf samples or isolated chloroplast membranes.

MATERIALS AND METHODS

Plant material. Pea (Pisum sativum cv. Meteor) plants were grown in moist Levington Universal compost in a glasshouse under natural daylight conditions and a mean air temperature of 22°C for 14-21 days.

Herbicide solutions. Stock solutions of rose bengal and paraquat were prepared in distilled water. Monuron was dissolved in methanol, and diphenyl ethers were prepared in acetone. Final concentrations of solvent in any experimental system never exceeded 0.5%.

Ethane. Discs (15 mm diameter) were cut from the first fully expanded leaves below the apex using a sharp cork borer. Batches of five leaf discs were floated on 10.0 ml of herbicide solution in 50 ml screw top conical

flasks, which had been fitted with a rubber septum to facilitate analysis of the gas headspace. Flasks were incubated in a controlled environment chamber for up to 120 hr under constant illumination (400 µmol $m^{-2}s^{-1}$ photon flux density) provided by warm white fluorescent tubes (65/85-W Thorn). A 1 ml sample of the flask gas headspace was removed using a gas tight syringe (Precision Sampling, St. Louis. Mo) and analysed for ethane by gas chromatography, using an alumina column at 125°C in a Pye Unicam (Cambridge U.K.) GCD chromatograph. Ethane present in the flask headspace was identified and quantified by comparison to authentic standards (Phase Separations Ltd, Queensferry, Clwyd).

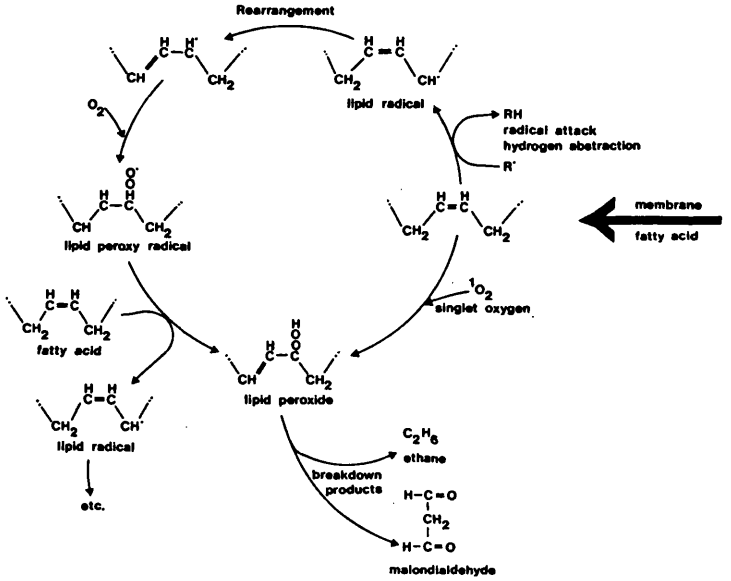


Figure 1. Mechanism of peroxidation of membrane lipids.

Malondialdehyde formation by isolated chloroplasts. Chloroplast membranes were isolated from 14-21 day old pea leaves as described elsewhere (Gillham & Dodge, 1986). Chloroplasts (50 µg chlorophyll ml^{-1}) were incubated in 50 mM phosphate buffer, pH 7.6, in a total volume of 20 ml in 50 ml screw capped conical flasks. Flasks were incubated in a water bath maintained at 20°C and illuminated from below for up to six hours. Illumination was provided by seven photoflood lamps (Thorn 100W), giving 500 µmol $m^{-2}s^{-1}$ photon flux density at the sample. Aliquots of the reaction mixture were analysed for malondialdehyde by the thiobarbituric acid method (Takahama & Nishimura, 1975). To determine malondialdehyde, 0.5 ml of 40% (w/v) TCA, 0.25 ml of 5M HCl and 0.5 ml of 2% (w/v) thiobarbituric acid were added to 2.0 ml of chloroplast fragments. After mixing, the chloroplasts were heated to 100°C for 10 min (water bath), cooled on ice and centrifuged at 2000 *g* for 5 min. Malondialdehyde was estimated from the absorbance of the resulting solution at 532 nm, after correction for non specific turbidity by subtracting the absorbance at 600 nm.

Chlorophyll. Chlorophyll was extracted using ethanol, and quantified according to Lichtenthaler & Wellburn (1983).

RESULTS

The quantification of short chain hydrocarbons emmanating from leaf tissue is a non-destructive method for estimating membrane damage. The results in Fig. 2 show the effect of monuron, paraquat, rose bengal and acifluorfen on chlorophyll bleaching (2a) and ethane generation (2b) from pea leaf discs. Ethane evolution from leaf discs was closely paralleled by chlorophyll loss, and this indicates that both processes are closely linked during photo-oxidative damage to green plants.

Ethane evolution has been used by some workers to assess lipid peroxidation in isolated membranes in response to herbicide treatments (Kunert & Böger, 1981; Percival & Dodge, 1984). Peroxidation of isolated membranes can also be followed by measuring malondialdehyde generation using the thiobarbituric acid method (Heath & Packer, 1968; Takahama & Nishimura, 1975). The results in Fig. 3a show that malondialdehyde was formed when isolated chloroplast membranes were illuminated. Addition of rose bengal (10 μM) to the reaction mixture induced a rapid increase in malondialdehyde formation. The herbicides monuron, paraquat and acifluorfen also promoted malondialdehyde formation, although no significant increase in generation was detected before 180 min incubation. Peroxidation of chloroplast membranes was also paralleled by chlorophyll bleaching (Fig. 3b).

Due to its simplicity and sensitivity, the thiobarbituric acid method can be used to compare damaging effects induced by different members of one group of herbicides. Results in Fig. 4 show peroxidation of illuminated chloroplasts is promoted to a similar extent by the diphenyl ether herbicides acifluorfen, fomesafen, nitrofen and bifenox. In addition, a range of other nitro and non-nitro diphenyl ether compounds were all active in inducing peroxidation of illuminated thylakoids (data not shown).

DISCUSSION

Assays of membrane lipid peroxidation have become widely used to assess damage to plant tissues induced by herbicides, environmental stress or senescence (Dhindsa et al., 1981; Kunert & Böger, 1981; Lambert et al., 1983; Horvath & van Hasselt, 1985). Methods employed include the determination of lipid breakdown products, for example hydrocarbons, aldehydes and lipid hydroperoxides. Other methods include measuring oxygen uptake, or the loss of lipid substrate from membrane preparations (Slater, 1984). Additionally, measurements of electrolytic conductivity changes or $^{86}Rb^{+}$ efflux from leaf tissue have been used to assess membrane disruption (Vanstone & Stobbe, 1977; Orr & Hess, 1982). However these methods are not assays of lipid peroxidation, but only of membrane permeability changes.

The two methods in this study to assess membrane lipid peroxidation have advantages in that both are simple to use, sensitive, and do not require complex preparative or analytical procedures. Ethane generation is a particularly suitable method of assessing lipid peroxidation in vivo because the technique is non-destructive, and therefore permits repeated observations to be made on the same tissue sample over a period of hours or days.

The thiobarbituric acid assay for malondialdehyde is probably the most frequently used technique for assessing membrane lipid peroxidation. However while this technique is suitable in vitro, it has become increasingly used in vivo, (Dhindsa et al., 1981; Orr & Hess, 1982; Horvath & van Hasselt, 1985) for which it is not suitable. Malondialdehyde is readily metabolised in

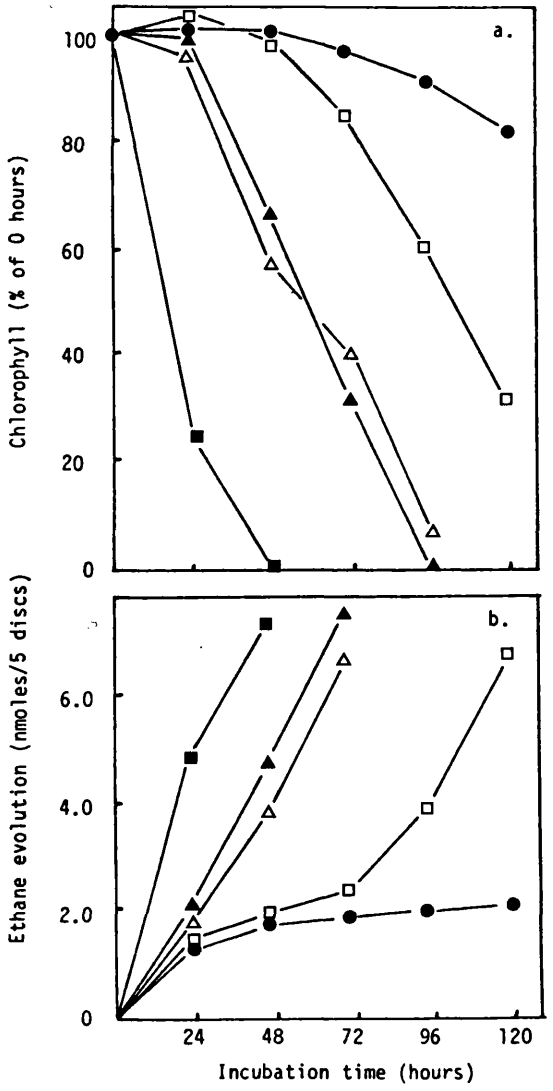


Figure 2. The effect of 0.1mM acifluorfen (△——△), paraquat (■——■), monuron (□——□) and rose bengal (▲——▲) on a) chlorophyll bleaching and b) membrane lipid peroxidation in pea leaf discs. Control (●——●).

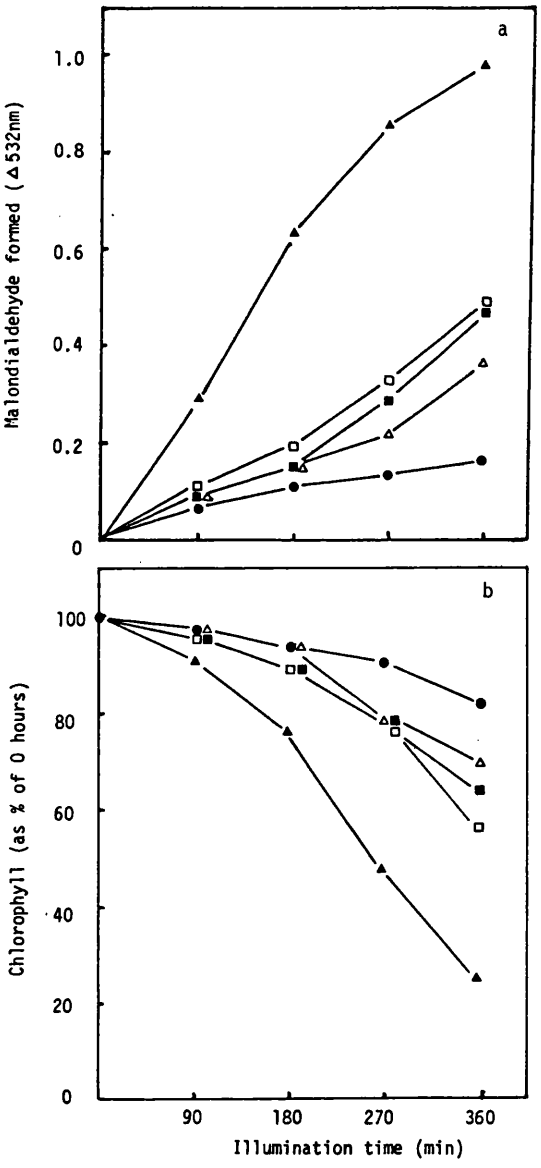


Figure 3. The effect of 10μM acifluorfen (△—△), paraquat (■—■), monuron (□—□) and rose bengal (▲—▲) on a) the peroxidation of illuminated chloroplast membranes and b) chlorophyll bleaching of chloroplast membranes. Control (●—●).

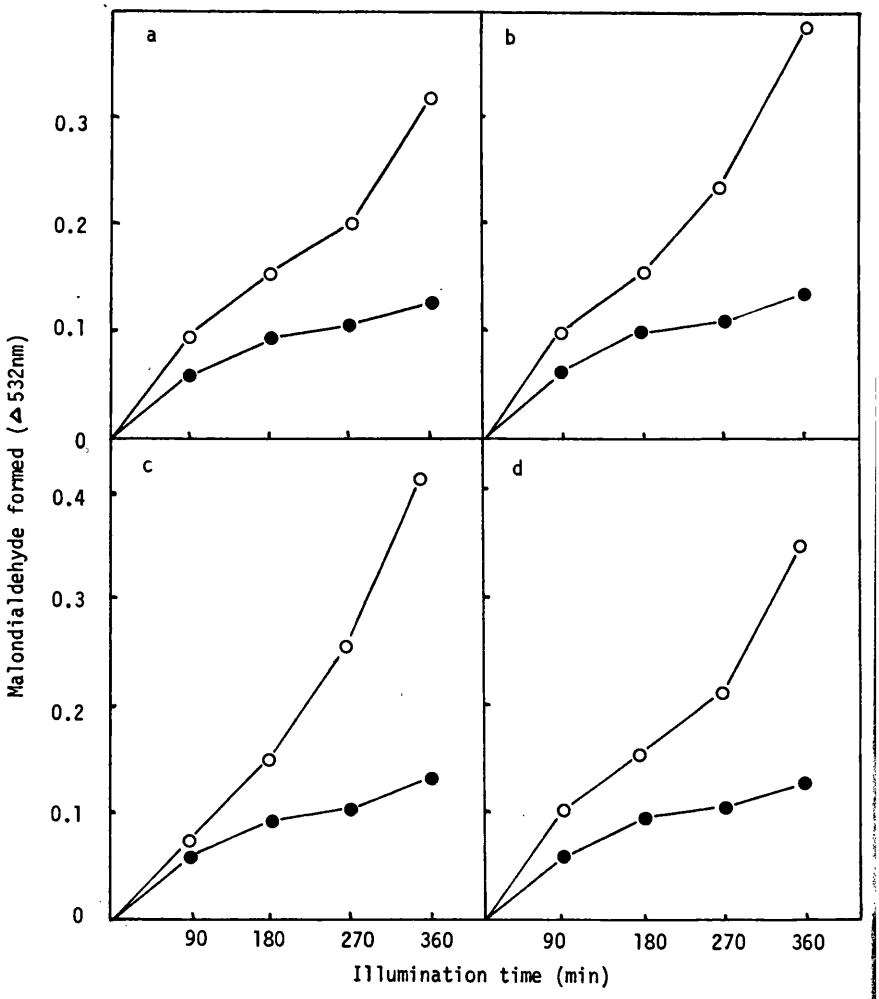


Figure 4. The effect of 25μM acifluorfen (a), fomesafen (b), bifenox (c) or nitrofen (d), on the peroxidation of illuminated chloroplast membranes. ●—● control and ○—○ herbicide.

vivo, and numerous substances interfere with the thiobarbituric acid reaction. These include haematoproteins and transition metals associated with biological membranes, which enhance colour formation. Tissue aldehydes and sugars also react with thiobarbituric acid forming a chromataphore that absorbs at 537 nm (Beuge & Aust, 1978). Despite these disadvantages, the simplicity and sensitivity of the thiobarbituric acid method makes it a useful tool for assessing lipid peroxidation in vitro, provided adequate care is taken to prevent interference by other substances.

REFERENCES

BEUGE, J. A. & AUST, S. D. (1978). Microsomal lipid peroxidation. Methods in Enzymology 52, 302-310.

DHINDSA, R. S., PLUMB-DHINDSA, P. & THORPE, T. A. (1981). Leaf senescence: Correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. Journal of Experimental Botany 32, 93-101.

DODGE, A. D. (1983). The influence of light on the action of herbicides. Aspects of Applied Biology 4, Influence of environmental factors on herbicide performance and crop and weed biology, pp. 217-226, 1983.

GILLHAM, D. J. & DODGE, A. D. (1986). Methods for investigating the action of light activated herbicides (ii) Discrimination between sites of activation. Aspects of Applied Biology 11, Biochemical and physiological techniques in herbicide research, pp. 105-110, 1986.

HALLIWELL, B. (1981). Chloroplast metabolism. Clarendon Press, Oxford.

HEATH, R. L. & PACKER, L. (1968). Photoperoxidation of isolated chloroplasts i. Kinetics and stoichiometry of fatty acid peroxidation. Archives of Biochemistry and Biophysics 125, 189-198.

HORVATH, I. & VAN HASSELT, P. R. (1985). Inhibition of chilling induced photo-oxidative damage to leaves of Cucumis sativus L. by treatment with amino alcohols. Planta 164, 83-88.

KUNERT, K. J. & BÖGER, P. (1981). The bleaching effect of the diphenyl ether oxyfluorfen. Weed Science 29, 169-173.

LAMBERT, R., SANDMANN, G. & BÖGER, P. (1983). Mode of action of nitrodiphenyl ethers affecting pigments and membrane integrity. In Pesticide chemistry and human welfare pp. 97-102. Eds J. Miyamoto & P. C. Kearney. Pergamon Press, Oxford.

LICHTENTHALER, H. K. & WELLBURN, A. (1983). Determinations of total carotenoids and chlorophylls a and b in leaf extracts in different solvents. Biochemical Society Transactions 11, 591-592.

MEAD, J. F. (1976). Free radical mechanisms of lipid damage and the consequences for cellular membranes. In Free Radicals in Biology. Vol. 1. pp. 51-68. Ed. W. A. Pryor. Academic Press.

ORR, G. L. & HESS, F. D. (1982). Mechanism of action of the diphenyl ether herbicide acifluorfen-methyl in excised cucumber (Cucumis sativus L) cotyledons. Plant Physiology 69, 502-507.

containing 30 mM NaCl and 5 mM $MgCl_2$.

Photosynthetic electron transport reactions. Light dependent oxygen uptake or evolution from illuminated thylakoids was determined in a Hansatech oxygen electrode, in a 3.0 ml reaction volume containing 30 mM Tricine-NaOH buffer, pH 7.6, 5 mM $MgCl_2$, 1.7 mM NH_4Cl and chloroplast membranes containing 50-100 µg chlorophyll.$^{-2}$ Illumination was provided by a 500 W tungsten lamp giving 500 µmol $m^{-2}s^{-1}$ photon flux density at the reaction chamber. Additions to the basic reaction mixture were 1.7 mM potassium ferricyanide (FeCN) for photosystem II, 10 µM paraquat for measuring electron flow through photosystem I and II in a Mehler reaction, 6.6 µM DCPIP, 13.3 mM ascorbate and 66 µM monuron for measuring the effect of herbicides on photosystem I oxygen uptake, or no additions to test the ability of herbicides to act as Mehler reaction catalysts. For $NADP^+$ reduction, 13.3 µM $NADP^+$ was reduced following periods of illumination.

Photosynthesis. Sub-apical pea leaves were floated on test solutions for 24 h in darkness prior to measuring photosynthetic activity. An Infra-Red gas analyser (model 225, Analytical Development Co, Hoddesdon, Herts) was used to measure CO_2 exchange in batches of 4 leaf discs. Plant material was maintained in a chamber at 24°C, and irradiation provided by a photoflood lamp (Thorn 400 W) giving 250 µmol $m^{-2}s^{-1}$ photon flux density at the sample chamber.

Chlorophyll bleaching. Pea leaf discs were incubated on H_2O or 0.1 mM monuron for 24 h in darkness prior to transfer to herbicide test solutions and incubated in a controlled environment cabinet for up to 72 h under constant illumination (400 µmol $m^{-2}s^{-1}$ photon flux density) provided by warm white fluorescent tubes (65/85-W; Thorn). Chlorophyll was extracted from leaf discs with ethanol, and quantified using the coefficients described by Lichtenthaler & Wellburn (1983).

Table 1. The effect of herbicides on CO_2 fixation in pea leaf discs. Leaf discs were incubated on herbicide solutions for 24 h in darkness prior to measurement of photosynthetic activity

	CO_2 fixation (µmol CO_2 uptake g^{-1} FW h^{-1})	
Control	71.88	(100%)
10 µM Paraquat	0	
10 µM Monuron	0	
25 µM Rose bengal	71.53	(99.5%)
25 µM Acifluorfen	55.27	(76.8%)
25 µM Fomesafen	51. 7	(71.9%)
25 µM Nitrofen	41. 6	(57.9%)
25 µM Bifenox	32. 1	(44.6%)

RESULTS

Both paraquat and monuron (10 µM) inhibited photosynthesis of pea leaf discs after 24 h dark incubation (Table 1). The diphenyl ether herbicides acifluorfen, nitrofen, bifenox and fomesafen (all at 25 µM) only partially inhibited photosynthesis after the same period of dark incubation. Rose bengal had no effect.

Table 2. The effect of herbicides on photosynthetic electron transport reactions of isolated pea thylakoids

	$H_2O \rightarrow FeCN$ (PS II)	$H_2O \rightarrow PQ$ (PS II→PSI)	Ascorb/DCPIP→O_2 (PS I)	$H_2O \rightarrow O_2$ (PS II → PS I)
Control	125.1[a] (100%)	134.5[b] (100%)	20.13[b] (100%)	29.5[b] (100%)
	Herbicide concentration			
	10uM %	50uM %	50uM %	10uM %	50uM %	50uM %
Monuron	1.6	0	0	-[c]	-	0
Paraquat	100.0	98.0	-[d]	997.0	992.0	837.0
Rose bengal	95.3	90.0	93.0	-[e]	-	107.0
Acifluorfen	101.6	100.0	91.6	106.1	100.0	108.3
Fomesafen	100.0	64.1	77.4	176.8	353.0	-
Nitrofen	65.1	23.3	21.4	100.0	100.0	-
Bifenox	96.9	68.7	73.2	159.2	271.0	-

a. as µmol O_2 evolution $mg^{-1}Chl.h^{-1}$
b. as µmol O_2 uptake $mg^{-1}Chl\ h^{-1}$
c. not assayed as reaction mixture contained monuron
d. not assayed as reaction mixture contained paraquat
e. no result obtained as rose bengal sensitises oxidation of ascorbate in the reaction mixture

The results presented in Tables 2 and 3 show the effect of paraquat, monuron, rose bengal and diphenyl ether herbicides on uncoupled photosynthetic electron flow reactions of isolated chloroplast membranes. Monuron abolished photosystem II ferricyanide reduction at low concentration. As a consequence, electron flow through photosystem I and II, and $NADP^+$ reduction by illuminated thylakoids was abolished. Paraquat promoted oxygen uptake by photosystem I, thus causing an inhibition of $NADP^+$ reduction (Table 3). Photosystem II activity was unaffected by paraquat. The diphenyl ether herbicide acifluorfen had no effect on electron flow through photosystem I and II, or on oxygen uptake by photosystem I (Table 2) at concentrations up to 50 µM. By contrast fomesafen, nitrofen, and bifenox all inhibited photosystem II ferricyanide reduction, although a higher concentration was required to elicit a response similar to monuron. Fomesafen and bifenox were additionally active Mehler reaction catalysts, promoting oxygen uptake from photosystem I (Table 2).

Table 3. The effect of herbicides on $NADP^+$ reduction by illuminated chloroplast membranes

	$NADP^+$ reduction ($\mu mol\ mg^{-1} Chl\ h^{-1}$)	
Control	33.53 (100%)	
	Herbicide concentration ($NADP^+$ reduction as % of control)	
	10μM	50μM
Monuron	16.3	0
Paraquat	14.8	0
Acifluorfen	81.7	63.4
Fomesafen	100	147.3
Nitrofen	58.4	20.8
Bifenox	88.5	62.4

While acifluorfen, nitrofen and bifenox were all inhibitory of $NADP^+$ reduction (Table 3), high concentrations of fomesafen catalysed electron flow to $NADP^+$. Rose bengal had no direct effect on any photosynthetic electron flow reactions.

Table 4. Requirement for photosynthetic electron transport in herbicide induced chlorophyll bleaching of pea leaf discs. Discs were pre-incubated on 0.1 mM monuron or H_2O for 24 h prior to illumination

	Chlorophyll after 48 h			
	H_2O		+Monuron (0.1 mM)	
	μg/leaf disc	% control		
Control	51.59	(100%)	50.78	(100%)
10 μM Rose bengal	16.46[1]	(31.9%)	15.5[1]	(30.6%)
10 uM Paraquat	11.93[2]	(23.6%)	29.7[2]	(58.5%)
25 μM Acifluorfen	26.3	(51.0%)	47.6	(93.6%)
25 μM Fomesafen	12.41	(24.1%)	27.2	(53.5%)
25 μM Bifenox	10.82	(20.9%)	25.6	(50.4%)
25 μM Nitrofen	21.3	(41.3%)	46.02	(53.5%)

1. Leaf discs illuminated for 72 h.
2. Leaf discs illuminated for 24 h.

The results presented in Table 4 demonstrate the role of photosynthetic electron flow in herbicide induced chlorophyll bleaching of pea leaf discs. Electron flow was inhibited by incubating discs on monuron for 24 h in darkness, prior to their transfer to herbicide test solutions and illumination. Monuron pre-treatment retarded bleaching induced by paraquat, acifluorfen, nitrofen, bifenox and fomesafen. This indicated that a functional electron flow system was necessary for herbicide activity. It is important to note, however, that monuron pre-treatment which abolished electron flow in leaf discs did not totally prevent paraquat induced chlorophyll bleaching, even though this herbicide is dependent on photosynthetic electron flow for activity. Bleaching of leaf discs induced by rose bengal was unaffected by monuron pre-treatment. Although rose bengal can stimulate the peroxidation of illuminated chloroplast membranes (Dodge & Gillham, 1986), these results show that the action, although light induced, is dependent of photosynthesis.

DISCUSSION

Although symptoms of injury induced by herbicides that promote membrane lipid peroxidation and chlorophyll bleaching are similar, results presented in this paper demonstrate that the requirement for photosynthetic electron flow in herbicide activity can be used to discriminate between sites of activation and modes of action of different herbicide classes.

The primary mode of action of the herbicides paraquat and monuron is to divert or inhibit photosynthetic electron flow thus stimulating the formation of superoxide and singlet oxygen in chloroplast respectively (Dodge, 1983). The increased formation of these oxygen species promotes lipid peroxidation.

Photosensitisers such as rose bengal, promote singlet oxygen formation but independently of photosynthesis (Dodge, 1983). Diphenyl ether herbicides are not strong inhibitors of photosynthetic electron flow. Furthermore the differences in response elicited by diphenyl ether herbicides tested in this study (Table 2) indicated that inhibition of photosynthetic flow was not their primary mode of action. Photosynthetic electron flow was necessary for herbicide activity with this group of chemicals (Table 4). Thus activation may occur by reduction of the diphenyl ether molecule to a radical capable of inducing lipid peroxidation, as proposed by Sandmann and Böger (1982). While acifluorfen appears to be activated by photosynthetic electron flow in the region of ferredoxin (Gillham & Dodge, 1986) the precise site of activation of other diphenyl ether herbicides, and the nature of the radical species formed that initiates lipid peroxidation remains unclear.

REFERENCES

DODGE, A. D. (1983). The influence of light on the action of herbicides. Aspects of Applied Biology 4, Influence of environmental factors on herbicide performance and crop and weed biology, pp. 217-226, 1983.

DODGE, A. D. & GILLHAM, D. J. (1986). Methods for investigating the action of light activated herbicides. (i) The assessment of lipid peroxidation. Aspects of Applied Biology 11, Biochemical and physiological techniques in herbicide research, pp. 97-104, 1986.

GILLHAM, D. J. & DODGE, A. D. (1986). Studies into the action of the diphenyl ether herbicides acifluorfen and oxyfluorfen. (ii) The interaction with photosynthetic electron transport. Pesticide Science. In press.

HALL, D. O. (1972). Nomenclature for isolated chloroplasts. Nature 235, 125-126.

LICHTENTHALER, H. K. & WELLBURN, A. (1983). Determinations of total carotenoids and chlorophyll a and b in leaf extracts in different solvents. Biochemical Society Transactions 11, 591-592.

SANDMANN, G. & BÖGER, P. (1982). Mode of action of herbicidal bleaching. In Biochemical responses induced by herbicides. ACS symposium series 181. pp. 111-130. Eds D. E. Moreland, J. B. St John and F. D. Hess.