THE MODE OF ACTION OF NITRODIPHENYL ETHER AND RELATED HERBICIDES

A thesis submitted in accordance with the requirements for
the degree of Doctor of Philosophy of the University of London

by

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Abstract

Nitrodiphenyl ether (NDPE) and related herbicides cause light and oxygen-dependent lipid peroxidation in susceptible green plants and algae. The exact mode of action of these herbicides has not yet been clearly established.

Experiments with the alga *Scenedesmus obliquus* indicate that the role of photosynthetic electron transport is principally to generate the oxygen required for NDPE toxicity, rather than having a more direct involvement. DPE I, an experimental NDPE, did not induce lipid peroxidation or chlorophyll bleaching in the alga *Chlamydomonas reinhardtii* but the herbicide did inhibit growth of the algal cultures.

The toxic effects of DPE I on chrysanthemum petals containing a mixture of carotenoids but no chlorophyll appeared to support the hypothesis that carotenoids are the photosensitisers in NDPE action. The bleaching of chromoplast fractions isolated from chrysanthemum petals, when incubated under light, was enhanced by DPE I. Experiments suggested that the NDPE-enhanced bleaching does not involve singlet oxygen or Fenton chemistry. Structure activity studies suggested that the mechanism of NDPE-induced bleaching of chromoplasts is different to that of the toxic effects induced by these herbicides in leaves.

DPE I treatment caused the abnormal accumulation of photodynamic tetrapyrrole compounds in cucumber, barley, *Scenedesmus* and *Chlamydomonas*. In *C. reinhardtii* the major tetrapyrrole accumulating was identified as protoporphyrin IX. Experiments with enantiomers of a phthalide DPE, phthalide DPE III showed that the S(-) isomer induced greater accumulation of tetrapyrroles than the R(+) isomer. This difference was reflected in the herbicidal activities of the S(-) and R(+) isomers and suggests the likely involvement of an enzymic binding process, possibly within the chlorophyll biosynthetic pathway, in the mode of action of the DPE herbicides.
Acknowledgements

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Abbreviations

ADP  Adenosine 5'-diphosphate  
AFM  Acifluorfen-methyl  
ALA  5-aminolevulinic acid  
ATP  Adenosine 5'-triphosphate  
BSA  Bovine serum albumin  
Chl  Chlorophyll  
DABCO  1,4-Diazabicyclo[2.2.2]octane  
DMSO  Dimethyl sulphoxide  
DPE  Diphenyl ether  
EDTA  Ethylenediaminetetraacetic acid  
ESR  Electron spin resonance  
FDA  Fluorescein diacetate  
GSH  Reduced glutathione  
HEPES  N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid  
MDA  Malondialdehyde  
Mg-Proto IX  Magnesium protoporphyrin IX  
MV  Paraquat  
NADPH  Nicotinamide adenine dinucleotide phosphate (reduced form)  
NDPE  Nitrodiphenyl ether  
'O₂'  Singlet oxygen  
'O₂'-'  Superoxide  
PAL  Phenylalanine ammonia lyase  
Proto IX  Protoporphyrin IX  
PS I  Photosystem I  
PS II  Photosystem II  
PUFA(H)  Polyunsaturated fatty acid  
R'  Free radical  
R value  Retention time  
SOD  Superoxide dismutase  
TBA  Thiobarbituric acid  
TCA  Trichloroacetic acid
## Herbicides

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<td>Acifluorfen-methyl</td>
<td>methyl 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoate</td>
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<td>Bifenox</td>
<td>methyl 5-(2,4-dichlorophenoxy)-2-nitrobenzoate</td>
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<td>DCMU</td>
<td>N''-(3,4-dichlorophenyl)-N,N-dimethylurea</td>
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CHAPTER 1

Introduction

Aliphatic ethers (APEs) have been established as industrial
constituents since the introduction of alkylated and fluoroalkyl
in 1958. These herbicides, which require light and oxygen to
display their toxic effects (Matsunaga, 1980; Faveyol & Varrez, 1979;
Giri & Dasa, 1983 & 86) are particularly prone to plant emergence and are
effective against broad-leaved weeds and grasses. When this project
began, the mode of action of APEs had not been established
although numerous hypotheses had been proposed to explain their
activity. The aim of this work therefore, was to determine the
primary mode of action of aliphatic ethers and related herbicides.

The task of determining the primary mode of action of any
herbicide is not a straightforward one. A given herbicide may have
multiple modes of action, which is a characteristic which is a widespread
phenomenon in herbicide research. Biphenyl ethers (BPEs) have been shown
to have several distinct effects on plant structures and processes;
many of which may be attributed to particular substitutions on the 4,4-
substituted biphenyl. Thus, a herbicide might cause many modes of action giving
rise to a range of effects on the plant, which would occur at
different herbicide concentrations or under different treatment
conditions. The researcher is also faced with the problem of
distinguishing between primary herbicidal effects and secondary
effects due to disturbed cell metabolism in general.

The first part of this chapter discusses the symptoms of BPE
activity in plant tissue, whilst in the second part, the proposed
mechanism of action which give rise to such effects are discussed.
Nitrodiphenyl ethers (NDPEs) have been established as commercial herbicides since the introduction of nitrofen and fluorodifen in the mid 1960s. These herbicides, which require light and oxygen to elicit their toxic effects (Matsunaka, 1969; Fadayomi & Warren, 1976; Orr & Hess, 1982a & b), are applied pre- or post- emergence and are effective against broad leaved weeds and grasses. When this project began, the mode of action of these herbicides had not been established although several hypotheses had been proposed to explain their activity. The aim of this work therefore, was to determine the primary mode of action of nitrodiphenyl ether and related herbicides.

The task of determining the primary mode of action of any herbicide is not a straightforward one. A given herbicide may have multiple modes of action, a characteristic which is a widespread problem in herbicide research. Diphenyl ethers (DPEs) have been shown to have several distinct effects on plant structures and processes, many of which may be attributed to particular substitutions on the DPE skeleton. Thus a herbicide might combine many modes of action giving rise to a range of effects on the plant, which could occur at different herbicide concentrations or under different incubation conditions. The researcher is also faced with the problem of distinguishing between primary herbicidal effects and secondary effects due to disturbed cell metabolism in general.

The first part of this chapter describes the symptoms of DPE activity in plant tissue, whilst in the second part, the proposed modes of action which give rise to such effects are discussed.
DPEs have been reported to cause the following effects in plant tissues:

(i) membrane lipid peroxidation
(ii) bleaching of chlorophyll and carotenoids
(iii) increased ethylene production
(iv) inhibition of photosynthetic electron transport
(v) inhibition of the plastidic ATP-synthase
(vi) inhibition of respiratory electron transport
(vii) inhibition of carotenogenesis

Since this work is mainly concerned with the p-nitro DPE herbicides, only the activity associated with this group of DPEs is discussed in detail here. The structures of some of the more commonly known NDPEs are shown in figure 1.1.
Figure 1.1 Structures of Some of the More Commonly Known NDPEs.
Lipid peroxidation is a complex process whereby unsaturated lipid molecules react with molecular oxygen leading to the formation of lipid hydroperoxides. Such processes generally proceed via a free radical mediated chain reaction (Slater, 1984). This is initiated by the abstraction of a hydrogen atom from the unsaturated lipid by the free radical, followed by a complex series of propagation reactions (Slater, 1972a). This is shown in equations 1.1 to 1.4. A hydrogen atom is abstracted from a polyunsaturated fatty acid (PUFA(H)) by a free radical (R'). Addition of oxygen yields a lipid peroxy free radical (PUFAO_2') which can itself abstract a hydrogen atom from a neighbouring molecule. This may also be a polyunsaturated fatty acid, thus propagating the reaction.

\[
\begin{align*}
PUFA(H) + R' & \rightarrow PUFA^* + RH \text{ equation 1.1} \\
PUFA^* + O_2 & \rightarrow PUFAO_2^* \text{ equation 1.2} \\
PUFAO_2^* + PUFA(H) & \rightarrow PUFAO_2H + PUFA^* \text{ equation 1.3} \\
PUFAO_2H & \rightarrow \text{PRODUCTS} \text{ equation 1.4}
\end{align*}
\]

In the presence of initiation factors, such reactions can occur.
within cell membranes (Mead, 1976). The orderly array of fatty acids in the hydrophobic matrix of the membrane allows maximum interaction of the individual molecules and readily propagates free radical reactions. Degradation of membrane PUFA(H)s causes considerable cell damage resulting in a disorganisation of membrane structure and disturbed membrane function (Slater, 1972b).

The lipid hydroperoxides formed in the above reactions are decomposed in the presence of metal ions in their lower oxidation states to give a range of breakdown products (Halliwell and Gutteridge, 1984). There is much experimental evidence to suggest that DPEs induce membrane destruction in plants and algae. Pereira et al (1971) showed that nitrofen caused leakage of vacuole-localised betacyanin from beetroot (Beta vulgaris L.) sections. DPE-induced membrane leakage was later confirmed by several other research groups using the electrolytic conductivity method. Vanstone and Stobbe (1977) used this technique to detect severe injury to buckwheat leaf (Fagopyrum esculentum Moench. cv. Tokyo) discs 8h after oxyfluorfen application. Cell membrane permeability was measured as a change in the electrolytic conductivity of solutions that contained leaf discs from treated plants. This method has been used to demonstrate DPE-induced membrane damage in green bean (Phaseolus vulgaris L. Spartan Arrow) leaves (Pritchard et al, 1980) and cucumber cotyledons (Cucumis sativus) (Kenyon et al, 1985).

A new technique to measure DPE-induced membrane damage was developed by Orr & Hess (1981, 1982a & b). Herbicidal injury was monitored by following the efflux of $^{86}$Rb$^-$ and other radiolabelled ions or organic molecules, $^{35}$Cl$^-$, $^{46}$Ca$^{2+}$, 3-0-methyl-$^{14}$C-glucose and
[\textsuperscript{14}C]-methylamine, from treated cucumber cotyledons. Using this assay it was possible to detect injury at concentrations as low as 0.01 \textmu M acifluorfen methyl (AFM) under illumination, after a 2-3 h lag period.

A structure-activity study was also undertaken using this method (Orr & Hess, 1981). The following decreasing order of relative activity was observed: AFM > MC-10982 > bifenox > oxyfluorfen. The use of the cucumber cotyledon bioassay for such studies is, however, limited because of probable differences in herbicide penetration and translocation. Differences in activity could also be related to the differential ability of cucumber cotyledons to detoxify these compounds.

Analysis of the breakdown products of lipid hydroperoxides can also be used to measure DPE-induced membrane damage. One such product is ethane. The use of this hydrocarbon gas as an indicator of DPE activity is discussed in Chapter 2. Malondialdehyde (MDA) which may be detected colorimetrically after reaction with thiobarbituric acid (TBA), has also been used to detect membrane damage after treatment with DPEs. Orr & Hess (1982b) assayed cucumber cotyledons for 'TBA reacting material' following AFM treatment and detected significant increases in the treated tissues after light activation. These experiments indicated that there was indeed direct physical damage to the membrane. This was confirmed by electron microscopy. The presence of 'TBA reacting material' provided the first evidence that NDPE-induced injury to membranes resulted from free radical mediated lipid peroxidation.

Lambert et al (1984) demonstrated that DPEs induced the formation of radicals by using EPR techniques. Phenyl-t-butyl nitrotrone was used to
trap radicals generated in isolated, illuminated chloroplasts. The radical signals were dramatically amplified in the presence of DPEs. A para-substitution of the DPE with NO$_2$, Cl or I was found to be necessary as well as an operating photosynthetic electron transport chain. The radicals were not identified but it was suggested that they might be alkyl or linolenic acid radicals.

1.1.2 Protein Damage.

When fatty acids are peroxidised, cell components including proteins, enzymes and pigments can be damaged by free radicals from the peroxidation process. Kunert et al (1985) investigated the effects of oxyfluorfen on induced redox reactions of chloroplast cytochromes and on protein degradation in *Scenedesmus acutus*. Light-induced redox reactions of the plastidic water-soluble cytochrome c-553 and the membrane bound cytochrome f-553 were totally inhibited after 6 h incubation of the cells with oxyfluorfen. The authors found that water-soluble proteins were rapidly damaged in oxyfluorfen-treated algal cells. Such damage can be detected either by production of fluorescent products, attributed to a cross linking reaction between malondialdehyde and proteins, or by loss of specific amino acid residues of proteins. Kunert et al (1985) found methionine and histidine to be among the amino acids most labile to oxyfluorfen-induced lipid peroxidation. Furthermore, the sensitivity of the water-soluble protein cytochrome c to oxyfluorfen under light was significantly higher than that of membrane-bound components such as
cytochrome $f$ and chlorophyll. Destruction of membrane bound components may be strongly dependent on their environment. $\alpha$-tocopherol and $\beta$-carotene, normal constituents of the thylakoid membrane, are powerful protectors against lipid peroxidation (Kunert & Boger, 1984; Lichtenthaler et al, 1981).

1.1.3 Toxic Oxygen Species and DPE Action.

As was shown in section 1.1.1, a free radical is required to initiate the lipid peroxidation chain reaction. Oxidation of polyunsaturated fatty acids by triplet oxygen is a spin-forbidden reaction but lipid peroxidation may be initiated by other, active oxygen species. The donation of an electron to the triplet oxygen molecule results in the generation of the superoxide molecule ($O_2^\cdot$). Such a reaction is involved in the mode of action of the bipyridynium herbicides (Bowyer et al, 1987). Although superoxide itself is relatively unreactive, in biological systems it can lead to the formation of hydrogen peroxide which, unlike superoxide, can readily diffuse through membranes and can oxidise lipids. Hydrogen peroxide is generated by the dimutation of superoxide either non-enzymically or by SOD:

$$2O_2^\cdot + 2H^+ \longrightarrow H_2O_2 + O_2$$ equation 1.5
The highly reactive hydroxyl radical may be derived from $O_2^\cdot$ \( \rightarrow \) $\overset{\cdot}{H}_2O_2$ in the Haber-Weiss reaction (Asada & Takahashi, 1987):

$$O_2^\cdot + Fe^{3+} \rightarrow O_2 + Fe^{2+} \text{ equation 1.6}$$

$$\overset{\cdot}{H}_2O_2 + Fe^{2+} \rightarrow OH^\cdot + OH^- + Fe^{3+} \text{ equation 1.7}$$

Fenton reaction

The high reactivity of hydroxyl radicals means that they react at or near their site of formation and thus they must be formed near the membrane lipid in order to initiate lipid peroxidation (Asada & Takahashi, 1987).

Hydrogen peroxide may be visualised at the ultrastructural level by cerium perhydroxide staining. Duke et al (1984) used this cytochemical technique with cucumber cotyledon discs treated with acifluorfen. After 3 h light exposure, extensive cerium hydroxide deposits were observed in the mitochondria but not in the chloroplasts or any other part of the cell. No deposits were observed in the controls. Similarly, localisation of superoxide by cytochemical techniques resulted in the staining of the mitochondrial matrix.

Duke et al (1984) found that copper penacillamine complex which has a high level of superoxide dismutating activity, partially protected against acifluorfen-induced ion leakage from cucumber cotyledons as did $N$-[2-(2-oxo-1-imidazolidinyl) ethyl]-$N'$-phenylurea (EDU), which induces high levels of SOD and catalase in plant tissues.
Ensminger and Hess (1985a) however, found no protection by copper penacillamine against the action of acifluorfen. Wettlaufer et al (1985) concluded that acifluorfen did not cause increased levels of $H_2O_2$ via superoxide, by measuring $H_2O_2$ levels in illuminated spinach chloroplasts treated with this herbicide.

Upham & Hatzios (1987) used the methional assay for detecting oxygen free radicals to study the involvement of toxic oxygen species in NDPE toxicity. The assay is based on the gas chromatographic determination of ethylene from the oxygen radical dependent oxidation of methional. Hydroxyl and alkoxyl radicals are able to oxidise methional to ethylene whereas $H_2O_2$, superoxide and alkyl radicals are not. It is, however, unclear whether singlet oxygen can carry out this oxidation. Isolated pea thylakoids were found to oxidise methional in the absence of NDPEs. Addition of 10 $\mu$M oxyfluorfen significantly increased the rate of methional oxidation. Addition of catalase inhibited the rate by 60% in both treated and untreated thylakoids suggesting that $H_2O_2$ is a direct precursor for the generation of hydroxyl / alkoxyl radicals. Addition of Fe-EDTA, a Fenton catalyst, to pea thylakoids in the presence or absence of oxyfluorfen caused a six-fold increase in the rate of methional oxidation. However, addition of 1 mM concentrations of nitrofen, nitrofluorfen and acifluorfen decreased the rate of methional oxidation and AFM had no effect, leading the authors to propose that oxyfluorfen has a different mode of action to other NDPEs.

The oxygen molecule has 2 unpaired electrons located in the $\pi^*$ antibonding orbitals (figure 1.2). Since the 2 electrons are in parallel spins the molecule is relatively unreactive. However,
Figure 1.2 The π^* Orbital of Ground and Singlet States of Oxygen, Superoxide and Peroxide.

Because of the extremely rapid decay of 'Σ_g O_2 in water (10^11 s^-1), it has little chance to interact with cell components and therefore in biological systems, the term 'singlet oxygen' is used to refer to 'Δ_g O_2 which decays more slowly in water (Asada & Takahashi, 1987).
reactivity can be increased by initiating spin reversal through energy input from, for example, a triplet photosensitiser such as chlorophyll, giving rise to singlet oxygen.

Haworth & Hess (1988) proposed that oxyfluorfen causes the generation of singlet oxygen in pea thylakoids. Using the bleaching of \( N,N \) dimethyl \( p \)-nitrosoaniline as a specific detector of singlet oxygen, production of this oxygen species over a concentration range of \( 10^{-4} \) to \( 10^{-9} \) M oxyfluorfen was demonstrated. There was an absolute dependence on an intact thylakoid membrane system for activity and the process was found to be independent of photosynthetic electron transport.

1.1.4 The Effects of DPEs on Plant Protective Mechanisms and Protection by Antioxidants.

Protection against destructive lipid peroxidation is provided by an array of antioxidative enzymes and a variety of small molecules including \( \alpha \)-tocopherol, ascorbate and glutathione.

Superoxide is probably generated during normal chloroplast electron transport and is scavenged by superoxide dismutase enzymes. All SOD enzymes are metalloproteins which catalyse the reaction shown in equation 1.5. The chloroplastic SOD is predominantly a Cu-Zn type that is partially bound to the thylakoid membranes (Jackson et al, 1978). The \( \text{H}_2\text{O}_2 \) produced by the enzyme must be removed since it is strongly inhibitory to a number of enzymes including SOD itself (Asada et al, 1975), fructose bisphosphatase and sedoheptulose bisphosphatase.
of the Calvin cycle (Kaiser, 1979). The removal of \( \text{H}_2\text{O}_2 \) within the chloroplast is achieved by the enzymes of the ascorbate:glutathione cycle: ascorbate peroxidase, dehydroascorbate reductase and glutathione reductase (Foyer & Halliwell, 1976). This is illustrated in figure 1.3. The integration of this system is also dependent on the presence of NADPH and also ascorbate and glutathione. Both of these antioxidants are present in millimolar concentrations in the chloroplast stroma (Gillham & Dodge, 1986). An alternative scavenging system for \( \text{H}_2\text{O}_2 \) would be via catalase but it is generally considered that this enzyme is not located in the chloroplast (Halliwell, 1974). Ascorbate and glutathione can also react directly with and inactivate radicals including superoxide, hydroxy radical and singlet oxygen (Halliwell, 1982; Wefers & Sies, 1983).

A high concentration of the lipid soluble \( \alpha \)-tocopherol has been found in the chloroplast envelope and the osmiophilic plastoglobuli of the plastid stroma (Lichtenthaler et al, 1981). \( \alpha \)-tocopherol is a very effective free radical quencher and there is evidence to suggest that its antioxidative action is determined by a synergism with ascorbate. Tappel (1968) suggested that ascorbate might reduce \( \alpha \)-tocopherol radicals formed by free radical reactions. Thus one molecule of \( \alpha \)-tocopherol would be able to scavenge many radicals derived from lipid peroxidation.

Carotenoids are vitally important as quenchers of both \( \text{Chl} \) and \( \text{O}_2 \). Plants which are deprived of carotenoids by mutation or by herbicides are rapidly bleached (Anderson & Robertson, 1960). Carotenoids present in the thylakoid membranes play an essential
Figure 1.3 The Ascorbate : Glutathione Cycle.

(i) ferredoxin-NADP+ reductase
(ii) glutathione reductase
(iii) dehydroascorbate reductase
(iv) ascorbate peroxidase
quenching role during normal photosynthesis and there is a whole class of herbicides dedicated to inhibiting carotenoid biosynthesis.

Orr & Hess (1982a) demonstrated a protective effect of \( \alpha \)-tocopherol against DPE-induced \( \equiv{\text{Rb}}^+ \) leakage from cucumber cotyledons. These authors (1982b) also found limited protection against AFM-induced injury with butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), exogenous antioxidants, but the concentrations necessary for protection were high (400 \( \mu \)M) and caused some damage to the controls.

Kunert (1984) found that levels of ascorbate and carotenoids were significantly lowered in oxyfluorfen-treated plants. Application of the herbicide decreased the carotenoid content approximately 53\%, but oxidation of ascorbate was greater than that of carotenoid. In another paper Kunert & Boger (1984) noted that addition of antioxidants to oxyfluorfen-treated \textit{Scenedesmus} cells inhibited the production of ethane. The most effective non-biological antioxidant tested was ethoxyquin which prevented ethane production to an extent comparable with \( \alpha \)-tocopherol. BHT and \( N,N' \)-diphenyl-1,4-phenylenediamine (DPPD) were less effective and no protection against lipid peroxidation and chlorophyll damage were observed when the hydroxyl radical scavenger mannitol was added to herbicide treated cells suggesting that hydroxyl radicals are not involved in the destructive process.

Finckh \textit{et al} (1985) investigated the relationship of \( \alpha \)-tocopherol and ascorbate to DPE-induced peroxidative cell damage. Generally the ascorbate content of plants was found to be significantly higher than that of \( \alpha \)-tocopherol. Plants with a substantial amount of both
vitamins and a concentration ratio of ascorbate to \( \alpha \)-tocopherol between 10 and 15 : 1 (wt/wt) were effectively protected against the phytotoxic action of oxyfluorfen. Herbicide-induced cell damage was found to increase in plants having a much lower or higher concentration ratio of ascorbate : \( \alpha \)-tocopherol or low levels of both antioxidants. Such evidence further supports the idea of a synergistic link between ascorbate and \( \alpha \)-tocopherol. Kenyon & Duke (1985) also found that DPE herbicides cause a decrease in the levels of ascorbate in plants under light. In acifluorfen-treated green cucumber cotyledons a 50% decrease in ascorbate within 1 h was observed, indicating that oxidation of ascorbate is one of the earliest effects of DPEs in plants. It was also noted that the reduced glutathione content in acifluorfen-treated cucumber tissue fell about 50% with respect to the control in the first 60 min of illumination.

Kenyon et al (1985) found that the activities of glutathione reductase, dehydroascorbate reductase, superoxide dismutase, ascorbate free radical reductase, peroxidase and catalase rapidly decreased in acifluorfen-treated tissue exposed to white light. However, none of these enzymes were inhibited in vitro by this herbicide. This loss of activity in vivo might, at least in part, be explained by general cellular disruption although the authors observe that the loss of enzyme activities was more rapid than the loss of electrolytes.
1.1.5 Chlorophyll and Carotenoid Bleaching.

Several groups have reported the destructive effect of DPEs on chlorophyll. Kunert & Boger (1981) noted a 90% decrease in the chlorophyll content of *Scenedesmus* within 24 h. The decrease of total carotenoids in these cultures was even more severe, 40% within 8 h and 95% within 24 h. Herbicide-induced pigment bleaching may be caused by inhibition of biosynthesis or pigment destruction. Kunert & Boger (1981) could not detect carotene precursors thought to be indicative of inhibited biosynthesis, nor did norflurazon treated *Scenedesmus* cultures produce ethane, indicating that oxyfluorfen has a different mode of action. Thus, these results suggested that the bleaching action of DPEs is to initiate pigment destruction. It is assumed that chlorophyll destruction in plant tissues is caused by photooxidation due to the lack of protection by carotenoids. In a later paper, Kunert & Boger (1984) demonstrated that chlorophyll damage was a consequence of the peroxidation process by showing that both lipid peroxidation and pigment damage were inhibited by α-tocopherol and ethoxyquin.

Kenyon *et al* (1985) found that the chlorophyll and carotenoid contents of cucumber cotyledons treated with acifluorfen diminished very slowly compared to other parameters of cellular disruption up until about 6 h (acifluorfen caused increases in ethane, ethylene and MDA production within 2 h and leakage of electrolytes was detected within 1 h). Thereafter, the chlorophylls and carotenoid began to bleach more rapidly. Gillham & Dodge (1987a) showed that acifluorfen and oxyfluorfen induced chlorophyll bleaching of pea leaf discs. DPE-
Induced chlorophyll bleaching was shown to be enhanced by oxygen and restricted by the electron transport inhibitor monuron.

1.1.6 DPE-Induced Ultrastructural Damage.

Orr & Hess (1982a) monitored the structural changes in the cellular membrane system of etiolated and green cucumber cotyledons treated with AFM. The ultrastructure of AFM-treated tissue was unchanged after 6 h dark incubation. However, after 30-45 min of illumination massive cellular and membrane damage was apparent. Etioplasts and chloroplasts were swollen, a characteristic of an organelle that is indiscriminately permeable to solutes (in particular, protons). Early signs of injury appeared in the outer chloroplast or etioplast membrane and in the tonoplast. The authors observed only limited damage to the thylakoids in tissue exposed to light for short periods of time. Similar observations were made by Kenyon et al, (1985) and Derrick et al, (1988).

Bowyer et al (1987) studied the ultrastructural effects of DPE I on wild-type barley and on two mutants, viridis-zea which lacks PSI and viridis-zd" which lacks PSII activity. The effects of DPE I on mesophyll cells were similar in both wild-type and mutants. Small vesicles appeared in the cytoplasm and the chloroplasts became swollen. In more severely affected cells the tonoplast disrupted, loss of chloroplast envelope membrane occurred and the plasma membrane detached from the cell wall in places. The mitochondria and peroxisomes appeared intact.
Early signs of injury to the chloroplast envelope would be consistent with a carotenoid involvement in the mode of action of DPEs (see section 1.2.1), since carotenoids are known to be present in the chloroplast envelope (Douce & Joyard, 1979). Destruction of the tonoplast would lead to the release of various hydrolytic enzymes from the vacuole, leading to secondary effects on the cell, induced by the herbicide.

1.1.7 Stress.

Increased production of the plant hormone ethylene is an indication that the plant is under stress. Gorske & Hopen (1978) noted that production of this hydrocarbon gas was raised in DPE-treated common purslane (Portulaca oleracea). Komives & Casida (1982, 1983) found that the phytotoxic action of acifluorfen was accompanied by the strong induction of phenylalanine ammonia lyase (PAL), a key enzyme in phytoalexin biosynthesis, and greatly increased levels of phytoalexins in several plant species. The authors found that inhibitors of ethylene production, silver nitrate and heat shock, counteracted the phytotoxic effect of acifluorfen and oxyfluorfen in spinach leaves. Increased production of ethylene (less than 3 h after treatment) was observed before increased PAL activity (8-10 h after treatment) or ethane production (15-20 h after treatment). Ethylene is an inducer of PAL. The authors suggested that ethylene itself is not directly involved in DPE phytotoxicity, but acifluorfen toxicity may be related to the stimulation of PAL activity and PAL-dependent
biosynthesis of aromatic stress metabolites. Stress metabolites from PAL induction or other pathways, for example, wyerone in broad bean and hemigossypol in cotton are highly toxic to plants. Alternatively, high PAL activity might divert phenylalanine from protein synthesis or other critical cellular processes.

Kunert (1984) measured ethane and ethylene evolution from oxyfluorfen treated mustard seedlings (*Sinapis alba*) under high and low light intensities. At high light intensity ethane produced via lipid peroxidation was the dominant product whereas ethylene production declined. However, under low light intensity lipid peroxidation was no longer the dominant reaction and oxyfluorfen stimulated the production of ethylene. Ethane and ethylene are produced by two different pathways. Methionine is the biological precursor of ethylene whether it is produced under normal or stress conditions (Yang & Adams, 1980), while ethane is only derived from the peroxidation of ω-3-polyunsaturated fatty acids. *In vivo* only traces of ethylene are produced peroxidatively. Kunert (1984) suggested that the decline in ethylene production under high light intensity was as a result of protein damage caused during lipid peroxidation since ethylene biosynthesis is regulated by an enzyme system that converts methionine to ethylene (Yang & Adams, 1980).

1.1.8 Inhibition of Photosynthetic Electron Transport by DPEs.

Many groups have reported that NDPE herbicides inhibit photosynthetic electron transport. In 1971 Periera et al noted that
Nitrofen at $7 \times 10^{-7}$ M inhibited electron transport by 10% and by 66% at $10 \times 10^{-8}$ M, in isolated spinach chloroplasts. Bugg et al. (1980) proposed that DPEs inhibit electron transport between plastoquinone and cytochrome f. Pritchard et al. (1980) found that oxyfluorfen slightly stimulated PSI electron transport ($\text{DCIPH}_2 \rightarrow \text{MV}$) (see figure 1.4) yet the herbicide caused a 30% reduction in electron transport when durohydroquinone ($\text{DQH}_2$) (an electron donor which must utilise the plastoquinone pool) was used as the electron donor to PSI. This result is consistent with the site of inhibition proposed above. Van den Berg & Tipker (1982) found that nitrofen inhibited photosynthetic electron transport in spinach chloroplasts at the plastoquinone region. Bowyer et al. (1987) reported that DPE I had a site of inhibition of electron transport at the cytochrome $b_6f$ complex. DPEs do, therefore, inhibit photosynthetic electron transport but in general at concentrations much higher than those required to show herbicidal activity (Pritchard et al., 1980). Thus Alscher & Strick (1984) showed that acifluorfen (250 $\mu$M) completely inhibited CO$_2$-dependent oxygen evolution whereas the inhibition of electron transport required an order of magnitude greater concentration of herbicide to produce inhibition.

1.1.9 Inhibition of Photophosphorylation and Plastidic ATP-Synthase Activity.

Pritchard et al. (1980) found oxyfluorfen to have little inhibitory effect on PSI-linked phosphorylations in spinach chloroplasts using
Figure 1.4 Simplified Scheme for Photosynthetic Electron Transport in the Chloroplast. DTE, dichlorophenolindophenol. For other abbreviations see text. Modified from Prichard et al., 1980.
phenazine methosulphate (PMS) -stimulated cyclic electron flow or non-cyclic electron flow from reduced 3,6-diamino durene (DADH\textsubscript{2}) to paraquat. However, when electron transport was mediated through DQH\textsubscript{2} it was found that ATP formation linked to this non-cyclic system was inhibited about 40\% by 10\textsuperscript{-4} M oxyfluorfen. This inhibition was probably linked to inhibition of electron transport by the herbicide.

A photosystem II linked phosphorylation (H\textsubscript{2}O $\longrightarrow$ 2,5 dimethyl-p-benzoquinone (DMQ)) was completely inhibited at 10\textsuperscript{-4} M oxyfluorfen.

In 1979 Lambert et al suggested that the main herbicidal effect of nitrofen was as an 'energy transfer inhibitor'. Working with chloroplasts from the microalga Bumilleriopsis, non-cyclic electron flow was not affected by concentrations of nitrofen up 10\textsuperscript{-5} M whereas non-cyclic and cyclic photophosphorylation were severely inhibited. Stimulation of electron flow by ADP in coupled chloroplasts was diminished by nitrofen. The author suggested that nitrofen competes with ADP but not with inorganic phosphate at the CF\textsubscript{1}-coupling factor (plastidic ATPase). In a later paper, Lambert et al (1983) using chlorinated p-nitrodiphenyl ethers again demonstrated energy transfer inhibition. Apparently such inhibition is restricted to p-nitro DPEs with 2- and 4- substituents at the phenyl ring not carrying the nitro group, most particularly the dichloro compounds.

The results of Huchzermeyer (1982) and Huchzermeyer & Loehr (1983) suggested that nitrofen binds to the plastidic coupling factor (CF\textsubscript{1}) inhibiting nucleotide exchange in the light. Such a nucleotide release (ADP) would activate the enzyme and allow for the re-binding of ADP plus inorganic phosphate and subsequent ATP formation. During illumination in the presence of active diphenyl ethers, the percentage
of nucleotide free CF₁ species would be enhanced and phosphorylation drops. Concurrently, an increased leakage of protons would be observed via the nucleotide free coupling factor leading to a decreased steady state proton gradient.

1.1.10 Inhibition of Respiratory Electron Transport.

DPEs inhibit mitochondrial electron transport but the $I_{50}$ values are about 5-10 times higher than those for inhibition of photosynthetic electron transport (Kunert et al., 1987).

1.1.11 Inhibition of Carotenogenesis.

The $m$-phenoxybenzamide type DPEs inhibit phytoene desaturase in the biosynthetic carotenoid pathway (for a review see Kunert et al., 1987). However, since this activity is the result of a specific substitution on the DPE skeleton rather than a general feature of diphenyl ether herbicides, this activity will not be discussed here in detail.

1.1.12 Structure Activity Studies.

Inhibition of ATP synthesis requires $p$-nitro DPEs carrying 1 or 2 chlorine atoms at the unsubstituted phenyl ring, with the exception of
the 3,4-dichloro compound which shows relatively low activity (Lambert et al, 1983). Typical $I_{50}$ values are around 10 $\mu$M (Kunert et al, 1987).

No particular structural features are assigned to inhibitors of photosynthetic electron transport. Van den Berg & Tipker (1982) found that the lipophilicity of the herbicide molecule was the most important parameter. In addition, substitution into the nitrophenyl ring was found to lower activity considerably. Typical $I_{50}$ values for good DPE inhibitors of photosynthetic electron transport are about 10 $\mu$M (Van den Berg & Tipker, 1982), with the exception of DNP-INT (2,4 dinitrophenyl-2'-iodo,4'-nitro,6'-isopropylphenyl ether) which has greater inhibitory activity than other known DPEs. Substituents on the DPE skeleton may affect the binding regions of the DPE in the photosynthetic electron transport chain (Draber et al, 1981).

Lambert et al (1983) studied the structural requirements for peroxidative activity of DPEs. In the case of the nitro DPEs, when the nitro group was moved from the para to the meta or ortho position, all of the peroxidative activity was lost. There was also a decrease in activity when the ether bridge was replaced by sulphur. Substituting the $p$-nitro group with Cl, I or even NH$_2$ lowered peroxidative activity but did not abolish it.

The ligand next to the $p$-nitro group is also an important structural feature. It is assumed that its electronic and lipophilic characteristics are responsible for this influence. NDPEs with the highest peroxidative activities were found to be those having a methyl or ethyl ester, methoxy or ethoxy substituent at the position adjacent to the nitro group (Lambert et al, 1983; Orr & Hess, 1981).
1.2 The Proposed Modes of Action of DPEs.

Several modes of action have been proposed over the years to explain the phytotoxic effects of these herbicides, some of which have already been discussed in this chapter. It quickly became clear that nitrofen and other DPEs required light for activation (Matsunaka, 1969) and perhaps the biggest debate has centred around the role of photosynthetic electron transport in this light activation.

1.2.1 The Involvement of Photosynthesis in DPE Phytotoxicity.

A large number of commercial herbicides including the phenylureas, s-triazines and uracils, inhibit photosynthetic electron transport at one major site of action on the acceptor side of PSII. The herbicides interact with the 32 kdalton D1 protein component which is part of the reaction centre core complex of PSII. The phenol-type herbicides, for example dinoseb and ioxynil, also interact with the D1 protein, but azido-derivatives photo affinity label a 41 kdalton protein of unknown function (Oettmeier et al, 1983). It had been proposed that the PSII inhibitor herbicides induce a change in the potential of the hypothetical component B making electron flow from $Q \rightarrow B \rightarrow$ plastoquinone (where Q is the primary electron acceptor for PSII) thermodynamically unfavourable (Pfister & Arntzen, 1979). However, there is considerable evidence to suggest that PSII inhibitor herbicides act as plastoquinone antagonists, that is, they compete with plastoquinone for the so-called $Q_a^-$ binding site at the reducing
side of PSII and hence block the forward oxidation of the primary acceptor plastoquinone $Q_a$, by the secondary acceptor $Q_b$ (Velthuys, 1981; Trebst & Draber, 1986). When electron flow is inhibited, the lifetime of singlet excited chlorophyll is increased, so that a greater proportion passes to the longer lived triplet state through inter-system crossing. Interaction between triplet chlorophyll and molecular oxygen leads to the generation of singlet oxygen which in turn induces the direct formation of lipid peroxides from membrane unsaturated fatty acids (Dodge, 1983).

The bipyridinium herbicides paraquat ($MV$) and diquat also require light and oxygen in order to exert their phytotoxic effects. The mode of action of PSI herbicides has been reviewed by Bowyer & Camilleri (1987). Paraquat is thought to compete with ferredoxin for electrons emanating from PSI. As a result, NADP$^+$ reduction is inhibited, leading to a rapid cessation of CO$_2$ fixation (Harris & Dodge, 1972). In vivo the paraquat radical so formed is immediately reoxidised by molecular oxygen, generating superoxide.

$$MV^{2+} + e^- \rightarrow MV^+$$  \hspace{1cm} \text{equation 1.8}

$$MV^{+} + O_2 \rightarrow MV^{2+} + O_2^-$$  \hspace{1cm} \text{equation 1.9}
Such enhanced production of superoxide may be detected by spin trapping techniques (Harbour & Bolton, 1975; Bowyer & Camilleri, 1985). The toxic effects of superoxide are discussed in section 1.1.3.

Several groups have indicated that photosynthesis is in some way involved in DPE toxicity. Alscher & Strick (1984) showed that red light was as effective as white light in the acifluorfen mediated inhibition of CO₂-dependent oxygen evolution in isolated intact chloroplasts. Kunert et al (1987) found that red light above 610 nm caused oxyfluorfen toxicity in *Scenedesmus*, suggesting chlorophyll as the important pigment in DPE toxicity.

Perhaps the strongest evidence for the involvement of photosynthesis in DPE toxicity comes from the protective effects of photosynthetic electron transport inhibitors. Kunert & Boger (1981) using ethane production as an index of herbicidal action, showed that diuron could protect against oxyfluorfen-induced lipid peroxidation in *Scenedesmus acutus*. The concentrations of oxyfluorfen used (1 μM) did not inhibit linear electron flow in isolated spinach chloroplasts. However, if DCMU was added to *Scenedesmus* cells that had been pretreated for 2 h with oxyfluorfen, the protective effect was no longer observed suggesting that electron transport was only required during the initial activation stage. The authors proposed that the light activation of oxyfluorfen was due to the photoreduction of the NDPE molecule by PSI to form a nitro-anion radical. Such a radical might then react with oxygen giving rise to superoxide, or itself initiate lipid peroxidation. Lambert et al (1984) used ESR to show that radicals were produced in oxyfluorfen-treated spinach.
thylakoids. The presence of DCMU eliminated the oxyfluorfen-dependent signal. However, relatively high concentrations (0.1 mM) of oxyfluorfen were used in these experiments and the ESR spectra obtained were not diagnostic of the radical. Orr et al (1983) and Ensminger et al (1985) found that analogues of AFM in which the p-NO₂ group was replaced by -Cl or -H were herbicidally active yet cyclic voltammetry showed that these molecules could not readily accept electrons to become free radicals. Thus, although it is plausible that AFM could be reduced by a biological system to the nitro-anion radical, in vivo such a reaction would not be available to the -Cl and -H analogs and therefore it is unlikely that the mode of action of DPEs involves the direct reduction and reoxidation of the DPE molecule. It cannot be completely discounted however, that the p-Cl or p-H DPE analogues were metabolised to the p-nitro compounds in vivo.

Draper & Casida (1985) observed the reduction of p-nitro DPE herbicides to p-nitroso derivatives and the binding of these derivatives to unsaturated lipids. This promoted the generation of nitroxide radicals that were sufficiently reactive to initiate lipid peroxidation. Nitroxide radicals were also detected in the lipid fraction of bean leaves that had been treated with nitrofen. Gillham & Dodge (1987a) found that preincubation of pea-leaf discs with monuron restricted DPE-induced chlorophyll bleaching and ethane generation. Oxyfluorfen and acifluorfen were found not to affect non-cyclic electron flow or promote superoxide formation (Gillham & Dodge, 1987b) and the authors suggested that an activation of NDPE herbicides by photosynthetic electron transport in the vicinity of ferredoxin was
necessary for light-dependent herbicide activation. It was proposed that \( p \)-NDPE herbicides would be reduced by ferredoxin to a radical species capable of initiating lipid peroxidation. This process would then be blocked by monuron through its inhibition of photosynthetic electron transport.

The protective effects of DCMU are consistent with the hypothesis of Wettlaufer et al (1985). It was proposed that acifluorfen stimulates a pathway for the inactivation of light activated chloroplast enzymes, resulting in an inhibition of carbon fixation. In the presence of acifluorfen therefore, electron transport would continue but carbon fixation would become inhibited as fructose 1,6-bisphosphatase and glyceraldehyde 3-phosphate dehydrogenase were inactivated. Reductant produced as a result would be shunted to the reduction of molecular oxygen in the Mehler reaction. The levels of superoxide and more reactive species, for example \( \text{OH}^- \), would increase thus leading to lipid peroxidation, but this would be blocked in the presence of DCMU.

Matringe et al (1986) found that incubation of DCMU plus AFM or LS 82-556 \((S)3-N\text{-}(\text{methyl-benzyl})\text{ carbamoyl-5-propionyl-2,6-lutidine})\) an experimental herbicide with DPE-like activity, with green cucumber seedlings suppressed herbicide-induced damage during 24 h incubation under high light intensity. It was concluded that AFM and LS 82-556 require an operating photosynthetic electron flow to elicit toxicity when applied to green tissue. In contrast, no such protective effect was afforded by DCMU when the herbicides were applied to etiolated tissue. Tissut et al (1987) found that in isolated cucumber cotyledon fragments floating on water, LS 82-556 or acifluorfen toxicity was
suppressed by various PSII inhibitors (DCMU, atrazine, phenmedipham). It was postulated that photosynthesis could be involved in acifluorfen and LS 82-556 toxicity insofar as it maintains sufficient amounts of soluble organic compounds in the cotyledon fragments, compounds among which could be found a necessary precursor for the phytotoxic action of the herbicides. The authors found that in the presence of 1% sucrose, DCMU-dependent protection against LS 82-556 was no longer observed, and similar observations were made for DPEs. The hypothesis of an osmotic artefact was ruled out by the use of 1% mannitol replacing sucrose which restored the diuron protective effect (Nurit et al, 1988). It was suggested that in the presence of sucrose, photosynthesis should no longer be needed for the toxic effect to occur as sucrose could act as a substrate for the synthesis of the precursor.

There is also a great deal of evidence to suggest that photosynthesis is not involved in the DPE mode of action. Vanstone & Stobbe (1977) concluded from the lack of bleaching and minimal herbicidal injury of DPE-treated buckwheat leaves (*Fagopyrum esculentum* Moench. cv. Tokyo) elicited by light in the spectral region of chlorophyll absorption, that chlorophyll is not involved in the light activation of oxyfluorfen. Orr & Hess (1982a) concluded that the mechanism of action of AFM requires neither photosynthetic electron transport nor chlorophyll. They demonstrated that DCMU and DBMIB (2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone), an inhibitor of cyclic photophosphorylation, did not interfere with AFM-induced $^{86}$Rb$^+$ efflux from green cucumber cotyledons. Furthermore, AFM was shown to be active in etiolated tissue which was found to contain less
than 1% of the chlorophyll content of green cotyledons. Duke et al (1984) also argued against a role of photosynthetic electron transport in DPE toxicity. Tentoxin, a fungal toxin which prevents chlorophyll, but not carotenoid synthesis, made cucumber cotyledon discs supersensitive to acifluorfen. Inhibitors of PSII (DCMU, atrazine and bentazon) were also found to have little or no effect on acifluorfen activity. Death of *Chlamydomonas eugametos* cells was not prevented by diuron when cells were treated with AFM (Ensminger & Hess, 1985b). Ensminger & Hess also demonstrated that the addition of DPE to chloroplast fragments is insufficient to initiate lipid peroxidation. Duke & Kenyon (1986) found that cucumber cotyledons grown under far red light, which were photosynthetically incompetent, were hypersensitive to acifluorfen. Ultrastructural damage became apparent within one hour of exposure to white light. Cotyledon tissues which had been allowed to green for various time periods showed no correlation between photosynthetic capacity and herbicidal efficacy of acifluorfen. Bowyer et al (1987) showed by ultrastructural studies and measurement of ethane formation resulting from lipid peroxidation, that barley mutants lacking PSI (*viridis-zb<sup><em>e</em>3)* or PSII (*viridis-zd<sup><em>e</em>w*) were resistant to paraquat but susceptible to DPE I. These results indicated that neither electron transfer through both photosystems nor cyclic electron transport around PSI are essential for the phytotoxicity of NDPE herbicides.

Matringe & Scalla (1987a) demonstrated the sensitivity to AFM and LS 82-556 of soybean cell cultures that were unable to synthesize chlorophylls. Both herbicides caused light and oxygen dependent membrane destruction. However, antimycin A, an inhibitor of
mitochondrial electron transport between cytochrome $b$ and cytochrome $c$., was found to have a protective effect leading the authors to suggest the involvement of the respiratory chain in the toxic process induced by these herbicides. This idea had previously been suggested by Duke et al (1984). As was discussed in section 1.1.3, cytochemical staining of cucumber cotyledon discs treated with acifluorfen suggested that this herbicide increased the mitochondrial production of superoxide. Antimycin A appeared to lessen the effect of acifluorfen induced electrolyte leakage, as did rotenone indicating that mitochondrial electron transport was necessary for full herbicidal activity. If superoxide production was dependent on electron flow then it might be expected that uncouplers would increase acifluorfen induced cell damage. However, dinitrophenol and cyanide-$m$-chlorophenylhydrazone slightly decreased herbicide-induced electrolyte leakage from cucumber cotyledon discs, suggesting that ATP may be involved in the toxic process. The authors proposed that in higher plants under illumination, acifluorfen causes the production of a toxic species, possibly using a carotenoid located in the outer chloroplast envelope as the photoreceptor. The toxic species would then act in the mitochondrion to cause excessive superoxide production.

Kenyon et al (1988) found that the effects of acifluorfen on disruption of the plasmalemma and photobleaching of chlorophyll in cucumber cotyledons were approximately the same at 3°C and 30°C, suggesting that there is no metabolic requirement for light-induced NDPE toxicity. It is however, important to note that the cotyledon discs were preloaded with acifluorfen by incubation for 20 h in the
dark at 25°C. The activity of paraquat was reduced by 75% at 3°C compared to 30°C. PSII inhibitors (atrazine and DCMU) were found to enhance the activity of acifluorfen at 30°C but had no effect at 3°C. This led the authors to suggest that a functional photosynthetic electron transport system provides some level of protection from acifluorfen by regeneration of antioxidants. Antimycin A provided almost equal protection against acifluorfen at 3°C and 30°C provided that antimycin A was added at the beginning of the light exposure rather than at the beginning of the preceding dark incubation period. Thus it would seem that the herbicidal activity of acifluorfen was reduced by inhibiting a process that was dependent on mitochondrial respiration during the dark period.

1.2.2 The Role of Carotenoids in the Photoactivation of DPEs.

Matsunaka in 1969 was the first to propose carotenoids to be sensitizers for the DPEs. Matsunaka found that a natural albino mutant and artificial white mutants of rice plants were tolerant to nitrofen, while yellow mutants were susceptible. The dominant yellow pigments extracted from the yellow mutants were found to be xanthophylls, present at the same level as in normal green seedlings, whereas the content of carotenes was nil or significantly lower than that of wild-type plants. This led to the proposal that xanthophylls were important as acceptors of light energy in the photoactivation of DPE herbicides. The author suggested that light energy absorbed by xanthophylls would be used to 'activate' the DPE, converting it to a
toxic compound with herbicidal action. Fadayomi & Warren (1976) similarly showed that on application of oxyfluorfen or nitrofen to albino soybean (Glycine max L. Merr.) seedlings no injury occurred, while mutant yellow and normal green corn (Zea mays L.) and soybean seedlings were injured in light. Carotenoids were again implicated. Vanstone & Stobbe (1979) found that oxyfluorfen caused the greatest injury to buckwheat leaves which were exposed to light of 565 to 615 nm wavelength. Xanthophylls absorb in the blue region (400-440nm). The authors suggested the existence of a xanthophyll-protein complex in membranes, absorbing at 565 to 615 nm which was responsible for light activation of the herbicide at these wavelengths.

Inhibitors of carotenoid biosynthesis have been used to demonstrate that carotenoids have a role in the photoactivation of DPEs. Orr & Hess (1982a) showed that pretreating cucumber seedlings with 10 µM fluridone (1-methyl-3-phenyl-5[(3-(trifluoromethyl)pentyl)-4-(H)-pyridinone) protected against AFM induced injury as measured by ^86Rb^ efflux from excised cotyledons. Devlin et al (1983) found that removal of carotenoids by treating corn (Zea mays L.) with norflurazon rendered them partially tolerant to oxyfluorfen and the DPE, RH-8817 (ethyl 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoate), even when grown in the light, again adding support to the hypothesis that carotenoid pigments are the light energy acceptors involved in the activation of DPEs. In algae however, inhibitors of carotenogenesis have been used to demonstrate that carotenoids are not essential for DPE toxicity. Lambert & Boger (1984) treated Scenedesmus cells with difunon, thereby lowering the total content of coloured carotenoids by 50%. In these cultures photosynthetic electron transport remained
effective. Herbicidal activity was measured by peroxidative formation of ethane. Both carotenoid-deficient and normal cells exhibited the same peroxidative activity with oxyfluorfen and the authors concluded that light activation of \( p \)-NDPEs took place through electron donation from the intact photosynthetic electron transport chain. Examination of the carotenoid fractions under the culture conditions of these experiments however, shows that in the difunon treated cells, whilst the content of \( \alpha \)- and \( \beta \)-carotene was lowered to about 17% of the control, xanthophylls were much less affected, being reduced to 73% of levels found in untreated cells.

In 1985, Ensminger & Hess published an action spectrum of the activity of AFM in *Chlamydomonas eugametos* (Ensminger & Hess, 1985b). Using the stain fluorescein diacetate (FDA) to record cell death they determined that the herbicide was most active at two peaks of light; 450 and 670 nm. These peaks of light correspond to known peaks of carotenoid and chlorophyll absorption. No herbicidal activity was found in the UV. The authors suggested that both carotenoids and chlorophyll are responsible for DPE herbicidal activity. Sato *et al* (1987) studied the wavelength requirements for the activity of acifluorfen and S-23144 (\( N \)-(4-chloro-2-fluoro-5-propargyloxy)phenyl-3,4,5,6-tetra-hydrophthalimide), an experimental herbicide with DPE-like activity, on cucumber cotyledons. Leakage of amino acids from the cotyledons was measured as an indication of phytotoxic activity. Major peaks of activity at 550 and 650 nm and a minor peak at 450 nm were observed for both herbicides. Light of wavelength 450 and 650 nm was relatively less effective in achlorophyllous tissue grown in far red light than in green tissue. From their results the authors
suggested that S-23142 and DPEs are more likely to interact with chlorophyll and related pigments than with carotenoids.

Matringe & Scalla (1987a) examined the role of carotenoids in DPE toxicity in nonchlorophyllous Soybean cells. The involvement of carotenoids was examined by monitoring fluorescein release and dry weight/fresh weight ratios in cells treated with norflurazon. Although deprived of carotenoids, norflurazon-treated cells were sensitive to 10 μM AFM and 100 μM LS 82-556 in white and blue light. Such a result is inconsistent with the proposed role of carotenoids in the phytotoxic mechanism. It is possible that in these experiments carotenoids were reduced to levels that were undetectable although sufficient to invoke a herbicidal response. However, as the authors point out, if this were so it might be expected to observe a decrease in sensitivity of carotenoid deprived cells and such an effect was not observed. Gaba et al. (1988) measured the effects of acifluorfen on tomato cell cultures. Action spectroscopy showed that 350 nm light was most effective for inhibiting the growth of white cells (obtained by continually culturing etiolated cells with norflurazon) in the presence of 1 μM acifluorfen, followed by 550 nm, 450 nm, cool-white fluorescent and 630 nm light. Far red light was ineffective. From their data the authors concluded that in this system, neither chlorophyll, carotenes, cryptochrome, flavins or phytochrome could be the sole receptor for acifluorfen action.
1.2.3 DPE-Induced Tetrapyrrole Accumulation.

In 1987 Halling & Peters suggested that some process associated with chlorophyll synthesis stimulated AFM activity. AFM-induced efflux of 3-o-methyl-[U-14C]glucose from excised cucumber cotyledons (Cucumis sativus L.) was significantly greater when the etiolated cotyledons were incubated under low light intensity versus high light intensity. It was shown that while carotenoid levels remained fairly similar under either light intensity, chlorophyll development was inhibited at high light intensity. It appeared that chlorophyll and/or its precursors were being photooxidised prior to being safely integrated in the developing lamellae under the more intense lighting. The authors also noted that AFM significantly inhibited the accumulation of green pigments under conditions in which no measurable loss of plasmalemma integrity was observed, indicating that AFM specifically inhibited some step in chlorophyll synthesis or that it was able to initiate photooxidation of green pigments without significantly disrupting the plasmalemma.

Matringe & Scalla (1987b, 1988a) were the first to note that DPE herbicides induce the abnormal accumulation of tetrapyrrole compounds in plant tissues. An action spectrum of AFM activity in nonchlorophyllous soybean cells revealed that the NDPE exerts most of its phytotoxicity in the blue region of the spectrum with lesser effects from 450-700 nm. This was found to be consistent with the absorption spectrum of protoporphyrin IX (Proto IX). Extracts from treated cells revealed relatively high levels of a component with an absorption spectrum that matched that of Proto IX. Proto IX is known
as a generator of singlet oxygen (Cannistraro et al., 1978). Accumulation of this pigment would therefore, under light, lead to lipid peroxidation, characteristic of DPE action. Pretreatment of the cells with 4,6-dioxoheptanoic acid (DHA), an inhibitor of tetrapyrrole biosynthesis, reduced the accumulation and simultaneously lessened AFM toxicity.

Further evidence that the herbicidal activity of DPE herbicides is dependent on porphyrin synthesis was presented by Lyndon & Duke (1988). Gabaculine (3-amino-2,3-dihydroxybenzoic acid) which inhibits porphyrin synthesis by inhibiting the biosynthesis of δ-aminolevulinic acid (ALA), greatly reduced the activities of acifluorfen, fluorodifen, oxyfluorfen and nitrofen (as determined by electrolyte leakage from cucumber cotyledon discs) while having no effect on the activities of paraquat and rose bengal. Gabaculine also provided protection against acifluorfen-induced damage in pigweed (Amaranthus retroflexus L.) and velvetleaf (Abutilon theophrasti Medic.) and tentoxin-treated cucumber seedlings. Whilst tentoxin-treated tissues normally accumulate very little chlorophyll, they retain the capacity for porphyrin synthesis. The authors found acifluorfen-treated cucumber cotyledon discs to contain abnormally high levels of a pigment that they also tentatively identified as Proto IX. Thus it was proposed that the DPE herbicides act by stimulating the synthesis or the accumulation of Proto IX which then acts as a photodynamic dye.
The aim of this work then, was to further investigate the roles of photosynthetic electron transport and carotenoids in DPE phytotoxicity and to examine the ability of these herbicides to induce the accumulation of photosensitising tetrapyrrole pigments.
CHAPTER 2

The Role of Photosynthetic Electron Transport in Nitrodiphenyl Ether Toxicity.

The role of photosynthetic electron transport in N1trodiphenyl Ether Toxicity.

DCMU had little or no effect on the action of DME, but caused an increase in inhibition of N1trodiphenyl ether degradation by tobacco leaf discs. This result agreed with the findings of other groups (Calkins and Kavan 1960, Evershed and How, 1966). However, Newton et al. (1962) reported that DCMU protected intact corn leaves from 85°C for 5 min, a compound with apparently the same mode of action as DME. In the green algae Scenedesmus obliquus, Pettway and Elyar (1961) showed that DCMU completely protected against light photolysis and chloroplast damage. This observation was taken as evidence to support the hypothesis that the initial event in N1trodiphenyl ether toxicity is reduction of the herbicide molecule by PSII to a radical.

Much of the data presented here features the nitrodiphenyl ether UPE 1 and a family of diphenyl ether analogues, whose structures are shown in figure 4. UPE 1 was introduced by Howarth et al. in 1962, who showed that it behaved as a typical N1trodiphenyl ether in causing light and dark-induced necrosis, chloroplast degradation and lipid peroxidation. Caillet et al. (1966) demonstrated that the phloemion that had reduced similar symptoms of photosynthetic rea.

53
2.1 Introduction.

The role, if any, of photosynthetic electron transport in NDPE toxicity has, as was discussed in Chapter 1, been much debated and there is a great deal of conflicting experimental evidence. This may be illustrated by considering the effects of the photosynthetic electron transport inhibitor DCMU. Bowyer et al (1987) found that DCMU had little or no effect on the action of DPE I as measured by chlorophyll degradation in tobacco leaf discs. This result agrees with the findings of other groups (Duke and Kenyon, 1986; Ensminger and Hess, 1985a). However, Matringe et al (1986) reported that DCMU protected intact green cucumber cotyledons from LS 82-556, a compound with apparently the same mode of action as DPEs. In the green alga Scenedesmus obliquus, Kunert and Boger (1981) showed that DCMU completely protected against lipid peroxidation and chlorophyll bleaching. This observation was taken as evidence to support the hypothesis that the initial event in NDPE toxicity is reduction of the herbicide molecule by PSI to a radical.

Much of the work presented here features the nitrodiphenyl ether DPE I and a family of diphenyl ether phthalides, whose structures are shown in figure 2.1. DPE I was introduced by Bowyer et al in 1987, who showed that it behaves as a typical NDPE herbicide in that it causes light and O₂-dependent necrosis, chlorophyll degradation and lipid peroxidation. Camilleri et al (1988) demonstrated that the phthalide DPEs induce similar symptoms of phytotoxicity (rapid chlorosis and necrosis) to those elicited by NDPEs. They were also
Figure 2.1 Structures of DPE I and the Phthalide DPEs.
shown to cause lipid peroxidation in French bean (*Phaseolus vulgaris* cv. Prince) as measured by ethane production. The phthalide DPEs are a useful tool for mode of action studies since their redox properties preclude reduction of the herbicide molecule by the photosynthetic electron transport chain.

In this chapter use has been made of two methods of detecting lipid peroxidation; measurement of ethane formation and the thiobarbituric acid assay.

Peroxidation of unsaturated membrane lipids via free radical reactions leads to the formation of lipid hydroperoxides (see Chapter 1), which are decomposed in the presence of metal ions in their lower oxidation state. In the case of ω-3 unsaturated fatty acid hydroperoxides eg. linolenic hydroperoxide, ethane is one of the products of this decomposition;

\[
R=\text{CH-CH}_\omega\text{-CH=CH-CH}_\omega\rightarrow H_\omega(\text{CH}_2)_m + R=\text{CH-CH=CH-CHO}
\]

\[
\text{ETHANE}
\]

\[
\text{equation 2.1}
\]

Riely and Cohen (1974) were the first to use ethane production as an index of lipid peroxidation, in spontaneously peroxidising mouse tissue. This assay has since been used by several groups to measure herbicide-induced peroxidation of plant membranes.

Another product of membrane lipid peroxidation is malondialdehyde
(MDA). Detection of MDA is probably the most widely used technique for assaying membrane lipid peroxidation. The assay is based on the reaction of MDA with thiobarbituric acid. One molecule of MDA reacts with two molecules of thiobarbituric acid with the elimination of two molecules of water to yield a pink pigment with an absorption maximum at 532 nm (Bird and Draper, 1984). It is known, however, that numerous substances interfere with the thiobarbituric acid reaction. These include haemoproteins and transition metals associated with biological membranes, which enhance colour formation. Tissue aldehydes and sugars also react with thiobarbituric acid forming a chromophore that absorbs at 537 nm (Beuge and Aust 1978). Provided that care is taken to prevent such interference this method of assessing lipid peroxidation is very useful because of its simplicity and sensitivity.
2.2 Materials and Methods.

2.2.1 Culture Conditions.

*Chlamydomonas reinhardtii.*

Cultures of *C. reinhardtii* were obtained from Dr. P. Bennoun, Institut de Biologie Physico-Chemique, Foundation Edmond de Rothschild, Paris. Suspension cultures were grown in TAP medium (Gorman and Levine, 1965) on an orbital shaker at 25°C, under light of intensity 200 Wm⁻² and subcultured every 5 days.

*Scenedesmus obliquus.*

Cultures of *S. obliquus* were obtained from Dr. N. Bishop, Oregon State University. Suspension cultures were grown on modified Kessler's medium (Bishop and Senger, 1971a) containing 0.25% (w/v) yeast extract (Bishop and Senger, 1971b). The cultures were grown at 22°C at a light intensity of 0.27 Wm⁻². All cultures were grown in the presence of 0.5% (w/v) glucose in order to duplicate the conditions described by Bowyer et al (1989). The cells were subcultured every 4 days.

2.2.2 Determination of Herbicide Effects on Chlorophyll a Content.

*Chlamydomonas reinhardtii.*

Five day old cultures were diluted with fresh medium to contain 10⁶ cells per ml. 10 ml aliquots were incubated with herbicides in
25 ml conical flasks stoppered with cotton wool. 1 ml samples were withdrawn from the flasks at various time intervals for assay. For each treatment, the Chl a content of at least 2 separate flasks was determined.

To determine Chl a content the samples were centrifuged and pigments extracted from the algal pellets by heating for 6 min in 96% ethanol at 80°C (van Rensen, 1975). The Chl a content was estimated spectrophotometrically by measuring the absorbance at 665 and 649 nm, in a 1 cm pathlength cuvette, using the equation of Lichtenthaler and Wellburn (1983);

\[
\text{Chl a} = 13.95 A_{665} - 6.88 A_{649}
\]

in µg per ml plant extract.

*S. obliquus.*

In order to compare herbicide-induced bleaching with herbicide induced ethane formation, similar conditions to those used for the latter by Bowyer et al (1989) were employed for determining Chl a bleaching. After 4 days growth, cultures were harvested and resuspended in fresh medium to give 30 mg wet weight cells per ml. 5 ml samples of concentrated algal suspension in 10 ml conical flasks were set up on an orbital shaker at 25°C. The flasks were stoppered either with a gas tight rubber seal (Subaseal, No. 33) or with cotton wool, permitting aeration during incubation with the herbicide. The flasks were illuminated with light of intensity 200 W. m\(^{-2}\) throughout the experiment. At intervals, 0.5 ml samples of the suspension were
removed by hypodermic syringe and their Chl a content determined as above. For each treatment, the Chl a content of at least 2 separate flasks was determined.

2.2.3 Hydrocarbon Formation in Herbicide Treated Algae.

For measurements of ethane formation arising from lipid peroxidation, 5 ml of concentrated cell suspension (30 mg wet weight cells per ml) was placed in a 10 ml conical flask and the appropriate herbicide added. The flask was sealed with a Subaseal, No. 33 rubber seal. If required, flasks were flushed for 10 min with air, oxygen or nitrogen. The flasks were incubated on an orbital shaker at 25°C under light of intensity 200 Wm⁻². At intervals, 0.5 ml samples of head space gas were removed from the flasks using a gas tight syringe, and analysed by gas chromatography using a Pye 104 Gas Chromatograph. The column was filled with activated alumina and was maintained at 110°C. The carrier gas (N₂) flow rate was 40 ml.min⁻¹. The column was calibrated using 1 ppm ethane in N₂ (Argo International). Ethane production was calculated from integrated peak areas using a value of 11 ml for the total volume of the headspace in each flask. Each time point was determined from the mean of the ethane formed in three separate flasks per treatment.
2.2.4 Oxygen Uptake and Evolution.

Rates of oxygen uptake or evolution by cell suspensions were measured at 20°C, using a Clark type O$_2$ electrode (Rank Brothers, Cambridge) and illumination was provided by a 150 W spotlight filtered by a Schott RG 610 red filter and 10 cm of water, giving saturating light intensity.

2.2.5 Preparation of Chloroplast Fragments from Pea.

The presence of sucrose interferes with the MDA assay, so methods of preparing chloroplast fragments and intact chloroplasts employing buffers containing sorbitol were used. Chloroplast fragments were prepared essentially according to the method of Ensminger and Hess (1985a), as described below.

Peas (Pisum sativum cv. Feltham first) were grown in Fisons Levington compost in a glasshouse for 2 weeks. 10 g of pea leaves were homogenised in an Atomix blender in 100 ml of 17 mM Tris.HCl buffer pH 7.4 for 30 s. The homogenate was filtered through 8 layers of muslin and centrifuged at 2,000 x g for 1 min. The pellet was discarded and the supernatant centrifuged at 10,000 x g for 10 min. The pellet obtained was resuspended in 17 mM Tris.HCl buffer, pH 7.4.

For experiments, the chloroplast fragment suspension was diluted to give a final concentration of 50 μg chlorophyll ml$^{-1}$. After appropriate additions, the suspensions were incubated under white light of intensity of 430 Wm$^{-2}$, provided by 2 x 150 W spotlights.
2.2.6 Preparation of Intact Chloroplasts from Pea.

Intact chloroplasts were prepared, as follows, by the method of Nakatani and Barber (1977), as described below.

35 g of pea leaves were harvested and homogenised in a modified Atomix blender (Kannangara et al., 1977) for 3 s in 100 ml of ice-cold 0.33 M sorbitol, 0.2 mM MgCl₂, 20 mM MES adjusted to pH 6.5 with Tris. The resulting slurry was filtered through 10 layers of muslin, the first 2 layers being separated by a thin layer of cotton wool. The filtrate was centrifuged at 2,200 x g (in a swing-out rotor) for 30 s. The supernatant was discarded and the pellet resuspended in 0.33 M sorbitol, pH 7.5 and centrifuged at 2,200 x g for 20 s. The supernatant was aspirated and the soft, upper portion of the pellet carefully removed and discarded before finally resuspending the pellet in a small volume of 0.33 M sorbitol, pH 7.5.

The percentage intactness of the chloroplast preparation was determined using an oxygen electrode, by taking advantage of the fact that ferricyanide is unable to act as a Hill acceptor in intact chloroplasts (Heber & Santarius, 1970). Final concentrations in the electrode chamber were: 25 μg chlorophyll per ml, 10 mM D,L-glyceraldehyde, 1.5 mM K₃Fe(CN)₆, and the volume made up to 4 ml with assay buffer containing 0.33 M sorbitol, 2 mM EDTA, 1mM MgCl₂, 1mM MnCl₂, 50 mM HEPES, 5mM PPi and 0.5mM Pi. The rate of light-dependent oxygen evolution was determined in the presence of 2 mM NH₄Cl.

The chloroplasts were lysed by suspension in water for 1 min prior to the addition of an equivalent volume of double strength assay
medium. The rate of oxygen evolution was measured as before and the %
intactness determined from the equation;

\[
\% \text{ intactness} = 100 - \frac{\text{Rate (intact)} \times 100}{\text{Rate (shocked)}}
\]

equation 2.3

For the experiments described below, the chloroplasts were
suspended in 0.33 M sorbitol pH 7.5 to a concentration of 50 μg. ml⁻¹
in a final volume of 2 ml. After appropriate additions the
suspensions were illuminated under light of intensity 430 Wm⁻².

2.2.7 Assay for Malondialdehyde.

The MDA content of the samples was determined by the method of
Takahama and Nishimura (1975).

To 2 ml of the chloroplast suspension was added 0.5 ml 40% (w/v)
TCA, 0.25 ml 5 M HCl and 0.5 ml 2% (w/v) thiobarbituric acid. After
mixing, the sample was placed in a boiling water bath for 10 min, then
cooled on ice. The suspension was then centrifuged at 2,000 x g for 5
min. The absorbance of the supernatant was recorded at 532 nm and, 
after subtraction of the absorbance at 600 nm for non-specific
turbidity, the MDA content was calculated using an extinction
coefficient of 1.52 x 10⁵ M⁻¹. cm⁻¹ (Placer et al, 1966).
2.2.8 Preparation of Pea Thylakoid Membranes and Measurement of Electron Transport.

15 g of pea leaves were homogenised for 15 s in an Atomix blender at full speed at 4°C in 200 ml of 0.4 M sucrose, 10 mM MgCl$_2$, 30 mM HEPES and 0.5% (w/v) BSA, pH 7.3. The homogenate was filtered through 4 layers of muslin and centrifuged at 300 x g for 3 min. The supernatant obtained was centrifuged at 4000 x g for 4 min and the resulting chloroplast pellet resuspended in 0.4 M sucrose, 0.5% (w/v) BSA. The chlorophyll content of the suspension was determined by the method of Arnon (1949). For the measurement of electron transport, thylakoids were suspended to 50 µg per ml Chl in 50 mM Tricine buffer (pH 8.0) containing 50 mM NaCl, 5 mM MgCl$_2$, 2 mM KH$_2$PO$_4$, 0.1 mM MV, 2 mM NH$_4$Cl and 1 mM sodium azide. Rates of electron transport were determined in the oxygen electrode at 20°C under the conditions described in section 2.2.4.

2.2.9 FDA Staining of Chlamydomonas.

Five day old light grown cultures were diluted to contain 10$^6$ cells per ml and appropriate additions of herbicides made. The cultures were incubated for 12 h in the dark. The cells were then counted and the cultures transferred to light conditions for 4 h. The cells were again counted and diluted back to 10$^6$ cells per ml. FDA was added to each culture to a final concentration of 5 µg.ml$^{-1}$. The cultures were then incubated for a further 15 min in the dark.
Fluorescence was determined using a Perkin Elmer 3000 fluorescence spectrometer, with excitation at 487 nm and emission at 508 nm, as determined using fluorescein as a standard.

2.2.10 Tetrapyrrole Accumulation in Herbicide-Treated Algae.

For these experiments, Scenedesmus cells were grown as described in section 2.2.1, either under low light (0.27 Wm$^{-2}$) or high light (200 Wm$^{-2}$) for several generations. Four day old cultures were harvested and resuspended to 30 mg wet weight per ml in 5 ml of fresh medium, and treated with appropriate herbicides. Flasks were stoppered with cotton wool or Subaseals and incubated in the dark at 25°C on an orbital shaker for 24 h. Tetrapyrroles were extracted using a modification of the method of Rebeiz et al (1975). The cell suspension was centrifuged at 3,000 x g for 5 min and the pellet resuspended in 1 ml 0.1 M NH$_4$OH. This suspension was added dropwise to liquid nitrogen in a mortar and the cells ground to a fine powder. The powder was resuspended in 9 ml of acetone and centrifuged at 3,000 x g for 5 min. The supernatant was removed and the extraction repeated twice. The acetone extracts were combined and centrifuged at 39,000 x g for 10 min at 4°C. The supernatant was washed with 2 equal volumes of hexane. The final volume of the acetone extract was adjusted to 10 ml with acetone:0.1 M NH$_4$OH (9:1). Fluorescence emission spectra of the hexane-washed acetone extracts were obtained using a Perkin Elmer 3000 fluorescence spectrometer, with excitation and emission slit widths of 10 nm and an excitation wavelength of 398 nm.
2.2.11 Measurement of Growth Inhibition in *C. reinhardtii*.

Five day old cultures were diluted with fresh growth medium to give $10^5$ cells per ml. 5 ml aliquots were transferred to 10 ml conical flasks and appropriate additions of herbicides made. The flasks were incubated on an orbital shaker under light of intensity 200 Wm$^{-2}$. 0.1 ml samples were removed at time intervals and the cells counted using a haemocytometer. Cell numbers were determined in three separate flasks per treatment.
2.3 Results.

2.3.1 Light Activation of the NDPE Molecule.

In order to eliminate the possibility that the NDPE molecule itself acts as its own light receptor, an absorption spectrum of the NDPE herbicide, DPE I, was recorded. Figure 2.2 shows that DPE I absorbs in the UV region of the spectrum but not in the visible region (although not shown in the figure, DPE I did not absorb between 600 and 700 nm). This observation has also been made for the phthalide DPE's (data not shown). The action spectrum obtained by Ensminger & Hess (1985b) showed that cell death of Chlamydomonas in the presence of AFM occurred maximally at two wavelengths, 450 & 670 nm. Other groups have also found peaks of activity for DPE's in the blue and red regions of the spectrum (Vanstone à Stobbe, 1979; Sato et al, 1987). Thus it appears that an additional light receptor is required for NDPE toxicity.

2.3.2 The Role of Electron Transport.

A possible mode of action of the NDPE herbicides could be that they act in a similar fashion to DCMU by inhibiting electron transport. Another possibility is that the NDPE molecules act as electron acceptors in the same way as paraquat. In the case of the phthalide diphenyl ethers, the latter possibility may be discounted,
Figure 2.2 Absorption Spectrum of 10 \( \mu \text{M} \) DPE I in Isopropanol.

A similar trend was obtained using the DPE II. Both results suggest that a chemical factor is the presence of the...
since Camilleri et al (1988) have shown that the redox properties of these molecules preclude reduction by photosystem I.

Figure 2.3 shows the effects of DPE I and phthalide diphenyl ethers II-IV on photosynthetic electron transport from water to paraquat. The concentration of DPE I required to cause a 50% reduction in the rate of electron transport compared to the control \( I_{50} \) is 42 \( \mu \text{M} \). This value is in the same order of magnitude as the \( I_{50} \) previously published for DPE I (Bowyer et al, 1987). The inhibitory effect of phthalide DPE III was almost identical to that of DPE I. Phthalide DPE IV was slightly more potent \( I_{50} = 22 \ \mu\text{M} \), and phthalide DPE II had the greatest inhibitory effect \( I_{50} = 6 \ \mu\text{M} \). Thus the effects of these herbicides on photosynthetic electron transport are similar to those reported for other NDPE's such as nitrofen \( I_{50} = 40 \ \mu\text{M} \) and bifenox \( I_{50} = 16 \ \mu\text{M} \) (Fedtke, 1982).

2.3.3 Malondialdehyde Formation.

Figure 2.4 shows the effect of paraquat and DPE I on MDA formation in chloroplast fragments. Incubation with 100 \( \mu\text{M} \) paraquat under white light caused increased MDA formation compared to the control. No such increase was observed in the case of DPE I, and increasing the concentration of this herbicide from 10 to 50 \( \mu\text{M} \) had no effect (data not shown). This result is in agreement with the findings of Ensminger & Hess (1985a), who obtained a similar result using the NDPE, AFM. Such results suggest that a stromal factor or the presence of the
Figure 2.3 The Effects of DPE I and the Phthalide Diphenyl Ethers on Photosynthetic Electron Transport from Water to Paraquat in Isolated Pea Thylakoids. Each herbicide was added from an appropriate stock solution in DMSO to give 0.1% (v/v) of the solvent. (○) DPE I, (♦) phthalide DPE II, (□) phthalide DPE III, (□) phthalide DPE IV.
Figure 2.4 Malondialdehyde Formation in Pea Chloroplast Fragments. Illuminated chloroplast fragments were incubated in the presence of 0.1% (v/v) DMSO (○), 10 μM DPE I (□), 0.1 mM MV (◇).
chloroplast envelope itself, are required in order to elicit a DPE herbicidal effect. In order to test this hypothesis, the effects of DPE I on intact chloroplasts were determined.

Suspensions of 60-80% intact chloroplasts could be obtained by the method described in section 2.2.6. However, incubation of the intact chloroplast suspensions with DPE I or paraquat (Fig. 2.5) did not result in increased MDA formation. It is surprising that paraquat did not elicit lipid peroxidation in intact chloroplasts since it had such an obvious effect on chloroplast fragments. Perhaps paraquat is unable to rapidly penetrate intact chloroplasts. However, Camilleri and Williamson (unpublished data) observed that paraquat induced ultrastructural effects on intact chloroplasts within 15 min of addition of the herbicide. Alscher & Strick (1984) observed that acifluorfen induced inhibition of CO$_2$-dependent oxygen evolution in intact spinach chloroplasts. This inhibition occurred within minutes of addition of the NDPE, suggesting that these herbicides also penetrate the chloroplast envelope quite easily. Bowyer et al (1987), reported that 50 μM DPE I completely inhibited CO$_2$-dependent O$_2$ evolution within 4 min of its addition to an illuminated chloroplast suspension. Therefore, it seems unlikely that the inability of DPE I to induce lipid peroxidation as measured by MDA formation is due to lack of entry of the herbicide into the chloroplast. One possible explanation for the results observed with paraquat and DPE I might involve the action of chloroplast protective mechanisms against lipid peroxidation. Such protection would be greater in the intact chloroplast. For example, the antioxidants glutathione and ascorbate are present in millimolar concentrations in the chloroplast stroma.
Figure 2.5 Malondialdehyde Formation in Intact Pea Chloroplasts.  Illuminated chloroplasts were incubated with 0.1% (v/v) DMSO (□), 10 μM DPE I (○), 0.1 mM MV (◇).
(Gillham & Dodge, 1986), which would be lost on preparation of chloroplast fragments. Thus, paraquat might have a more rapid peroxidising effect in chloroplast fragments than in intact chloroplasts. Similarly, although NDPEs may penetrate the intact chloroplast and cause inhibition of CO$_2$-dependent oxygen evolution, substantial lipid peroxidation might be delayed by the protective mechanisms of the intact chloroplast.

2.3.4 The Effects of DPEs in *Chlamydomonas reinhardtii*.

Kunert & Boger (1981) showed that in *Scenedesmus*, simultaneous incubation of cells with DCMU suppressed the peroxidising effects of the NDPE oxyfluorfen. In order to see whether this was the case in other algae, the effect of DPE I on *Chlamydomonas reinhardtii* was investigated. *Chlamydomonas reinhardtii* was chosen since a double mutant lacking both PSI & PSII activity was available. Bowyer *et al* (1987) were able to show with barley mutants lacking either PSI or PSII activity that neither photosystem alone was essential for NDPE toxicity. However, their results did not preclude the possibility that either PSI or PSII is required.

Figure 2.6 shows the effect of various concentrations of paraquat on Chl a content of *C. reinhardtii*. *C. reinhardtii* appeared to be very sensitive to paraquat, 10 μM causing complete destruction of Chl a by 48h after addition of the herbicide. 0.1 μM paraquat had no effect on chlorophyll content over the time period tested (96 h), but 1 μM paraquat led to complete destruction of Chl a after an initial
Figure 2.6 The Effects of Paraquat on the Chlorophyll a Content of C. reinhardtii.
Five day old cultures were diluted with fresh growth medium to give $10^5$ cells per ml. Ten ml aliquots were incubated under light with 0.1% (v/v) DMSO (□), 0.1µM MV (■), 1 µM MV (○), 10 µM MV following 30 min incubation with 10 µM DCMU (●), 10 µM MV (◇), 10 µM MV following 30 min incubation with 10 µM DCMU (◆).
accumulation. Incubation with 10 μM DCMU afforded some protection against the bleaching effect of 10 μM paraquat, but suppressed the initial phase of Chl a accumulation seen in the presence of 1 μM paraquat alone. As is shown in fig. 2.5, DCMU itself has an inhibitory effect on the rate of Chl a accumulation in light-grown cultures.

*C. reinhardtii* is able to synthesise chlorophyll in the absence of light. Figure 2.7 shows the effect of 50 μM DPE I on chlorophyll accumulation in the dark. A high concentration of DPE I was used in this experiment since 10 μM DPE I did not prevent (but delayed) chlorophyll accumulation under light conditions (see figure 2.8). Under dark conditions, such accumulation appears to be completely inhibited by DPE I. This may reflect an inhibition of chlorophyll synthesis or inhibition of growth. Bowyer et al (1989) showed that DPE I and oxyfluorfen completely inhibited the heterotrophic growth of *Scenedesmus* in darkness. When the algal cells were incubated with 10 μM DPE I under light, there appeared to be a small bleaching effect during the first 24 h, after which the cultures seemed to recover and began to accumulate Chl a (Figure 2.8). When the herbicidal concentration was increased to 50 μM, there was a more marked bleaching effect over the first 24 h, but again the cultures appeared to recover and slowly began to accumulate Chl a. This result might suggest that the herbicide is being metabolised by *C. reinhardtii*. Preincubation with 10 μM DCMU before addition of both concentrations of DPE I appeared to cause protection from bleaching over the first 24 h. However, under these conditions the cultures did not recover, and bleaching occurred after 48 h.
Figure 2.7 The Effect of DPE I on the Chlorophyll a Content of C. reinhardtii in the Absence of Light.
Five day old cultures were diluted with fresh growth medium to contain $10^6$ cells per ml. Ten ml aliquots were incubated with 0.1% (v/v) DMSO (□), 50 μM DPE I (○), in darkness.
Figure 2.8 The Effects of DPE I and DCMU on the Chlorophyll a Content of *C. reinhardtii*.

Five day old cultures were diluted with fresh growth medium to contain $10^6$ cells per ml. Ten ml aliquots were incubated under light with 0.2% (v/v) DMSO ($\square$), 10 μM DCMU ($\blacksquare$), 10 μM DPE I ($\bigcirc$), 10 μM DPE I following 30 min incubation with 10μM DCMU ($\blacklozenge$), 50μM DPE I ($\bigcirc$), 50 μM DPE I following 30 min incubation with 10μM DCMU ($\bullet$).
DPE I inhibited growth of *C. reinhardtii* in the light (Fig. 2.9). Again, it appeared that the cultures started to recover from the initial effects of the herbicide. DPE I similarly inhibited growth of *C. reinhardtii* cultures in the dark (result not shown).

Ensminger *et al* (1985) used the stain fluorescein diacetate to detect AFM-induced cell death in *Chlamydomonas eugametos* after 2 h exposure to light, following a 12 h dark incubation period. Table 2.1 shows the effect of 10 µM oxyfluorfen on *C. reinhardtii*. Oxyfluorfen caused an inhibition of growth after 12 h in the dark. However, after a subsequent 4 h light incubation, and dilution of the culture to contain 1 x 10^5 cells ml^-1, no cell death could be detected by FDA staining. Similar results were obtained with DPE I (not shown).

Figure 2.10 shows that DPE I does not induce ethane formation in *C. reinhardtii*. A similar observation was made with 10 µM concentrations of oxyfluorfen and nitrofen (not shown). This result suggests that NDPE herbicides are unable to induce lipid peroxidation in *C. reinhardtii*. It might be the case that *C. reinhardtii* cells contain unusually high concentrations of antioxidants. However, the sensitivity of the alga to paraquat would suggest that this is not so.

In view of the results obtained with the wild-type, it was decided not to proceed with studies on the mutant lacking PSI and PSII. The only light-dependent herbicidal effect of DPE I found was short-term bleaching, from which the cultures quickly recovered. Inhibition of chlorophyll accumulation and/or growth could occur in the absence of light. Therefore, it would seem that *C. reinhardtii* is not a suitable organism with which to study the role of photosynthesis in NDPE toxicity.
Figure 2.9 The Effect of DPE I on the Growth of *C. reinhardtii* Cultures.
Five day old cultures were diluted with fresh growth medium to give $10^6$ cells per ml. Five ml aliquots were incubated under light with 0.1% (v/v) DMSO (○), 10 μM DPE I (●).
Table 2.1 The Effects of Oxyfluorfen on *C. reinhardtii* as Determined by FDA Staining.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>x $10^6$ cells per ml after 12 h dark incubation</th>
<th>Relative fluorescence after 4 h light incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>4.54</td>
<td>2.27</td>
</tr>
<tr>
<td>10μM oxyfluorfen</td>
<td>1.02</td>
<td>2.17</td>
</tr>
</tbody>
</table>

Boiled cells showed no fluorescence.
Figure 2.10 Herbicide-Induced Ethane Formation in *C. reinhardtii*. Five day old cultures were resuspended in fresh growth medium to give a wet weight of 0.03 g per ml. Five ml aliquots were incubated with 0.2% (v/v) DMSO (●), 10 μM MV (○), 10 μM DPE I (■), 10 μM DPE I following 30 min incubation with 10 μM DCMU (□). The cells were sealed into conical flasks and incubated under light.
2.3.5 The Effects of DPEs in *Scenedesmus obliquus*.

Bowyer *et al* (1989) showed that incubation with 10 μM DPE I caused ethane formation in *Scenedesmus obliquus*, a process which was suppressed by the photosynthetic electron transport inhibitors DCMU and prometyn. Furthermore, very little lipid peroxidation was induced by DPE I in mutants of *Scenedesmus* lacking PSI, PSII or the cytochrome b₆f complex. These results support the idea that photosynthetic electron transport has an essential role in the toxic action of DPEs in *Scenedesmus*. Phthalide DPE II was shown to induce marked ethane formation, even though pulse radiolysis studies show that it cannot be reduced by PSI, and concurrent treatment with DCMU provided complete protection. This suggests that the essential role of photosynthetic electron transport in *Scenedesmus* is not to act as a reductant for the herbicide as suggested by Kunert and Boger (1981).

Figure 2.11 examines the effect of oxygen on Phthalide DPE II and paraquat-induced bleaching of Chl a in *Scenedesmus*, incubated in sealed flasks. Figure 2.11B shows that no bleaching occurred with paraquat in the absence of oxygen and little or no bleaching with Phthalide DPE II. Bowyer *et al* (1989) showed that with either DPE I or Phthalide DPE II, flasks flushed with air showed greater rates of ethane formation than those flushed with nitrogen. The presence of oxygen is therefore an important factor in DPE toxicity. However the conditions employed by Bowyer *et al* (1989) for measuring DPE-induced ethane formation (in unflushed flasks) would be likely to lead to conditions of limiting oxygen. Under the conditions of the experiments in which the algae were suspended in heterotrophic growth
Figure 2.11 The Effects of Oxygen on Paraquat and Phthalide DPE II -Induced Bleaching of Chlorophyll a in S. obliquus.
Cells were incubated in sealed flasks with 10μM phthalide DPE II (◇), 2mM MV (□), 0.1% (v/v) DMSO (○). At 24 h intervals the flasks were flushed for 10 min with air (A) or nitrogen (B).
medium, respiration would lower the oxygen concentration, particularly if photosynthetic oxygen evolution is chemically blocked in flasks sealed with rubber seals. To try and overcome this problem the flasks were flushed with air every 24 h (figure 2.12). However, under these conditions DCMU still protected against DPE I-induced ethane formation. Gassing with oxygen appeared to cause less DPE I-induced ethane formation and again the protective effect of DCMU was maintained (figure 2.12B). The rate of respiration of S. obliquus cells in fresh medium at the concentration used for ethane formation studies was then measured in the oxygen electrode and found to be 100 μmol O₂.mg Chl⁻¹.h⁻¹. The total oxygen content of a flask equilibrated with air would be about 95 μmol. Therefore, in the absence of photosynthesis, all the oxygen in the flask could be consumed by respiration in approximately 3 h. Thus, flushing with air every 24 h may not be sufficient to overcome protection by DCMU against DPE I-induced lipid peroxidation. In cultures not treated with DCMU, there is net oxygen release as a result of photosynthesis.

The assay for ethane production requires that the cultures are sealed, preventing exchange of gases. This is not essential in experiments designed to measure chlorophyll bleaching. Figure 2.13 shows the effect of DPE I on the Chl a content of Scenedesmus. The flasks were lightly stoppered with cotton wool and shaken at high speed on an orbital shaker (200 revs per min), in order to achieve high aeration. After a lag period of about 30 h net bleaching was induced by both DPE I and paraquat in the vigorously aerated cultures. In rubber sealed flasks (figure 2.14), as was found with phthalide DPE II, under conditions of low O₂ (figure 2.11B) no chlorophyll bleaching
Figure 2.12 The Effects of Air and Oxygen on DPE I-Induced Ethane Formation in *S. obliquus.*

Cells were incubated in sealed flasks with 0.2% (v/v) DMSO (■), 10 μM DPE I (○), 10 μM DCMU (□), 10 μM DPE I following 30 min incubation with 10 μM DCMU (●). Flasks were flushed at 24 h intervals for 10 min with air (A) or oxygen (B).
Figure 2.13 The Effects of DPE I, DCMU and Paraquat on the Chlorophyll a content of *S. obliquus*. Four day old cultures were resuspended to give a wet weight of 0.03 g per ml. Five ml aliquots were incubated with 0.2% (v/v) DMSO (□), 10 μM DCMU (■), 2mM MV (♦), 10 μM DPE I (○), 2 mM MV following 30 min incubation with 10 μM DCMU (◇), 10 μM DPE I following 30 min incubation with 10 μM DCMU (●). The flasks were lightly stoppered with cotton wool and incubated under light.
Figure 2.14 The Effects of DPE I and DCMU on the Chlorophyll a Content of *S. obliquus* Under Conditions of Limiting Oxygen.

Four day old cultures were resuspended in fresh growth medium to give a wet weight of 0.03 g per ml. Five ml aliquots were incubated in sealed flasks with 0.2% (v/v) DMSO (□), 10 μM DCMU (■), 10 μM DPE I (○), 10 μM DPE I following 30 min incubation with 10 μM DCMU (●).
was observed although DPE I did inhibit chlorophyll accumulation. This result is interesting since under identical conditions, DPE I induced ethane formation in *Scenedesmus*. Perhaps more vigorous aeration is needed to elicit chlorophyll bleaching than lipid peroxidation. Figure 2.13 also shows that in vigorously aerated suspensions, DCMU inhibited chlorophyll bleaching in cells treated with paraquat, but not with DPE I. One possible explanation why DCMU is able to protect *Scenedesmus* cells from DPE I-induced bleaching but unable to prevent DPE I-induced chlorophyll bleaching, might be that these destructive processes are initiated by different mechanisms. Thus, perhaps singlet oxygen could be responsible for chlorophyll destruction which might not be prevented by DCMU, unlike lipid peroxidation which could be initiated by a different toxic oxygen species. Wettlaufer et al (1985) suggested that the presence of DPEs leads to reduced activities of the Calvin cycle enzymes fructose-1,6-bisphosphatase and glyceraldehyde-3-phosphate dehydrogenase, thus inhibiting carbon fixation. Electrons from PSI might then be channelled to molecular oxygen, generating H$_2$O$_2$. If DPEs were also able to block the reduction of H$_2$O$_2$ (say for example, by inhibiting the ascorbate peroxidase/glutathione reductase pathway), then H$_2$O$_2$ would accumulate in illuminated chloroplasts. DCMU would then prevent DPE I-induced lipid peroxidation by blocking the accumulation of H$_2$O$_2$. In order to test this hypothesis, the effects of exogenous H$_2$O$_2$ and catalase on lipid peroxidation in *Scenedesmus* were determined (figure 2.15). H$_2$O$_2$ was able to induce lipid peroxidation in *Scenedesmus*, but only in the presence of DCMU. DCMU blocks the reduction of H$_2$O$_2$ by the ascorbate peroxidase/glutathione reductase
Figure 2.15  The Role of Hydrogen Peroxide in DPE I -Induced Lipid Peroxidation in *S. obliquus.*
Four day old cultures were resuspended in fresh growth medium to give a wet weight of 0.03 g per ml. Five ml aliquots were incubated with 0.2% (v/v) DMSO (◇), 1.5 mM H₂O₂ (◆), 10 μM DPE I (●), 10 μM DPE I following incubation for 30 min with 10 μM DCMU (□), 1.5 mM H₂O₂ following incubation for 30 min with 10 μM DCMU (○), 10 μM DPE I + 800 units / ml catalase (■).
pathway (this pathway would be active in the absence of DPE I), by preventing the photoreduction of dehydroascorbate. It has been shown that on addition of $H_2O_2$ to intact chloroplasts in the dark, the capacity to photoreduce $H_2O_2$ is lost, but addition of the same amounts of $H_2O_2$ to illuminated chloroplasts is without effect (Anderson et al, 1983; Asada & Badger, 1984). The addition of catalase did not inhibit DPE I-induced lipid peroxidation. This result argues against the above hypothesis since catalase would be expected to eliminate DPE I-induced lipid peroxidation if it were due to the accumulation of $H_2O_2$, assuming that exogenous catalase is able to significantly lower the intracellular concentration of $H_2O_2$.

2.3.5 Tetrapyrrole accumulation in *Scenedesmus obliquus*.

The results of Matringe and Scalla (1987b, 1988a) showed that AFM induced abnormal tetrapyrrole accumulation in non-chlorophyllous soybean cells, which could then photosensitize singlet oxygen formation leading to cellular damage. The ability of DPE I to induce tetrapyrrole accumulation in *Scenedesmus* was examined under various conditions. The fluorescence emission spectra (figure 2.16) of hexane-washed acetone extracts of *Scenedesmus* show that the cells incubated with DPE I in darkness accumulate a pigment (figure 2.16 B) which had the same spectral characteristics as protoporphyrin IX (figure 2.16 A), that is an emission maximum of 628 nm and an excitation maximum of 398 nm. In the algal extracts, the 628 nm emission is seen as a shoulder on a much larger emission band peaking
Figure 2.16 DPE I - Induced Tetrapyrrole Accumulation in S. obliquus.

Cells were incubated with 0.1% (v/v) DMSO (i), 10 µM DPE I (ii), 10 µM DCMU + 10 µM DPE I (iii) and 2mM MV (iv) for 24 h in the dark, then extracted into 9:1 acetone : 0.1 M NH₃OH. Fluorescence emission spectra were elicited by excitation at 398 nm.

A. Protoporphyrin IX. B. Wild-type cells, grown under low light intensity (0.27 Wm⁻²), incubated in flasks stoppered with cotton wool. C. Mutant cells lacking PSI grown under low light intensity, incubated in flasks stoppered with cotton wool. D. Wild-type cells grown under low light intensity, incubated in flasks stoppered with Subaseals. E. Wild-type cells grown under high light intensity (200 Wm⁻²), incubated in flasks stoppered with Subaseals.
at 670 nm, which is attributed to more polar chlorophyll derivatives. The accumulation of tetrapyrrole in the dark in *Scenedesmus* grown under dim light (intensity 0.27 W.m\(^{-2}\)) which had been treated with DPE I, was only seen in flasks lightly stoppered with cotton wool, and not in Subasealed flasks (figure 2.16 B and D). In the Subasealed flasks, an unidentified component with an emission maximum at 588 nm appeared, which was suppressed by DPE I. These experiments were not carried out with cells incubated with DPE I under light since Proto IX can be destroyed under these conditions. Thus Sandmann and Boger (1988) found no accumulation of Proto IX after treatment of autotrophic *Scenedesmus* cells with the peroxidising herbicide chlorophthalim, a cyclic imide herbicide which induces Proto IX accumulation in dark, heterotrophically grown *Scenedesmus* cells. Matringe and Scalla (1988b) have, however, shown that under certain conditions, porphyrin accumulation can be demonstrated in tissue incubated with DPEs under light.

DCMU slightly suppressed DPE I induced tetrapyrrole accumulation (figure 2.16 B and E). This could not be related to inhibition of photosynthetic electron transport since the incubations were carried out in darkness. This is in agreement with the results of Matringe and Scalla (1988b), who demonstrated that DCMU prevented AFM or LS 82-556 -induced tetrapyrrole accumulation in green cucumber cotyledons when incubated under light or dark conditions. In a mutant of *Scenedesmus* lacking PSI, tetrapyrrole accumulation was also induced by DPE I (figure 2.16 C). In contrast to cells grown under dim light (dark grown cells), light grown cells incubated in darkness with DPE I in Subasealed flasks, did show tetrapyrrole accumulation but to a
considerably lower extent than dark grown cells lightly stoppered with cotton wool. The rate of respiration in light grown cells (99 μmol O₂.g wet weight⁻¹.h⁻¹) was found to be almost 40% less than that of dark grown cells (158 μmol O₂.g wet weight⁻¹.h⁻¹) and thus the oxygen content of the sealed flasks would be less rapidly reduced by the light grown cells.

2.3.7 Singlet Oxygen and DPE I Toxicity in *Scenedesmus*.

In order to determine whether or not singlet oxygen is the toxic oxygen species involved in initiating DPE-induced lipid peroxidation, *Scenedesmus* cells were incubated with DPE I in the presence of compounds which would either enhance or reduce the effects of singlet oxygen. The lifetime of singlet oxygen is extended in D₂O (Kearns, 1979). Figure 2.17 shows that DPE I-induced lipid peroxidation was not enhanced in D₂O, rather it was reduced. The singlet oxygen quencher DABCO (Takahama & Nishimura, 1975), however, appeared to provide some protection against DPE I activity. Such a protective effect was not observed when cells were incubated with paraquat and DABCO (Fig. 2.18). The lack of effect of DABCO on paraquat-induced lipid peroxidation (which is caused by oxygen radical attack rather than singlet oxygen) suggests that the suppression observed with DPE I is specific and indicative of the involvement of singlet oxygen.
Figure 2.17 The Role of Singlet Oxygen in DPE I -Induced Ethane Formation in S. obliquus.
Four day old cultures were resuspended in fresh growth medium made up in H₂O or 90% D₂O to give a wet weight of 0.03 g per ml. Five ml aliquots were incubated with 0.1% (v/v) DMSO (●), 0.1% (v/v) DMSO (D₂O) (◇), 0.1% DMSO + 10 mM DABCO (■), 10 µM DPE I (□), 10 µM DPE I (D₂O) (●), 10 µM DPE I + 10 mM DABCO (□).
Figure 2.18  The Effect of DABCO on Paraquat-Induced ethane Formation in *S. obliquus.*

Four day old cultures were resuspended in fresh growth medium to give a wet weight of 0.03 g per ml. Five ml aliquots were incubated with 2mM MV (○), 10 mM DABCO (□), 2mM MV + 10 mM DABCO (■), control (●).
2.4 Discussion.

DPE I and the phthalide DPEs inhibit photosynthetic electron transport, but such inhibition as determined by $I_{50}$ values is at least one order of magnitude lower than that of commercial PSII herbicides, for example DCMU with $I_{50} = 0.24 \mu\text{M}$ (Kakkis et al., 1984). However, DPE I elicits necrosis and chlorophyll bleaching \textit{in vivo} at a concentration $10^2$ to $10^3$ times lower than that of DCMU and other PSII herbicides (Bowyer et al., 1987). Furthermore, Camilleri et al. (1988) showed that on foliar application, the phthalide DPEs are fast acting (1-2 days) whereas PSII inhibitors act over a relatively long period (5-10 days). Thus, although most diphenyl ethers can act as inhibitors of photosynthetic electron transport in isolated chloroplast thylakoids, this type of activity is not expected to play an important role in determining the phytotoxicity of these compounds. A similar conclusion was reached by Pritchard et al. (1980).

Unlike paraquat, DPE I did not induce MDA formation in chloroplast fragment preparations, suggesting that the mode of action of NDPEs is different to that of paraquat. This is confirmed by the fact that the phthalide diphenyl ethers have NDPE-like activity (Camilleri et al., 1988; Bowyer et al., 1987; and Figure 2.11), even though their redox properties preclude reduction of the molecule by PSI (Camilleri et al., 1988). Ensminger & Hess (1985) also reported that neither AFM, MC 15608 or oxyfluorfen elicited MDA formation in chloroplast fragments. These results suggest that DPE herbicides do not solely require electron transport processes for induction of lipid peroxidation and
the chloroplast thylakoids do not appear to be the primary site of DPE herbicidal activity. The findings of Kunert & Boger (1981), and Lambert et al (1984), however, suggest that in *Scenedesmus* at least, photosynthetic electron transport plays an essential role in DPE toxicity.

The results presented here and those of Bowyer et al (1989) suggest that the role of photosynthetic electron transport in DPE toxicity in *Scenedesmus* may be to provide oxygen. Oxygen is necessary for DPE-induced lipid peroxidation (Bowyer et al, 1989), and DPE-induced chlorophyll bleaching. Thus photosynthetic electron transport would have the dual role of generating oxygen and reducing the herbicide in the case of paraquat toxicity, but would only have the former role in the case of DPEs. This hypothesis would be consistent with the different effects of blocking photosynthetic electron transport on paraquat and DPE-induced chlorophyll bleaching in vigorously aerated cell suspensions. In experiments with barley seedlings, DPEs did induce ethane formation in mutants unable to evolve oxygen photosynthetically (Bowyer et al 1987 & Chapter 3). However, oxygen limitation is less likely in these experiments since leaves (unlike algae) were not submerged, and they were only sealed into small tubes to measure ethane formation for a 4 h period at each time point.

The experiments on tetrapyrrole accumulation further support the secondary role of photosynthetic electron transport in DPE toxicity in *Scenedesmus*. If, as seems likely, DPE toxicity is linked to tetrapyrrole accumulation, then since DPE I did elevate the amount of tetrapyrrole in the mutant lacking PSI, failure to observe ethane
formation in this mutant may be a secondary consequence of the mutation. Tetrapyrrole accumulation was, however, suppressed in algal suspensions in which oxygen would have been depleted as a result of respiration. It is known that oxygen is required as the oxidant in the oxidation of protoporphyrinogen to protoporphyrin (Porra & Falk, 1964). The increased accumulation of tetrapyrrole in light-grown versus dark-grown cells in Suba-sealed flasks could then be attributed to the higher respiration rate of the latter. Photosynthesis may also have a role in the provision of ATP and NADPH for porphyrin synthesis. Inhibition of photosynthesis could reduce ATP and NADPH levels in chloroplasts which could in turn depress ALA synthesis (Kannangara et al, 1984). Such a role would, however, be of greater importance in completely autotrophic cells.

Protection against DPE I-induced lipid peroxidation by DABCO and the lack of protection by catalase, suggest that singlet oxygen is the active oxygen species involved in this process. This would be consistent with the hypothesis of Matringe & Scalla (1988a). Thus, for NDPE toxicity oxygen is required in both the synthesis of tetrapyrroles and as the substrate for singlet oxygen formation photosensitised by the tetrapyrrole; the role of photosynthesis is to provide a source of oxygen from water oxidation under conditions of oxygen limitation. Haworth & Hess (1988) reported that oxyfluorfen caused the generation of singlet oxygen in intact thylakoid membranes. This could not be as a result of tetrapyrrole accumulation, since these preparations are obviously devoid of tetrapyrrole precursors, to say nothing of ATP and the enzymes of the biosynthetic pathways (Smith & Rebelz, 1979). As shown in this chapter, DPE I did not induce lipid
peroxidation in chloroplast fragment preparations containing thylakoids, although Gillham and Dodge (1986) have reported that acifluorfen promotes lipid peroxidation in illuminated thylakoids.

The experimental conditions of Kunert & Boger (1981) (autotrophically grown cells bubbled with air) would be less likely to lead to oxygen limitation under conditions of inhibited photosynthesis, yet they still observed an inhibition by DCMU of oxyfluorfen-induced lipid peroxidation and, in contrast to the results presented here, chlorophyll bleaching. However, in a later paper, Kunert et al (1985) observed that the destruction of cytochromes induced by oxyfluorfen was only slightly diminished by DCMU. This led the authors to propose that protection by DCMU might be related to a lower oxygen content of the cells.

The results obtained with *C. reinhardtii* are difficult to explain, since after 48 h incubation, rather that protect the alga from DPE I-induced chlorophyll bleaching, DCMU seemed to enhance its effect. This might be explained by the cumulative toxic effect of the herbicides. Incubation of *C. reinhardtii* with DCMU and DPE I did not induce lipid peroxidation, but again this could be explained by conditions of oxygen limitation. Inhibition of growth of *Chlamydomonas* cultures in the dark may be related to the inhibition of chlorophyll accumulation. *Chlamydomonas*, like *Scenedesmus*, synthesises chlorophyll and assembles a complete photosynthetic apparatus in darkness. Thus if growth is necessarily linked to this process, then DPE's would inhibit growth if the induced tetrapyrrole accumulation results from an inhibition of chlorophyll biosynthesis. This is consistent with the observation that LS82-556 does not inhibit the
dark heterotrophic growth of a non-chlorophyllous soybean cell suspension (Matringe & Scalla, 1987a). It is also possible that DPEs inhibit both chlorophyll and haem biosynthesis, which would prevent the assembly of a complete respiratory chain needed for aerobic heterotrophic growth.
CHAPTER 3

The Role of Carotenoids in Diphenyl Ether Phytotoxicity.

Carotenoids are a class of lipophilic compounds that are important in the photosynthesis of higher plants. Carotenoids have a basic C35 linearly condensed structure, which may be identified by deamidation, cyclization, and introduction of oxygen reactions. Carotenoids are biosynthesized from mevalonate with a conserved toosan-3-pyrophosphate (TPP) and mevalonate-5-pyrophosphate reductase precursor. A series of condensation reactions yields precursors that are then converted to the probable C35 precursor of all other carotenoids. The biosynthesis of carotenoids has been reviewed by Goodwin (1970b).

The primary action of the 2-phenylbenzylamine type diphenyl ethers in the 2-pyrimidinyl inhibition of phytoene dehydrogenase (Lambert et al. 1980) acting in a similar manner to pentachlorophenol (PCP) and other diphenyl ethers. Such inhibitory activity is, however, not a property of the corresponding pentaphenyl ethers.

In photosynthesizing plants, carotenoids are vital in the absorption of both CHL and "O2". Photosynthesis of CHL and "O2" generation requires complex systems which are probably elucidated by heat digestion.
3.1 Introduction

The carotenoids represent one of the most important groups of natural pigments. Carotenoids are a class of hydrocarbons (carotenes) and their oxygenated derivatives (xanthophylls). They are important constituents of chloroplasts and are responsible for almost all the yellow to red colours found in the flowers and fruits of higher plants. Structurally, carotenoids have a basic C₄₀ tetraterpenoid skeleton, which may be modified by desaturation, cyclization and introduction of oxygen functions. Carotenoids are biosynthesised from mevalonic acid which is converted to isopentenyl pyrophosphate (IPP), the basic C₆ isoprene precursor. A series of condensation reactions yields phytoene, the probable C₄₀ precursor of all other carotenoids. The biosynthesis of carotenoids has been reviewed by Goodwin (1980a).

The primary action of the m-phenoxylbenzamide type diphenyl ethers is the light-independent inhibition of phytoene desaturation (Lambert and Boger, 1983), acting in a similar manner to norflurazon (see figure 3.1). Such inhibitory activity is, however, not a property of the peroxidizing p-nitrodiphenyl ethers.

In photosynthesising plants carotenoids are vitally important as quenchers of both ^3Chl and ^1O₂. Quenching of ^1O₂ and ^3Chl generates triplet carotenoid which is probably de-excited by heat emission.

\[
^3\text{Chl} + \text{Car} \rightarrow \text{Chl} + ^3\text{Car} \quad \text{equation 3.1}
\]

\[
^3\text{Car} \rightarrow \text{Car} + \text{heat} \quad \text{equation 3.2}
\]
FIGURE 3.1 The biosynthesis of carotenoids showing inhibition of phytoene desaturase
Orr and Hess (1982a) proposed the following model for the mechanism of action of AFM:

\[
{\cdot}O_2 + \text{Car} \rightarrow {\cdot}O_2 + {\cdot}\text{Car} \quad \text{equation 3.3}
\]

Light absorbed by the carotenoids would activate the AFM molecule and the carotenoid molecule involved would be destroyed following the activation of the herbicide. The light-activated form of the AFM molecule would then be involved directly or indirectly, in initiation
of a radical chain reaction through the abstraction of a hydrogen atom from the divinyl methane structure in PUFA(H).

Carotenoids in flower petals are localised in plastids called chromoplasts. These plastids have irregular shape and in many cases develop from chloroplasts, carotenoids accumulating as the lamellar structure breaks down. This change is apparently irreversible. There are at least five different structural categories of chromoplast: globulous, tubulous, reticulo tubulous, membranous and crystalline (Whatley and Whatley, 1987).

The structure of daffodil chromoplasts has been well documented (Goodwin, 1980b). The mature organelle is composed solely of numerous concentric membranes. Liedvogel et al (1976) found that the lipid and protein patterns of these membranes resemble those of the plastid envelope more closely than those of thylakoids and proposed that the peripheral membranes originated from the plastid envelope. The plastid envelope has been shown to be one of the earliest sites of DPE action (Derrick et al, 1988) and thus, the chromoplast system should be a useful model for studies on the mode of action of these herbicides.

Bowyer et al (1987) showed DPE I activity in mutants of barley lacking PSI or PSII activity but were unable to eliminate the possibility that NDPE toxicity requires electron transport through PSI or PSII. In order to eliminate the possible involvement of photosynthetic electron transport in NDPE action, some of the experiments to be described in this chapter were performed on the alb-e's albino mutant of barley, which is unlikely to be capable of carrying out this process. This lethal mutant has a specific block in
the conversion of protoporphyrin IX to protochlorophyllide and has reduced levels of porphyrin and carotenoid synthesis in general (Gough, 1972). If this mutant is unable to synthesise chlorophyll then it is reasonable to assume that it does not have an operating photosynthetic electron transport chain.

Chrysanthemum

Chrysanthemum flowers were chosen for these experiments because of their all-year-round availability. Horticultural chrysanthemum plants (‘Bright Golden Saucers Mix’) in pots were used. For studies on intact petals, detached open blooms with their cut sides in water were coated with a solution of chlorophyll in 0.01% (v/v) aqueous Triton X-100. Control plants were treated with 0.35% (w/v) DMSO, 0.01% (v/v) Triton X-100. The flowers were exposed to a light intensity of 600 μmol m⁻² s⁻¹ for 1 h. Damage was assessed visually.

For all experiments with chrysanthemum, only the outer portion of the petals were treated, or removed, for pigment extraction or chromatograph isolation. This was because the bases of the petals appeared slightly green and probably contained chlorophyll.

Bulbs of dwarf daffodils (Narcissus, cv. Golden Harvest) growing in the University of London Botanic Garden were treated as above.

Daffodils

The outer portion of each daffodil (Narcissus, cv. Golden Harvest) growing in the University of London Botanic Garden were treated as above and kept in a 16 h photoperiod with a light intensity of 100 μmol m⁻² s⁻¹ at 25°C. After 5 days the bulbs were sliced
3.2 Materials and Methods.

3.2.1 Treatment of Whole Plant Material.

**Chrysanthemum.**

Chrysanthemum flowers were chosen for these experiments because of their all-year round availability. Horticultural chrysanthemum plants ('Bright Golden Princess Anne') in pots were used. For studies on intact petals, detached open blooms with their cut stems in water were coated with a solution of herbicide in 0.01% (v/v) aqueous Triton X-100. Control plants were treated with 0.1% (v/v) DMSO, 0.01% (v/v) Triton X-100. The flowers were exposed to a light intensity of 600 ± 50 Wm⁻² for 51 h. Damage was assessed visually.

For all experiments with chrysanthemum, only the outer portions of the petals were treated, or removed for pigment extraction or chromoplast isolation. This was because the bases of the petals appeared slightly green and probably contained chlorophyll.

**Daffodil.**

The coronae of daffodils (*Narcissus* cv. Golden Harvest), grown in the University of London Botanic Garden, were treated as above.

**Barley**

Wild-type barley seeds ('Golden Promise') and the mutant *alb-e'c* were germinated and grown in vermiculite, with a 16 h photoperiod, light intensity 35 Wm⁻², at 25°C. After 7 days herbicide solutions
containing 0.01% (v/v) Triton X-100 were painted on to the seedlings and the light intensity increased to 200 Wm$^{-2}$. After appropriate periods of illumination, damage was assessed visually and shoots were removed for ethane determination.

3.2.2 Measurement of Ethane Formation.

Barley.

Two shoots per treatment were removed and sealed into a 100mm x 10mm diameter test tube with a Subaseal number 13 rubber seal. The tubes were illuminated with light of intensity 900 ± 100 Wm$^{-2}$ for 4 h. Sample heating was prevented using a water filled chromatography tank. The 4 h time period is included in the time after herbicide application. A 0.5 ml sample of headspace was removed using a gas tight syringe and analysed as in Chapter 2, section 2.1.3.

To obtain dry weights, the seedlings were dried at 70°C for 48 h.

Chrysanthemum.

Flowers were treated as in 3.2.1 and sealed into glass jars, 9 cm x 5.4 cm diameter, specially adapted with a septum. Each jar contained 10 ml of water, into which the cut stem was dipped. The jars were incubated under light of intensity 900 ± 100 Wm$^{-2}$. At 24 h intervals, 1 ml headspace was removed for analysis as above, and the lids removed to allow equilibration with air (so that the oxygen content in the jars did not become limiting). After 5 min, the jars were resealed and returned to the light.
3.2.3 Electron Microscopy.

Chrysanthemum Petal.

Sections of petal were fixed for 2 h in 4% (w/v) glutaraldehyde in 0.1M sodium cacodylate, 0.05M calcium chloride, pH 7.2. After washing for 1h in 0.1M sodium cacodylate, 0.05M calcium chloride, pH 7.2, the samples were post-fixed for 45 min in 1% (w/v) osmium tetrachloride in the same buffer. They were dehydrated through a series of ethanol dilutions (15 min each in 30%, 50%, 70%, 95% v/v ethanol:water, then three times for 10 min in absolute ethanol), infiltrated with propylene oxide (2 x 30 min) and left in 1:1 (v/v) propylene oxide:araldite medium mix resin overnight. The samples were then transferred to 100% araldite resin, which was allowed to polymerise for several days at 60°C. Electron microscopy was carried out at Shell Research Ltd. Ultrathin sections were cut on an ultratome, stained for 25 min with uranyl acetate solution, then 10 min in lead citrate solution and viewed under the electron microscope.

Chromoplasts.

Concentrated chromoplast suspensions (prepared as in section 3.2.5) were centrifuged at 16,500 x g and the pellet resuspended in 5% sodium alginate. The suspension was transferred to a syringe and extruded dropwise into 0.3 M calcium chloride solution. The resulting beads were left in 0.3 M calcium chloride for 30 min, then washed twice with distilled water. The alginate beads in which chromoplasts were trapped, were fixed and stained as above.
3.2.4 Extraction of Pigments and HPLC Analysis.

Barley.

*alk-e* and wild-type barley were grown in vermiculite under constant illumination (intensity 35 Wm$^{-2}$) at 25°C. Leaves were then harvested, cut into small pieces, and homogenised in 80% (v/v) acetone using a Polytron homogeniser. The homogenate was then centrifuged at 3,000 x g for 5 min. The supernatant was then centrifuged to a separating funnel and the pigments extracted by shaking with an equal volume of diethyl ether and two volumes of 10% (w/v) KCl. Extraction from the aqueous layer was repeated twice. The 3,000 x g pellet was extracted with diethyl ether, the ether extracts combined, washed with distilled water and dried under a stream of nitrogen.

Chrysanthemum.

The upper parts of the outer petals only were extracted as above.

Daffodil.

Coronae were extracted as above.

Spectra were recorded on samples taken up in petroleum ether (40-60°C). For HPLC, samples were dissolved in hexane. With daffodil, on dissolving the extract in pet. ether, a precipitate was formed which was removed by centrifuging the sample for 5 min in a benchtop centrifuge. The supernatant was removed and termed Extract 1. The pellet was resuspended in dichloromethane and again centrifuged. A whitish layer formed on top of the supernatant. The supernatant was
removed by Pasteur pipette and termed Extract 2. Extracts 1 and 2 were dried down and analysed separately. Extract 2 would not dissolve in pet. ether and was dissolved in dichloromethane for analysis.

Samples were separated by HPLC (Hewlett Packard HP1090 liquid chromatograph equipped with a 150 x 4.6 mm i.d. Spherisorb 5CN column) and analysed using a diode array detector. The solvent systems used were dichloromethane/2% methanol (A) and hexane/0.1% tributylamine (B). Conditions: 80% B for 15 min; 80 to 40% B for 25 min; 40 to 80% B for 5 min, at a flow rate of 0.5 ml/min. Injection volume: 15 µl except for alb-euffers (white phenotype), where the injection volume was increased to 20 µl because of the low concentration of pigments in the sample, and the chromoplast extracts where the injection volume was 25 µl.

For separation of the chromoplast extracts, an identical but shorter HPLC column (100mm) was used.
3.2.5 Isolation of Chromoplasts and Determination of Carotenoid Content.

Chromoplasts were isolated as previously described (Falk et al., 1974).

The upper parts of chrysanthemum petals (or daffodil coronae) were homogenised in a MSE Atomix blender at ¼ max speed for 4 x 5 s in 0.47 M sucrose, 5mM MgCl₂, 0.2% polyvinylpyrrolidone, 0.067 M phosphate, pH 7.5. The homogenate was filtered through 4 layers of muslin and the cellular debris removed by centrifugation at 1000 x g for 15 min. The supernatant obtained was centrifuged at 16,000 x g for 20 min. The pellet was then resuspended using a Potter-Elvehjem glass homogeniser in 5 mM MgCl₂, 0.067M phosphate, pH 7.5 containing 50% sucrose and overlaid with equal volumes of 40% sucrose, 30% sucrose and 15% sucrose dissolved in the same buffer. The sucrose gradients were centrifuged at 50,000 x g in a swing out rotor (Beckmann SW28) for 1 h.

Chromoplasts were visible as yellow bands between the 30% and 40% and 15% and 30% sucrose layers. The bands were removed by Pasteur pipette and diluted with 5 mM MgCl₂, 0.067 M phosphate, pH 7.5 to give a final concentration of 15% sucrose.

For bleaching experiments, the chromoplast suspension was adjusted to contain approximately 2.4 x 10⁻⁴ mg carotenoid per ml. Two ml of the carotenoid suspension were placed into glass tubes stoppered with cotton wool. After appropriate additions of herbicide had been made (in DMSO to give a final DMSO concentration of 1% v/v), the tubes were incubated under light of intensity 430 ± 20 Wm⁻². Bleaching was
determined as below with reference to a control suspension treated with 1% (v/v) DMSO.

Carotenoid content was determined as follows:
The chromoplasts were pelleted by centrifugation at 100,000 x g for 1 h in a swing out rotor (Beckmann SW60), and the supernatant discarded. The pellet was resuspended in ethanol and the white precipitate formed removed by centrifugation at 8,000 x g for 8 min. An optical absorption spectrum was taken and the extinction coefficient \(E^\varepsilon_{\lambda}\) 2500 for the predominant peak (absorbing between 437-440 nm) was used to calculate carotenoid content.

3.2.6 Difference Spectra.

Chromoplasts were prepared as above and centrifuged at 16,000 x g. The pellet obtained was resuspended in 5 mM MgCl\(_2\), 0.067 M phosphate pH 7.5. One ml of the chromoplast suspension was put into each of 2 quartz cuvettes which were placed in the reference and reading compartments of a spectrophotometer. The spectrophotometer was set to zero over a range of wavelengths. Equivalent volumes of DMSO and DPE I in DMSO were added to the reference and reading cuvettes respectively and a spectrum recorded.

Protein content of the chromoplast suspension was estimated by the modified Folin-Lowry method (Markwell et al, 1978).
3.3 Results.

3.3.1 Barley.

Germination of alb-e<sup>16</sup> seeds produced approximately 25% of the white phenotype and 75% of the green phenotype. Treatment of white and green phenotypes of alb-e<sup>16</sup> and wild-type barley with 10 μM DPE I produced symptoms of injury (wilting, necrosis) after 24-48 h light incubation. Figure 3.3 shows the effects of DPE I and paraquat on ethane formation in both albino and wild-type seedlings. Paraquat caused marked ethane formation in the wild-type up to 28 h after treatment, after which hydrocarbon production declined. This is consistent with the observations of Bowyer et al (1987) and is probably accounted for by rapid necrosis after 28 h. Paraquat did not induce ethane formation in the albino mutant and experiments with alb-e<sup>16</sup> thylakoid preparations in the oxygen electrode (results not shown) suggest that this mutant does not have an operating electron transport chain. DPE I induced ethane formation in both the wild-type and, after 52h, in the albino mutant. Table 3.1 shows that the white mutant contains less than 1% of the total carotenoid per gram fresh weight of wild-type barley. Thus if carotenoids are the photosensitisers for DPE activity, a delay in the response to the herbicide might be expected in a system depleted in these pigments.

The pigment contents of alb-e<sup>16</sup> (green and white phenotypes) and wild-type barley were qualitatively examined. The HPLC elution profiles of these extracts are shown in figures 3.4, 3.5 and 3.6.
Figure 3.3 Ethane Formation in Herbicide-Treated *als-e* Seedlings. White phenotype: 10 μM DPE I (■), 0.1% (v/v) DMSO (●), 0.1 mM MV (◆). Green phenotype: 10 μM DPE I (□), 0.1% (v/v) DMSO (○), 0.1 mM MV (◆).
TABLE 3.1
Amount of Carotenoid Extracted from Each Tissue Sample (calculated using the absorbance at 445 nm of samples dissolved in pet. ether).

<table>
<thead>
<tr>
<th>Sample</th>
<th>mg carotenoid / g fresh weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>alb-e&lt;sup&gt;1&lt;/sup&gt; (white phenotype)</td>
<td>1.289 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
<tr>
<td>alb-e&lt;sup&gt;2&lt;/sup&gt; (green phenotype)</td>
<td>0.185</td>
</tr>
<tr>
<td>Wild type</td>
<td>0.156</td>
</tr>
<tr>
<td>Chrysanthemum</td>
<td>0.039</td>
</tr>
<tr>
<td>Daffodil (Ext. 1)</td>
<td>0.028</td>
</tr>
<tr>
<td>Daffodil (Ext. 2)*</td>
<td>0.088</td>
</tr>
</tbody>
</table>

* in dichloromethane
TABLE 3.2
Possible Identities of the Peaks Obtained from alb-e<sup>16</sup> (White Phenotype). See figure 3.4.

<table>
<thead>
<tr>
<th>Peak no.</th>
<th>R&lt;sub&gt;t&lt;/sub&gt; (min)</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt; (nm)</th>
<th>Possible identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.62</td>
<td>n. d.</td>
<td>β-carotene</td>
</tr>
<tr>
<td>2</td>
<td>3.75</td>
<td>n. d.</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>17.70</td>
<td>n. d.</td>
<td>chlorophyll a</td>
</tr>
<tr>
<td>4</td>
<td>28.50</td>
<td>425,448,476</td>
<td>lutein</td>
</tr>
<tr>
<td>5</td>
<td>29.43</td>
<td>432,452,480</td>
<td>zeaxanthin</td>
</tr>
<tr>
<td>6</td>
<td>32.74</td>
<td>428,448,480</td>
<td>antheraxanthin</td>
</tr>
<tr>
<td>7</td>
<td>35.52</td>
<td>n. d.</td>
<td>violaxanthin</td>
</tr>
</tbody>
</table>

n.d. = not detected
Figure 3.4  HPLC Elution Profile from *alb-e* (White Phenotype). 1.269 g of tissue were extracted as in section 3.2.4. The extract was dissolved in 100 μl hexane and 20 μl injected on to the column.
### TABLE 3.3
Possible Identities of Peaks Obtained from alb-e\(_r\) (Green Phenotype). See figure 3.5.

<table>
<thead>
<tr>
<th>Peak no.</th>
<th>Rt (min)</th>
<th>( \lambda_{\text{max}} ) (nm)</th>
<th>Possible identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.23</td>
<td>452, 476</td>
<td>( \beta )-carotene</td>
</tr>
<tr>
<td>2</td>
<td>3.70</td>
<td>n.d</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>9.12</td>
<td>410</td>
<td>phaeophytin a</td>
</tr>
<tr>
<td>4</td>
<td>11.63</td>
<td>434</td>
<td>chlorophyll isomer</td>
</tr>
<tr>
<td>5</td>
<td>14.05</td>
<td>n.d</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>17.75</td>
<td>428</td>
<td>chlorophyll a</td>
</tr>
<tr>
<td>7</td>
<td>23.96</td>
<td>454</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>25.81</td>
<td>456</td>
<td>chlorophyll b</td>
</tr>
<tr>
<td>9</td>
<td>28.39</td>
<td>422, 448, 474</td>
<td>lutein</td>
</tr>
<tr>
<td>10</td>
<td>32.57</td>
<td>426, 442, 472</td>
<td>antheraxanthin</td>
</tr>
<tr>
<td>11</td>
<td>35.45</td>
<td>421, 442, 472</td>
<td>violaxanthin</td>
</tr>
<tr>
<td>12</td>
<td>40.22</td>
<td>410, 434, 465</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>44.87</td>
<td>416, 439, 467</td>
<td>neoxanthin</td>
</tr>
</tbody>
</table>
Figure 3.5  HPLC Elution Profile from alb-e^+ (Green Phenotype).
2.268 g of tissue were extracted as in section 3.2.4.
The extract was dissolved in 5 ml hexane and 15 µl
injected onto the column.
### TABLE 3.4
Possible Identities of the Peaks Obtained from Wild-Type Barley.
See figure 3.6.

<table>
<thead>
<tr>
<th>Peak no.</th>
<th>Rt (min)</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>Possible identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.21</td>
<td>452,476</td>
<td>$\beta$-carotene</td>
</tr>
<tr>
<td>2</td>
<td>3.67</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>9.10</td>
<td>410</td>
<td>phaeophytin a</td>
</tr>
<tr>
<td>4</td>
<td>11.61</td>
<td>434</td>
<td>chlorophyll isomer</td>
</tr>
<tr>
<td>5</td>
<td>14.10</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>17.81</td>
<td>428</td>
<td>chlorophyll a</td>
</tr>
<tr>
<td>7</td>
<td>24.17</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>25.79</td>
<td>456</td>
<td>chlorophyll b</td>
</tr>
<tr>
<td>9</td>
<td>28.46</td>
<td>422,448,475</td>
<td>lutein</td>
</tr>
<tr>
<td>10</td>
<td>32.66</td>
<td>442</td>
<td>antheraxanthin</td>
</tr>
<tr>
<td>11</td>
<td>35.49</td>
<td>421,444,472</td>
<td>violaxanthin</td>
</tr>
<tr>
<td>12</td>
<td>40.26</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>43.61</td>
<td>434</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>44.77</td>
<td>415,440,467</td>
<td>neoxanthin</td>
</tr>
</tbody>
</table>
Figure 3.6  HPLC Elution Profile from Wild-Type Barley.  
1.785 g of tissue were extracted as in section 3.2.4.  
The extract was dissolved in 2 ml hexane and 15 μl injected on to the column.
major peaks from each sample have been tentatively identified from their optical spectra and polarity, assuming that the least polar compounds were eluted first (see Appendix I). The results are shown in tables 3.2-3.4. Previous reports of the pigment content of wild-type barley (Lichtenthaler, 1968; Grumbach, 1985) indicate that chlorophyll a, chlorophyll b, lutein, β-carotene and violaxanthin are the major pigments present. The results presented here agree with this. However, relatively large amounts of another pigment, tentatively identified as phaeophytin a were also shown to be present. This may be a result of changes which have occurred during the extraction process. Phaeophytin a is not normally present in such relatively large amounts. All the other pigments identified in this study have previously been shown to occur in wild-type barley. The elution profile of the albino mutant (figure 3.4) shows a small peak at 17.7 min, indicating the presence of chlorophyll a. There was no evidence to suggest that chlorophyll b was present. The alb-e16 mutant is therefore a 'leaky' mutant. Gough (1972) noted that the alb-e16 mutant contains about 10% of wild-type levels of protochlorophyllide. In view of this evidence it cannot be completely ruled out that photosynthesis is operating in the mutant at undetectable levels and thus contributing in some way to DPE toxicity.

Only 5 types of carotenoid could be distinguished and tentatively identified in the albino. If carotenoids are involved in NDPE activation then it is possible that the presence of one or more of these carotenoids is essential to NDPE toxicity. β-carotene, lutein, antheraxanthin and violaxanthin but not zeaxanthin were also shown to be present in alb-e16 (green phenotype) and wild-type barley.
3.3.2 Chrysanthemum and Daffodil.

Figure 3.7A shows the effect of 10 µM DPE I on chrysanthemum petals after 51 h incubation. In the light, DPE I caused severe necrosis, but had no effect in the dark. Paraquat had little or no effect on chrysanthemum petals under conditions of light or darkness (figure 3.7B). Figure 3.7C demonstrates the effects of some other NDPEs on chrysanthemum. DPE V (an experimental NDPE) caused severe damage to the petals whilst acifluorfen had a lesser effect. No damage was observed after bifenox treatment nor, interestingly, after incubation with nitrofen. Non-nitro diphenylethers did not appear to have any herbicidal effects on chrysanthemum petals (figure 3.7D). Phthalide DPEs II and III did not cause necrosis, nor did 169, a phenylamide derivative which has similar effects on plants to NDPEs (P. Camilleri, personal communication). DPE I did not cause necrosis in daffodil coronae.

The effects of DPE I on the ultrastructure of chrysanthemum petals are shown in figure 3.8. In untreated cells (figure 3.8A and B), there is a large central vacuole with the chromoplasts arranged in a thin layer of cytoplasm around the perimeter of the cell, bounded by the tonoplast. The chromoplasts are membrane bound structures containing globules which may be lipid or carotenoid. No chloroplasts are present. Incubation with DPE I under light (figure 3.8C) causes swelling of the chromoplasts and rupture of the tonoplast, leading to extensive cellular disruption. Similar types of effect have been observed in DPE I treated barley mesophyll cells (Bowyer et al, 1987). It must be mentioned that some damaged cells could also be found in
Figure 3.7 The Effects of Herbicides on Chrysanthemum Petals.

A. Petals were treated with:
1. 0.1% (v/v) DMSO
2. 10 μM DPE I
3. 10 μM DPE I, incubated in darkness.

The treated flowers were incubated for 51 h.
Figure 3.7 B. Petals were treated with:
1. 0.1 mM MV
2. 0.1 mM MV, incubated in the dark.
The treated flowers were incubated for 51 h.
Figure 3.7 C. Petals were treated with:
1. 10μM DPE V
2. 10μM acifluorfen
3. 10μM bifenox
4. 10μM nitrofen.
The treated flowers were incubated for 51 h.
Figure 3.7 D. Petals were treated with:
1. 10 μM phthalide DPE II
2. 10 μM phthalide DPE III
3. 10 μM 169.
The treated flowers were incubated for 51 h.
Figure 3.8  Ultrastructural Effects of DPE I on Chrysanthemum Petals.
A. Untreated tissue.

v: vacuole.
ch: chromoplast.
The scale bar represents 1 μm.
Figure 3.8B. Untreated tissue.
v: vacuole
ych: chromoplast.
g: carotenoid/ lipid globule.
The scale bar represents 1 μm.
Figure 3.8C. Tissue incubated with 10 μM DPE I for 51 h. 
ch: chromoplast. 
The scale bar represents 0.5 μm.
control tissue when viewed under the electron microscope, but damage was far more prolific in the herbicide-treated cells.

DPE I induced ethane formation in chrysanthemum petals (figure 3.9). This effect was not seen in paraquat-treated tissue. Thus, it appears that DPE I causes light-dependent necrosis and lipid peroxidation in chrysanthemum flowers.

Pigments were extracted from chrysanthemum petals and daffodil coronae in an attempt to see whether the non-susceptible tissue (daffodil) was deficient in any particular carotenoid, present in all of the different types of susceptible tissue tested (alb-e watc^e green phenotypes, wild-type barley and chrysanthemum). Figure 3.10 shows that chrysanthemum petals contain a complex mixture of about 40 pigments. There is no evidence to suggest the presence of Chl a, indicating the absence of photosynthetic reaction centres (Witt, 1978). The absence of a peak around Rr 25.8 min suggests that Chl b is not present.

In this study, lutein and vioaxanthin appear to be the predominant pigments in daffodil coronae (figures 3.11 & 3.12). This is in agreement with the results of Liedvogel et al (1976) who found that lutein and violaxanthin accounted for 57% and 24% respectively of the total carotenoid in daffodil. Comparison of the HPLC elution profiles of the pigments of barley (wild-type and mutant alb-e^) and chrysanthemum with daffodil showed that the affected species did not contain any pigment which was not present in daffodil.
Figure 3.9  DPE I -Induced Ethane Formation in Chrysanthemum Petals. Chrysanthemum petals were treated with 0.1% (v/v) DMSO (◇), 10 μM DPE I (○), 0.1 mM MV (□).
### TABLE 3.5
Possible Identities of Some of the Peaks Obtained from Chrysanthemum. See figure 3.10

<table>
<thead>
<tr>
<th>Peak no.</th>
<th>Rt (min)</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt; (nm)</th>
<th>Possible identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>27.07</td>
<td>434</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>27.65</td>
<td>410, 436, 466</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>28.49</td>
<td>422, 446, 475</td>
<td>lutein</td>
</tr>
<tr>
<td>4</td>
<td>29.61</td>
<td>412, 438, 465</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>30.82</td>
<td>415, 442, 473</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>32.54</td>
<td>420, 442, 472</td>
<td>antheraxanthin</td>
</tr>
<tr>
<td>7</td>
<td>35.41</td>
<td>422, 444, 472</td>
<td>violaxanthin</td>
</tr>
</tbody>
</table>
Figure 3.10  HPLC Elution Profile from Chrysanthemum.  
10.12 g of tissue were extracted as in section 3.2.4.  
The dry weight of the extract was 22 mg.  10.35 mg were 
dissolved in 100 μl hexane and 15 μl was injected on to 
the column.
### TABLE 3.6
Possible Identities of Some of the Peaks Obtained from Daffodil Extract 1.
See figure 3.11.

<table>
<thead>
<tr>
<th>Peak no.</th>
<th>Rt (min)</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>Possible identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.18</td>
<td>n.d.</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>28.29</td>
<td>448</td>
<td>lutein</td>
</tr>
<tr>
<td>3</td>
<td>30.65</td>
<td>442</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>32.43</td>
<td>442</td>
<td>antheraxanthin</td>
</tr>
<tr>
<td>5</td>
<td>35.30</td>
<td>444</td>
<td>violaxanthin</td>
</tr>
<tr>
<td>6</td>
<td>36.65</td>
<td>420</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>37.45</td>
<td>420</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>37.95</td>
<td>413, 440, 467</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>40.02</td>
<td>438</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>44.62</td>
<td>440</td>
<td>neoxanthin</td>
</tr>
</tbody>
</table>
Figure 3.11  HPLC Elution Profile from Daffodil (Extract 1).
38.64 g tissue were extracted as in section 3.2.4.
The dry weight of Extract 1 was 172.42 mg. 10.44 mg were dissolved in 1 ml hexane and 15 μl was injected onto the column.
TABLE 3.7
Possible Identities of Some of the Peaks Obtained from Daffodil Extract 2.
See figure 3.12.

<table>
<thead>
<tr>
<th>Peak no.</th>
<th>Rt (min)</th>
<th>λmax (nm)</th>
<th>Possible identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>28.05</td>
<td>448</td>
<td>lutein</td>
</tr>
<tr>
<td>2</td>
<td>32.41</td>
<td>450</td>
<td>antheraxanthin</td>
</tr>
<tr>
<td>3</td>
<td>35.19</td>
<td>444</td>
<td>violaxanthin</td>
</tr>
<tr>
<td>4</td>
<td>44.65</td>
<td>442</td>
<td>neoxanthin</td>
</tr>
</tbody>
</table>
Figure 3.12. HPLC Elution Profile from Daffodil (Extract 2). The dried extract was dissolved in 5 ml dichloromethane and 100 µl diluted 1 in 10. 15 µl was injected on to the column.
3.3.3 Chromoplasts.

Figure 3.13 shows an electron micrograph of the 'chromoplast' preparation obtained from chrysanthemum petals, by the method described. As might be expected, the suspension did not contain intact chromoplasts but appeared to consist of the globular structures observed inside whole chromoplasts. It is difficult to determine whether or not the plastid envelope or fragments of it, are present in the preparation. A comparison of this figure with sections of chrysanthemum petal shows that the globules in the chromoplast preparation are about twice the size of those found in the intact organelle. This is presumably the result of fusion of globules during the isolation procedure.

When the chromoplast suspensions were incubated under light (intensity 430 ± 20 Wm⁻²), their carotenoid content decreased. This bleaching process was greatly accelerated by the presence of DPE I (figure 3.14). No bleaching occurred in the dark with any of the herbicides tested here.

DPE I-induced bleaching was shown to be dependent on light intensity (table 3.8). Bleaching was also dependent on the presence of oxygen. When chromoplasts were incubated with DPE I under nitrogen, the herbicide-induced bleaching was reduced by 64%.

Pigments were extracted from both control and DPE I -treated chromoplasts and analysed by HPLC (figures 3.15, A & B). The chromoplast preparations appeared to contain several carotenoids amongst which lutein, antheraxanthin and violaxanthin were tentatively identified. If the model of Orr & Hess (1982a) were correct then any
Figure 3.13  Electron Micrograph of a Chromoplast Preparation from Chrysanthemum Petals. The scale bar represents 0.1 µm.
Figure 3.14. The Bleaching Effect of DPE I on Chrysanthemum Chromoplasts. Chromoplast suspensions were incubated with 1% DMSO (v/v) (□), 10 μM DPE I (■).
**TABLE 3.8**

The Effect of Light Intensity on DPE I-Induced Bleaching of Chrysanthemum Chromoplasts.

<table>
<thead>
<tr>
<th>Light intensity (Wm$^{-2}$)</th>
<th>Bleaching (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>52</td>
</tr>
<tr>
<td>150</td>
<td>67</td>
</tr>
<tr>
<td>200</td>
<td>83</td>
</tr>
</tbody>
</table>
TABLE 3.9
Possible Identities for Some of the Peaks from Chrysanthemum Chromoplast Preparation (DMSO-Treated).
See figure 3.15 A

<table>
<thead>
<tr>
<th>Peak no.</th>
<th>Rt (min)</th>
<th>( \lambda_{\text{max}} ) (nm)</th>
<th>Possible identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10.94</td>
<td>408, 428, 463</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>12.33</td>
<td>412, 436, 464</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>28.41</td>
<td>n.d.</td>
<td>lutein</td>
</tr>
<tr>
<td>4</td>
<td>32.85</td>
<td>422, 447, 474</td>
<td>antheraxanthin</td>
</tr>
<tr>
<td>5</td>
<td>35.61</td>
<td>418, 443, 466</td>
<td>violaxanthin</td>
</tr>
</tbody>
</table>

Pigments in the DPE I-treated extract were present at concentrations too low to be detected by the diode array detector.
Figure 3.15  HPLC Elution Profile from Chromoplasts Incubated Under Light for 16 h.
Ten ml of a chromoplast suspension containing 2.4 x 10^{-4} mg carotenoid per ml, was incubated with 0.1% (v/v) DMSO (A) and 10μM DPE I for 16 h and the pigments extracted. Extract A was dissolved in 100 μM hexane and 25 μl injected on to the column. Extract B was dissolved in 50 μl hexane and 25 μl injected on to the column.
carotenoid involved in DPE activation could be selectively destroyed in the process. A comparison of the HPLC elution profiles of control and treated chromoplast pigment extracts indicates that there is no selective destruction of any particular carotenoid, rather, the bleaching effect is spread over the whole range of carotenoids present.

The bleaching effects of a range of DPE herbicides on chrysanthemum chromoplast preparations were investigated (table 3.10). In this study it was observed that the nitrodiphenyl ethers induced bleaching whereas the phthalide DPEs had little or no effect. Acifluorfen is perhaps the exception here since this NDPE only induced 12% bleaching compared to the control. This might be due to problems of accessability of the herbicide molecule to its target site. If the induction of bleaching by these herbicides involves an interaction of the NDPE molecule with a receptor in a lipophilic environment, then the negative charge on the carboxyl group of acifluorfen might restrict access of the herbicide to such a site. Orr & Hess (1981) suggested that the lack of ability of acifluorfen and MC-7783 (the salt of bifenox) to induce efflux from pre-loaded excised cucumber cotyledons was due to the charges on these compounds preventing them from getting to the site of activation and/or action within the cell. Nitrofen was found to be the most potent inducer of chromoplast bleaching. This order of activity was not reflected in green plants as determined by the primary herbicide screen score. The results shown in table 3.10 suggest that the mechanism of action of diphenyl ethers in isolated chromoplasts may be
### TABLE 3.10
Comparison of the Efficacy of Compounds in Causing Bleaching in the Chromoplast Assay with Primary Herbicide Screen Data.

<table>
<thead>
<tr>
<th>Herbicide</th>
<th>% Bleaching induced by 5 μM compound on chromoplasts after 18 h.</th>
<th>Primary screen score (sum of scores on 8 plant species, for 1 kg/ha foliar spray, max score 72. *)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrofen</td>
<td>76</td>
<td>58</td>
</tr>
<tr>
<td>DPE I</td>
<td>43</td>
<td>68</td>
</tr>
<tr>
<td>Bifenox</td>
<td>30</td>
<td>57</td>
</tr>
<tr>
<td>Acifluorfen</td>
<td>12</td>
<td>n.a.**</td>
</tr>
<tr>
<td>Phthalide DPE II</td>
<td>9</td>
<td>70</td>
</tr>
<tr>
<td>169</td>
<td>3</td>
<td>27</td>
</tr>
<tr>
<td>Phthalide DPE III</td>
<td>0</td>
<td>66</td>
</tr>
</tbody>
</table>

* Data supplied by Shell Research Ltd.
** n.a. = not available
different to that in green plants. It is also interesting to note the inconsistencies between the action of these herbicides on whole flowers and on the bleaching of chromoplasts. Nitrofen, the most potent bleaching agent of chromoplasts did not appear to damage whole petals whereas acifluorfen induced only minor bleaching of the chromoplasts but caused necrosis in the intact flower. The former effect might be explained by difficulties of penetration of the herbicide into the intact petal.

DPE I did not cause necrosis in daffodil coronae and therefore it was expected that the herbicide would not cause enhanced bleaching of daffodil chromoplasts. Once again however, a conflicting result was obtained (table 3.11) and DPE I caused 50% bleaching of the chromoplast preparation. As was seen with chrysanthemum chromoplasts, nitrofen was the more potent inducer of bleaching.

3.3.4 Mechanism of Action of NDPEs in Chromoplasts.

Since ethane was released from whole flowers treated with DPE I, it would seem that the herbicide induces lipid peroxidation in the petal tissue. Treatment of chromoplast preparations with the free radical scavenger \( \alpha \)-tocopherol (table 3.12) protected against DPE I-induced bleaching, suggesting that radicals are involved in this process.

If the effect of NDPEs on chromoplast preparations was to inhibit carotenoid biosynthesis, then the bleaching observed might be due to a
TABLE 3.11
Herbicide-Induced Bleaching of Daffodil Chromoplasts. The table shows bleaching after 18 h light incubation.

<table>
<thead>
<tr>
<th>Herbicide (10 μM)</th>
<th>Bleaching (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrofen</td>
<td>65</td>
</tr>
<tr>
<td>DPE I</td>
<td>50</td>
</tr>
</tbody>
</table>
TABLE 3.12
The Effects of MV, Norflurazon and α-tocopherol on Chrysanthemum Chromoplasts. The table shows bleaching after 18 h incubation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Bleaching (% of controls)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 μM DPE I</td>
<td>89</td>
</tr>
<tr>
<td>10 μM DPE I (dark)</td>
<td>0</td>
</tr>
<tr>
<td>10 μM DPE I + α-tocopherol (60 μg/ml)</td>
<td>11</td>
</tr>
<tr>
<td>100 μM MV</td>
<td>7</td>
</tr>
<tr>
<td>10 μM norflurazon</td>
<td>5</td>
</tr>
</tbody>
</table>

In order to determine whether or not tannin catalyzed Fenton-type reactions are involved in DPE-induced bleaching of chromoplasts, the effects of several tannins and phenolic compounds on this process were examined. Fenton chemistry is a potent free radical generator in DPE. The results are shown in Table 3.12. Although fusicoccin gave slight protection from bleaching, the effect of enhancement by Fe(II), and the relatively small effect of ascorbic acid indicate that the Fenton reaction is not part of the primary bleaching mechanism.

Singlet oxygen does not seem to be involved in DPE-induced bleaching of chromoplasts (Table 3.12). Photoreduction of DPE I increases the lifetime of singlet oxygen and should therefore enhance bleaching by singlet oxygen. DPE I-induced bleaching was in fact reduced in the presence of SyM, AzM and DABCO quench singlet oxygen and should diminish bleaching by singlet oxygen. Even effects were not observed when chromoplasts were incubated with these compounds and DPE.
blocking of carotenoid turnover. To test this hypothesis, chromoplasts were incubated with norflurazon, a known inhibitor of carotenoid synthesis. Norflurazon induced very little bleaching of chromoplasts (table 3.12) and therefore NDPEs must have a genuine bleaching effect.

The Fenton reaction involves the reduction of hydrogen peroxide by an iron complex in its lower oxidation state, with the formation of hydroxyl ions (see section 1.1.3, equation 1.7). Such a reaction is thought to be involved in paraquat toxicity, where \( \text{Fe}^{2+} \) is generated from \( \text{Fe}^{3+} \) on reduction by \( \text{O}_2^- \) (the Haber-Weiss reaction) (see Chapter 1). In order to determine whether an iron catalysed Fenton-type reaction is involved in NDPE-induced bleaching of chromoplasts, the effects of added \( \text{FeCl}_3 \) and desferrioxamine on this process were examined. Desferrioxamine is a potent iron chelator known to block Fenton chemistry (Halliwell & Gutteridge, 1981). The results are shown in table 3.13. Although desferrioxamine gave slight protection from bleaching, the lack of enhancement by \( \text{FeCl}_3 \), and the relatively small effect of desferrioxamine indicate that the Fenton reaction is not part of the primary bleaching mechanism.

Singlet oxygen does not seem to be involved in DPE I -induced bleaching of chromoplasts (table 3.14). \( \text{D}_2\text{O} \) increases the lifetime of singlet oxygen and should therefore enhance bleaching by singlet oxygen. DPE I-induced bleaching was in fact reduced in the presence of \( \text{D}_2\text{O} \). Azide and DABCO quench singlet oxygen and should diminish bleaching by singlet oxygen. Such effects were not observed when chromoplasts were incubated with these compounds and DPE I.

Figure 3.16A shows difference spectra obtained from chromoplast
### TABLE 3.13
The Role of the Fenton Reaction in DPE I-Induced Bleaching of Chromoplasts.
The table shows bleaching after 18 h incubation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Bleaching (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 µM DPE I</td>
<td>90</td>
</tr>
<tr>
<td>10 µM DPE I + 50 µM FeCl₃</td>
<td>90</td>
</tr>
<tr>
<td>10 µM DPE I + 100 µM desferrioxamine</td>
<td>80</td>
</tr>
<tr>
<td>10 µM DPE I + 50 µM FeCl₃ + 100 µM desferrioxamine</td>
<td>86</td>
</tr>
</tbody>
</table>
TABLE 3.14
The Role of Singlet Oxygen in DPE I-Induced Bleaching of Chromoplasts. The table shows bleaching after 18 h incubation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Bleaching (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 μM DPE I (in H$_2$O buffer)</td>
<td>80</td>
</tr>
<tr>
<td>10 μM DPE I (in 90% D$_2$O buffer)</td>
<td>61</td>
</tr>
<tr>
<td>10 μM DPE I + 10mM DABCO</td>
<td>81</td>
</tr>
<tr>
<td>10 μM DPE I + 1mM sodium azide</td>
<td>82</td>
</tr>
</tbody>
</table>
Figure 3.15 Difference Spectra of DPE I.
Difference spectra were recorded for DPE I in chromoplast suspensions (containing 10 µg protein per ml) (A) and in buffer minus chromoplasts (B).
suspensions (10 μg protein. ml⁻¹) treated with 5 and 10 μM DPE I. Addition of the herbicide to buffer in the absence of chromoplasts caused an increase of absorption in the visible region of the spectrum (figure 3.16B). In DMSO (as in propanol), the herbicide itself only absorbs substantially in the ultra-violet region (see Chapter 1). This result might suggest that the herbicide has different absorption properties in aqueous solutions but it is more probable that the lipophilic herbicide is precipitating in the aqueous buffer and scattering visible light. DPE I induced a decrease in absorption in the chromoplast suspension at about 500 nm. To investigate this effect further, the experiment was repeated with a more concentrated chromoplast suspension (70 μg protein. ml⁻¹). Figure 3.17 shows troughs at 478, 442 and 420 nm. Peaks occur at 462 and 432 nm. Increasing the herbicide concentration from 5 to 10 μM seemed to make these absorption changes more pronounced. Such effects are difficult to explain. However, since the apparent decreases in absorption occur in the region of the spectrum where carotenoids would be expected to absorb, these changes might reflect rapid destruction of carotenoid components, induced by the herbicide. Alternatively, these absorption changes could be an indication of an interaction between the herbicide and the chromoplast membrane. When the experiment was repeated with phthalide DPE II (figure 3.18), similar changes in absorption were observed (troughs at 480, 448 and 420 nm, peaks at 428 and 466 nm).
Figure 3.17  Difference Spectra from Chromoplast Suspensions Treated with DPE I.
Spectra were recorded against chromoplast suspensions (containing 70 µg protein per ml) treated with DMSO. The numbers show the positions of peaks and troughs in the spectrum (in nm).
Figure 3.18 Difference Spectrum from a Chromoplast Suspension Treated with Phthalide DPE II.
The spectrum was recorded against a chromoplast suspension (containing 70 μg protein per ml) treated with DMSO. The numbers show the positions of peaks and troughs in the spectrum (in nm).
3.4 Discussion.

It had been intended to make a comparison of carotenoid content of tissue susceptible and non-susceptible to DPE herbicides, in order to determine whether the presence of a particular carotenoid was essential to elicit DPE toxicity. The albino mutant alb-e'" (white phenotype) is susceptible to DPE I, and only contains a few types of carotenoid. However, the non-susceptible tissue tested here, daffodil, could be resistant to the herbicide because the herbicide molecule is unable to penetrate through the cuticle or cell wall, or because it is metabolised after entry into the cell. The results obtained with daffodil chromoplasts suggest that one of the above is likely. Thus it cannot be ruled out from this study, that β-carotene, lutein, antheraxanthin or violaxanthin are required for NDPE action.

DPE I clearly induces herbicidal effects in chrysanthemum petals and isolated chrysanthemum chromoplasts. No herbicidal effects were observed after incubation with MV. Since no Chl a was detected on pigment analysis and no chloroplasts could be observed in the tissue under the electron microscope, it seems reasonable to assume that there is no photosynthetic electron transport activity in chrysanthemum petals and thus, in this tissue at least, photosynthetic electron transport is not involved in NDPE-toxicity. Greater than 50% bleaching occurred in chromoplast suspensions which had been treated with DMSO alone. This might have been induced by the relatively high concentrations of solvent used in these experiments. However, similar levels of bleaching occurred in the absence of DMSO (result not shown).
when chromoplast suspensions were incubated under light. It is assumed that the bleaching observed in the controls is as a result of photo-oxidation of carotenoid under the high light intensity used in these experiments.

The mechanism of DPE-induced bleaching of chromoplasts remains a mystery. Although the observed protection against DPE I-induced bleaching by α-tocopherol suggests that free radicals are important in this bleaching process, Fenton chemistry does not seem to play a role. Furthermore, singlet oxygen does not appear to be involved in DPE-induced bleaching of chromoplasts which makes doubtful the possibility that accumulated protoporphyrin IX is responsible for this effect. DPE-induced tetrapyrrole accumulation might be involved in the initiation of the lipid peroxidation observed in intact chrysanthemum petals. The results obtained with intact flowers and chrysanthemum chromoplasts however, suggest that the mode of action of the herbicides tested is different in chrysanthemum petals to that in green plants. The phthalide DPEs are potent herbicides in the latter yet they have no effect on whole chrysanthemum petals and cause little or no bleaching of chromoplasts. It is possible that there is more than one primary mode of action for DPE herbicides in leaf tissue, only one of which might be available in chrysanthemum petals.

The results obtained from the difference spectra may indicate some kind of interaction between DPE I and the chromoplast membranes. Similar effects were obtained with phthalide DPE II, suggesting possible interaction occurs with this herbicide, even though it induced only minor bleaching of chromoplasts after 18 h incubation (10 μM phthalide DPE II caused < 9% bleaching of chromoplasts after 18 h
incubation). The changes in absorption observed on addition of DPE I to chromoplast suspensions may indicate rapid destruction of carotenoid. However, the peaks and troughs observed (see figure 3.17) do not correspond to those seen in the absorption spectrum of total carotenoid from chromoplasts (peaks: 412, 437 & 467 nm; troughs: 418 & 453 nm), rather they correspond more closely to those observed in the absorption spectra of peaks identified as antheraxanthin (peaks: 420, 442 & 472 nm; troughs: 424 & 458 nm) or lutein (peaks: 422, 446 & 475 nm; troughs: 427 & 463 nm) from the chrysanthemum petal extract.

(N.B. for a carotenoid spectrum to correspond to the difference spectrum, a peak of the former would correspond to a trough of the latter and vice versa.) The HPLC elution profiles of extracts from untreated and DPE I-treated chromoplasts however, showed that there was no selective destruction of any particular carotenoid.

The results presented in this chapter are consistent with the hypothesis that carotenoids are the photosensitizers in NDPE toxicity. However, all the work presented on chrysanthemum was from one strongly pigmented variety, 'Bright Golden Princess Anne'. White-petalled chrysanthemums (variety unknown) were obtained from the market and the petals treated with DPE I. After 2 days incubation under light, severe necrosis of the petals was observed. Pigment extraction revealed that the white chrysanthemum contained 41 times less total carotenoid than the yellow variety, yet DPE I appeared to cause equally severe damage in both types. It is possible that only small amounts of carotenoid are required for the initiation of a NDPE-induced radical chain reaction, which then gives rise to the observed necrosis. The albino barley mutant alb-ε contains less than 1% of...
the total carotenoid of wild-type barley, yet DPE I was still able to elicit necrosis and lipid peroxidation in the mutant.
CHAPTER 4

Herbicide-Induced Tetrapyrrole Accumulation.

This reaction involves energy transfer from the excited singlet state of the porphyrin to ground state oxygen. In solution, porphyrin is photooxidized to a variety of products which may be as a result of the reaction of the porphyrin molecule with singlet oxygen. (See Ref. Whiting, 1983).

The photosensitizing properties of porphyrins have long been studied in the medical world. Porphyrins are an inherited disease in which there is a defect in heme synthesis. This enzyme, known as the formation of heme from protoporphyrin IX, is involved in the formation of heme from protoporphyrin IX and iron (iron from Haase et al., 1993).
4.1 Introduction.

Matringe and Scalla (1987b) were the first to demonstrate that diphenyl ether herbicides induce abnormal accumulation of tetrapyrrole compounds in plant tissues. Extracts from non-chlorophyllous soybean cells and etiolated cucumber hypocotyls treated with AFM were shown to contain unusually high concentrations of a pigment with the fluorescence characteristics of protoporphyrin IX.

Most porphyrins and some metalloporphyrins are good sensitisers for the formation of singlet oxygen (Cannistraro et al., 1978):

\[
P \quad \text{by} \quad \rightarrow \quad P^{*1} \quad \text{inter-system crossing} \quad \rightarrow \quad P^{*3} \quad \text{equation 4.1}
\]

\[
P^{*3} \quad + \quad ^{3}O_{2} \quad \rightarrow \quad P \quad + \quad ^{1}O_{2} \quad \text{equation 4.2}
\]

This reaction involves energy transfer from the excited triplet state of the porphyrin to ground state oxygen. In solution, Proto IX is photooxidised to a variety of products which may be as a result of the reaction of the porphyrin molecule with singlet oxygen (Cox and Whitten, 1983).

The photosensitising properties of porphyrins have long been known in the medical world. Protoporphyria is an inherited disease in which there is a defect in ferrochelatase, the enzyme which catalyses the formation of haem from Proto IX and ferrous iron (Kapas et al., 1983). The disease results in cutaneous photosensitivity. The photosensitising properties of porphyrins have been exploited in the
treatment of cancer. Haematoporphyrin is retained by malignant tissue conferring photosensitivity on the tumor cells (Christensen et al, 1983). That photoactivated Proto IX is able to cause membrane damage was shown by Girotti and Deziel (1983), who demonstrated that this compound induced permeability and lipid peroxidation of resealed erythrocyte membranes.

The idea of using induction of tetrapyrrrole accumulation as a herbicidal mode of action is not new. In 1984 Rebeiz et al described the design of 'laser herbicides', compounds targeted at the chlorophyll biosynthetic pathway, with the aim of exploiting the possible photodynamic properties of porphyrin, Mg-porphyrin and protochlorophyll intermediates. The authors observed the accumulation of tetrapyrrroles and photodestruction after spraying with δ-aminolevulinic acid (ALA), a chlorophyll precursor, and 2,2'-dipyridyl, an activator of the chlorophyll synthetic pathway.

Figure 4.1 shows a schematic diagram of chlorophyll synthesis from δ-aminolevulinic acid to Chlorophyll a. Proto IX is the last common precursor to haems and chlorophylls, the chlorophyll branch of the pathway beginning with the insertion of Mg into the Proto IX nucleus. All of the steps from ALA to Proto IX have been reported to occur in the soluble stroma phase of developing plastids (Smith and Rebeiz, 1979). Proto IX subsequently becomes associated with the plastid membranes where it is converted to Mg-protoporphyrin IX (Mg-Proto IX). Richter and Rienäis (1982) demonstrated that membranes from lysed, washed cucumber etioplasts could synthesise 14C-Mg-Proto IX from 14C-Proto IX. Maximal activity in these preparations was dependent upon added EDTA, GSH, ATP and MgCl2. There was an absolute
Figure 4.1  Schematic Diagram of Chlorophyll a Biosynthesis in Higher Plants.
Reaction A is catalysed by protoporphyrinogen oxidase.
Reaction B is catalysed by Mg-chelatase.
Modified from Castelfranco and Beale, 1983.
requirement for ATP. Fuesler et al (1982) used ion pair HPLC to show that chelation of Mg$^{2+}$ into Proto IX precedes methylation. There was no evidence for the coupling of these 2 steps.

If, as the results of Matringe and Scalla (1987b) suggest, DPEs induce abnormal accumulation of Proto IX in plant tissues, then inhibition of Mg-chelatase activity is the most obvious target for these herbicides.

Another possible effect of these herbicides is that they interfere with the regulation of the chlorophyll biosynthetic pathway. There do not appear to be any regulatory steps between ALA and protoporphyrinogen (Castelfranco and Beale, 1983). Protoporphyrinogen can be converted non-enzymatically to Proto IX in the presence of oxygen and light, but under normal conditions, this conversion is catalysed by a specific enzyme, protoporphyrinogen oxidase (Jacobs et al, 1982). If DPE-induced tetrapyrrole accumulation is due to enhanced synthesis, then stimulation of the activity of ALA synthesising enzymes would be the more likely target.

A third possibility might be that DPEs interfere with the conversion of Proto IX into haem, perhaps by inhibiting ferrochelatase activity.
4.2 Materials and Methods.

4.2.1 Treatment of Plant Material.

Cucumber.

For MDA determinations, cucumber (Cucumis sativus L. Telegraph) seedlings were germinated in Vermiculite in the dark at 25°C for 3 days. The seedlings were coated with a solution containing 10 μM DPE I, 0.1% (v/v) Triton X-100 (0.1% [v/v] Triton X-100, 0.1% [v/v] DMSO in the control). The treated seedlings were incubated in the dark for 22 h, then transferred to white light of intensity 600 ± 50 Wm⁻². At appropriate time intervals seedlings were harvested and assayed for MDA, as described below.

For tetrapyrrole extractions, seedlings were grown for 6 days in Levington compost, in a Conviron controlled environment chamber at 25°C, either in complete darkness or with a 16 h photoperiod. The seedlings were then harvested and the cut ends placed in a solution containing 10 μM DPE I (0.1% [v/v] DMSO, control), 0.1% (v/v) Triton X-100. After 17 h, tetrapyrroles were extracted from the plant tissue, as described below.

Barley.

The mutant barley alb-e's was grown in Vermiculite in the dark for 7 days. The seedlings were then carefully dipped into a solution containing 10 μM DPE I (0.1% [v/v] DMSO, control), 0.1% (v/v) Triton
The dipped seedlings were incubated in the dark for 24 h at 25°C.

*Chlamydomonas.*

For growth inhibition experiments, 5 day old cultures of *C. reinhardtii* (grown under the conditions described in Chapter 2, section 2.2.1) were resuspended in fresh growth medium to give $10^6$ cells per ml. 5 ml aliquots were transferred to 10 ml conical flasks and appropriate additions of herbicides made. The flasks were incubated for 24 h in the dark on an orbital shaker, then transferred to light of intensity 200 Wm$^{-2}$ for 24 h. The cells were then counted using a haemocytometer and $I_{50}$ values (the concentration of herbicide required to reduce the population increase by 50%) determined.

For tetrapyrrole extraction, 5 day old cultures of *C. reinhardtii* were harvested by centrifugation and resuspended to give 0.03 g wet weight cells per ml. 5 ml aliquots were transferred to 10 ml conical flasks and appropriate herbicidal additions made. The flasks were incubated in the dark for 24 h.

4.2.2 MDA Assay of Cucumber Seedlings.

MDA content was determined as described by Matringe *et al* (1986). Tissues were homogenised in a Potter Elvehjem homogeniser in 10 % (w/v) TCA containing 0.25 % (w/v) TBA. The homogenate was heated for 10 min in a boiling water bath, cooled on ice and clarified by centrifugation. MDA content was determined by the absorbance of the
supernatant at 532 nm as described in Chapter 2, section 2.2.7. Dry
weights were determined by drying extracted tissue at 60°C for 48 h.

Cucumber

Tissue samples were homogenized by the method of Davis et al. (1976).
1 or 2 g of tissue were homogenized in 10 ml 0.1 M triethanolamine, pH 7.4
in a Potter-Elvehjem homogenizer, at 4°C. The homogenate was
centrifuged at 20,000 x g for 10 min, then the aqueous supernatant
mixed with 2 equal volumes of ethanol. The final volume was adjusted
to 9 ml with 0.1 M triethanolamine, pH 7.4 and fluorescence
spectrum recorded on a Perkin Elmer 2040 fluorescence
spectrophotometer.

Barley

0.5 g of treated tissue were chopped into fine pieces and extracted
as above. The levels of corrinoids associated were calculated by
determining the fluorescence emission of each extract at 580 nm and
comparing to a standard curve prepared by measuring the fluorescence
emission at 582 nm (excited at 360 nm) of known concentrations of
standard fluid.

4. Discussion

Tissue culture were collected by centrifugation at 3,000 x g and
resuspended in an equal volume of the medium. After 24 h the
extracts were centrifuged at 6000 x g for 10 min. The aqueous
supernatant was mixed with 2 equal volumes of ethanol, the final

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4.2.3 Extraction of Tetrapyrroles.

Cucumber.

Tetrapyrroles were extracted by the method of Rebeiz et al (1975). 1 or 2 g of tissue were homogenised in 10 ml 9:1 (v/v) acetone : 0.1 M NH₄OH in a Potter Elvehjem homogeniser, at 4°C. The homogenate was centrifuged at 39,000 x g for 10 min, then the acetone supernatant washed with 2 equal volumes of hexane. The final volume was adjusted to 5 ml with 9:1 (v/v) acetone : 0.1 M NH₄OH, and fluorescence spectra recorded on a Perkin Elmer 3000 fluorescence spectrophotometer.

Barley.

0.5 g of treated tissue was chopped into fine pieces and extracted as above. The levels of porphyrin accumulated were calculated by determining the fluorescence emission of each extract at 628 nm and comparing to a standard curve prepared by measuring the fluorescence emission at 628 nm (elicited at 398 nm) of known concentrations of standard Proto IX.

C. reinhardtii.

Treated cells were collected by centrifugation at 3,000 x g and resuspended in 10 ml 9:1 v/v acetone : 0.1 M NH₄OH. After 30 min, the extracts were centrifuged at 39,000 x g for 10 min. The acetone supernatant was washed with 2 equal volumes of hexane. The final
volume was adjusted to 5 ml and fluorescence spectra recorded as above.

4.2.4 HPLC Analysis of *Chlamydomonas* Extracts.

To each acetone extract was added 1/17 th volume of 0.25 M maleic acid and the pH adjusted to 6.8 using NaH₂PO₄. Tetrapyrroles were then extracted into peroxide-free ether. The ether extract was washed 3 times with water and concentrated to 1 ml under a stream of nitrogen.

HPLC: The ether extracts were analysed by ion-pair HPLC (Fuesler et al, 1982) on a Spherisorb S5 ODS-EXL C₁₈ reverse phase column (25 cm long, 4.6 mm internal diameter) coupled to a Waters 600E HPLC system. 5 μl of the ether extract was injected on to the column which had been pre-equilibrated using 70% (v/v) methanol : 30% 5 mM PIC A reagent (Waters). Tetrapyrroles were eluted using 70 % methanol : 30 % 5 mM PIC A reagent which was changed to 70 % methanol : 30 % H₂O after 3 min. The flow rate was 0.75 ml/min. Tetrapyrroles were detected with a photodiode array detector (Waters Associates model 990). For further identification peaks were collected and fluorescence spectra recorded.
4.3 Results.

4.3.1 DPE-Induced Tetrapyrrole Accumulation in Higher Plants.

In 1987 Matringe and Scalla (Matringe & Scalla, 1987b) demonstrated tetrapyrrole accumulation in non-chlorophyllous soybean cell cultures and etiolated cucumber seedlings, induced by the NDPE herbicide AFM and the chemically unrelated LS 82-556. The light-dependent peroxidising activity of DPE I in cucumber was demonstrated by incubating etiolated seedlings with 10 μM DPE I in the dark for 22 h, then exposing them to light. Table 4.1 shows that DPE I induced lipid peroxidation in the treated seedlings, but had caused no damage relative to the control during the dark incubation.

Figure 4.2 shows DPE I induced tetrapyrrole accumulation in etiolated cucumber hypocotyl. As was demonstrated with AFM by Matringe and Scalla (1987b), the accumulated pigment has similar fluorescence emission properties to Proto IX. Tetrapyrrole accumulation was also induced in pre-greened cucumber hypocotyl incubated in the dark with DPE I (figure 4.3). Thus NDPE herbicides can induce tetrapyrrole accumulation in non-chlorophyllous and chlorophyllous tissues. This conclusion was also reached by Matringe and Scalla in a later paper (1988b).

If the primary mode of action of DPE herbicides is based on their ability to induce abnormal accumulation of Proto IX, then such accumulation must occur in all tissues susceptible to these herbicides. The albino mutant of barley alb-ε has a reduced
Table 4.1 MDA Formation in DPE I -Treated Etiolated Cucumber Seedlings.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>DMSO (nmol MDA / 0.1 g dry wt)</th>
<th>DPE I (nmol MDA / 0.1 g dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6.59</td>
<td>4.84</td>
</tr>
<tr>
<td>2</td>
<td>5.06</td>
<td>3.74</td>
</tr>
<tr>
<td>4</td>
<td>10.86</td>
<td>183.82</td>
</tr>
</tbody>
</table>

Etiolated seedlings were incubated for 22 h in darkness with 0.1% (v/v) DMSO and 10 µM DPE I then exposed to light.
Figure 4.2 DPE-Induced Tetrapyrrole Accumulation in Etiolated Cucumber Hypocotyl.
Etiolated cucumber seedlings were incubated with 10 μM DPE I or 0.1% (v/v) DMSO in the dark for 24 h. Tetrapyrroles were extracted and fluorescence emission spectra elicited at 398 nm were recorded. The fluorescence emission spectrum for 8 x 10^-7 M Proto IX on the same scale, is also shown.
Figure 4.3 DPE I -Induced Tetrapyrrole Accumulation in Greened Cucumber Hypocotyl. Greened cucumber seedlings were incubated in the dark with 10 μM DPE I or 0.1% (v/v) DMSO for 17 h. Tetrapyrroles were extracted and fluorescence excitation (elicted at 628 nm) and emission (elicted at 398 nm) spectra recorded. The excitation and emission spectra of a standard Proto IX solution are also shown.
synthetic ability for porphyrins (Gough, 1972). The mutation has also resulted in a block in protochlorophyllide formation after Proto IX, apparently at the Mg-chelatase. After treatment with DPE I, both the white and green phenotypes of alb-\textsuperscript{e}\textsuperscript{15} accumulated tetrapyrroles on dark incubation (figure 4.4, table 4.2). It is interesting to note however, that more tetrapyrrole accumulated in the green phenotype control than in the DPE I-treated white phenotype, yet the green control showed no sign of injury when incubated with DPE I under light (see Chapter 3). This could imply that the herbicidal effects observed in the albino mutant when incubated with DPE I under light are not as a direct result of tetrapyrrole accumulation. However, the tetrapyrrole levels shown in table 4.2 are those accumulated after 24 h dark incubation, under conditions where the conversion of protochlorophyllide to chlorophyllide is inhibited, and tetrapyrroles would not be expected to accumulate in untreated, green seedlings in the light. It must also be noted that green tissue has higher levels of carotenoid (see Chapter 3) which would protect against Proto IX-induced \textsuperscript{1}O\textsubscript{2} formation.
Table 4.2 Tetrapyrrole Accumulation in alb-e′ After 24 h Dark Incubation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>nmol Proto IX/ g dry weight tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>white phenotype (DMSO)</td>
<td>0.79</td>
</tr>
<tr>
<td>white phenotype (10 μM DPE I)</td>
<td>2.52</td>
</tr>
<tr>
<td>green phenotype (DMSO)</td>
<td>2.80</td>
</tr>
<tr>
<td>green phenotype (10 μM DPE I)</td>
<td>92.52</td>
</tr>
</tbody>
</table>

Figure 4.4 DPE I -Induced Tetrapyrrole Accumulation in the Barley Mutant alb-e′.
White and green phenotypes of alb-e′ were treated with 10 μM DPE I or 0.1% (v/v) DMSO, and incubated in the dark for 24 h. Tetrapyrroles were extracted and fluorescence excitation (elicited at 628 nm) and emission (elicited at 398 nm) spectra were recorded.
4.3.2 DPE-Induced Tetrapyrrole Accumulation in Algae.

The ability of DPE I to induce abnormal accumulation of tetrapyrroles in *Scenedesmus* has been shown in Chapter 2.

Figures 4.5 and 4.6 show that on dark incubation, tetrapyrroles accumulate in DPE I -treated *C. reinhardtii*. An examination of the visible spectrum of the acetone extract from DPE I -treated *C. reinhardtii* (figure 4.5) shows a large absorption peak at 402 nm with small peaks at 505, 537, 572 and 627 nm. Similar peaks are observed in the absorption spectrum of Proto IX. The fluorescence emission spectrum of the DPE I treated extract shows a peak at 628 nm, similar to that observed for Proto IX.

Figure 4.7 shows accumulation of tetrapyrroles in DPE I treated *C. reinhardtii* cells over a 30 h time period. Tetrapyrroles appeared to accumulate rapidly for the first 10 h, after which tetrapyrrole levels increased more slowly and begin to 'level-out'.

Figure 4.8 shows the effect of increasing DPE I concentration on tetrapyrrole accumulation (determined as in section 4.2.3). At low concentrations (<0.25 μM) the amount of tetrapyrrole accumulated appeared to increase linearly with herbicide concentration. At concentrations above 1 μM, increasing the herbicide concentration had little effect on the amount of tetrapyrrole accumulated after 24 h dark incubation. Halling and Peters (1987) observed similar dose response effects with 3-o-methyl-[C\(^14\)]-glucose leakage from etiolated cucumber cotyledons which had been pre-incubated with herbicide in darkness for 6 h prior to illumination. It must be noted that the results presented in Figure 4.8 were obtained from 1 batch of
Figure 4.5 Absorption Spectrum of a Crude Extract from DPE I-Treated *C. reinhardtii* Cells.
*C. reinhardtii* cells were incubated with 5 μM DPE I or 0.1% (v/v) DMSO. Tetrapyrroles were extracted and an absorption spectrum recorded of the extract from DPE I-treated cells against the extract from DMSO-treated cells. The absorption spectrum of standard Proto IX dissolved in 9:1 (v/v) acetone : 0.1 M NH₄OH is also shown.
Figure 4.6 Fluorescence Spectrum of Tetrapyrroles Extracted from DPE I-Treated *C. reinhardtii* Cells. *C. reinhardtii* cells were incubated for 24 h in the dark with 0.1% DMSO or 5 μM DPE I. Tetrapyrroles were extracted and fluorescence excitation (elicited at 628 nm) and emission (elicited at 398 nm) spectra recorded.
Figure 4.7 Time Course for DPE I -Induced Tetrapyrrole Accumulation in *C. reinhardtii* Cells.
*C. reinhardtii* cells were incubated in the dark with 10 µM DPE I. Tetrapyrroles were extracted and fluorescence emission (elicited at 398 nm) at 628 nm recorded.
Figure 4.8 The Effect of Herbicide Concentration on Tetrapyrrole Accumulation in *C. reinhardtii*.
*C. reinhardtii* cells were incubated in the dark with different concentrations of DPE I, for 24 h. Tetrapyrroles were extracted and the fluorescence emission (elicited at 398 nm) at 628 nm recorded.
C. reinhardtii cells. The maximum amount of tetrapyrrole accumulating at high herbicide concentrations was subject to much variation between different C reinhardtii cultures, presumably due to different rates of metabolism in different cultures. However, the relationship between herbicide concentration and relative amounts of tetrapyrrole accumulating was always the same, with a linear increase at low herbicide concentrations.

An experimental protocol designed to quantify the relative ability of compounds to elicit tetrapyrrole accumulation had to take into account variations in maximum amounts of tetrapyrrole produced from one batch of cells to the next, as well as the fact that the establishment of a maximal level of tetrapyrrole accumulation might simply reflect the limited solubility of the herbicide. Thus the ability of a particular herbicide to induce tetrapyrrole accumulation over the linear region of the dose response graph was directly related to that of DPE I as a reference standard for each batch of C. reinhardtii cells. The protocol and results are shown in table 4.3. Phthalide DPE II appeared to be the most potent herbicide for inducing tetrapyrrole accumulation, followed by oxyfluorfen. Nitrofen was the least effective herbicide of those tested in this respect. The primary screen data (see Chapter 3, table 3.3) showed that phthalide DPE II was a more effective herbicide against 8 plant species than DPE I. Nitrofen was shown to be less effective than DPE I. Although C. reinhardtii proved useful for structure activity studies on the ability of herbicides to induce tetrapyrrole accumulation, it has proved to be less useful for studying the other effects of NDPE-type herbicides. As the results in Chapter 2 show, DPE I (and other
Table 4.3: Comparison of the Ability of DPEs to Induce Tetrapyrrole Accumulation with that of DPE I.

<table>
<thead>
<tr>
<th>Herbicide</th>
<th>Relative ability to induce accumulation compared to DPE I</th>
</tr>
</thead>
<tbody>
<tr>
<td>phthalide DPE II</td>
<td>27.66</td>
</tr>
<tr>
<td>oxyfluorfen</td>
<td>12.55</td>
</tr>
<tr>
<td>phthalide DPE III</td>
<td>3.76</td>
</tr>
<tr>
<td>phthalide DPE III (S(-)) isomer</td>
<td>4.67</td>
</tr>
<tr>
<td>phthalide DPE III (R(+)) isomer</td>
<td>1.06</td>
</tr>
<tr>
<td>phthalide DPE IV</td>
<td>1.28</td>
</tr>
<tr>
<td>DPE I</td>
<td>1.00</td>
</tr>
<tr>
<td>nitrofen</td>
<td>0.22</td>
</tr>
</tbody>
</table>

Tetrapyrrole accumulation in *C. reinhardtii* was determined with a range of herbicide concentrations. Where plots of herbicide concentration vs tetrapyrrole accumulation were linear (see figure 4.8, dashed line), the slope was determined. The relative ability to induce tetrapyrrole accumulation was derived by dividing this value for each herbicide by that obtained for DPE I in the particular batch of cells used.
DPEs) did not induce ethane formation in this alga and cultures of *C. reinhardtii* recover from DPE I -induced bleaching. Thus, in order to relate tetrapyrrole accumulation to herbicidal toxicity, it was decided to use inhibition of growth as a measure of herbicidal activity. Hess (1980) previously used a similar assay to measure the activity of bifenox and other herbicides in *C. eugametos*. The results are presented in table 4.4. Nitrofen caused the least inhibition of growth of *C. reinhardtii* cultures. However, in this study, oxyfluorfen was more potent at inhibiting growth than phthalide DPE II.

The tetrapyrrole containing extracts from *C. reinhardtii* were analysed by ion pair HPLC. Incubation of *C. reinhardtii* in the dark with DPE I resulted in the abnormal accumulation of at least 4 components (the peak with *R*ₜ 32.10 min appeared to consist of more than one component, see Appendix II) (figure 4.9). The major pigment accumulating (*R*ₜ 23.70 min) was identified as Proto IX by comparison of its retention time, absorption and fluorescence spectra with standard Proto IX (figure 4.10). When the DPE I-treated extract and standard Proto IX were co chromatographed, the peak identified as Proto IX was enhanced (figure 4.11). It can be seen from this figure that the peaks have shifted to a longer retention time. Such shifts are also shown in figure 4.12. Although this HPLC system consistently separated the tetrapyrroles, shifting retention times were a problem and for future work perhaps different HPLC conditions should be considered. In the absence of specific standards it was not possible to positively identify the other pigments accumulating. However, from its relative retention time and absorption spectrum.
Table 4.4  \(I_{50}\) Values for the Inhibition of Growth of \textit{C. reinhardtii}.

<table>
<thead>
<tr>
<th>Herbicide</th>
<th>(I_{50}) ((\mu M))</th>
</tr>
</thead>
<tbody>
<tr>
<td>oxyfluorfen</td>
<td>0.0026</td>
</tr>
<tr>
<td>phthalide DPE III</td>
<td>0.023</td>
</tr>
<tr>
<td>phthalide DPE III S(-) isomer</td>
<td>0.019</td>
</tr>
<tr>
<td>phthalide DPE III R(+) isomer</td>
<td>0.083</td>
</tr>
<tr>
<td>phthalide DPE II</td>
<td>0.030</td>
</tr>
<tr>
<td>DPE I</td>
<td>0.160</td>
</tr>
<tr>
<td>nitrofen</td>
<td>0.340</td>
</tr>
</tbody>
</table>

\(I_{50}\) is defined as the concentration of herbicide required to give a 50% inhibition of growth of the culture, monitored by the effect on increase in cell density 24 h after transfer to light of intensity 200 \(Wm^{-2}\).
Figure 4.9  HPLC Analysis of Tetrapyrroles Extracted from DPE I-Treated *C. reinhardtii* Cells.

Pigment extracts from *C. reinhardtii* were separated by ion pair HPLC. Elution of chromophores was monitored at 405-460 nm.

A  Cells incubated 24 h in the dark with 10 μ DPE I.

B  Cells incubated with 0.1% (v/v) DMSO.

C  Protoporphyrin IX standard.
Figure 4.10 Absorption Spectra of the Peak with $R_k$ 23.7 min (A) and Standard Protoporphyrin IX (B). The inserts show corresponding fluorescence emission spectra (elicited at 388 nm) recorded between 550 and 730 nm.
Figure 4.11 Co-Chromatography of an Extract from DPE I-Treated C. reinhardtii Cells with Standard Protoporphyrin IX. Ten ml of $8 \times 10^{-7}$ M Proto IX dissolved in 9:1 (v/v) acetone : 0.1 M NH$_4$OH was extracted into ether and reduced to a volume of 1 ml under N$_2$. Ten µl of the standard Proto IX solution plus 5 µl of the cell pigment extract were co-injected on to the column.
(absorption maximum 415 nm, see Appendix II) (Witkowski & Halling, 1988; Fuesler et al, 1982), the peak of Rₜ 21.40 min has been tentatively identified as Mg-Proto IX. Phthalide DPE III is a mixture of 2 enantiomeric forms. The S(-) and R(+) isomers have been separated using a chiral stationary phase HPLC column (Camilleri et al, 1989). By measuring ethane production in french bean (Vicia faba) and barley (Hordeum vulgare), Camilleri et al (1989) demonstrated that the biological activity of the S(-) isomer was substantially greater than that of the R(+) isomer. This difference in activity is reflected in the relative abilities of the isomers to inhibit growth of C. reinhardtii cultures (table 4.4). Figure 4.12 shows tetrapyrrole accumulation against herbicide concentration for phthalide DPE III. For experiments to compare the abilities of the R(+) and S(-) isomers of phthalide DPE III, concentrations of herbicides from the linear part of the above graph were chosen. Figure 4.13 shows that the S(-) isomer induced greater accumulation of tetrapyrroles than the R(+) isomer. Analysis by HPLC of extracts from the algal cells treated with the isomers (figure 4.14), revealed that at 0.075 μM the S(-) isomer induced approximately a six-fold greater accumulation of tetrapyrroles than the R(+) isomer.
Figure 4.12 The Effect of Herbicide Concentration on Phthalide DPE III -Induced Tetrapyrrole Accumulation in C. reinhardtii. C. reinhardtii cells were incubated with various concentrations of phthalide DPE III in darkness, for 24 h.
Figure 4.13 Accumulation of Tetrapyrroles by Enantiomers of Phthalide DPE III.
C. reinhardtii cells were incubated with 0.025 μM R(+) isomer, 0.025 μM S(-) isomer or 0.1% (v/v) propanol, in darkness for 24 h. Tetrapyrroles were extracted and fluorescence emission spectra (elicited at 398 nm) recorded.
Figure 4.14  HPLC Separation of Tetrapyrroles Accumulated by C. reinhardtii Cells Treated with Enantiomers of Phthalide DPE III.
Pigment extracts from C. reinhardtii were separated by ion-pair HPLC, and elution of chromophores monitored 405 nm.
A Cells treated with 0.075 μM S(-) isomer.
B Cells treated with 0.075 μM R(+) isomer.
C Untreated cells.
The peak with Rₜ 25.8 min was tentatively identified as Proto IX.
4.4 Discussion.

DPE-induced tetrapyrrole accumulation has to date been reported in non-chlorophyllous soybean suspension cultures (Matringe & Scalla, 1987b), etiolated and greened cucumber seedlings (this chapter, Matringe & Scalla, 1988; Lyndon & Duke, 1988; Witkowski & Halling, 1988), tobacco cell suspensions (Kouji et al., 1988) and in the algae *Scenedesmus* and *Bumilleriopsis filiformis* (Sandmann & Boger, 1988; Bowyer et al., 1989). Also, induction of tetrapyrrole accumulation has been demonstrated by herbicides with DPE-like activity, including LS 82-556 (Matringe & Scalla, 1987b) and the cyclic imides (Sandmann & Boger, 1988). In this chapter it has been shown that the NDPEs can also induce tetrapyrrole accumulation in barley and the alga *C. reinhardtii*. The ability of the phthalide DPEs to induce tetrapyrrole accumulation has also been demonstrated. Furthermore, the data presented here support the previous suggestion that the major tetrapyrrole accumulating on incubation with NDPEs is Proto IX. Witkowski & Halling (1988) analysed extracts from ALA-fed, AFM treated cucumber (*Cucumis sativus*) by HPLC. The authors could find no evidence for the accumulation of Mg-Proto IX, leading them to propose that the NDPEs interfere with Mg-chelatase activity. However, analysis of DPE I-treated *C. reinhardtii* extracts suggests that the herbicide does induce slightly increased levels of this porphyrin in *Chlamydomonas* at least, suggesting that Proto IX accumulation might be induced by some mechanism other than inhibition of Mg-chelatase. The results obtained with the *alb-e*e* mutant of barley would support this conclusion since
DPE I caused tetrapyrrole accumulation in this mutant even though Mg-chelatase activity is already impaired by the mutation. However, it could be argued that the DPE I -induced tetrapyrrole accumulation in alb-e occurs as a result of inhibition of residual Mg-chelatase activity. Gough (1972) found that on feeding with ALA, this mutant retained some ability to synthesise protochlorophyllide (15 % of total porphyrin synthesised). With the exception of phthalide DPE II (in relation to oxyfluorfen and phthalide DPE III), the relative order of toxicity of the herbicides tested (as determined by growth inhibition) in C. reinhardtii was reflected in the relative order of the ability of these herbicides to induce tetrapyrrole accumulation in this alga. Inhibition of growth might have been a result of damage induced by the accumulated tetrapyrrole, when the cultures were transferred to light. However, since DPEs are able to inhibit growth of C. reinhardtii in darkness (Chapter 2, table 2.1), it is possible that growth inhibition could be related to an inhibition of chlorophyll synthesis, haem synthesis, or both.

Of the phthalide DPEs, Camilleri et al (1988) found that phthalide DPE II (methoxyphthalide II) was the most active as determined by ethane generation in french bean plants, followed by phthalides III and IV respectively. Replacing the methoxy group in the 3 position of the phthalide ring with a H atom or propoxy group decreased activity considerably. Furthermore, the above order of activity was consistent on a number of broad leaf and grass species treated with these compounds in a primary screen bioassay. These results indicated that the substituent at the 3 position of the phthalide ring is extremely important in determining the herbicidal activity of these compounds.
In a subsequent paper Camilleri et al (1989) described the separation of 2 enantiomeric forms of phthalide DPE III differing only by their absolute configuration at the 3 position of the phthalide ring. The difference in activity between the enantiomeric forms as demonstrated in wheat, barley, french bean, cleavers, chickweed, speedwell (Camilleri et al, 1989) and here in C. reinhardtii indicate that either the initial step in the action of DPEs involves the interaction of these molecules with an enantiotopically discrete site or that the 2 enantiomers are metabolised at different rates. The results of Camilleri et al (1989) show that french bean is very susceptible to both NDPEs and the S(-) isomer of phthalide DPE III and presumably lacks a NDPE-metabolising enzyme which is present in resistant species, for example, soybean (Frear et al, 1983). It is extremely unlikely that a metabolising enzyme with a uniquely high specificity for the R(+) isomer of phthalide DPE III would exist, especially over the range of species studied. Thus although differential metabolism cannot be completely ruled out as an explanation for the differences in activity of the enantiomers, it seems more likely that such differences reflect the interaction of these molecules with an enantiotopically specific active site, suggesting the likely involvement of a protein binding process in the mode of action of DPE herbicides.

The greater ability of the S(-) isomer of phthalide DPE III to induce Proto IX accumulation and inhibit growth in C.reinhardtii compared to the R(+) isomer further links herbicidal activity to the ability of a DPE to induce tetrapyrrorole accumulation, and suggests that the phthalide DPEs (NDPEs) may act by inhibiting the activity
of an enzyme of the chlorophyll biosynthetic pathway. The enantiomers should prove to be a useful tool in the identification of the primary \textit{in vivo} target enzyme of the DPEs.

It remains a mystery as to why DPE I did not induce ethane formation or cell death as determined by FDA staining in illuminated \textit{C. reinhardtii} cells, despite being able to induce similar accumulation of tetrapyrrole in darkness to that induced in \textit{S. obliquus} (10 \textmu M DPE I induced 50.9 nmol tetrapyrrole per g wet weight cells in \textit{S. obliquus}, 5 \textmu M DPE I induced 48.3 nmol tetrapyrrole per g wet weight cells in \textit{C. reinhardtii}, after 24 h dark incubation). In an attempt to observe a synergistic effect, \textit{C. reinhardtii} cells were incubated with 10 \textmu M DPE I and 10 \textmu M ALA, under illumination. However, even under these conditions no ethane production was observed. It must be assumed either that a different situation occurs in the light in \textit{C. reinhardtii} (for example, a very high rate of destruction of Proto IX before it can cause significant photodamage) or, that the protective mechanisms specific to singlet oxygen are enhanced in \textit{C. reinhardtii} compared to \textit{S. obliquus}.
CHAPTER 5

Discussion

At the time that this work was started, the ability of DPE
barbiturates to induce abnormal accumulation of tetraporphyrins in plants
was known. With the publication of the work of Mahaffey &
Scalia (1967, 1969), opinions rapidly moved away from theories
involving tetraporphyrins as a major source for the DPE activity, to
newer ideas involving changes in the photosynthetic electron transport chain. The hypothesis of Mahaffey &
Scalia that the DPE-induced activity of DPE-DPE barbiturates results
from their ability to interfere with the synthesis of tetraporphyrins,
no different from most of what is known of DPH activity. DPE
barbiturates have been shown to be active in red, orange, green and blue
light (Green et al., 1923; Venkatesara & Stobbe, 1965; Smalley &
Drew, 1953; Dole et al., 1967; Mahaffey & Scalia, 1967, 1969) which are
consistent with the absorption spectrum of Prat IX. Tetraporphy
accumulation may occur in the dark but light is required for the
photosynthetic reactions that occur prior to membrane accumulation,
thereby explaining the light requirement for DPE toxicity. This may also
explain the observations of Dole et al. (1963) who noted that a
long period of 10 hours incubation with actinomycin was necessary for
effective activity of the DPE in barley. The observation regarding
sufficient time for the accumulation of the reactive porphyrins to
cause various photodestruction.

Antioxid-A has been demonstrated to have a protective effect
against the action of both actinomycin and dibenzylhydrazine (DBH) as
well as other aromatic amines. These findings by Bell et al. (1969) found that
Antioxid-A was able to protect in vitro cell membranes more effectively during incubation
period than antipyrine induced oxidative damage (Green 1970).
At the time that this work was started, the ability of DPE herbicides to induce abnormal accumulation of tetrapyrroles in plant tissues was unknown. With the publication of the work of Matringe & Scalla (1987b, 1988a), opinions rapidly moved away from theories involving carotenoids as the photosensitizers for DPE activity, or mechanisms involving reduction of the DPE to a radical by the photosynthetic electron transport chain. The hypothesis of Matringe & Scalla that the herbicidal activity of DPE-type herbicides results from their ability to interfere with the metabolism of tetrapyrroles, is consistent with most of what is known of DPE action. DPE herbicides have been shown to be active in red, orange, green and blue light (Gabba et al, 1988; Vanstone & Stobbe, 1979; Ensminger & Hess, 1985; Sato et al, 1987; Matringe & Scalla, 1987b, 1988) which is consistent with the absorption spectrum of Proto IX. Tetrapyrrole accumulation may occur in the dark but light is required for the photooxidative reactions that occur prior to membrane destruction, thus explaining the light requirement for DPE toxicity. This may also explain the observations of Duke et al (1984, 1986) who noted that a long (about 20 h) dark incubation with acifluorfen was necessary for maximal activity of the herbicide, the dark period providing sufficient time for the accumulation of the required porphyrin to cause maximum photodestruction.

Antimycin A has been demonstrated to have a protective effect against the action of both acifluorfen and acifluorfen methyl (Duke et al, 1984; Matringe & Scalla, 1987a). Kenyon et al (1988) found that if antimycin A was added at the beginning of a 20 h dark incubation period, then acifluorfen-induced electrolyte leakage was reduced on
subsequent exposure to light. No such protection was observed if the antimycin A was added at the beginning of the light exposure. The authors suggested that full herbicidal activity of acifluorfen was dependent upon a process which required mitochondrial respiration during the dark incubation period. Matringe & Scalla (1988a) subsequently showed that AFM-induced tetrapyrrole accumulation was reduced in the presence of antimycin A. A requirement for ATP in the synthesis of ALA could explain why antimycin A inhibits the DPE-induced Proto IX accumulation in dark-treated plant tissue. In such tissue, respiratory electron transport, which is inhibited by antimycin A, presumably provides much of the ATP required.

The protective effects of inhibitors of porphyrin synthesis, 4,6-dioxoheptanoic acid (Matringe & Scalla, 1988a & b) and gabaculine (Lydon & Duke, 1988) against DPEs, further connect the activity of these herbicides with tetrapyrrole accumulation. This theory could also explain the failure to demonstrate DPE-induced lipid peroxidation in thylakoid preparations (Chapter 2; Ensminger & Hess, 1985) which do not have soluble precursors and are therefore probably unable to perform any significant tetrapyrrole synthesis. The demonstration of oxyfluorfen-induced singlet oxygen formation in isolated illuminated thylakoids (Haworth & Hess, 1988) is anomalous in this respect. Matringe & Scalla (1988b) also examined the protective effect of the carotenoid biosynthesis inhibitor norflurazon against DPE activity in cucumber seedlings. Norflurazon prevented or significantly lowered the accumulation of porphyrin in AFM treated cotyledons. Previously it had been assumed that the protective effect of norflurazon was a direct consequence of its ability to inhibit carotenoid biosynthesis.
It has now been proposed that the role of norflurazon is also to disrupt chloroplast biogenesis and thus inhibit tetrapyrrole synthesis. Presumably a similar explanation holds for the tolerance to DPEs of carotenoid-free mutants (Matsunaka, 1969).

Cultures of *S. obliquus* incubated with DPE I in the dark showed reduced tetrapyrrole accumulation in the presence of DCMU. However, in the presence of light it is expected that inhibition of photosynthetic electron transport by this compound leading to conditions of oxygen limitation, is more important in the protective effect of DCMU against DPE activity. Molecular oxygen is obviously required for the generation of singlet oxygen, for the processes of lipid peroxidation and indeed for tetrapyrrole synthesis itself (Castelfranco & Schwarcz, 1978). A further role for photosynthesis in DPE toxicity might be to provide ATP for ALA synthesis. Kannangara and Gough (1977) found that the strong stimulation by light of the synthesis of ALA from glutamate or α-ketoglutarate in isolated barley and spinach plastids, was due to the increased generation of ATP and NADPH. 10 μM DCMU was found to inhibit ALA formation by 60% in isolated plastids from immature spinach leaves whilst 3 μM DCMU inhibited ALA formation by 92% in isolated barley plastids. Furthermore, Boger and co-workers (data presented at the meeting on Herbicides Active in the Chloroplast, Monheim, West Germany, 1989) have shown that while autotrophically grown and heterotrophically grown *Scenedesmus* cells both accumulate Proto IX during a 4 h aerobic light incubation (the Proto IX disappears on further illumination), DCMU has a much greater effect on Proto IX accumulation in the autotrophically grown cells than in the heterotrophic cells supplied
with glucose. Under these conditions, the primary role of photosynthesis is probably to provide ATP and reducing power for ALA biosynthesis; glucose metabolism provides these substrates in the heterotrophic cells.

The tetrapyrrole accumulating ability of the DPEs is now established but the question of how this occurs has not yet been satisfactorily answered. The most obvious hypothesis, that of Witkowski & Halling (1988), is that these herbicides inhibit the activity of the Mg-chelatase. This enzyme appears to be situated at or near the surface of the plastid envelope (Fuesler et al, 1984), and ultrastructural studies show that the plastid envelope is an early site of DPE-induced damage (Orr & Hess, 1982; Kenyon et al, 1985; Bowyer et al, 1987; Derrick et al, 1988). However, the results in this study suggest, that in C. reinhardtii at least, incubation with NDPEs might also cause the elevation of Mg-Proto IX levels, suggesting that the effect of these herbicides may not purely be to inhibit Mg-chelatase activity. Furthermore, Scalla and Matringe (1989b) have shown using corn etioplasts, that AFM inhibits the synthesis of Mg-Proto IX from ALA but not from Proto IX, suggesting an inhibitory site between ALA and Proto IX rather than an inhibition of Mg-chelatase. The differential effects of the enantiomers of phthalide DPE III point to the likely involvement of an enzymic binding process in the mode of action of these herbicides.

A second possible effect of DPEs on the porphyrin synthesis pathway could be that these herbicides inhibit ferrochelatase. However, Kouji et al (1988) found that AFM and nitrofen did not inhibit the activity of this enzyme in a spinach homogenate. This
result was confirmed by Matringe et al (1989b) who found that AFM and its analogues showed very little inhibitory activity towards the mitochondrial ferrochelatases of yeast, potatoes and mouse liver.

A third possibility is that DPEs stimulate porphyrin synthesis, possibly by stimulating ALA synthesising enzymes (Kouji et al., 1988). Kouji et al (1988) found that AFM-treated green cucumber cotyledons accumulated large amounts of protochlorophyllide in the dark (a result in direct contrast with that of Witkowski and Halling (1988) who reported that AFM inhibited protochlorophyllide synthesis in dark incubated, ALA-fed cucumber cotyledons), suggesting that the herbicide did not inhibit Mg-chelatase. Furthermore, when the transformation of ALA was inhibited by levulinic acid, the amount of ALA synthesised was increased in the presence of AFM, suggesting that AFM was stimulating the ALA synthesising enzymes.

Since the completion of this work, Matringe et al (1989a & b) have shown that DPEs (and other herbicides with DPE-like activity) have a strong inhibitory effect on the enzyme protoporphyrinogen oxidase, which catalyses the conversion of protoporphyrinogen to Proto IX. Thus, the accumulation of Proto IX which occurs in DPE-treated plants would be the result of the non-enzymic oxidation of protoporphyrinogen. Such an explanation requires that the non-enzymically produced Proto IX is not readily available to the membrane bound ferrochelatase and Mg-chelatase. Matringe et al (1989b) suggest that the accumulating protoporphyrinogen (which does not absorb or fluoresce in the visible spectral region) diffuses from its site of synthesis before non-enzymic oxidation to Proto IX. Because of its abnormal sub-cellular location, the Proto IX so formed is not
available to the metal chelatases. Since a major site of protoporphyrinogen synthesis is likely to be in the plastid, which also houses the Mg-chelatase activity, it could be assumed that Proto IX generated in the cytoplasm by spontaneous oxidation of protoporphyrinogen diffusing out of the plastid, is unable to re-enter this organelle. However, this would be somewhat inconsistent with the demonstration of Mg-Proto IX formation from added Proto IX in etioplasts (Matringe et al, 1989b) and an alternative sequestration mechanism appears to be required. In this model there would also be no accumulation of Mg-porphyrins and the results from C. reinhardtii do suggest that DPEs can cause a slight accumulation of Mg-Proto IX. Although an inhibition of protoporphyrinogen oxidase would explain the inhibition of Mg-Proto IX formation from ALA in etioplasts (this would be limited by the spontaneous rate of oxidation of protoporphyrinogen to Proto IX), it cannot account for the enhanced rate of ALA synthesis in green cucumber cotyledons (Kouji et al, 1988) and indeed is inconsistent with the accumulation of protochlorophyllide. It remains to be determined whether the enantiomers of phthalide DPE III differentially inhibit protoporphyrinogen oxidase but this would provide a critical test of the Matringe model.

Matringe et al (1989b) also showed that DPEs are strong inhibitors of mitochondrial protoporphyrinogen oxidase in preparations from potato, yeast and mouse liver. Duke et al (1984) first suggested the involvement of the mitochondrion in the mechanism of action of acifluorfen. The toxic oxygen species implicated was however, superoxide radical rather than singlet oxygen. It is possible that singlet oxygen might have damaged the respiratory chain leading to the
increased superoxide formation. It would be clearly of considerable interest to determine the sub-cellular location of accumulated Proto IX in DPE treated plant tissues.

It is difficult to imagine that the induction of tetrapyrrole accumulation is the single major mode of action of NDPE herbicides, when the effects of these herbicides on isolated chromoplasts are considered. In whole petals, NDPEs may lead to tetrapyrrole accumulation in plastids, giving rise to the observed necrosis and lipid peroxidation. However, it seems unlikely that the chromoplast fragments isolated from chrysanthemum, would be capable of significant tetrapyrrole synthesis, and even if they were, this does not explain why the phthalide DPEs, potent herbicides in other plant tissues, were ineffective in this system. Furthermore, no evidence could be found for the involvement of singlet oxygen in NDPE-induced chromoplast bleaching. It would appear that there is a different mode of action for these herbicides in isolated chromoplasts and possibly intact petals, one that is not available to non-nitro DPEs. However, this mechanism remains a mystery and it is possible that carotenoids have a role in such a mode of action. Orr and Hogan (1985) described the stimulation of β-carotene bleaching in Triton X-100 micelles by lipophilic NDPE herbicides. Very little activity, even at high concentrations, was observed with a chloro analogue of AFM. This bleaching however, was shown to be dependent on UV-A light, white light alone (400-700 nm) being without effect. It would be interesting to determine the effects of porphyrin synthesis inhibitors on NDPE-induced chromoplast bleaching, in order to completely eliminate tetrapyrrole accumulation as the mode of action in these
organelles.

In conclusion then, it would appear that the NDPE and related herbicides have more than one possible mode of action in plant tissues. However, there is very strong evidence to suggest that the major mechanism of action in higher plants and algae is the ability of these herbicides to induce the abnormal accumulation of Proto IX. This photosensitising pigment then reacts with molecular oxygen under light, to produce singlet oxygen which gives rise to membrane lipid peroxidation, leading to cellular disruption. The major questions remaining relate to the actual mechanisms by which Proto IX accumulates in dark-treated tissue, and the modifications to this process in the light.
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Appendix I

Absorption Spectra from the HPLC Separation of the Carotenoids of alb-e\textsuperscript{a} (White Phenotype), and of Chl a and Chl b of Wild Type Barley.
(see Chapter 3)

<table>
<thead>
<tr>
<th>Carotenoid</th>
<th>λ\text{max} (nm)</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>δ-carotene</td>
<td>230, 448, 478</td>
<td>pet, ether</td>
</tr>
<tr>
<td>(β,δ-carotene)</td>
<td>429, 448, 478</td>
<td>ethanol</td>
</tr>
<tr>
<td>lutein</td>
<td>421, 444, 474</td>
<td>pet, ether</td>
</tr>
<tr>
<td>(β,γ-carotene-3,3'-dial)</td>
<td>422, 445, 474</td>
<td>ethanol</td>
</tr>
<tr>
<td>anthoxanthin</td>
<td>423, 448, 472</td>
<td>pet, ether</td>
</tr>
<tr>
<td>(γ,β-caroxy-3,6-dihydro)</td>
<td>427, 447, 472</td>
<td>ethanol</td>
</tr>
<tr>
<td>(γ,β-carotene-3,3'-dial)</td>
<td>430, 485, 484</td>
<td>chloroform</td>
</tr>
<tr>
<td>violaxanthin</td>
<td>416, 440, 485</td>
<td>pet, ether</td>
</tr>
<tr>
<td>(γ,β-caroxy-3,6'-dihydro)</td>
<td>416, 440, 485</td>
<td>ethanol</td>
</tr>
<tr>
<td>5',5'-tetrahydro-α,δ</td>
<td>475, 485, 495</td>
<td>chloroform</td>
</tr>
<tr>
<td>caroten-3,3'-dial</td>
<td></td>
<td></td>
</tr>
<tr>
<td>neoxanthin</td>
<td>416, 440, 487</td>
<td>pet, ether</td>
</tr>
<tr>
<td>(β,γ-caroxy-3,7-dihydro)</td>
<td>416, 440, 487</td>
<td>ethanol</td>
</tr>
<tr>
<td>5,5'-tetrahydro-α,δ</td>
<td>425, 440, 475</td>
<td>chloroform</td>
</tr>
<tr>
<td>violaxanthin</td>
<td>426, 440, 475</td>
<td>pet, ether</td>
</tr>
<tr>
<td>(β,γ-carotene-3,3'-dial)</td>
<td>426, 440, 475</td>
<td>ethanol</td>
</tr>
<tr>
<td>neoaxanthin</td>
<td>426, 440, 475</td>
<td>acetone</td>
</tr>
<tr>
<td>(β,γ-carotene-3,3'-dial)</td>
<td>426, 440, 475</td>
<td>acetone</td>
</tr>
</tbody>
</table>

adapted from Arlt, 1980.
Table AI.1 Light Absorption by the Carotenoids.

<table>
<thead>
<tr>
<th>Carotenoid</th>
<th>λ max (nm)</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-carotene (β,β-carotene)</td>
<td>425, 449, 476</td>
<td>pet. ether</td>
</tr>
<tr>
<td></td>
<td>450, 476</td>
<td>ethanol</td>
</tr>
<tr>
<td></td>
<td>429, 452, 478</td>
<td>acetone</td>
</tr>
<tr>
<td>lutein (β,ε-carotene-3,3'-diol)</td>
<td>421, 445, 474</td>
<td>pet. ether</td>
</tr>
<tr>
<td></td>
<td>422, 445, 474</td>
<td>ethanol</td>
</tr>
<tr>
<td>antheraxanthin (5,6-epoxy-5,6-dihydro-β,β-carotene-3,3'-diol)</td>
<td>422, 445, 472</td>
<td>pet. ether</td>
</tr>
<tr>
<td></td>
<td>422, 444, 472</td>
<td>ethanol</td>
</tr>
<tr>
<td></td>
<td>430, 456, 484</td>
<td>chloroform</td>
</tr>
<tr>
<td>violaxanthin (5,6,5',6'-diepoxyl-5,6,5',6'-tetrahydro-β,β-carotene-3,3'-diol)</td>
<td>416, 440, 465</td>
<td>pet. ether</td>
</tr>
<tr>
<td></td>
<td>419, 440, 470</td>
<td>ethanol</td>
</tr>
<tr>
<td></td>
<td>426, 449, 478</td>
<td>chloroform</td>
</tr>
<tr>
<td>neoxanthin (5',6'-epoxy-6,7-diepoxyl-5,6,5',6'-tetrahydro-β,β-carotene-3,5,3'-triol)</td>
<td>416, 438, 467</td>
<td>pet. ether</td>
</tr>
<tr>
<td></td>
<td>415, 439, 467</td>
<td>ethanol</td>
</tr>
<tr>
<td></td>
<td>423, 448, 476</td>
<td>chloroform</td>
</tr>
<tr>
<td>zeaxanthin (β,β-carotene-3,3'-diol)</td>
<td>424, 449, 476</td>
<td>pet. ether</td>
</tr>
<tr>
<td></td>
<td>428, 450, 478</td>
<td>ethanol</td>
</tr>
<tr>
<td></td>
<td>430, 452, 479</td>
<td>acetone</td>
</tr>
<tr>
<td></td>
<td>433, 462, 493</td>
<td>chloroform</td>
</tr>
</tbody>
</table>

Adapted from Britton, 1985.
Figure A1.1 Absorption Spectra of the Peaks of $R_t$ 28.50 min and $R_t$ 29.43 min from the HPLC Separation of a Pigment Extract from $alb-e^{lo}$ (White Phenotype). See figure 3.4.
Figure A1.2  Absorption Spectrum of the Peak of $R_t$ 32.74 min from the HPLC Separation of a Pigment Extract from $alb-e'$ (White Phenotype). See figure 3.4.
**Figure A1.3** Absorption Spectra of the Peaks Identified as Chl a ($R_t$ 17.81 min) and Chl b ($R_t$ 25.79 min) from the HPLC Separation of Wild-Type Barley. See figure 3.6.
Appendix II

Absorption Spectra From the HPLC Separation of the Extract Obtained From DPE I-Treated *C. reinhardtii* Cells.

(see Chapter 4)
Figure AII.1 Absorption Spectrum of the Peak $R_t$ 21.05 min from the HPLC Separation of the extract obtained from DPE I -Treated C. reinhardtii Cells. See figure 4.9.
Figure AII.2 Absorption and Fluorescence Spectra of the Peak of $R_t$ 21.40 min from the HPLC Separation of the Extract Obtained from DPE I-Treated *C. reinhardtii* Cells. See figure 4.9. The fluorescence spectrum represents a mixture of the 2 peaks $R_t$ 21.05 min and $R_t$ 21.40 min. The fluorescence spectrum was elicited at 398 nm.
Figure AII.3 Absorption and Fluorescence Spectra of the Peak of Rt 27.05 min from the HPLC Separation of the Extract Obtained from DPE I -Treated C. reinhardtii Cells. See figure 4.9. The fluorescence spectrum was elicited at 398 nm.
Figure AII.4. Absorption and Fluorescence Spectra of the Peak of $R_t$ 28.65 min from the HPLC Separation of the Extract Obtained from DPE I-Treated *C. reinhardtii* Cells. See figure 4.9. The fluorescence spectrum was elicited at 398 nm.
Figure AII.5 Absorption Spectrum of the Peak of Rₜ 32.10 min from the HPLC Separation of the Extract Obtained from DPE I -Treated C. reinhardtii Cells. See figure 4.9. Also shown is an absorption spectrum taken through the 'tail' of this peak at Rₜ 33.45 min.