AN ABSTRACT OF THE THESIS OF

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Title: Characterization of Strains of Scenedesmus Resistant to the Herbicide DCMU, (3-(3,4-dichlorophenyl)-1,1-dimethylurea).

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Three DCMU resistant mutants of the green alga Scenedesmus obliquus D_3 are characterized. The mutants exhibit a ten fold greater resistance to DCMU than the wild type strain; this is comparable to the level of DCMU resistance detected in other DCMU resistant algae. In whole cell reactions, as well as isolated chloroplast reactions, the resistance is maintained. Altered cell wall permeability is shown not to be the cause of resistance. In the presence of increasing concentrations of DCMU the wild type shows a large and rapid increase in fluorescence while the resistant mutants show a slow continual rise. The wild type and the resistant mutants show a comparable susceptibility to atrazine. Polypeptide analysis demonstrates that during chloroplast development and in fully matured cells the polypeptide patterns of the wild type and the resistant mutant thylakoids are the same. Two dimensional thin layer

chromatograms of thylakoid lipids of the wild type and resistant strains are qualitatively comparable. A notable difference is detected in the unsaturation ratio of fatty acids found in the wild type versus the resistant mutants; the wild type contains proportionately less unsaturated fatty acids than the mutants.

The DCMU resistant mutants characterized in this study exhibit a ten fold increase in resistance to DCMU. Atrazine resistant higher plants exhibit a significantly larger magnitude of resistance; because of this large difference it is probable that the resistance phenomena observed in Scenedesmus mutants is not the same as that occurring in atrazine resistant higher plants. The results of this study indicate that lipid composition influences the binding of the herbicide DCMU in thylakoid membranes of DCMU resistant mutants of Scenedesmus obliquus.

Characterization of Strains of *Scenedesmus* Resistant to the Herbicide DCMU, (3-(3,4-dichlorophenyl)-1,1-dimethylurea)

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A	Absorbance
ATP	Adenosine triphosphate
Atrazine	2-chloro-4(ethylamino)-6-(isopropylamino)-1,3,5-triazine
B	The secondary electron carrier of Photosystem II
Cyt f	Cytochrome f
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DGDG	Digalactosyl diglyceride
EMS	Ethyl methane sulfonate
Fd	Ferredoxin
FeS	A nonheme iron sulfur center
I_{50}	Concentration of inhibitor required to give 50% inhibition
kDa	Kilodaltons
LDS	Lithium dodecyl-sulfate
M	Molar
mA	milliAmpere
MGDG	Monogalactosyl diglyceride
mW	milliWalt
NADP	Nicotinamide adenine dinucleotide phosphate
nm	Nanometers
PAGE	Polyacrylamide gel electrophoresis
PC	Phosphatidyl choline
PCy	Plastocyanin
PCV	Packed cell volume
PE	Phosphatidyl ethanolamine
PQ	Plastoquinone
PSI	Photosystem I
PSII	Photosystem II
Q	The primary electron acceptor to Photosystem II
WT	Wild type, the unmutated strain
W/V	Weight/Volume
Z	The primary electron donor to Photosystem II

Characterization of Strains of Scenedesmus Resistant to the Herbicide DCMU, (3-(3,4-dichlorophenyl)-1,1-dimethylurea).

INTRODUCTION

The extensive utilization of the herbicidal action of numerous chemicals to control the growth of undesirable weed species has become a standard practice in modern agriculture. Both from an industrial and a scientific viewpoint there is an increasing desire to understand the mechanism of herbicidal action. Many of the more widely applied herbicides inhibit photosynthesis, specificially photosynthetic electron transport, as their primary mode of action. Researchers in photosynthesis have utilized herbicides such as DCMU (3-(3,4-dichlorophenyl)-1, 1-dimethylurea) and atrazine (2-chloro-4-(ethylamino)-6-(isopropylamino)-1,3,5-triazine) as fundamental tools in dissecting the photosynthetic apparatus. Although this approach has been valuable in extending the understanding of many aspects of the photosynthetic process, only limited knowledge has been gained about the specific mode of action of the individual herbicides.

The discovery of higher plant mutants resistant to atrazine (Ryan 1970) has offered new insight into the herbicidal mechanism of action. As a result of extensive biochemical studies on these higher plant mutants it is currently believed that atrazine and possibly DCMU inhibit photosynthetic processes by binding to a chloroplast membrane

protein with an approximate molecular mass, M, of 32 kilodaltons (kDa) (Mullet and Arntzen, 1981; Pfister et al., 1981; Steinback et al., 1981). There is evidence that indicates that DCMU and atrazine have separate binding sites or at least spatially separated binding sites on the same protein (Oettmeier et al., 1982; Shochat et al., 1982). Because there are no known, naturally occurring, DCMU resistant plants, resistant phenotypes were developed in algae, which are relatively easy to mutate. Attempts at controlled mutagenesis in higher plants have not been successful because mutants are usually deficient in several Photosystem II polypeptides necessary for normal photosynthetic functioning.

This thesis was undertaken in an effort to determine the mechanism of DCMU resistance in mutated strains of the green alga Scenedesmus obliquus. It is hoped that through the characterization of these mutants more can be learned about resistance to herbicides affecting electron transport.

Photosynthesis and the Electron Transport Chain

Photosynthesis involves the conversion of light energy to chemical energy and the subsequent use of that chemical energy to reduce CO_2 to such compounds as glucose. The process of photosynthesis can be divided into two reactions. The first reaction is a 'light' reaction; Light energy is captured and converted into the chemical energy of ATP and NADPH. The second reaction is a 'dark' reaction; The products of the

light reaction, ATP and NADPH, are used to bring about the fixation of carbon via the Calvin-Benson cycle. The light reaction involves the capture of light energy, the splitting of water, and an electron transport chain which produces ATP and NADPH. Presently, the most widely accepted model of the electron transport chain is the Z scheme, first introduced by Hill and Bendall (1960) (Figure 1). The Z scheme can be described as two light gathering photocenters, Photosystem II (PSII) and Photosystem I (PSI) connected by a series of electron carriers. When PSII is excited by light energy it transfers an electron to Q, the primary electron acceptor of PSII. As a result of this transfer the PSII reaction center becomes oxidized and must be reduced before it can once again reduce Q. The electrons used to reduce PSII are obtained from the splitting of water. The PSII reaction causes the oxidation of a primary electron donor, Z. Z, a yet unidentified entity, is a strong oxidant and contains bound manganese. Sufficient oxidizing power can be generated by Z to cause water to be split and to produce this reaction: $2H_2O \longrightarrow 4H^+ + 4e^- + O_2$. The electrons generated in this reaction are used to reduce PSII. (This reaction is also responsible for the O_2 evolved during photosynthesis.) Q, the primary electron acceptor of PSII, is a bound plastoquinone molecule (Van Gorkum, 1974), and a one electron carrier (Malkin and Kok, 1966). Q reduces B, a secondary electron carrier between Q and plastoquinone (Bouges-Bouquet, 1973; Velthuys and Amesz, 1974). B, also

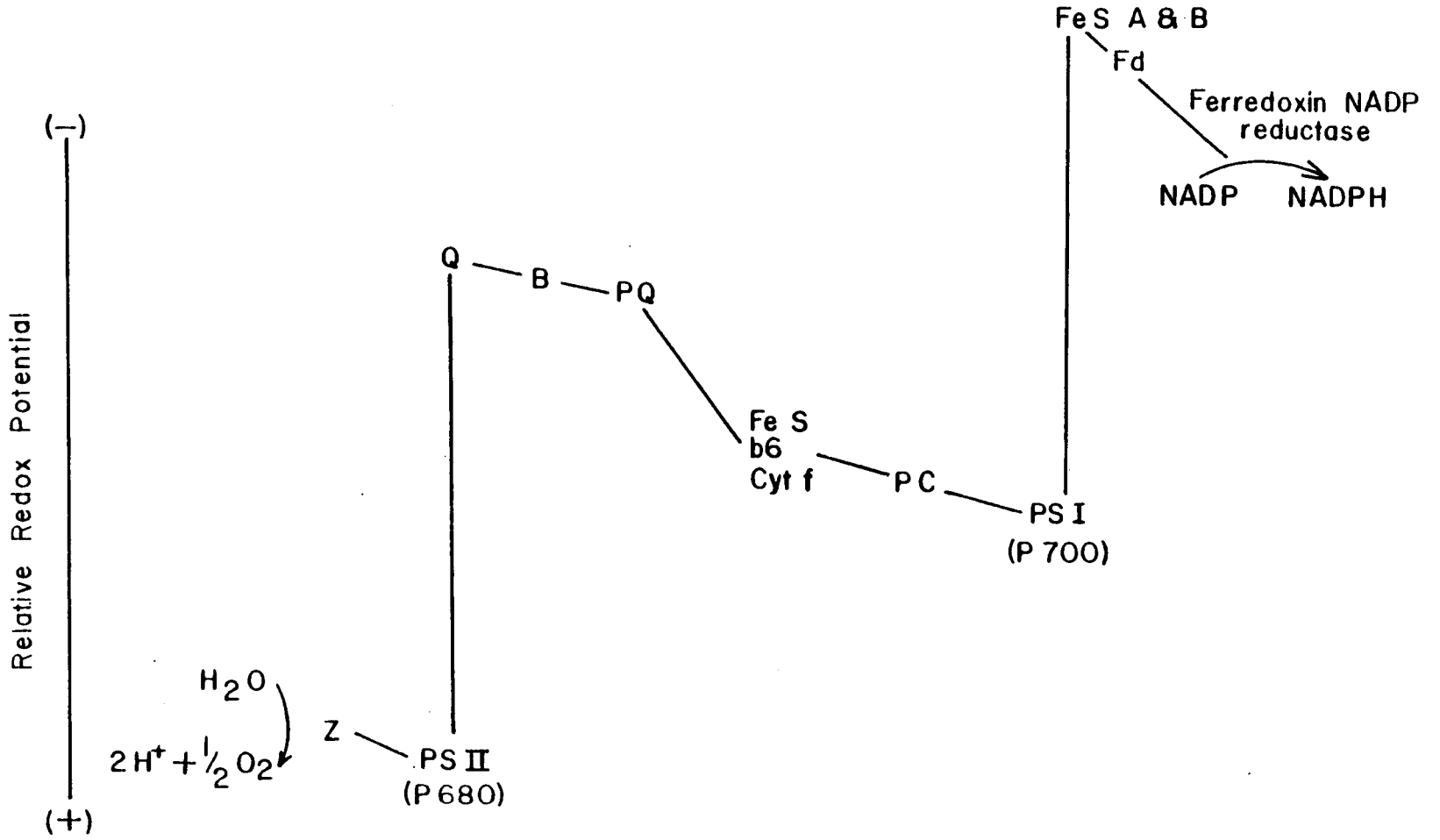

Figure 1. The Z scheme for noncyclic photosynthetic electron transport.

thought to be a bound quinone, is a two electron carrier, accumulating a pair of electrons before transferring them on to plastoquinone. Plastoquinone molecules are thought to exist as a large pool, with electrons from the plastoquinone pool reducing the cytochrome f-b6-FeS complex. This complex in turn reduces plastocyanin and plastocyanin reduces PSI. (The electron carriers from Q to plastocyanin are often referred to as the reducing side of photosystem II.)

When PSI is excited by light energy it reduces two nonheme iron sulfur centers (FeS A and B). These complexes act as the primary electron acceptors for PSI. Oxidized PSI is then reduced by the electron being transferred from PSII via the electron transport chain. The iron sulfur centers reduce ferredoxin, which transfers its electron in a reaction catalyzed by ferredoxin-NADP reductase, to $NADP^+$ to form NADPH.

Classes of Inhibitors

This thesis deals with a select group of herbicides that act as inhibitors of photosynthetic electron transport on the reducing side of PSII. These herbicides can be divided into two classes of inhibitors. The first class is composed of a variety of compounds which have two main features in common: the structural element N-C=X, where X signifies N or O (but not S) and a lipophilic group in the close vicinity of the essential structural element (Draber et al., 1974). (Differences in activity within a group are caused by the various lipophilic

side chains). The chemical groups of ureas, amides, triazines, triazinones, pyridazinones and carbamates belong to this class of inhibitors. The herbicides DCMU, from the urea group, and atrazine and simazine, from the triazine group, will be discussed in this thesis (Figure 2). (The above class of inhibitors will be referred to as DCMU-type herbicides.)

The second class of inhibitors consists of phenolic herbicides which, in addition to an aromatic hydroxyl group, bear nitro and/or halogen and/or nitrile substituents (Trebst and Draber, 1979). These herbicides inhibit electron transport on the reducing side of PSII as do the DCMU-type herbicides and in addition they are also uncouplers of photophosphorylation. This class is mentioned because it has been assumed that these inhibitors interfere with electron transport in a manner similar to the DCMU-type herbicides (Tischer and Strotmann, 1977). Recent evidence indicates that these herbicides actually interfere at a different site. Dinoseb is the phenolic herbicide which will be discussed (Figure 2).

Herbicide Inhibition

To cause interference with photosynthetic electron flow an herbicide must first enter the plant, be transported to cells carrying out photosynthesis, and finally reach receptor sites on or in the chloroplast. Differential uptake of the applied chemical by roots, stems, or leaves, differences in translocation and distribution of the herbicide within the plant, or metabolic reactions within the plant which modify the herbicide

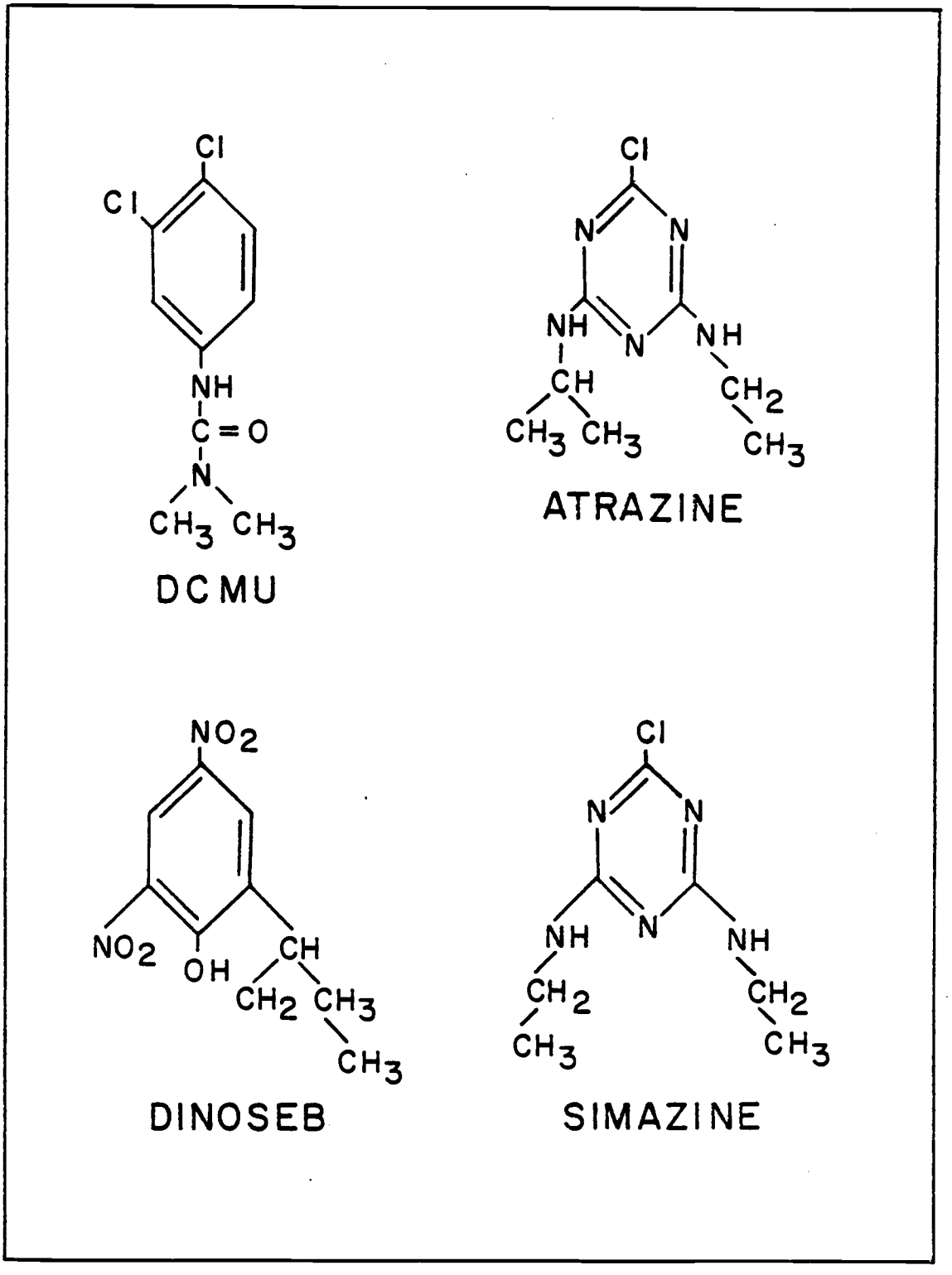

Figure 2. Chemical structures of some photosynthetic electron transport inhibitors acting on the reducing side of Photosystem II.

to produce nontoxic derivatives can affect the tolerance or susceptibility of a plant. Metabolic alterations and sorption to inactive sites can convert the herbicide to an inactive form or effectively titrate out large amounts, lessening its effect on the plant. Through metabolic pathways herbicides can be hydroxylated, demethylated, or conjugated with peptides. Cotton, (Gossypium hirsutum L.) can degrade DCMU into 1-(3,4 dichlorophenyl) urea by demethylation (Smith and Sheets, 1967). Corn, (Zea mays) can use two pathways to convert atrazine to a less toxic compound. The formation of an atrazine-glutathione conjugate, via the enzyme glutathione s-transferase, is the primary pathway used (Shimabukuro et al., 1971) but corn is also capable of carrying out a non-enzymatic hydroxylation reaction where atrazine is converted into hydroxy-atrazine. This reaction is catalyzed by a cyclic hydroxamate, benzoxazinone (Hamilton and Moreland, 1962). The effect of an herbicide can also be lessened by binding to unspecific receptors. Sorption to lipids could effectively titrate out large amounts of an herbicide (St. John and Hilton, 1974).

Plants resistant to the herbicide atrazine were described by Ryan (1970). In a nursery where the herbicide had been sprayed annually for several years certain weedy species had become atrazine resistant. Plants of the same species found outside the nursery spray area were not resistant. Early attempts to study the herbicide resistant biotype focused on altered herbicide uptake and metabolism. Jensen et al.

(1977) treated resistant and susceptible biotypes of common groundsel (Senecio vulgaris) with ^{14}C labeled simazine. After 96 hours, 80-90% of the radioactivity was found as unmetabolized simazine, 10-15% was found as water soluble simazine metabolites. There was no differential increase in water soluble simazine metabolites between the two biotypes. Comparable studies were also carried out on lambsquarters (Chenopodium album) and redroot pigweed (Amaranthus retroflexus) with similar results (Radosevich, 1977). These findings produced no evidence that could account for the mechanism of resistance in triazine resistant weeds.

Evidence that could account for resistance, however, was being collected as early as 1956. Wessels and Van der Veen (1956) demonstrated that DCMU inhibited the Hill reaction of isolated chloroplasts. Bishop (1958, 1962) showed that DCMU and atrazine behaved similarly to poisons which inhibit oxygen evolution. They did not influence photoreduction and they prevented deadaptation which normally occurs at higher light intensities. It was therefore concluded that DCMU and atrazine acted somewhere in the region of the oxygen evolving system. The work of Duysens and Sweers (1963) helped to pinpoint the actual site of inhibition. They proposed that the stimulation of fluorescence by DCMU was caused by the inhibitor preventing the reoxidation of reduced Q. The site of inhibition was proposed as being between Q and cytochrome. In 1974, Velthuys and Amesz proposed that DCMU inhibited electron transport by

altering the redox potential of B, a secondary electron carrier between Q and plastoquinone (Bouges-Boucquet, 1973) which therefore prevented electron transfer to plastoquinone.

Experiments with the water soluble enzyme, trypsin, have provided evidence that PSII inhibitors interact with a polypeptide of the PSII complex, possibly the apoprotein of B (Renger 1976). Regitz and Ohad (1976) and Renger (1976) showed that DCMU sensitivity could be removed by proper treatment of chloroplasts with trypsin. Boger and Kunert (1979) carried out similar experiments with triazines. It has been proposed that trypsin treatment removes the receptor site to which the herbicides bind (Trebst, 1979; Tischer and Strotmann, 1979; and Steinback et al., 1981). Trypsin treatment has been found to increase inhibition caused by the phenolic herbicide dinoseb (Boger and Kunert, 1979).

The affinity of various inhibitors for the herbicide binding site was examined (Tischer and Strotmann, 1977; Pfister et al., 1979). The binding of labeled atrazine was found to be inhibited competitively by other chemically different, unlabeled inhibitors such as DCMU and dinoseb. The relative concentration of specific binding sites was 1/300-500 chlorophyll molecules or about 1/electron transport chain. These results indicated that triazines, ureas and phenolic inhibitors interfered with the same electron carrier and according to the same molecular mechanism.

The association of PSII inhibitors with their binding site is noncovalent and for this reason attempts at physical isolation

of a protein labeled with radioactive inhibitor have failed; detergent fractionation or electrophoretic separation rapidly lead to dissociation of the acceptor-inhibitor complex. Photoaffinity labeled herbicides have been used in the attempt to identify the herbicide receptor protein in chloroplast membranes (Oettmeier et al., 1980; Pfister et al., 1981). Azido ^{14}C atrazine was used to form a covalent attachment of labeled inhibitor to resistant and susceptible biotypes of Amaranthus hybridus (Pfister et al., 1981). No covalent binding was found in the resistant biotype, but the susceptible biotype showed inhibitor binding to polypeptides of the 32-34 kDa size class. Some binding was also seen in 25 kDa and 16 kDa peptides. A photoaffinity label was also created for the phenolic herbicide, i-dinoseb, whose binding site is not identical to the DCMU type herbicides but was considered to be on the same protein (Oettmeier and Masson, 1980). The results of this study showed that the strongest binding of the inhibitor was on a polypeptide of approximately 41 kDa with some binding on a 25 kDa and 12.5 kDa protein.

Electrophoretic analysis of trypsin treated membranes reveals partial or complete digestion of the 32 kDa protein. In isolated thylakoids of Spirodela, the 32 kDa and 26 kDa proteins were partially digested by trypsin, yielding PSII electron transport that was DCMU insensitive (Mattoo et al., 1981). However, Mullet and Arntzen (1981) found that while trypsin digestion resulted in atrazine resistant electron

flow, DCMU was able to cause partial inhibition of PSII activity. Croze et al. (1979) reported that thylakoids treated with trypsin showed the disappearance of two proteins at 32 kDa and 27 kDa and the almost complete loss of DCMU sensitivity. The different results in these experiments are probably due to differences in trypsin concentrations and digestion times and while these results are somewhat contradictory they do indicate that it is possible to affect inhibitor binding by digestion of proteins on the surface of the thylakoid.

The 32 kDa protein has been selectively depleted from the chloroplast membrane in several experiments. Weinbaum (1979), using chloramphenicol to deplete the level of 32 kDa protein in Spirodela 84%, found that photosynthetic CO_2 fixation remained at 85% of normal, indicating that the 32 kDa protein is probably not a direct and integral part of the photosynthetic pathway. In the absence of polypeptides in the range of 32-35 kDa it was observed that DCMU still bound and inhibited electron transport but the affinity was significantly reduced (Shochat et al., 1982).

The data reported indicate that the absence of the 32 kDa protein is not necessary to achieve resistance to DCMU type herbicides. Mattoo et al. (1982) reported that it was clear that atrazine resistance was not due to a loss of expression of the gene for the 32 kDa protein as synthesis of the 32 kDa polypeptide occurred at similar rates in both triazine resistant and susceptible biotypes. Trypsin and photoaffinity labeling

experiments implicate at least a portion of the 32 kDa protein as being necessary for DCMU-type herbicide binding. Evidence from these same experiments indicates that phenolic herbicides have a different binding site, since trypsin digestion increases their inhibitory power and photoaffinity labeling shows binding to a 41 kDa protein. Also, chloroplasts from atrazine resistant mutants show a 10-fold increase in sensitivity towards phenolic herbicides in electron transport (Pfister and Arnzten, 1979). Competitive binding experiments indicate however, that atrazine, DCMU, and dinoseb all competitively inhibit the binding of each other, implying a mutual site of inhibition. A model has been proposed which predicts that there are probably two polypeptides (41 kDa and 32 kDa) involved in forming the herbicide binding site (Oettmeier et al., 1982).

Some recent studies indicate that while the herbicide binding protein shows high affinity binding sites, additional low affinity binding is indicated (Laasch et al., 1982; Shochat et al., 1982). For high affinity binding, a direct correlation between binding and inhibition of electron flow has been shown. Low affinity binding probably represents a partitioning of the herbicide into the lipophilic membrane. Shochat et al. (1982) found that formation of the high affinity binding site for DCMU in Chlamydomonas required participation of two types of polypeptides: the 32-35 kDa polypeptide and polypeptides participating in the formation of the PSII reaction centers. These latter polypeptides seemed to affect the conformation of the former

polypeptide in such a way as to give rise to the high affinity site.

Herbicides and Membrane Lipids

The herbicides DCMU and atrazine are known to inhibit lipid synthesis. Treatment of Chlorella with DCMU causes large changes in the lipid composition of cells (Sumida et al., 1973). In Senecio vulgaris L., atrazine inhibits lipid synthesis in susceptible biotypes, but does not appear to affect resistant biotypes (Radosevich, 1977). Treatment with atrazine or DCMU results in the failure of the electron transport chain to produce ATP and NADPH. The lack of these important sources of chemical energy reduces the levels of biosynthetic precursors for lipid synthesis. Sumida et al. (1975) found that by providing ATP, NADPH, and other cofactors exogenously that gross lipid synthesis was not significantly affected by DCMU. These results suggest that the preferential inhibition of galactolipid synthesis by DCMU or atrazine is not caused by direct attack on lipid biosynthesis enzyme systems but instead is a secondary result of the primary inhibition of electron transport.

The inhibition of lipid synthesis in susceptible but not resistant biotypes can be reasonably explained, but the actual lipid composition of the biotype membranes could be a factor in resistance or susceptibility. The physical state of membrane lipids has been shown to be an important factor in modulation of protein function (Heron et al., 1980; and Borochov and Shinitzky,

1976). The vertical displacement of a protein in a membrane is influenced by interactions with lipids of that membrane. The degree of exposure of a protein to outer surroundings is influenced by changes in lipid microviscosity. Increased lipid microviscosity may cause a protein to be 'squeezed' out of the membrane, a decrease in microviscosity may cause a protein to sink deeper into the membrane. Therefore, it is entirely possible that the environment of the 32 kDa protein is responsible for changes observed in its binding capabilities. Membrane lipids could affect access to binding sites on the protein.

The lipid composition of the thylakoid membranes of several herbicide resistant biotypes have been investigated. Pillai and St. John (1981) reported that there were differences in the lipid composition of resistant and susceptible biotypes of Senecio vulgaris L., Chenopodium album L., and Amaranthus retroflexus L. Chloroplasts from resistant biotypes contained higher proportions of monogalactosyl diglyceride (MGDG) and phosphatidyl ethanolamine (PE) and lower proportions of digalactosyl diglyceride (DGDG) and phosphatidyl choline (PC) than did chloroplasts from susceptible biotypes. MGDG and PE were also quantitatively higher in membranes of resistant versus susceptible biotypes. The major lipid classes of resistant chloroplast membranes contained lipids comparatively richer in unsaturated fatty acids (with the exception of DGDG).

In Brassica campestris L. biotypes, there was no significant difference in the proportion of lipids between biotypes (Burke et al., 1982). Significant differences were found in the individual

fatty acid composition of each biotype. In general, it was found that phospholipids but not glycerolipids in the resistant biotype had a higher linolenic acid concentration and lower levels of oleic and linoleic fatty acids than the susceptible biotype (Burke et al., 1982). The results of these studies (Pillai et al., 1981; and Burke et al., 1982) indicate definite changes in the bulk lipid of the thylakoids of resistant biotypes. Resistant biotypes have chloroplast lipids higher in unsaturated fatty acids than susceptible biotypes.

DCMU Resistant Mutants

Several herbicide resistant strains of algae have been developed in an attempt to obtain more information on resistance patterns. Mutants have been isolated from Aphanocapsa, Bumerilliopsis, Chlamydomonas, and Euglena.

Astier (1979) isolated two different DCMU resistant strains of Aphanocapsa 6714. The resistant strains were obtained by growing normal cells in the presence of 10^{-5} M DCMU. Strain DCMU r-1 showed resistance after being grown in the presence of DCMU. (The strain did not show higher resistance than the wild type before the adaptation phase.) Thylakoids made from these cells showed no resistance and therefore the whole cell resistance appeared to be due to some alteration of cell permeability. Strain DCMU r-2 had a higher initial resistance than the wild type (WT, the original unmutated strain) and after an adaptation phase its resistance was further increased. Resistance decreased however,

in the isolated thylakoids of the adapted DCMU r-2. These DCMU resistant strains showed comparable resistance to atrazine. Astier (1981) reported that the polypeptide composition of the DCMU r-2 adapted cells showed no 33 kDa polypeptide. Aphanocapsa WT cells subjected to trypsin treatment acquired resistance comparable to the DCMU r-2 adapted thylakoids.

Boger (1981) developed DCMU resistant Bumerilliopsis filiformis by growing cells in the presence of DCMU. Again, as in the Aphanocapsa mutants, a higher degree of resistance was found in the whole cells of mutants than in their chloroplasts. Trypsin treatment of resistant thylakoids resulted in complete loss of inhibition by DCMU or atrazine. The WT, even with trypsin treatment, could not be caused to become more than 50% resistant. The Bumilleriopsis mutants isolated exhibited resistance to DCMU but not to atrazine. The resistance of the mutants to DCMU could not be lowered below a factor of 10 when compared to the wild type.

Lien et al. (1977) used mutagenesis with nitrosoguanidine to develop a DCMU resistant strain of Chlamydomonas reinhardi. No altered permeability for inhibitors at the whole cell level was found. The mutant was not resistant to simazine and was 10 times more resistant to DCMU than the WT.

Laval-Martin et al. (1977) induced a DCMU resistant Euglena gracilis strain by growth of normal cells in DCMU. Whole cells of the mutant proved to be more resistant to the herbicide than the WT cells but significant ferricyanide or DPIP mediated O_2

evolution could not be satisfactorily measured with chloroplast preparations.

In all of the DCMU resistant algae isolated the maximum level of resistance obtainable is approximately 10 fold greater than that of the wild type. In Bumerilliopsis and Chlamydomonas 10 fold increases in resistance have been observed. In Aphanocapsa a 7 fold increase in resistance is observed. Higher plants resistant to atrazine however, show an enormous increase in resistance, ranging from 430 fold in Chenopodium album (Boger, 1981) to 1000 fold in Amaranthus retroflexus (Pfister et al., 1979). It is not known why this difference exists.

At present no extensive work has been done on a DCMU resistant mutant. The Aphanocapsa mutants have been the subject of several papers but they present several drawbacks. They are prokaryotic. They are resistant to both DCMU and atrazine, a phenomena which is not observed in green algae and higher plants. And resistant strains show complete loss of a 33 kDa polypeptide, a loss which is not observed in higher plants or green algae. The development of DCMU mutants more consistent with higher plants could potentially provide information that would help in the establishment of a more complete model of the herbicide binding site. This thesis undertakes the characterization of DCMU resistant phenotypes of Scenedesmus obliquus in an effort to learn more about the actual site of inhibition of DCMU.

MATERIALS AND METHODS

Mutant Isolation and Selection

The photosynthetic mutants used in this study were induced by treatment of Scenedesmus obliquus, strain D_3, (to be referred to as WT), with ethyl methane sulfonate (EMS) following the method of Bishop (1971). Initial selection and isolation of mutants resistant to DCMU was performed by Dr. Bishop by plating treated cells on agar plates (see Algal Culture) containing 6×10^{-6} M DCMU. Survivors were collected and listed as DCMU R-1 through 50 (where R denotes resistant). Further screening of these resulted in the decision to use the phenotypes designated DCMU R-1, DCMU R-28, and DCMU R-32 for further analysis of their mechanism of resistance to DCMU.

This study also utilized developmental mutants, strains which show only traces of chlorophyll and no detectable photosynthesis when grown heterotrophically. On light exposure, these strains develop chlorophyll and photosynthetic competence comparable to the WT. A developmental mutant of WT, designated 2A', and a DCMU resistant strain, designated R-7, were prepared by Dr. Bishop using X-ray mutation and EMS treatment respectively.

Algal Culture

Heterotrophic cultures were grown on nitrate medium (Kessler, Arthur, and Brugger, 1957) enriched with 0.5% glucose and 0.25% yeast extract. These cultures were grown at 28°C in 250 ml of

medium in a 500 ml screw cap Erlenmeyerflask on a rotary shaker. Two day old cultures were harvested for experimentation since these cultures display optimal photochemical activity (Berzborn and Bishop, 1973).

Autotrophic cultures were grown in nitrate medium at 25°C in glass bubble tubes (Bishop and Senger, 1971). Fluorescent light banks of warm white and grow lux bulbs provided light intensity of 1 mW/cm^2 at the surface of the bubble tubes. A mixture of 4% CO_2 in air was bubbled through the tubes. Agar plates, for autotrophic growth, were prepared with nitrate medium and 1.5% agar.

Packed cell volume (PCV) was determined by centrifuging an aliquot of a sample in a modified cytocrit centrifuge tube (a gift to Dr. Bishop from Dr. Horst Senger) using a Sorvall table top centrifuge (Model GLC-1) for five minutes at 300 xg.

Chlorophyll Determinations

Chloroplast suspensions of Scenedesmus were extracted with 80% acetone. Debris was removed by centrifuging five minutes at 300 xg. A Zeiss PMQ II spectrophotometer was used to measure optical densities at specific wavelengths. A modified version of the equation developed by Arnon (1949) was used to determine the chlorophyll concentration (mg/l). The equation used was as follows:

Chlorophyll (a + b) = (17.75 X A_{647})+(7.01 X A_{664}).

A_{647} and A_{664} indicate the absorbance of the chlorophyll extract at 647 nm and 664 nm.

Amperometric Measurement of O_2

A Clark (Ag-AgCl) electrode was used to monitor dissolved O_2 concentrations (Hanus et al., 1980). The electrode was calibrated with air-saturated water and argon. The sample was placed in a 1.4 ml electrode curvette similar to that described by Jones and Bishop (1976). The cuvette was illuminated by light from a tungsten filament, 8 volt, 5 amp projection lamp (Tiyota microscope lamp) filtered through 2.5 cm of a $CuSO_4$ solution (2% w/v). Light intensity at the cuvette surface was 9.5 mw/cm^2.

For photosynthesis and respiration measurements cells were suspended in 0.05 M KH_2-K_2HPO_4 buffer (pH 6.5) at 10 µl PCV/ml and rates of O_2 uptake and production measured. Various concentrations of the inhibitor, DCMU, were added to the cells where noted. A stock solution of 1 X 10^{-3} M DCMU in ethanol was used. The total amount of ethanol introduced with the inhibitor was 1% or less in all reactions; at this level ethanol is not inhibitory (Senger and Bishop, 1979).

For the Hill reaction, with p-benzoquinone as electron acceptor, cells at 10 µl PCV/ml were suspended in 0.05 M KH_2-K_2HPO_4 buffer (pH 6.5) made to 10 mM $MgCl_2$, 20 mM KCl and

4.6 mM p-benzoquinone (Bishop 1972). Various concentrations of DCMU were added where noted.

Fluorescence

Fluorescence measurements were made with the instrumentation and methods employed by Senger and Bishop (1972). An excitation wavelength of 470 nm was used which provided a light intensity of 30 $\mu w/cm^2$ at the sample cuvette. Variable yield fluorescence (relative quantum efficiency) was measured by adding increasing amounts of DCMU. The cells were used at a final concentration of 1 µl PCV/ml in 0.05 M KH_2-K_2HPO_4 buffer (pH 6.5).

Absorption Spectra

Absorption spectra of whole cells of the WT and mutant strains were determined using an Aminco-Chance DW-2 Spectrophotometer operated in the split beam mode. The sample cuvette contained cells (10 µl PCV) suspended in 3 ml of 0.05 M KH_2-K_2HPO_4 buffer (pH 6.5) with the reference cuvette containing only buffer.

Chloroplast Isolation

Chloroplast fragments were prepared by the method of Berzborn and Bishop (1973) with the following modifications. The breaking and rinsing solutions were the same and consisted of 20 mM tricine-KOH buffer (pH 7.5), 10 mM KCl, 0.05 mM EDTA, 0.5 mM dithiothreitol and 0.4 M sucrose (Berzborn buffer). The glass beads used were 1 mm

in diameter rather than 0.35 mm. The glass beads were removed following breakage by filtering the solution through a fritted glass funnel. The filtrate collected was centrifuged at 6000 xg for 2 minutes to remove unbroken cells and cell debris. The supernatant from this step was centrifuged at 54,000 xg for 30 minutes. The resulting green pellet was diluted to the desired chlorophyll concentration in an appropriate buffer, suspended using a glass homogenizer and used immediately for chloroplast reactions (see specific reaction description).

Thylakoid Purification

Chloroplast fragments were prepared as above in the Chloroplast Isolation procedure. The resulting membrane pellet was suspended in 3 ml of Berzborn buffer, and layered on a step gradient consisting of 5 ml of 2 M sucrose, 10 ml of 1.2 M sucrose, and 15 ml of 0.4 M sucrose (all in Berzborn buffer). This gradient was then centrifuged in a Beckman SW-27 rotor for 1 hour at 55,000 xg at 4°C. After centrifugation a dark green layer at the 1.2 M interface was present; the solution above this region was gently removed with suction. The green layer was carefully removed with a pipette, transferred to a clean centrifuge tube, diluted with additional Berzborn buffer and centrifuged at 54,000 xg for 30 minutes. The supernatant was discarded and the pellet was used immediately or stored frozen until needed. Pellets for electrophoresis were prepared following

this procedure with one modification; the Berzborn buffer used also contained the following: 5 mM e-aminocaproic acid, 1 mM p-aminobenzamidine, 1 mM a-toluenesulfonyl flouride, and 25% ethylene glycol.

Chloroplast Reactions

The water to methyl viologen Hill reaction, a cell free reaction requiring both photosystems, was originally described by Kok, Ruranski, and Owens (1965). The methodology of Harvey (1974) was followed for this assay. Chloroplasts were prepared according to the Chloroplast Isolation procedure described above. The membrane pellet was suspended in 20 mM tricine-KOH (pH 7.5), 30 mM KCl, 0.4 M sucrose and 1% (W/V) BSA). The reaction mixture contained 0.1 mM methyl viologen, 0.3 mM NaN_3, 90 µg chlorophyll/sample and various concentrations of DCMU as noted. A Clark (Ag-AgCl) electrode (as described in Amperometric Measurement of O_2) was used to monitor this reaction.

Polyacrylamide Gel Electrophoresis

The polypeptide composition of purified thylakoid membranes was examined using lithium dodecyl-sulfate (LDS) polyacrylamide gel electrophoresis (PAGE). This procedure is based on a sodium dodecyl sulfate gel system originally described by Lammeli (1970). The methods used were as described by Delepelaire and Chua (1979) except for the following modifications. Thylakoid membranes to be used in electrophoresis were prepared as stated above in

Thylakoid Purification. Protein concentration was measured instead of chlorophyll concentration. Protein determinations were made using the Bio-Rad protein assay, a modified Bradford assay (Bradford, 1976). A final concentration of 125 µg protein/sample was applied to each gel slot. Denatured samples were prepared by heating a solubilized thylakoid membrane preparation in boiling water for two minutes. The gel was 1.5 mm thick and the stacking gel contained 4.5% acrylamide instead of 5%. Electrophoresis was performed on a Protean cell, a vertical dual slab gel apparatus (Bio-Rad), at a constant current setting of 8 mA/plate. Following electrophoresis the gel slabs were stained in a solution of 0.2% Coomassie brilliant blue R-250 (Bio-Rad), 10% acetic acid and 50% methanol. Gels were stained overnight and destained in several washes of 10% acetic acid and 50% methanol. Gels were then photographed and stored at 4°C in plastic bags. Some gels were silver stained using the Gel-Code system (Upjohn Diagnostics), a system derived from the work of Sammons et al., (1981). In this staining procedure 10 µg of protein was used per slot.

Molecular Weight Estimation

The molecular weights of the proteins separated by LDS-PAGE were estimated by comparing migration distances of unknown molecular weight proteins to those of protein standards of known molecular weight (Weber and Osborn, 1969). Protein standards were dissolved in the same solubilizing solution used for

chloroplast membrane preparations. The protein standards were run on each gel for calibration. Six protein standards were used: bovine serum albumin, 66,000 Da; ovalbumin, 45,000 Da; DNAsel, 31,000 Da; trypsinogen, 24,000 Da; B-lactoglobulin, 18,400 Da; and lysozyme, 14,300 Da. A standard calibration curve was prepared for each gel.

LDS-PAGE of ^{35}S Labeled Membranes

Heterotrophic cultures of developmental mutants were grown in nitrate medium from which the normal source of sulfur had been removed, $MgSO_4$ had been replaced by $MgCl_2$. Sulfur was provided in the form of Na_2-$^{35}SO_4$, with a specific activity of approximately 800 mC/mmole. As greening progressed, cultures were harvested at intervals and prepared as purified thylakoids. These samples were electrophoresed and the gel was stained with Coomassie brilliant blue, destained, photographed, and dried on Whatmann 3 mm filter paper in a gel slab drier (Hoefer Scientific instruments). The dried gel was then sandwiched with X-ray film (Kodak X-Omat RP) and stored at -20°C for 10 to 14 days depending on the number of counts incorporated into the sample. The film was then removed and developed.

Thylakoid Lipid Extraction

The lipid composition of the thylakoid membranes of the various Scenedesmus mutants was examined using the methodology of Allen and Good (1971). Purified thylakoids were prepared and

the resulting pellet was suspended in 10 ml of 2:1 methanol-chloroform. After 5 minutes, 3 ml of chloroform and 3 ml of water were added and the mixture agitated by bubbling argon. The mixture was centrifuged and the upper layers removed with suction. The chloroform layer was removed with a pipette and evaporated to dryness with argon. The residue was dissolved in 2 ml of chloroform and used immediately or deoxygenated with argon and stored frozen at -20°C.

Thylakoid Lipid Analysis: Thin Layer Chromatography

Glass plates, 20 X 20 cm, were coated with a 0.025 cm layer of Silica gel G (Merck) and activated by heating in a 100°C oven for 1 hour. For two dimensional development, the plates were spotted with an aliquot of the thylakoid membrane lipid extract approximately 3 cm from the bottom and 3 cm from the right-hand corner. The plates were developed first in $CHCl_3$:CH_3OH:H_2O (65:35:4) removed, dried, and then chromatographed in the second solvent, $CHCl_3$:CH_3OH:$(CH_3)_2CHNH_2$:NH_4OH (65:35:0.5:5). The plates were dried and lipid spots visualized by exposure to iodine vapor. Identification of the individual lipid spots was made by co-chromatography of lipid standards (P. L. Biochemicals).

Fatty Acid Analysis

An aliquot of the total thylakoid lipid extract was spotted on 20 X 20 cm silica gel plates. The plates were developed in $CHCl_3$:CH_3OH:H_2O (65:35:4). Lipid bands running

below the pigmented solvent front were collected and trans-esterified using the methodology of Allen and Good (1971). Behenic acid (100 µg) was added to each vial to act as an internal standard. The pentane extract resulting from trans-esterification was evaporated with argon to a final volume of 0.25 ml. The fatty acid methyl esters were analyzed on an HP 5830A Gas Chromatograph. A 6 foot, 1/8 inch column packed with 10% SP-2340 on 100/120 Supelcoport B-2414 (Supelco) was used. A multilevel temperature program was used: 6 minutes at 175°C, a temperature increase of 10°C/min until 195°C was reached, and then 6 minutes at 195°C. The nitrogen carrier gas had a flow rate of 24 ml/min and the flame ionization detector was operated at 220°C. The fatty acid methyl esters were identified and quantitated by area comparisons to Supelco standards (RM-6, 4-7025).

RESULTS

Selection of Resistant Mutants

The DCMU resistant mutants used in this study were initially selected on the basis of their ability to survive, after mutagenesis with EMS, when grown autotrophically on plates containing 6×10^{-6} M DCMU. The strains isolated from this procedure were further screened for their ability to carry out photosynthesis, their level of resistance to DCMU, the permeability of their cell wall to the herbicide and their fluorescence characteristics in the presence of the herbicide. On the basis of these experiments the three resistant strains, DCMU R-1, R-28 and R-32, were chosen.

Whole Cell Reactions

Whole cells of the mutants DCMU R-1, R-28 and R-32 show normal growth and carry out photosynthesis at a rate equal to the WT. Even at varied light intensities the performance of the mutants and the WT are equivalent.

Since DCMU inhibits the electron transport chain in the region between Q and plastoquinone, herbicide inhibition causes a cessation of electron transport and concommitantly, O_2 production. The inhibitory effect of the herbicide was quantified by subjecting whole cells of the WT and the resistant strains to varing concentrations of DCMU and observing the change in O_2 production (Figure 3). The rate of oxygen evolution without DCMU was comparable for the WT and the resistant strains. The

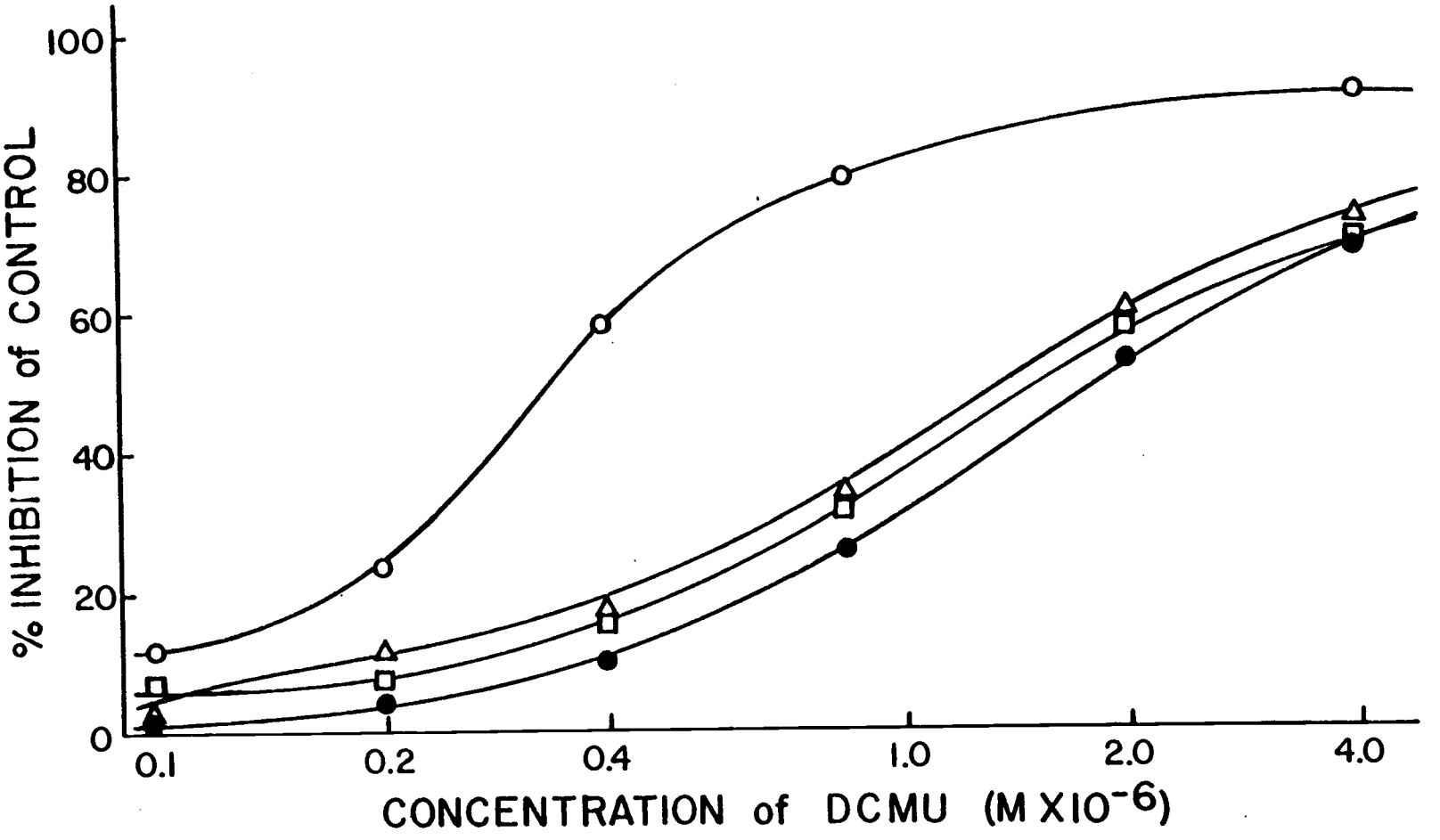

Figure 3. The effect of varying concentrations of DCMU on whole cell photosynthetic oxygen production of the wild type Scenedesmus (○-○) and the mutants DCMU R-1 (△-△), DCMU R-28 (●-●) and DCMU R-32 (□-□). Cells were incubated with DCMU for 5 minutes prior to analysis.

photosynthesis of WT cells is very sensitive to low levels of DCMU reaching 50% inhibition of the control (I_{50}) at 3.5 X 10^{-7} M DCMU. The DCMU resistant mutants are less susceptible to inhibition with I_{50}'s at 1.5 X 10^{-6} M DCMU, 2.0 X 10^{-6} M DCMU, and 1.75 X 10^{-6} M DCMU for DCMU R-1, R-28 and R-32 respectively.

To test the resistant strains for the possibility of altered cell wall permeability the chemical p-benzoquinone was added to whole cell reactions. P-benzoquinone has been found to enter algal cells by reacting with the cell membrane; the remaining unreacted p-benzoquinone acts as an electron acceptor causing a whole cell Hill reaction (Gimmler and Avron, 1971); see Chloroplast Reactions for a description of the Hill reaction. P-benzoquinone acts without affecting components involved in photosynthetic electron transport from water to PSI (Gimmler and Avron, 1971); oxygen evolution may be measured in the presence of this chemical.

Whole cells of WT and the resistant phenotypes were treated with p-benzoquinone and varying concentrations of DCMU (Figure 4). This treatment increased the sensitivity to DCMU in both the WT and the resistant phenotypes causing a decrease in the I_{50} concentration for both groups. Even though the herbicide sensitivity of both the WT and the resistant mutants increased, the mutants displayed approximately ten fold greater resistance than the WT. This experiment indicates that altered cell wall permeability is not the mechanism of resistance employed by these mutants.

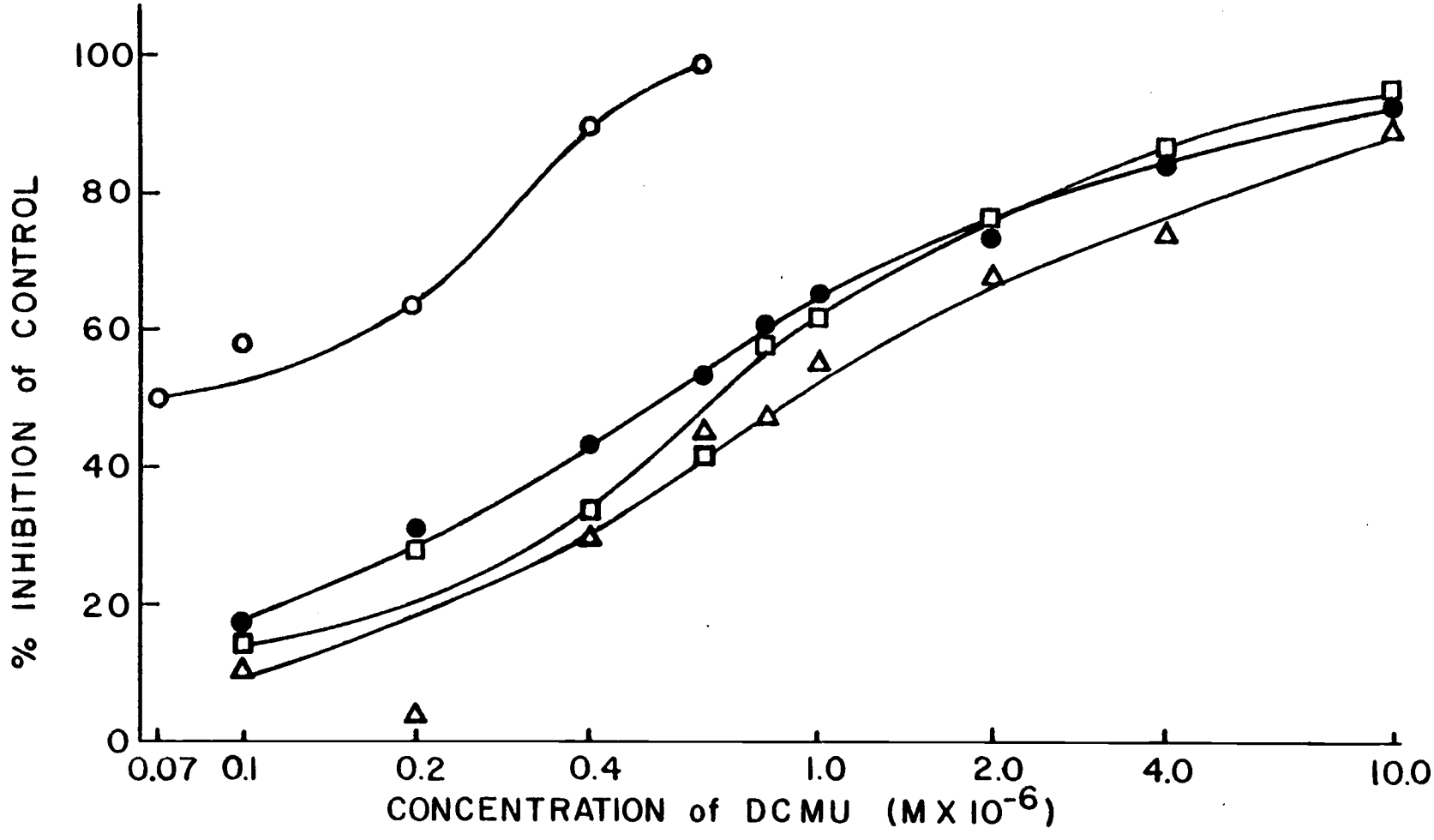

Figure 4. The effect of increasing concentrations of DCMU on the p-benzoquinone Hill reaction in whole cells of the wild type Scenedesmus (○-○) and the mutants DCMU R-1 (△-△), DCMU R-28 (●-●) and DCMU R-32 (□-□).

Fluorescence

Chlorophyll fluorescence can be used as a monitor of PSII activity. Absorption of a photon by a chlorophyll molecule results in the creation of an excited singlet state chlorophyll. The excitation energy of this state is transferred to the PSII reaction center where it initiates the transfer of an electron to Q, the primary acceptor of PSII. Fluorescence yield is dependent on the state of the quencher Q. If Q is reduced and cannot be oxidized, the PSII reaction center becomes reduced and the chlorophyll-donated excitation energy decays to its ground state by release of heat or reemission of a photon at a lower energy level, resulting in fluorescence.

Fluorescence can be used to observe the redox state of Q. Blue light is used to cause chlorophyll excitation in the sample and a photomultiplier tube detects the red fluorescence near 685 nm. A normal suspension of cells produces low level fluorescence as most of the chlorophyll excitation energy is transferred through the electron transport chain. This base level of fluorescence is comparable in the WT and the resistant mutants. If an inhibitor such as DCMU is added to the system the transfer of electrons from Q to PQ is blocked. Q is reduced by an electron from PSII but cannot be oxidized by electron donation to PSI thus the fluorescence yield rises. The effect of additions of varying concentrations of DCMU on the fluorescence of the WT and the three mutants was measured

(Figure 5). The fluorescence of the wild type increased rapidly with low concentrations of DCMU; the fluorescence rise observed in the mutants was much slower than the wild type and did not reach as high a level over the course of DCMU concentrations tested. The extent of the DCMU induced fluorescence may be taken as an measure of DCMU inhibition. The ability of DCMU to inhibit the oxidation of Q by PSI and thus increase fluorescence is more pronounced in the WT than in the mutants.

Chlorophyll Content of Cells

Chloroplasts from triazine resistant biotypes of Brassica campestris have a lower chlorophyll a/b ratio than the susceptible biotype (Burke et al., 1982). To examine the chlorophyll content of the Scenedesmus WT and resistant mutants, absorbancy difference spectra for WT versus DCMU R-1, R-28 and R-32 were measured (Figure 6). No significant variation in the pigment content was observed. This test indicates that the amount of the chlorophyll a/b light harvesting complex is comparable in both the WT and the resistant mutants.

Chloroplast Reactions

Herbicides that inhibit the electron transport chain have been rountinely called inhibitors of the Hill Reaction. The Hill Reaction is defined as the evolution of oxygen by a

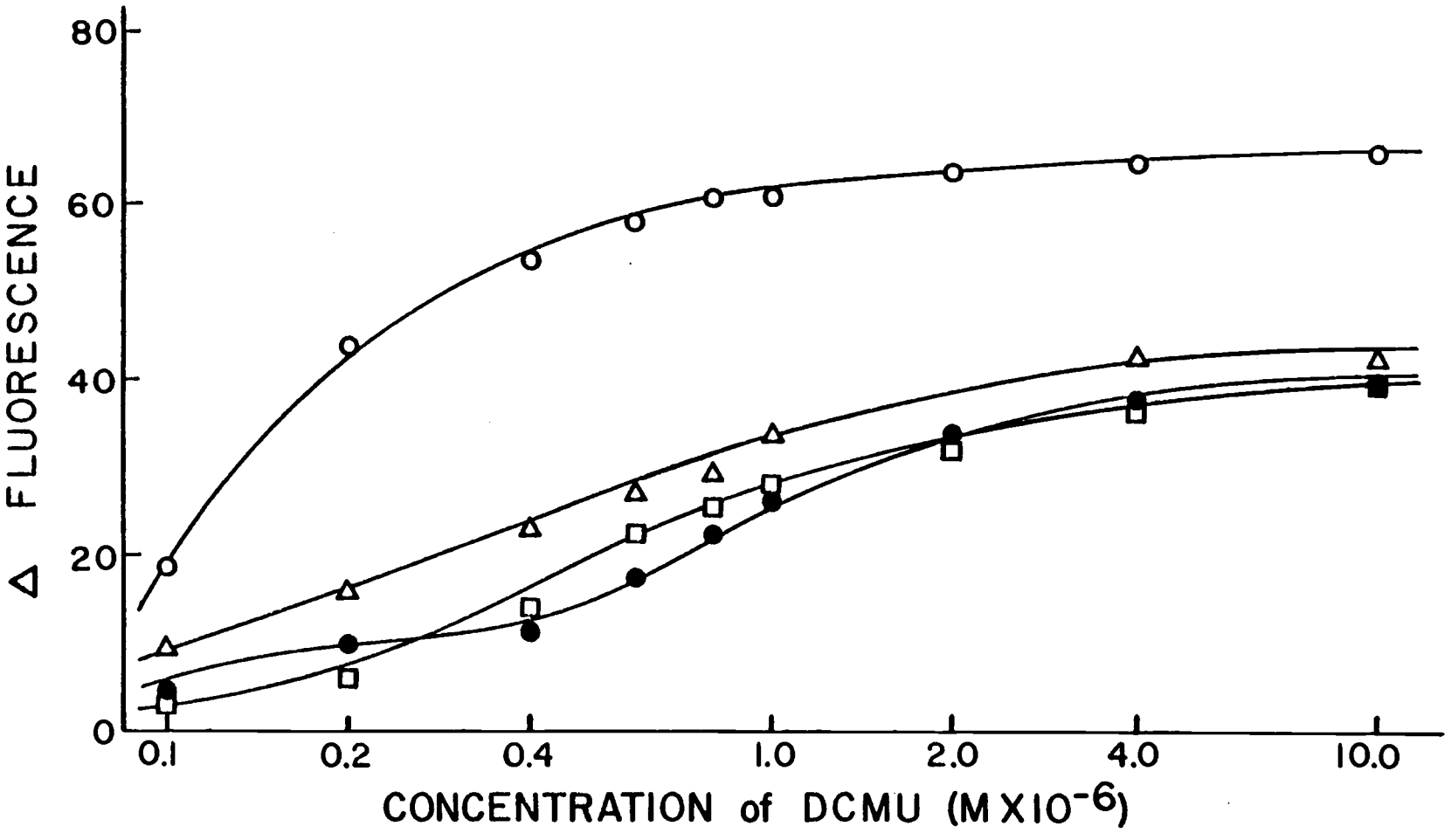

Figure 5. Stimulation of fluorescence by varying concentrations of DCMU in wild type Scenedesmus (○-○) and mutants DCMU R-1 (△-△), DCMU R-28 (●-●) and DCMU R-32 (□-□). For experimental details see Materials and Methods.

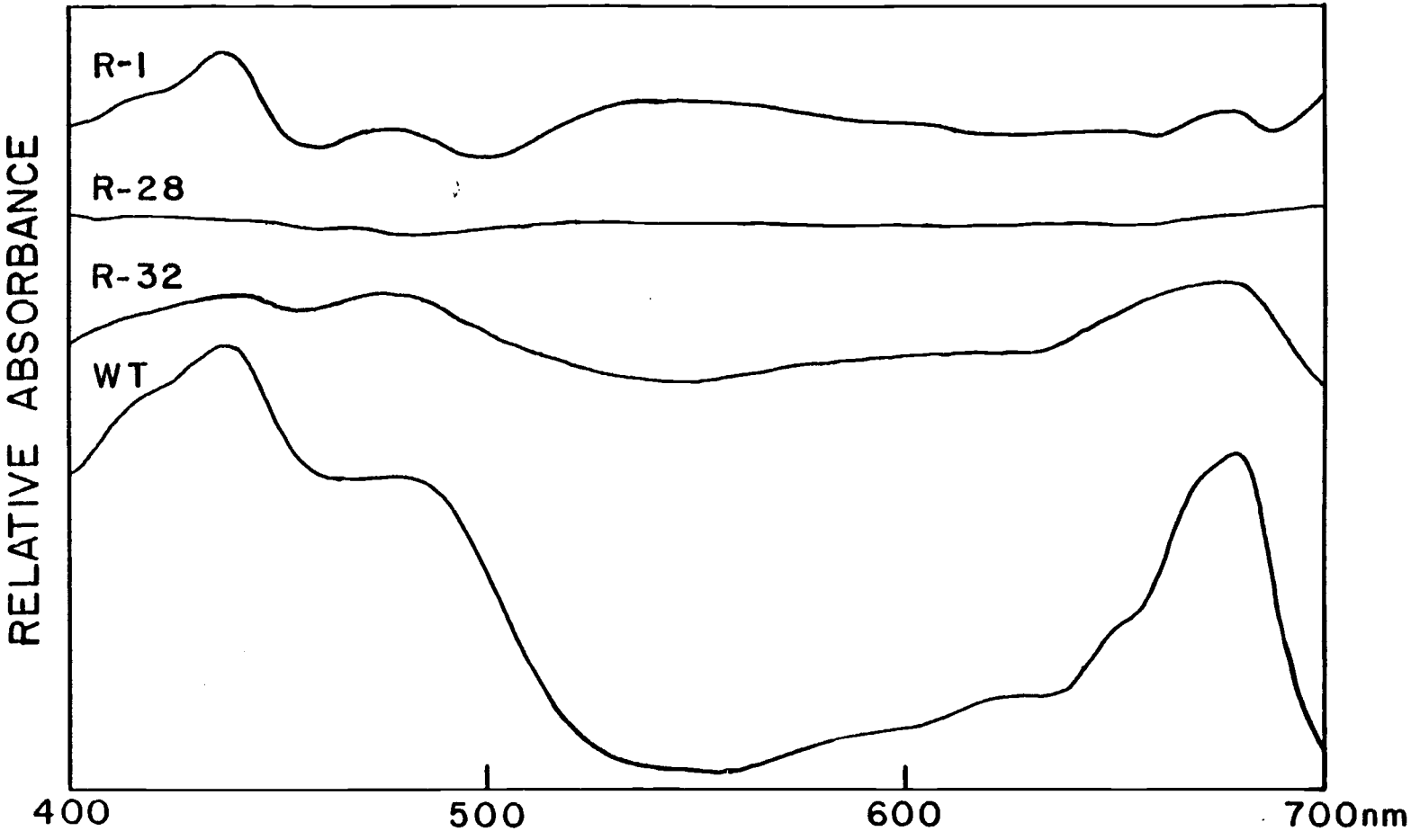

Figure 6. Absorbancy difference spectra of the wild type Scenedesmus versus DCMU R-1 (R-1), DCMU R-28 (R-28) and DCMU R-32 (R-32). An absorption spectrum of the wild type is shown for reference.

suspension of isolated chloroplasts when illuminated in the presence of an artificial electron acceptor. This reaction allows the separation of the light reaction from CO_2 fixation, and it also allows the substitution of various electron acceptors for the in vivo acceptor, NADP. By using various acceptors it is possible to monitor the activity of PSII. Partial reactions can be used to localize the region of the electron transport chain where a specific inhibitor exerts its influence or to quantify the efficiency of an inhibitor. The methyl viologen Hill Reaction was used to measure the effect of varying concentrations of DCMU on chloroplasts of the wild type and the resistant mutants (Figure 7). The results obtained were similar to those observed in whole cell measurements of DCMU sensitivity. The resistance ratio, I_{50} resistant phenotype/I_{50} WT, is 10 for the chloroplast reaction indicating that DCMU tolerance is not reduced in thylakoids of the mutants.

A methyl viologen assay using atrazine as the inhibitor was carried out (Figure 8). The assay indicated that DCMU resistant phenotypes were not more resistant to atrazine than the WT. The WT and DCMU R-1, R-28 and R-32 show comparable susceptibility to atrazine.

Astier et al. (1979) reported that upon growth in sublethal concentrations of DCMU, resistant phenotypes of Aphanocapsa showed an adaptation phase after which, the strains were more resistant to DCMU. Cultures of Scenedesmus were grown in sublethal concentrations of DCMU for three days, WT in 2×10^{-7} M DCMU and R-32

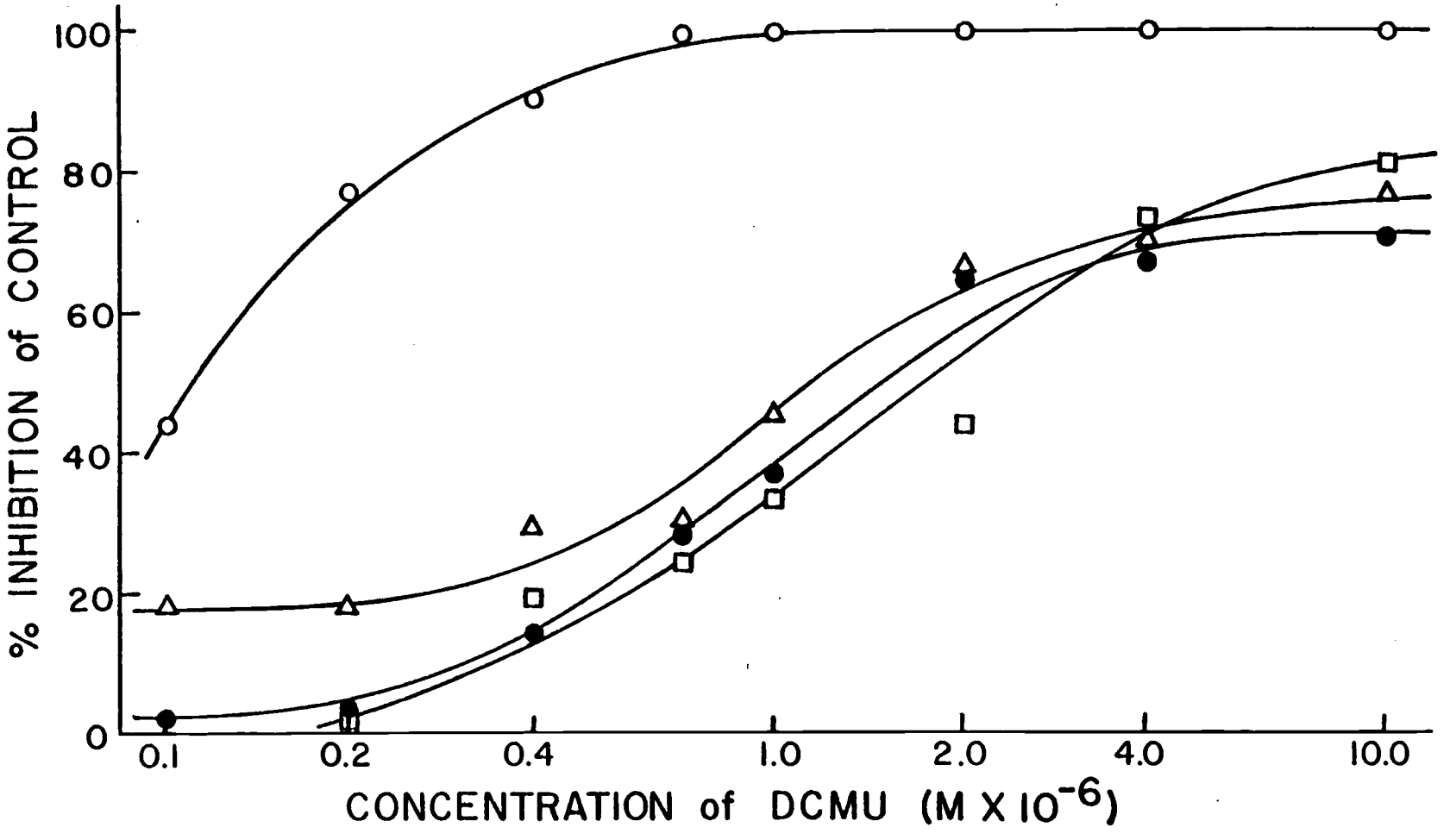

Figure 7. The effect of increasing concentrations of DCMU on the methyl viologen Hill reaction in isolated chloroplasts of wild type Scenedesmus (○-○) and the mutants DCMU R-1 (△-△), DCMU R-28 (●-●) and DCMU R-32 (□-□). Cells were incubated with DCMU for 5 minutes prior to analysis.

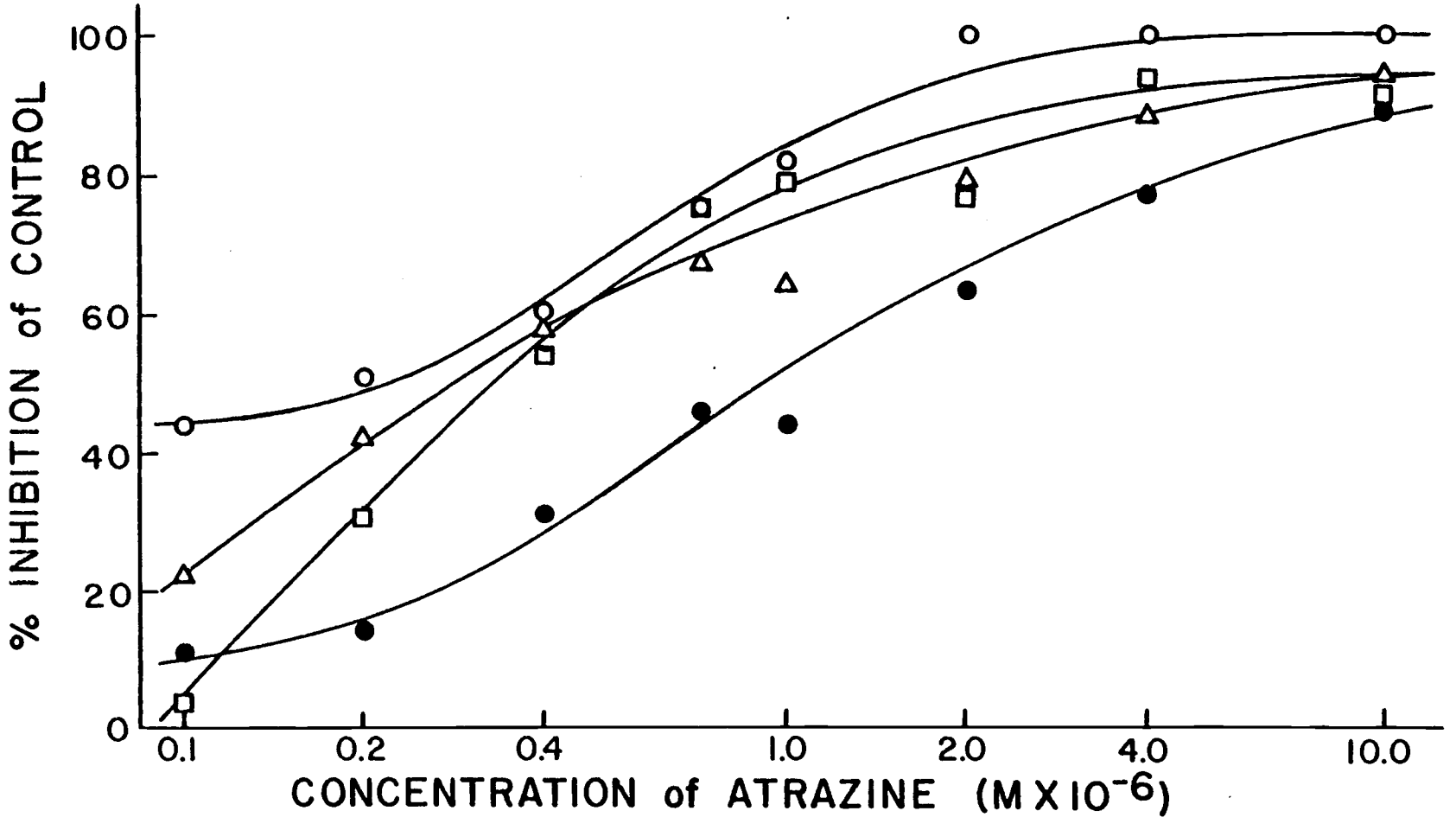

Figure 8. The effect of increasing concentrations of atrazine on the methyl viologen Hill reaction in isolated chloroplasts of the wild type Scenedesmus (○-○) and the mutants DCMU R-1 (△-△), DCMU R-28 (●-●) and DCMU R-32 (□-□). Cells were incubated with atrazine for 5 minutes prior to analysis.

in 2 X 10^{-6} M DCMU. Chloroplasts isolated from these cultures showed no increase in resistance to DCMU when tested in a methyl viologen assay.

Trypsin Treatment of Chloroplast Membranes

Trypsin digestion has been used extensively to remove the herbicide binding protein. Renger (1976), Regitz and Ohad (1976), Croze et al. (1979) and many others have used this digestion procedure to effect removal of all or part of DCMU sensitivity. The trypsin treatment most commonly employed involves the incubation of 50 µg chlorophyll/ml with 50 µg/ml of trypsin for periods of 4 to 20 minutes. A Hill Reaction system using water as the electron donor and ferricyanide as the electron acceptor is most commonly used to monitor the digestion. Under these conditions Scenedesmus thylakoids showed complete inactivation of the water splitting apparatus. No oxygen evolution could be detected in thylakoid particles active before treatment with trypsin, until the trypsin level was lowered to 5 µg/ml or less. When significant levels of O_2 evolution were detected, DCMU was still inhibitory. Problems with this assay stem from the fact that Scenedesmus cells are very difficult to break. The most commonly used method is breakage by use of vibrating glass beads. This method is harsh and Scenedesmus thylakoids are ruptured. It is apparent that the water splitting apparatus itself is exposed to trypsin digestion in this case. Regitz and Ohad (1976) were successful in carring out trypsin digestion on

Chlamydomonas thylakoids but as they demonstrated they were able to isolate them intact. In an attempt to obtain intact thylakoids the methodology of Regitz and Ohad (1976) was followed very closely. A French pressure cell was used and active thylakoids were prepared. As in previous experiments when the trypsin concentration was low enough to permit oxygen evolution, DCMU was still inhibitory.

Lithium Dodecyl-sulfate Polyacrylamide Gel Electrophoresis

Electrophoretic analysis of the atrazine resistant higher plants reveals no polypeptide differences when they are compared to normal biotypes; both atrazine resistant and susceptible biotypes actively synthesize a 32 kDa protein (Mattoo et al., 1982). No differences could be detected between the polypeptide profile of the WT Scenedesmus and the profiles of the DCMU resistant mutants, even with the improved sensitivity of silver staining (Figure 9).

Astier et al. (1981) reported that upon growth in high concentrations of DCMU, DCMU resistant Aphanocapsa mutants exhibited a loss of a 33 kDa polypeptide. Scenedesmus mutants were grown autotrophically and heterotrophically in 10^{-5} M DCMU for 2 weeks. No changes were seen in the polypeptide composition of these mutants.

Figure 9. Polypeptide profiles of the thylakoid membranes of the wild type Scenedesmus and the mutants DCMU R-1, R-28 and R-32. Analysis was by LDS-PAGE at 4°C using a 7.5% to 15% polyacrylamide gradient in the separating gel. The gel was stained with Bio-Rad silver stain.

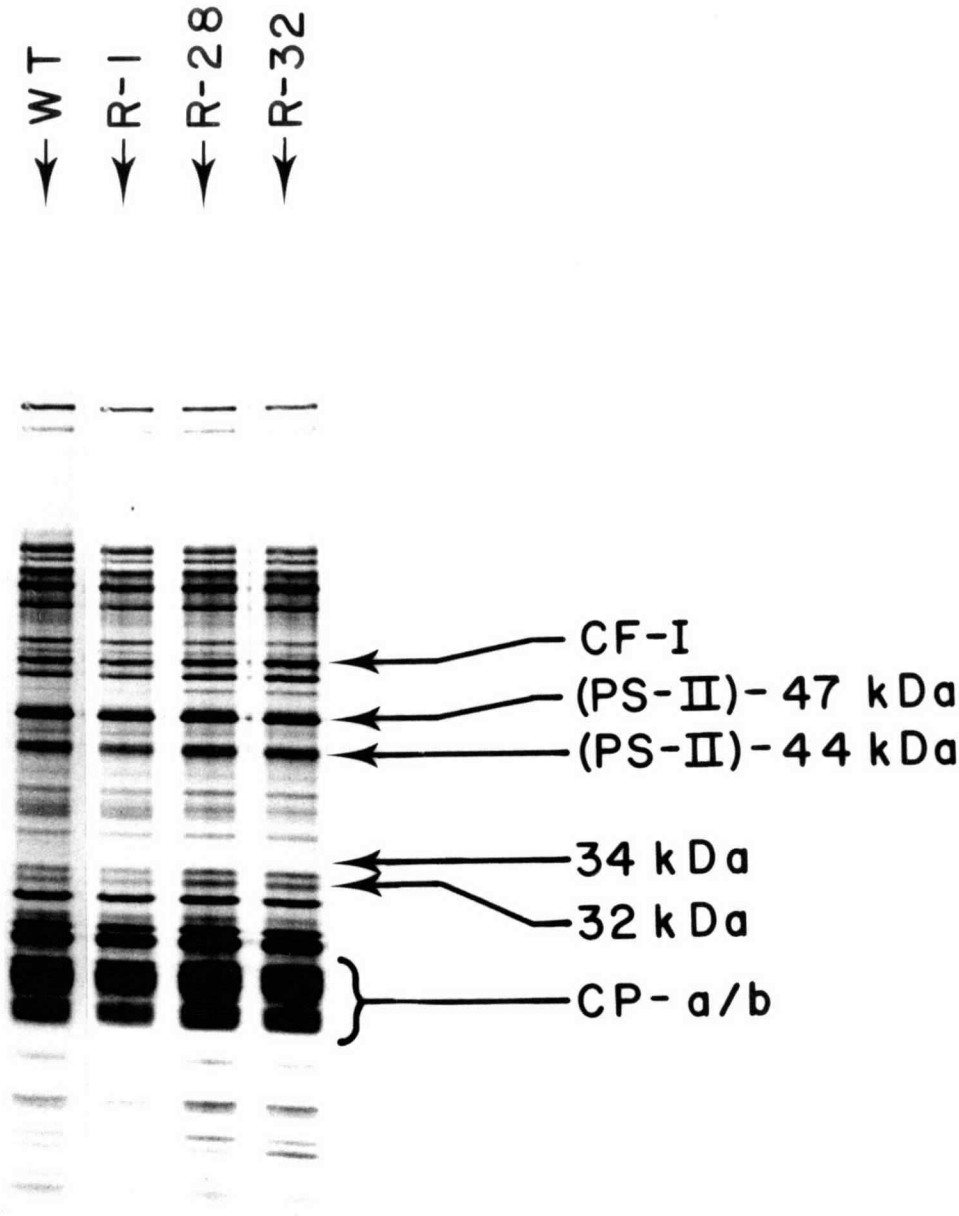

Figure 9.

Developmental Mutants

Developmental mutants were used to follow chloroplast development in the WT and resistant strains. These mutants were used to view the processing of polypeptides during the development of the chloroplast. Cultures were placed in the light and harvested at 2 hour intervals as they greened. No significant differences in electrophoretic patterns of the purified chloroplast thylakoids could be observed between the developmental mutant of the WT, 2A', and the DCMU resistant developmental mutant, R-7 (Figure 10).

The developmental mutants were labeled with ^{35}S and the incorporation of label monitored by electrophoresis. The resulting gel was used to expose X-ray paper; the autoradiogram yielded no polypeptide differences.

Selection for Increased Resistance

Attempts were made by Dr. Bishop to increase the resistance level of the mutants. Strain DCMU R-32 was mutated with ethyl methane sulfonate (EMS) and plated on agar plates containing μM DCMU. Several mutants were recovered but the effect of DCMU on photosynthetic oxygen evolution was similar to that of the original DCMU resistant mutants.

Figure 10. Polypeptide profiles of thylakoid membranes of developmental mutants 2A' and R-7 before (0 hrs) and during (4 hrs) greening and completely greened (48 hrs). Analysis was by LDS-PAGE at $4^{o}C$ using a 7.5% to 15% polyacrylamide gradient in the separating gel. The gel was stained with Coomassie brilliant blue R-250.

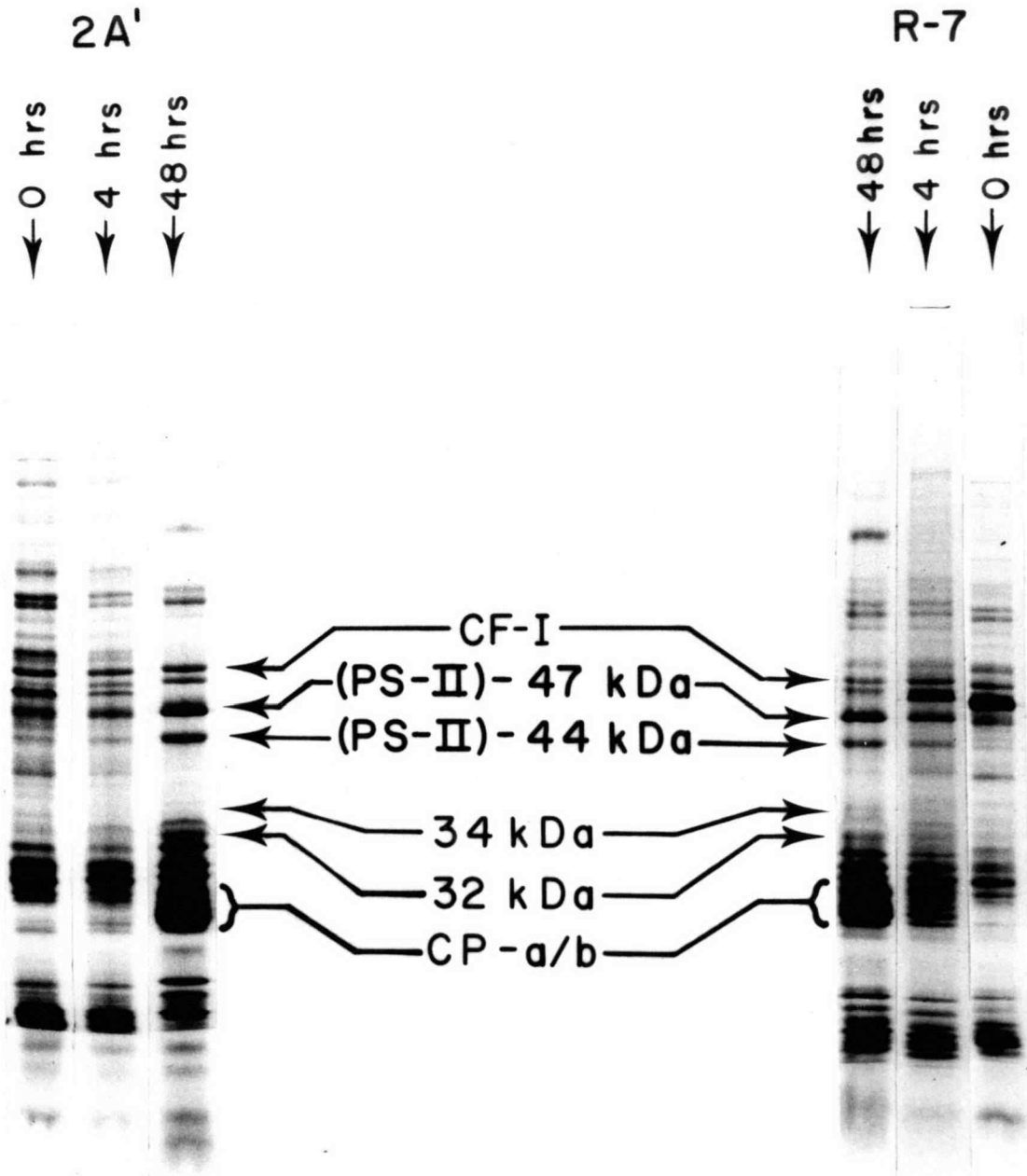
2A'
R-7
0 hrs
4 hrs
48 hrs
48 hrs
4 hrs
0 hrs
CF-I
(PS-II)- 47 kDa
(PS-II)- 44 kDa
34 kDa
32 kDa
CP-a/b

Figure 10.

Chloroplast Lipid Analysis

Studies investigating the mechanism of herbicide resistance have focused on alterations in the structure of the 32 kDa herbicide binding protein. The influence of lipids on the binding protein has received little attention. Analysis of thylakoid lipids in the Scenedesmus mutants was carried out to ascertain if the lipid composition of the thylakoid could be responsible for the resistance of the mutants.

Two dimensional thin layer chromatographic analysis was performed on thylakoid lipid extracts of heterotrophic WT, DCMU R-1, R-28, and R-32 (Figure 11). The lipid composition was determined to be qualitatively equivalent.

The fatty acid side chains of lipids are very important in determining the fluidity of a membrane. The microviscosity of a membrane can be affected by alterations in the saturation of the fatty acids present. Pillai et al. (1981) and Burke et al. (1982) have observed differences in the acyl side chains of lipids present in herbicide resistant mutants. Analysis of the fatty acid composition of the thylakoid lipids of the WT Scenedesmus and DCMU resistant strains was carried out (Table 1). The resistant phenotypes exhibited a larger ratio of unsaturated/saturated fatty acids. They had a higher relative proportion of 16:1 (palmitoleic acid), 18:1 (oleic acid) and 18:2 (linoleic acid). The mutants contained lower relative proportions of 16:0 (palmitoleic acid) and 18:3 (linolenic acid).

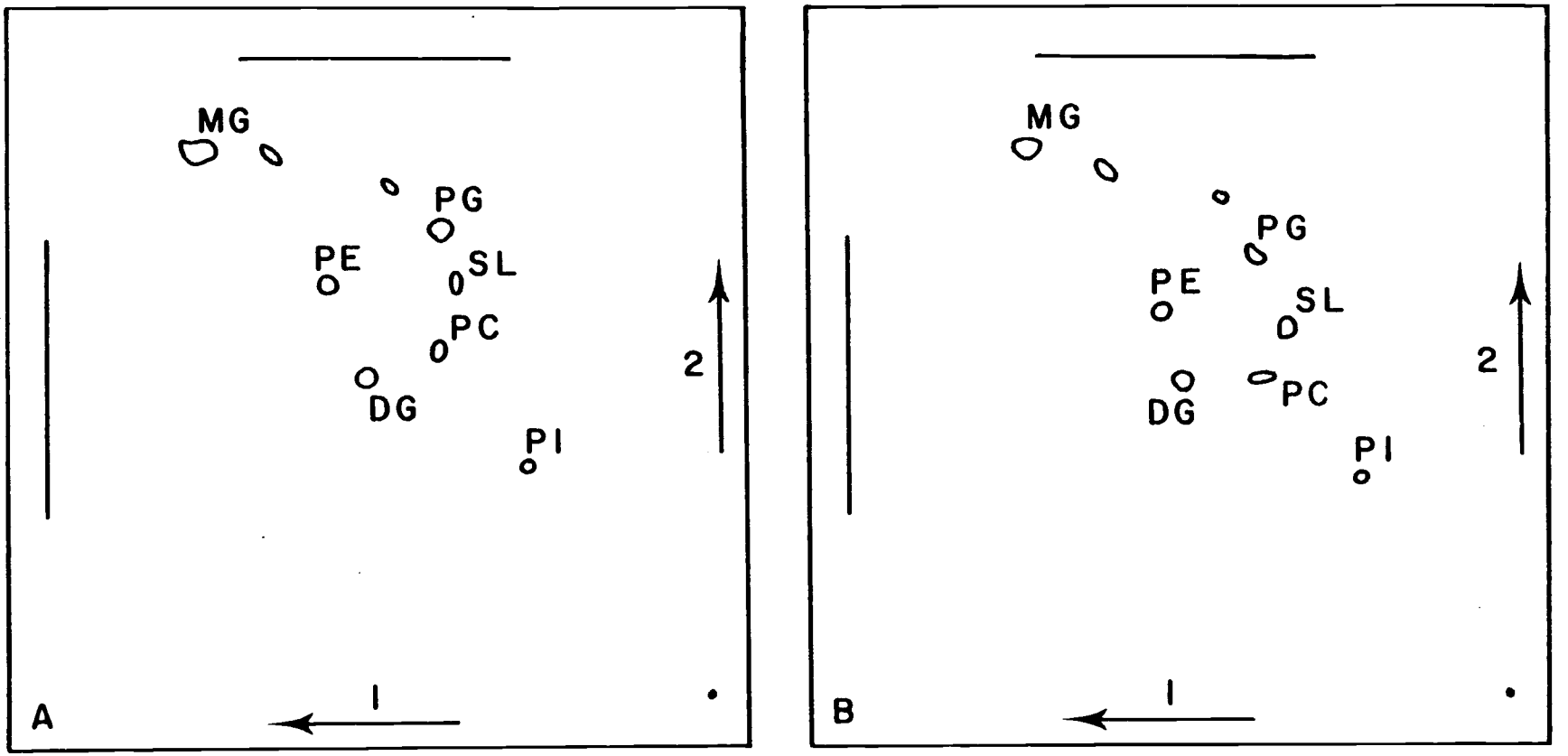

Figure 11. Two dimensional thin layer chromatograms of CH_3OH-$CHCl_3$ (2:1) extracts of thylakoid lipids of wild type Scenedesmus (A) and DCMU R-28 (B). Solvent, direction 1: $CHCl_3$-CH_3OH-H_2O (65:25:4). Solvent, direction 2: $CHCl_3$-CH_3OH-$(CH_3)_2CHNH_2$-NH_4OH (65:35:0.5:5). Lipids were detected with iodine vapor and identified with standards from P. L. Biochemicals. MG-monogalactosyl diglyceride, DG-digalactosyl diglyceride, PE-phosphatidyl ethanolamine, PC-phosphatidyl choline, PI-phosphatidyl inositol and PG-phosphatidyl glycerol. SL-sulfolipid was identified by comparison with published results using the same solvent system (Allen and Good, 1971 p. 534). Pigmented regions have been deleted.

TABLE 1. Fatty acid composition of thylakoid lipids from Scenedemus obliquus wild type and mutants DCMU R-1, R-28 and R-32.

FAME[1]	WT	DCMU R-1	DCMU R-28	DCMU R-32
		mol %		
16:0	26.8	16.2	19.7	19.8
16:1c	4.7	10.3	8.9	12.4
18:0	1.6	tr	0	0
18:1c	26.0	44.1	38.8	33.1
16:4[2]	+	tr	+	+
18:2c	0	5.9	4.9	6.6
18:3	41.0	23.5	27.8	28.1
Total	100.1	100.0	100.1	100.1
$\frac{\text{Unsat}}{\text{Sat}}$	2.5	5.1	4.1	4.1

[1]FAME: Fatty Acid Methyl Ester

[2]This FAME has been tentatively identified as hexadecatetraenoic acid methyl ester. Peaks of small but notable size were detectable in WT, DCMU R-28 and DCMU R-32; a trace amount of this FAME was present in DCMU R-1.

CONCLUSIONS

This study has focused on the characterization of Scenedesmus mutants resistant to the urea herbicide, DCMU. This chemical is known to inhibit the reoxidation of Q, the primary electron acceptor of photosystem II (Duysens and Sweers, 1963). Inhibition results from the noncovalent binding of one inhibitor molecule per electron transport chain, (i.e., per photosynthetic unit). DCMU has been shown to inhibit competitively the binding of other herbicides such as atrazine and dinoseb. It has been proposed that these herbicides act by altering the redox potential of B, the secondary electron acceptor of PSII. This phenomenon may occur by herbicide binding to the apoprotein of B. That herbicide inhibition involves binding to a protein has been demonstrated in several experiments where trypsin was employed to digest proteins on exposed thylakoid surfaces. This procedure removed most or all sensitivity to atrazine and DCMU. A 32 kDa herbicide binding protein has been implicated in herbicide binding through electrophoresis of trypsin digests and photoaffinity labeling experiments. The binding protein may have several binding sites or there may be two proteins involved in the formation of the binding site(s). Either of these models could explain why the herbicides competitively inhibit the binding of each other and it could also explain the development of resistance to one herbicide and not another.

It has not been possible in this study to determine the exact site of DCMU inhibition in the Scenedesmus mutants. Fluorescence

experiments show that DCMU prevents the reoxidation of Q by plastoquinone indicating that the point of inhibition on the electron transport chain is the same as has been found in other systems. Whether DCMU binds to a 32 kDa protein in Scenedesmus has not been established; trypsin digestion experiments were not successful and a photoaffinity label for DCMU is not available.

The DCMU resistant mutants exhibit the following characteristics: They show photosynthesis comparable to the wild type (WT) and possess a similar complement of accessory pigments. They demonstrate a 10 fold greater resistance to DCMU than the WT. In whole cell reactions as well as isolated chloroplast reactions the resistance is maintained. Altered cell wall permeability is shown not to be the mechanism of resistance. The ability of DCMU to inhibit the electron transport chain and thus increase fluorescence is more pronounced in the WT than in the mutants. The WT and the resistant mutants exhibit comparable susceptibility to the herbicide atrazine. Polypeptide analysis demonstrates that during chloroplast development and in fully matured cells the complement of proteins of both the WT and the mutants are the same. Two dimensional thin layer chromatograms of thylakoid lipids of resistant mutants and the WT are qualitatively comparable. The only difference detected in this study is in the fatty acid composition of the lipids. There is a notable difference in the unsaturation ratio of fatty acids found in the WT versus the resistant mutants; the WT contains less unsaturated

fatty acids than the mutants. It is possible that the mechanism of resistance in Scenedesmus may involve the environment of the herbicide binding protein. The fluidity of the lipid environment of a protein has been indicated in the vertical displacement of membrane proteins (Borochov and Shinitzky, 1976). The resistant mutants have a higher proportion of unsaturated fatty acids and thus could provide a more fluid environment for the herbicide binding protein; the protein could lie deeper in the membrane in the resistant mutants making it less accessible for herbicide binding. It is possible however, that DCMU is binding unspecifically to lipids in the mutants. More work is needed before a complete interpretation can be presented.

The DCMU resistant Scenedesmus mutants show a resistance pattern somewhat similar to that of atrazine resistant higher plants. Resistance is not due to altered permeability; the 32 kDa polypeptide is present in both resistant and susceptible types and there is an increase in unsaturated fatty acids in the lipids of the resistant biotypes, a fact that has been reported for some atrazine resistant higher plants (Pillai and St. John, 1981; Burke et al., 1982). The Scenedesmus mutants however show a magnitude of resistance similar to other resistant algal strains and significantly less than that of atrazine resistant higher plants. Because of this large difference it is probable that the resistance phenomenon observed in DCMU resistant algae is not the same as that occurring in atrazine

resistant higher plants. The work of this study indicates that the lipid composition of the thylakoid membrane influences the binding of the herbicide DCMU in DCMU resistant mutants of Scenedesmus obliquus.

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