INTERACTION OF HERBICIDES WITH THE CYANOBACTERIA, *Anacystis nidulans*

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SAGARIKA MISHRA

SCHOOL OF LIFE SCIENCES
JAWAHARLAL NEHRU UNIVERSITY
NEW DELHI - 110 067

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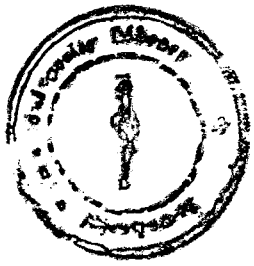
CERTIFICATE

The research work emobdied in this dissertation has been carried out in the School of Life Sciences, Jawaharlal Nehru University, New Delhi. This work is original and has not been submitted so far, in part or in full, for any other degree or diploma of any university.



(Sagarika Misra)
Candidate

(Gauri Shanker Singhal)
Supervisor

(Prasanna Mohanty)
Dean
21/12/87

SCHOOL OF LIFE SCIENCES
JAWAHARLAL NEHRU UNIVERSITY
NEW DELHI-110 067

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Sagarika Misra

ABBREVIATIONS

ADP	-	Adenosine diphosphate
APC	-	Allo phycocyanin
ASC	-	Ascorbate
ATP	-	Adenosine triphosphate synthase
Chl a	-	Chlorophyll a
Chl b	-	Chlorophyll b
Cyt b_6/f	-	Cytochrome b_6/f
DCMU	-	3-(3,4-dichlorophenyl)-1, 1-dimethyl urea
DCPIP	-	Dichlorophenyl indophenyl
DGDG	-	Digalactosyl diglyceride
DPC	-	Diphenyl carbazide
EDTA	-	Ethylene diamine tetra-acetic acid
ESR	-	Electron spin resonance
ETC	-	Electron transport chain
Fo	-	Coupling factor
F_1	-	Coupling factor 1
Fd	-	Ferrodoxin
kD	-	Kilodatton
MGDG	-	Monogalactosyl diglyceride
MV	-	Methyl viologen
NADP	-	Nicotine adenine dinucleotide phosphate
OEC	-	Oxygen evolving complex
PBS	-	Phycobilisome
PBPS	-	Phycobiliproteins
PBQ	-	Para benoquinone

PCB	-	Phycocyanobilin
PQ	-	Plastoquinone
PC	-	Plastocyanin
PC	-	Phycocyanin
PE	-	Phycoerythrin
PEG	-	Polyethylene glycol
PS I	-	Photosystem I
PS II	-	Photosystem II
PQ	-	Plastoquinone
Q_A	-	Quinone A
Q_B	-	Quinone B
San 133-410H	-	[4-chloro-5)methylamino)-2-phenyl-3(2H)-pyridazinone]
SDS-PAGE	-	Sodium dodecyl sulphate polyacryl-amide gel electrophoresis
TMPD	-	N-tetramethyl paraphenyl diamine

CONTENTS

INTRODUCTION

INTRODUCTION

The process of photosynthesis involves the conversion of light energy into chemical energy by chlorophyll containing organisms. It consists of two phases, the light reaction and the dark reaction. In the former, light energy is absorbed and converted to chemical energy, leading to the production of ATP and reduced NADP. These products of light reaction are used up during the dark reaction to reduce carbon dioxide to carbohydrates. The overall reaction may be represented as :

$$CO_2 + 2H_2O \xrightarrow[Chl]{h\nu} (CH_2O) + H_2O + O_2$$

Cyanobacteria have been extensively used for studies on photosynthesis (Stanier and Cohen-Bazire, 1977) since they are also oxygen-evolving photosynthetic organisms containing two photosystems (Ho and Krogman, 1983) which function as depicted in the figure (Fig.1). But the striking difference between the green plants and cyanobacteria is that the former contain Chl b whereas the latter contain phycobilin pigments as the major light-harvesting pigments. Chlorophyll a and carotenoids are present in both the types (Thornber, 1986).

ORGANIZATION OF CYANOBACTERIAL PHOTOSYNTHETIC APPARATUS

Cyanobacterial thylakoids are lamellar in nature and traverse the cytoplasm. The exact organization of these thylakoids depends on the species as well as the physiological state of cells (Wolk, 1973). The thylakoids are not enclosed by a bilayered

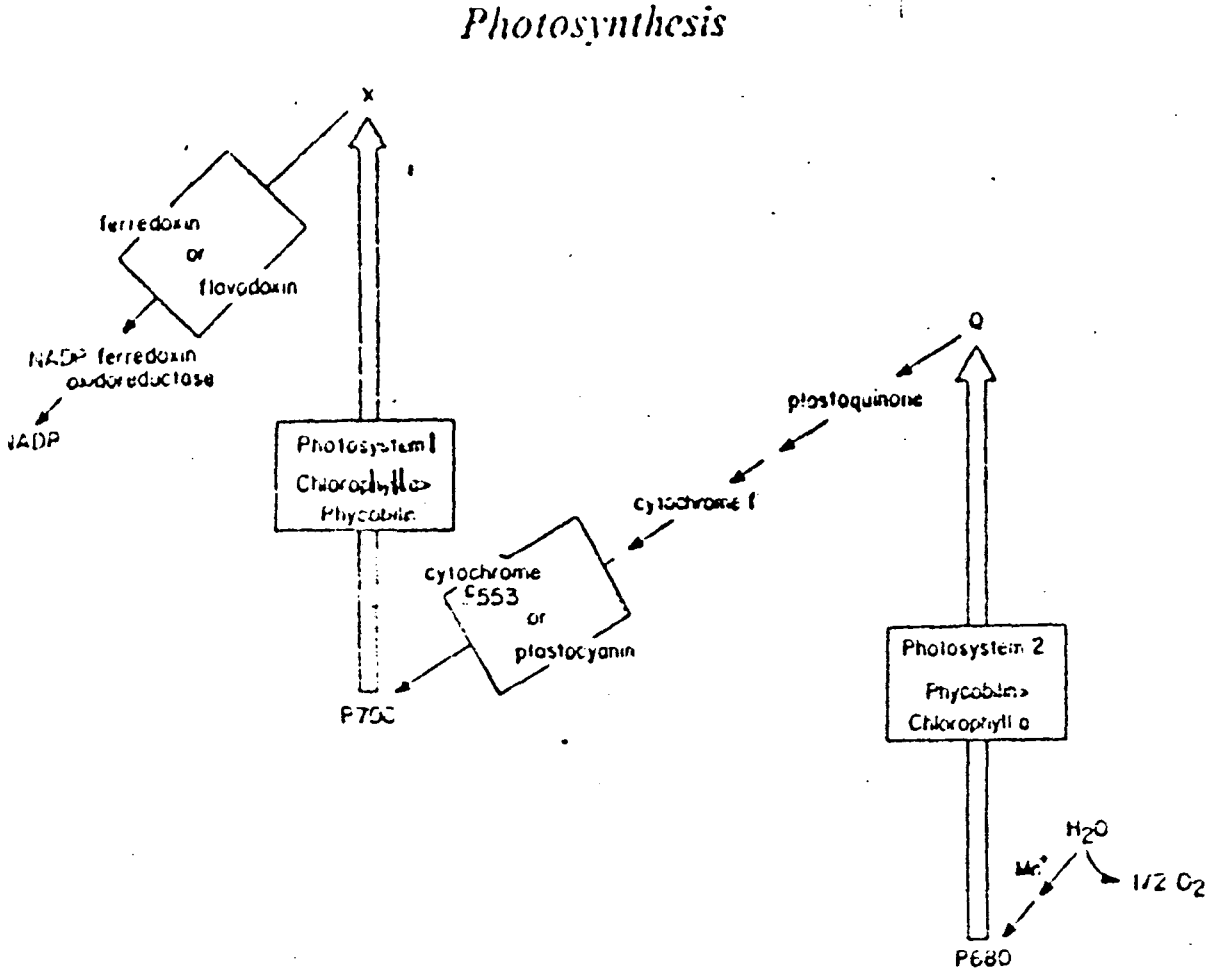


Fig. 1. Photosynthetic electron transfer sequence in cyanobacteria (From Ho and Krogmann, 1982).

unit membrane like the chloroplasts of photosynthetic eukaryotes. In some species the membranes are arranged in concentric layers, in others they are highly curved and show a contorted appearance. Several workers reported that the thylakoids of cyanobacteria can be physically continuous with the cytoplasmic membrane. But recently Staehelin (1986) and Golecki and Drews (1982) have found that in most cyanobacteria the thylakoids are arranged peripherally in three concentric layers and appear to act as separate units. They are neither differentiated into stacked (grana) membranes and unstacked (stroma) membranes nor do they show any lateral heterogeneity of the structural regions like the chloroplast thylakoids. The cyanobacterial thylakoids are thought to house the respiratory apparatus also (Bricker et al. 1986).

Lipid composition of the membrane fraction is very similar to that of the whole cell (Hiramaya, 1967). The major lipids of cyanobacteria are monogalactosyl diglyceride (MGDG), digalactosyl diglyceride (DGDG), sulphoquinovosyl diglyceride and to a minor degree, the phospholipid, phosphatidyl glycerol (Nichols et al. 1965; Hiramaya, 1967; Nichols and Wood, 1968a; Fork et al. 1979).

The fatty acid patterns of cyanobacteria are variable. The unicellular cyanobacteria, have only saturated (myristic, palmitic and stearic acid) and mono-unsaturted (palmitoleic, trans-3 hexadecanoic and oleic) fatty acids, that are characteristic of the other prokaryotes. Occasionally linoleic acid is also present. Several unicellular cyanobacteria also contain large amounts of polyunsaturated fatty acids (Kenyon, 1972). The cyanobacterial thylakoids contain phycobiliproteins, the light harvesting complex which are

not present in chloroplasts of higher plants. These water soluble proteins are attached to the outer surface of thylakoid membrane of cyanobacteria (Zilinskas and Greenwald, 1986). Also the photosystem II complex, the photosystem I complex, cyt b_6/f complex and the coupling factors in cyanobacterial thylakoids are not spatially segregated as in chloroplasts of higher plants since there is no grana or stroma formation in cyanobacteria.

THE LIGHT HARVESTING COMPLEX OF CYANOBACTERIA

The cyanobacteria contain the light-harvesting pigments called phycobiliproteins, which are packaged in multimeric pigment protein complexes called phycobilisomes (Zilinskas and Greenwald, 1986). The major components of phycobilisomes are the bilin-containing proteins: phycoerythrin (PE, λ_{max} 565 nm), phycocyanin (PC, λ_{max} 620 nm), allophycocyanin (APC, λ_{max} 670 nm). The last two pigment proteins are universally found in all cyanobacteria and red algae (Bryant et al. 1979; Gantt, 1986). The phycobiliproteins (PBPs) collectively absorb light in green, orange and red regions of the visible spectrum. The phycobilisomes are assembled together through linker polypeptides (Zilinskas and Greenwald, 1986).

The phycobiliproteins contain an apoprotein portion to which varying numbers and types of chromophores, having linear tetra-pyrrole structure are attached by thioether cysteine bonds (Brown et al. 1979). PC and APC carry the phycocyanobilin (PCB) chromophore (Zilinskas and Greenwald, 1986). In cyanobacteria the phycobilisomes are approximately 7×10^{-6} daltons in size (Glazer, 1983).

The protein portion of phycobiliproteins (PBPs) have two dissimilar polypeptides, α and ß, which occur in a ratio of 1:1. The building block for the PBPs is the monomer (αß), with the most common aggregation states being the trimer $(\alpha ß)_3$ and hexamer $(\alpha ß)_6$. The absorption and emission maxima of PBPs show that the energy pathway in vivo is from PE to PC to APC (Fig.2). Recent studies reveal the association of a chromophore-containing linker polypeptide with the thylakoid and the phycobilisome core (Lundell and Glazer, 1983; Lundell et al. 1981; Redlinger and Gantt, 1982; Rusckowski andZilinskas, 1982; Zilinskas, 1982). All these workers suggested a two fold function for the polypeptide i.e. attaching PBsome to the membrane and transferring the excitation energy to chlorophyll a (Chl a). The ultrastructural studies show that the PBsomes are hemidiscoidal in cyanobacteria (Bryant et al. 1979). The model for PBsome (Fig.3) shows that morphologically these particles have two distinct domains; a core made up either of three (Bryant et al. 1979; Morschel et al. 1977) or two (Glazer et al. 1979) cylindrical objects from which six rods made up of stacked discs (120 x 60 Å) extend in a hemidiscoidal pattern. The discs proximal to the core contain phycocyanin and the peripheral discs contain phycoerythrin and the core itself contains allophycocyanin (Bryant et al. 1980; Gingrich et al. 1982). Kursar et al. (1983) have put forth a PBsome model with three fold axis of symmetry, which accomplishes better self assembly and efficient energy transfer.

The molecular organization of the phycobilisome is such that excitation energy absorbed by any one of the chromophore is

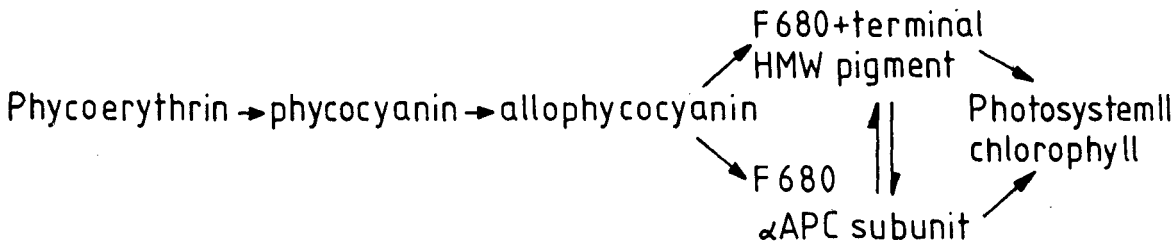


Fig. 2. Energy transfer pathway in phycobilisome

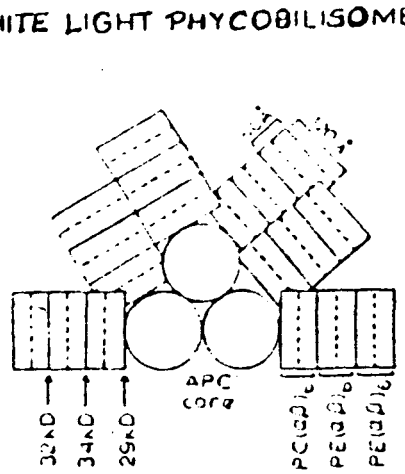


Fig. 3. A model of the typical three cylinder core phycobilisomes, isolated from the cyanobacterium Nostoc sp. (From Zitinskas and Greenwated, 1986.

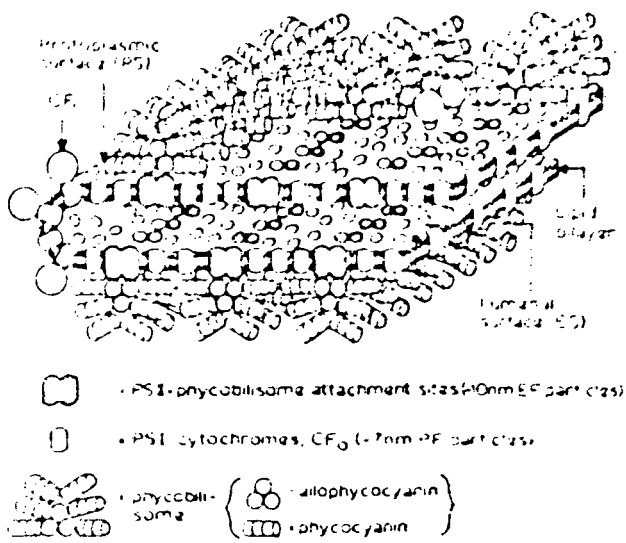


Fig. 4. A model depicting phycobilosome - thylakoid associations. Phycobilisomes with allophycocyanin at the base, are aligned in rows in contact with the surface exposed-portion of the presumptive PS II centers. Each PS II center appears to be composed of two subunits. (From Gidding et al. 1983).

transferred to photosystem II (PS II) reaction center with approximately 100% efficiency. In fact, it reduces the random transfer of energy and channelises the directional flow of energy through the few steps to the final emitter in the core of the PBsome. The spatial distribution of phycobilisomes in the thylakoid membrane is represented in the figure (Fig.4).

CHLOROPHYLL PIGMENTS

The cyanobacteria contain only chlorophyll a. It is present in the thylakoid membrane with the carrier proteins forming intramembrane complexes. Most of the chlorophyll is localised in photosystem I (PS I) whereas photosystem II (PS II) contains only a small fraction of it (Mimuro and Fujita, 1977; Tel-Or and Malkin, 1977). In in vivo condition Chl a is present in several spectroscopic forms such as Chl a 660, Chl a 670, Chl a 680, Chl a 685, Chl a 690 and Chl a 700-720. The cyanobacteria lack Chl b which is present in all eukaryotic photosynthetic organisms.

OTHER ACCESSORY PIGMENTS

Besides phycobilin pigments, carotenoids and xanthophylls also serve as accessory pigments in cyanobacterial photosynthesis. ß-carotenes are most predominantly present accompanied by either or both of the oxycarotenoids zeaxanthin (3,3',-dihydroxy-ß-carotene) and echinone (4-keto-ß-carotene) (Hertzberg et al. 1971). The myxoxanthophylls (1'-2'-dihydro-3'-4' didehydro-3', 1'-dihydroxy-carotene) are also present.

PHOTOSYSTEM I

The photosystem I (PS I) complex contains a reaction center comprising of a dimer of Chl a molecules, absorbing at 700 nm (P 700). The PS I complex of higher plants contains six subunits whereas those of green algae and cyanobacteria have only four subunits (Okamura et al. 1982). A dimer of subunit I constitutes the reaction center. The two 70 kDa polypeptides of the RC (subunit I) may be minor structural variants of a single polypeptide (Bar-Nun et al. 1977; Chua et al. 1975). Another subunit binds plasto-cyanin to the PS I comkplex (Haehnel et al. 1980). The remaining subunits are involved in binding and orientation of the light-harvesting complexes. The two primary electron acceptors of PS I, namely A_0 and A_1, are present in higher plants as well as in cyanobacteria. The position and shape of the ESR signal from cyano-bacterial PS I suggest that A_1 is also a phylloquinone in cyano-bacteria, like that of the hgiher plants (Smith et al. 1987). A_0 has been suggested as a Chl a monomer identified by ESR and optical studies (Bonnerjea and Evans, 1982; Gast et al. 1983; Mansfield and Evans, 1985).

PHOTOSYSTEM II

The reaction center of PS II, P 680 contains Chl a molecule absorbing at 680 nm. The PS II core complex consists of several membrane spanning polypeptide subunits of 47, 44, 34 and 10 kD molecular weights. According to earlier reports the polypep-tide doublet (of 47 kDa and 43 kDa) is the reaction center of PS II whereas Green and Camm (1984) and Nakatani et al. (1984)

suggest that the 43 kDa protein binds Chl a and 47 kDa protein acts as the reaction center. But current evidence suggests that P 680, Pheo and Q_A are all bound to the 34 and 32 kD core proteins (Michel and Deisenhofer, 1985; Arntzen and Pakrasi, 1985). The core complex also contains an electron donor, Z, which is an anionic plastoquinone (Dutton, 1986; Ort, 1986). At the reducing side it contains a tightly bound quinone, Q_A and a loosely bound quinone acceptor, Q_B. There is now extensive evidence that a 32 kD polypeptide is the site of Q_B binding (Vermaas et al. 1983). A second polypeptide known as D_2 protein (34 kD) is also present in the PS II complex (Chua and Gillham, 1977; Arntzen and Pakrasi, 1986) whose function is still obscure. Cyt b_{559} which is a heterodimer of two polypeptides of about 10 and 4.5 kD is present in PS II core complex (Hermann et al. 1984; Babcock et al. 1985).

OXYGEN EVOLVING COMPLEX

The oxygen evolving complex of PS II accomplishes the photolysis of water. According to the S-state model (Joliot and Kok, 1975) of water oxidation (Fig.5) four electrons are removed from two water molecules in four sequential one-electron steps. Three water soluble polypeptides of 33 kDa, 23 kDa and 17 kDa and cofactors such as Mn^{2+}, chloride (Cl^{-1}) and Ca^{2+} play an important role in the water splitting complex. Z is the primary physiological electron-donor to the reaction center, P 680 of PS II.

CYTOCHROME b_6/f COMPLEX

Cyt b_6/f complex oxidises the plastoquinol and reduces plastocyanin, which in turn is oxidised by PS I. Hence it is often called

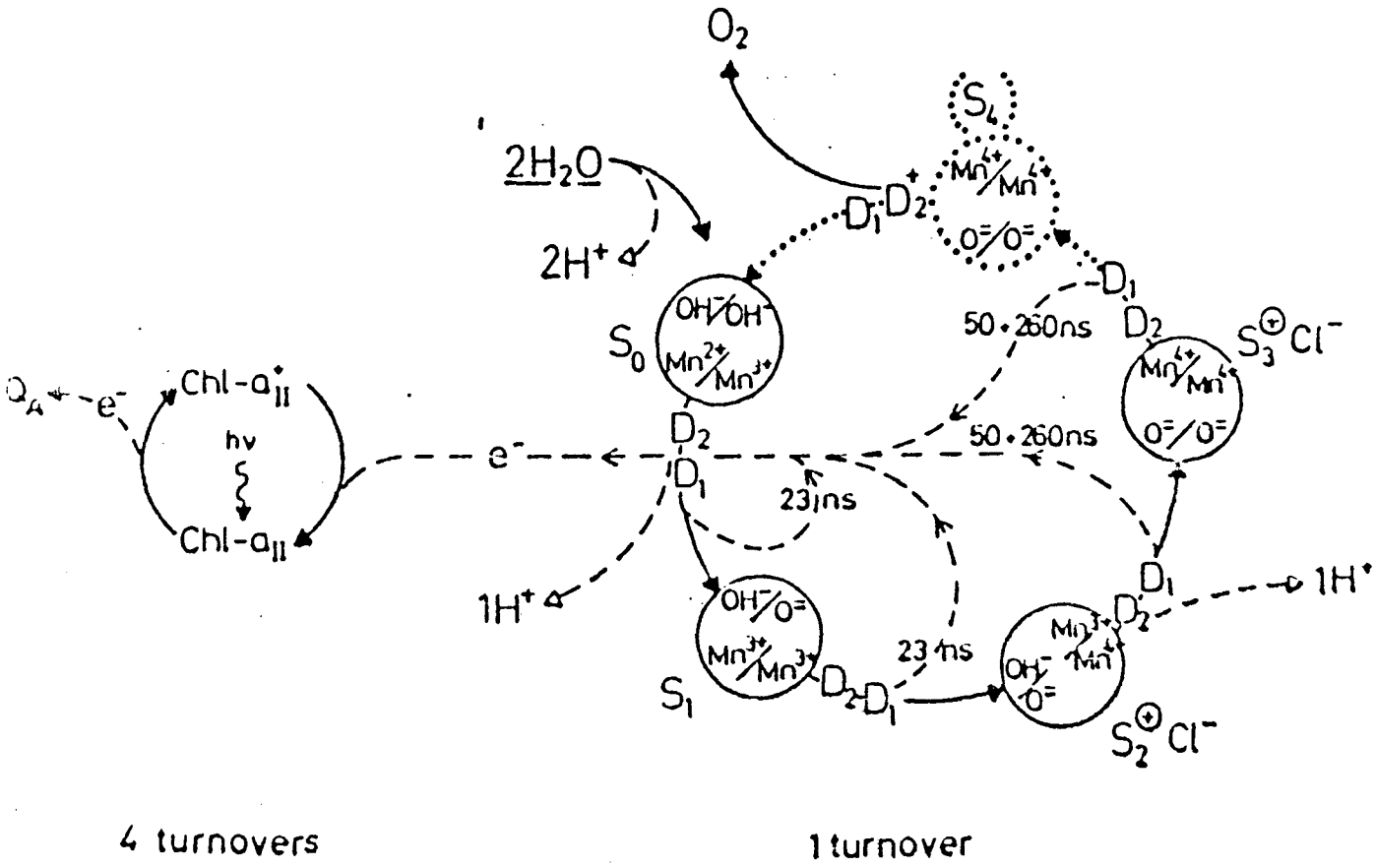


Fig. 5. S-state water oxidation model (From Witt et al. 1986).

as plastoquinol : plastocyanin oxidoreductase. It consists of five polypeptides of 34 kDa, 33 kDa, 23 kDa and 17 kDa (Hurt et al. 1981).

F_o - F_1 ATP SYNTHASE

The adenosine triphosphate synthase (ATPase) is able to use the electrochemical gradient across the membrane to drive the endothermic phosphorylation of adenosine diphosphate (ADP). It consists of two parts: the extrinsic coupling factor, F_1 and an aggregate of hydrophobic proteins termed F_o. F_1 contains the active sites of H^+-ATPase synthase whereas the F_o functions to bind F_1 to the membrane and as a proton channel. The F_o contains three polypeptides of 15, 12 and 8 kDa. The F_1 contains 5 subunits of 59, 54, 37, 17.5 and 13 kDa respectively (Nelson, 1982).

PLASTOCYANIN

It is a copper containing protein with a molecular weight of 10.5 kD (Katoh, 1960 and Katoh et al. 1962). It functions as electron carrier between cytochrome b_6/f complex and the photosystem I (PS I) complex. Two plastocyanin molecules, one bound to P 700 and another mobile, are present per P 700 (Malkin and Bearden, 1973 and Haehnel, 1977).

FERREDOXIN

Ferredoxin (M_r = 11 kDa) is a water soluble protein present on the outer surface of thylakoid. It contains 2 Fe-2S centers (Hall and Rao, 1977). It mediates the electron transport between PS I

and the final acceptor NADP. Ferredoxin-NADP oxidoreductase (40 kDa) is a flavonoid protein catalyzing the reduction of $NADP^+$ to NADPH.

FUNCTIONING OF THE ELECTRON TRANSPORT CHAIN

The photosystem II is associated with the oxidation of H_2O and photosystem I (PS I) is associated with the reduction of $NADP^+$. Light energy (λ is) absorbed by the reaction center P 680 of PS II and primary charge separation takes place leading to reduction of the primary electron acceptor pheophytin and the production of a strong oxidant Z^+ capable of oxidising H_2O to O_2. Similarly the light absorbed by PS I antenna is trapped in the reaction center P 700 of PS I, thereby oxidising it and reducing the primary electron acceptor A_0. Due to the electron migration along the electron transport chain the components are re-oxidised and re-reduced alternately. The net output of the simultaneous operation of the two photosystems is the transfer of electrons from H_2O to $NADP^+$. At the same time the adenosine triphosphate synthase (ATPase) drives the phosphorylation of ADP to ATP using the electrochemical gradient of H^+ across the membrane. The products (ATP and NADPH) of the light reaction are utilized in the dark reaction to reduce carbon dioxide to carbohydrates.

INTERACTION OF HERBICIDES WITH PHOTOSYNTHESIS

Chemicals used to kill the weeds are known as herbicides. In principle herbicides can cause damage to cultivated plants, too. Their selectivity depends on differences in susceptibility of the

organism owing to such factors as developmental stage, morphological and anatomical features and different rates of penetration and metabolism of the herbicides. The margin between herbicidal action on the weeds and phytotoxicity to the crop is often narrow. Studies on the mode of action of photosynthetic herbicides provide a better scope for understanding the mechanism of electron transport, fluorescence quenching and light distribution phenomena.

I. CLASSIFICATION OF HERBICIDES

Buchel (1972) has classified the herbicidal photosynthetic inhibitors into the following groups : (i) phenyl urea; (ii) heterocyclic ureas; (iii) phenylcarbamates; (iv) acylanilides; (v) S-triazines and (vi) 1,2,4-triazinones. Trebst (1980) has classified the electron transport inhibitors on the basis of their mode and site of action as follows :

(i) inhibitors of oxidising side of plastoquinone;

(ii) inhibitors of reducing side of plastoquinone;

(iii) inhibitors operating after plastocyanin;

(iv) inhibitors and inactivators of the oxygen evolving site.

According to the recent report by Trebst and Draber (1986) the herbicides can be grouped into classical and non-classical types. The classical inhibitors of the acceptor side of PS II are triazines, ureas, triazinones, anilides etc. These belong to different chemical groups but act on the same functional site, at Q_B protein (Trebst and Draber, 1986). These herbicides can displace each other from the membrane (Tischer and Strotman, 1977) revealing the fact that they are functionally related but chemically different compounds

having overlapping binding sites on Q_B protein (Pfister and Arntzen, 1979; Trebst and Draber, 1979) (See Fig.9).

The non-classical type of inhibitors include nitro and halogen substituted phenols like dinoseb and ioxynil (van Rensen, 1977), pyridazinones uracils, 4-hydroxybenzonitriles, benzimidazoles and other π-excessive heterocycles, benzimidazo-isoquinolinediones.

Recently, a number of phenol analogs are attributed as potent PS II inhibitors such as pyridones (Trebst et al. 1985), quinolones (Draber, 1987), benzoquinones (Bauer and Kocher, 1979 and Oettmeier et al. 1978), pyrones (Kuwabara et al. 1980), a natural occurring chromone (Stigmatellin) (Oettmeier et al. 1985) and cyanoacrylates (Phillips and Huppatz, 1984). A chemical element common to all herbicides of the classical type (triazine, urea family) is a Sp2 hybrid orbital bound to N, O or CH and attached to a lipophilic substituent (Buchel, 1972; Trebst and Draber, 1979).

We have investigated two herbicides which act at the reducing side of PS II. These are DCMU and SAN 133-410H.

(i) Sites of action of herbicides

Herbicides affect the metabolic processes of susceptible plants. The photosynthetic herbicides interfere with the function and organization of chloroplasts and pigments. A detailed account of the inhibitory mode of action of herbicidal compounds on photosynthetic electron transport, photophosphorylation, inhibition of carotenoids and chlorophyll biosynthesis and the peroxidative destruction of chloroplast components is schematically outlined in Figure 6 (Sandmann and Boger, 1986).

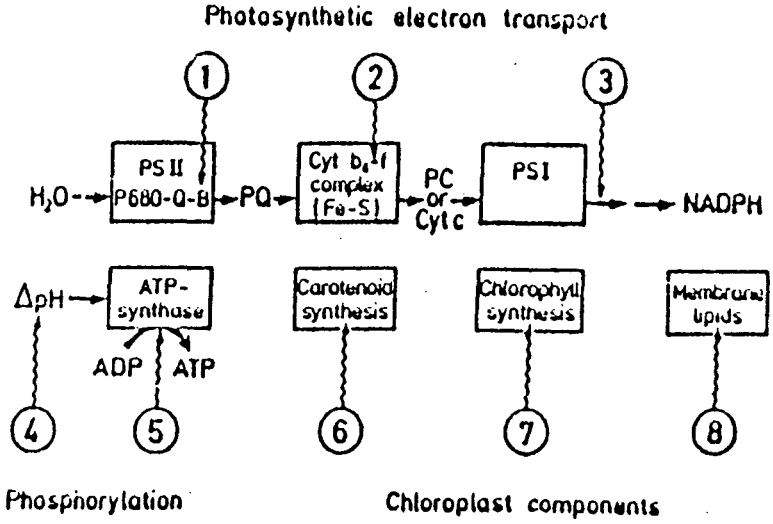


Fig. 6. Bioenergetic reactions in the chloroplast affected by phytotoxic compounds: 1) Inhibition of electron transport at the reducing side of photosystem II primarily at B = Q_B (binding of, e.g., ureas, triazines); 2) inhibition of electron flow through the cytochrome b_6/f complex (diphenyl ethers, e.g., DNP-INT; DBMIB); 3) deviation of electrons from the reducing side of photosystem I (bipyridyliums, radical formation); 4) dissipation of the proton gradient (e.g., phenylhydrazones, carbanilates, diphenylamines); 5) inhibition of ATP formation at the ATPase (nitrofen); 6) inhibition of carotenoid biosynthesis (e.g., norflurazon, difunon, m-phenoxybenzamides); 7)inhibition of chlorophyll biosynthesis (oxidation, DTP, MK-616); 8) peroxidative desdtruction of membrane lipids (bipyridyliums, diphenyl ethers). Cyt cytochrome (Cyt c denotes cytochrome c-553 present in pro- and eukaryotic algae); PC plastocyanin; Q primary quencher; P-680 reaction center chlorophyll of PS II (From Sandmann and Boger, 1986).

II. EFFECT OF HERBICIDES ON ELECTRON TRANSPORT

(i) The herbicide binding protein

The proteinaceous nature of DCMU binding site (Q_B) was proved by trypsin-treatment experiments. But binding studies reveal that various herbicides bind partly to overlapping sites on the same protein (van Rensen and Snel, 1985). By radioactive photo-affinity studies it was established that the herbicides bind to a 32,000 M_r polypeptide, located at the reducing side of PS II and after binding, the electron transport from Q_A to Q_B is inhibited. Further research would reveal the possible reasons behind the inhibited electron flow, whether it is due to :

(i) a conformational change of Q_B protein caused by the binding of herbicide;

(ii) the displacement of Q_B from the Q_B protein by herbicide; or

(iii) a herbicide induced loss of bicarbonate which is essential for electron flow (van Rensen, 1982).

III. MECHANISM OF INHIBITION OF PS II ACTIVITY BY HERBICIDES

The PS II inhibitors (I) displace the secondary quinone acceptor Q_B, from its binding site, as schematically shown in Figure 7. It was proposed for the PS II complexes of purple photosynthetic bacteria (Wraight, 1982b) and higher plants (Velthuys, 1982). The binding to thylakoids or to PS II enriched membrane fragments is reversible (Varmaas et al. 1984). The plastoquinol is weakly bound to the quinone binding site on Q_B protein so it is easily displaced

by the herbicide than the Q_B (Fig.7b). But the semiquinone is very tightly bound than the Q_B (Crofts and Wraight, 1983). From the equilibrium between Q_A AND Q_B it is evident that the inhibitor traps the electron from Q_A thereby displacing Q_B (Fig.7c). Recently, it has been suggested that a change in the amino acid sequence of 32 kDa PS II protein (Hirschberg et al. 1984) inhibits the binding affinity of triazine herbicides (and related herbicides) to the PS II complex (Pfister and Arntzen, 1979). Due to the change in the conformation of the 32 kDa PS II protein the herbicides no more compete with Q_B for the binding site. But recently, it was demonstrated that the herbicides and quinones do not occupy identical binding sites on the Q_B-protein (Oettmeier et al. 1984) although binding of one affects that of the other possibly by some allosteric mechanisms (Arntzen and Pakrasi, 1986). It is inferred from the experiments conducted using azidoquinone (Q_B analog), which reduces the binding affinity for atrazine but not for ioxynil. Moreover, the absolute number of binding sites for either herbicide remained the same (Vermaas et al. 1983).

IV. ROLE OF DCMU AND SAN-TYPE HERBICIDES

(i) The urea herbicide

DCMU is one of the most commonly used commercial urea herbicides. In 1946, Thomason et al. surveyed the growth inhibitory activities of several substituted urea compounds (Fig.8). DCMU (1,3-bis(2,2,2-trichloro0l-hydroxyethyl) urea) was the first substituted urea to be used commercially for weed control. Most urea herbicides are relatively non-selective and are usually applied to the soil,

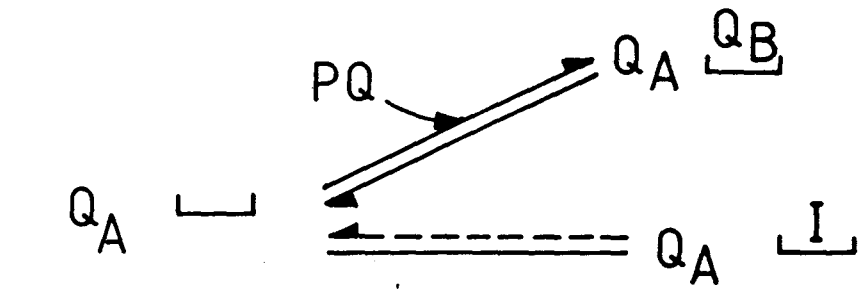


Fig. 7.(a)

Fig. 7.(b)

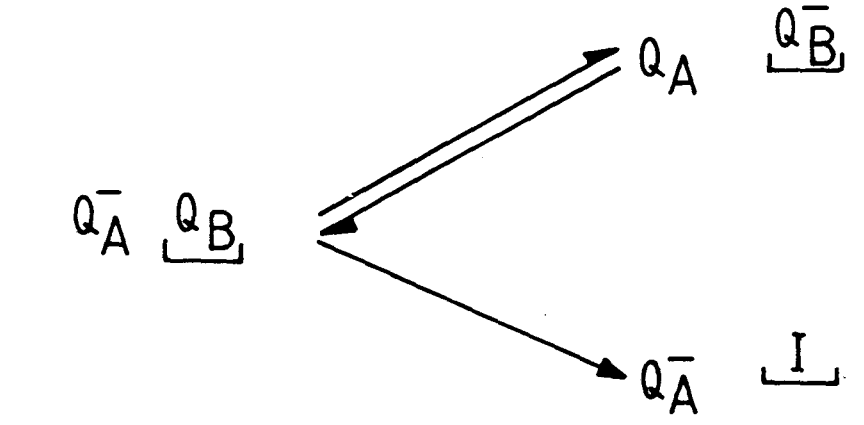


Fig. 7.(c)

Fig. 7 The figures show the various interactions between Q_A, Q_B and the inhibitors (From: Sandmann and Boger, 1986).

however, some act by foliar application. The absorption and translocation of diuron from leaves or roots of several species show that its pattern of movement is restricted to the apoplast (Bayer and Yamaguchi, 1965; Leonard et al. 1966; Leonard and Glenn, 1968). Histoautoradiographic studies revealed that DCMU enters into the cell walls completely, even those of the stele.

It induced stomatal closure in higher plants (Soybean) due to an increase in the CO_2 concentration in the intercellular spaces as a result of the blockage of photosynthesis (Lay and Ilnicki, 1972). In Euglena gracilis, DCMU (10^{-9}M - 10^{-6}M) caused significant loss of chlorophyll and disorganization of chloroplasts (Laval-Martin et al. 1977). After growing in that condition for two months the cultures acquired resistance to DCMU. Eudorina elegans colonies were killed at 10^{-4}M DCMU and growth was inhibited at 10^{-9}M to 10^{-7}M of DCMU (Orr et al. 1976). Stanger and Appleby (1972) proposed that diuron acts by catalyzing lethal photosensitized oxidations by an intervention of electron flow and an inhibition of NADPH formation, which is necessary to maintain a functional carotenoid protective mechanism. But Ridley (1977) was critical of Stanger-Appleby hypothesis and suggested that blocking of electron transport by DCMU, prevents conformational changes in the chloroplast membrane that are necessary for the transfer of excitation energy from Photosystem II to Photosystem I. The energy is transferred from chlorophyll to carotenoids, but the input is greater than the dissipation. Thus photooxidative destruction of the pigment complexes begins and eventually lipids also undergo destruction, which causes further modification of pigment-protein

complexes (Ashton and Crofts, 1981). Therefore, the primary symptom of photosynthetic inhibitors is chlorosis. The herbicides also inhibit the biosynthesis of photosynthetic pigments.

Wessels and Van der Veen (1956) for the first time showed that DCMU inhibits the Hill reaction in isolated chloroplasts. The stimulation of fluorescence by DCMU was due to the inhibition of reoxidation of Q (Duysens and Sweers, 1963). Extensive studies on the electron transport chain of photosynthesis show that the site of inhibition of DCMU is located between Q and the PQ pool (van Rensen, 1982). Photosystem I dependent electron transport is inhibited only at very high concentrations and cyclic electron flow can be stimulated by relatively low concentration of these inhibitors by redox poising (Avron and Neumann, 1968). In respiratory electron flow in Saccharomyces the cytochrome b/c segment is sensitive to DCMU (Convent and Briquet, 1978). An effect of DCMU on the donor side of photosystem II and on the S-states of oxygen evolving complex has been proposed (Etienne, 1974; Bouges-Bocquet et al. 1975). By replacement technique using radiolabelled herbicides Tischer and Strotman (1977) showed that phenylureas, triazines, triazinones, pyridazionones and biscarbamates compete for the same binding site. Therefore, these herbicides though different chemically are known as urea analogs. Recently Thiel and Boger (1986) proposed closely located binding sites for DCMU and atrazine on the basis of trypsin digestion studies. The relative concentration of specific binding sites was 1 per 300-500 chlorophyll molecules that is about 1 per electron transport chain. van Rensen and van Steeklenberg (1965) found that inhibition of oxygen evolution in algae by DCMU

can be removed by washing. Izawa and Good (1965) showed that DCMU is reversibly bound to chloroplasts by weak bonds due to interaction of the herbicides and the receptor molecule of the thylakoid membrane. Shipman (1981) suggested that polar components of the herbicides bind via coulombic interactions at or near to a highly polar protein site, probably a protein salt-bridge or the terminus of an α-helix of the Q-B protein. Thus the effects of herbicidal inhibitors is reversible.

The proteinaceous nature of the binding site of urea herbicides was proved by Regitz and Ohad (1975) and Renger (1976) by trypsin digestion of chloroplasts. It was found that trypsin treatment removes the receptor sites for herbicides (Trebst, 1979; Tischer and Strotman, 1979; Steinback et al. 1981). It appears probable that Q_B, bicarbonate, formate, DCMU-type and phenol-type herbicides all interfere with the protein environment, thus affecting electron flow at the reducing side of PS II. In this protein environment probably more than one polypeptide participate (van Rensen and Snel, 1985). Q_B exchanges rapidly (except when semireduced) with free PQ, diffusing in the membrane. Thus the Q_B-binding site is often left vacant, giving access to other compounds for binding. Because of the possible allosteric interaction the binding of one compound causes a conformational change in the binding protein resulting in a reduced affinity for a second compound. It was suggested that (Khanna et al. 1981; van Rensen, 1982) the binding of a herbicide and also the absence of bicarbonate cause a conformational change of the protein environment to which they bind. The change in conformation would have two consequences:-

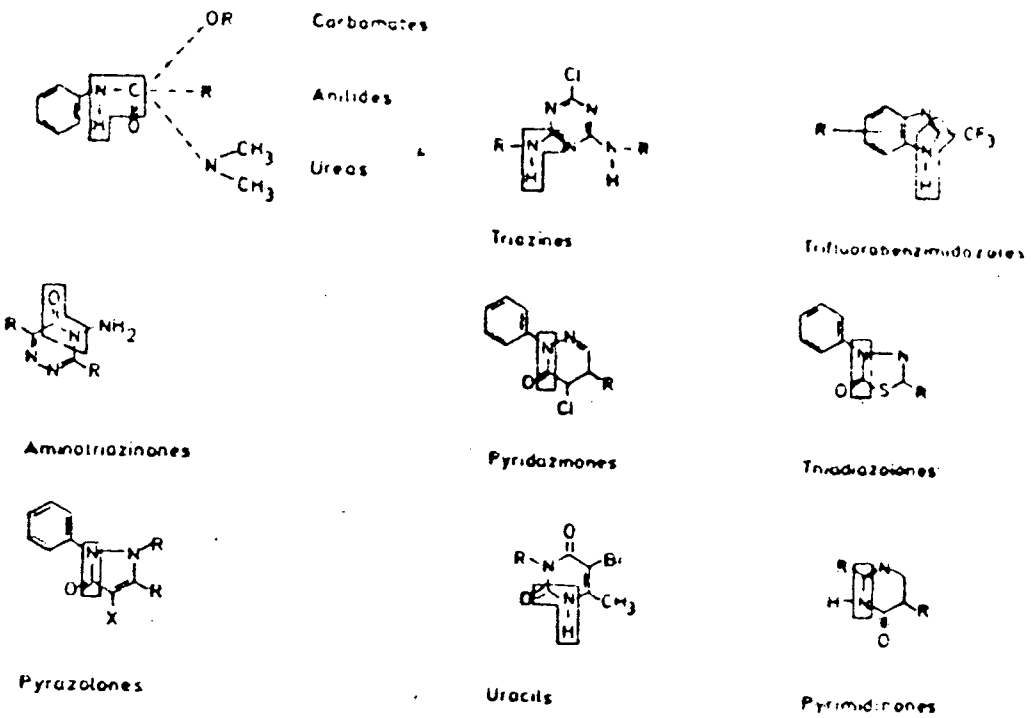


Fig. 8. Molecular structure of DCMU analog herbicides (From (Trebst, 1981).

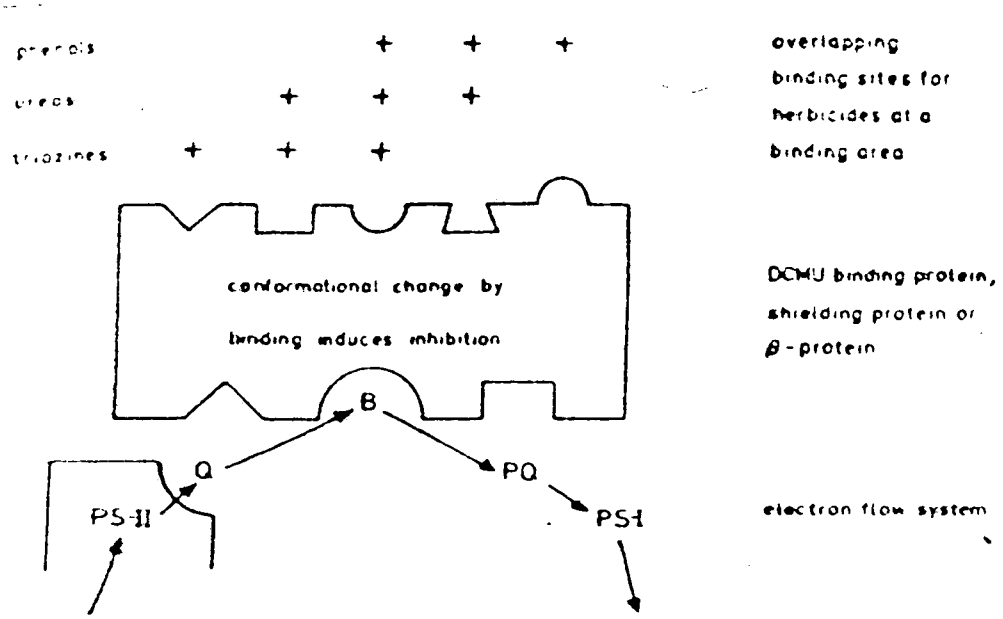


Fig. 9. Overlapping binding sites for different herbicides (From Trebst, 1981).

1. The binding of another herbicide or bicarbonate is impaired; and
2. Electron transfer is inhibited by decreasing the affinity of Q_B to its binding protein.

Although it is well established that the main site of action of PS II inhibiting herbicides is located between Q and PQ pool, there are reports that they also inhibit at the water splitting side. Schmid and Lehmann-Kirk (1977) have demonstrated in the thylakoids of Anabaena cylindrica and of Oscillatoria chalybea, the photoreduction of ferricyanide was insensitive to DCMU when DPC served as electron donor. This leads to the conclusion that DCMU acts before the site of electron donation of DPC, i.e. at the water splitting side of photosystem II. But Spiller contradicted the above finding because of the use of high concentrations of DPC. He demonstrated high rates of oxygen evolution in thylakoids of Anabaena 7119 in a DCMU-insensitive Hill reaction with SiMo as an electron acceptor (SiMo accepts electrons from Q). But Codd and Schmid (1980) showed complete inhibition of Hill reaction by DCMU in the thylakoids of Anabaena cylindrica using SiMo. Their conclusion that DCMU acts at the oxygen evolving side of photosystem II was supported by fluorescence studies.

(ii) The pyridazinone herbicides

The pyridazinone herbicides have the following inhibitory activities : inhibition of Hill reaction and photosynthetic CO_2 fixation; inhibition of carotenoid biosynthesis accompanied by photo-destruction of chlorophyll; and interference with the formation of

chloroplast membrane polar lipids (John and Hilton, 1976). The parent compound, pyrazon (5-amino-4 chloro-2 phenyl-3(2H)-pyridazinone), inhibits the Hill reaction and photosynthetic CO_2 fixation. Trifluoromethyl substitution of the phenyl ring of pyrazon, monomethyl substitution of the amine or substitutions at both positions result in inhibition of carotenoid biosynthesis. Membrane polar lipids are altered when the molecular structure of pyrazon is changed. Dimethyl substitution of amine of pyrazon brings about a decrease in linolenic acid accompanied by an increase in linoleic acid without any change in the proportion of saturated to unsaturated fatty acids of the membrane lipids. The trifluoromethyl substitution of the phenyl ring and monomethyl substitution of the amine are related to a shift towards a higher proportion of saturated fatty acids of chloroplast membrane lipids (John and Hilton, 1976). The grades of pyrazon used by John and Hilton (1976) were San 133-410H (4-chloro-5-(Methylamino)-2-phenyl-3-(2H)-pyridazinone), San 9785 (4-chloro-5-5(dimethylamine)-2-phenyl-3-(2H)-pyridazinone), norflurazon, San 6706 (Fig.10).

In fact the primary action of pyridazinone herbicides is to inhibit the electron transport catalyzed by PS II. The phenylpyridazinone herbicides act as efficient bleaching agents by reducing the carotene and chlorophyll content of growing algae and higher plants (Kummel and Grimme, 1975; Boger and Schlue, 1976; Bartels and Watson, 1978). The initial action of pyridazinones in the bleaching process is still obscure. Some workers propose that the biosynthetic pathway of carotene formation is blocked (Vaisberg and Schiff, 1976; Urbach et al. 1976) leading to the photoreduction of chlorophylls

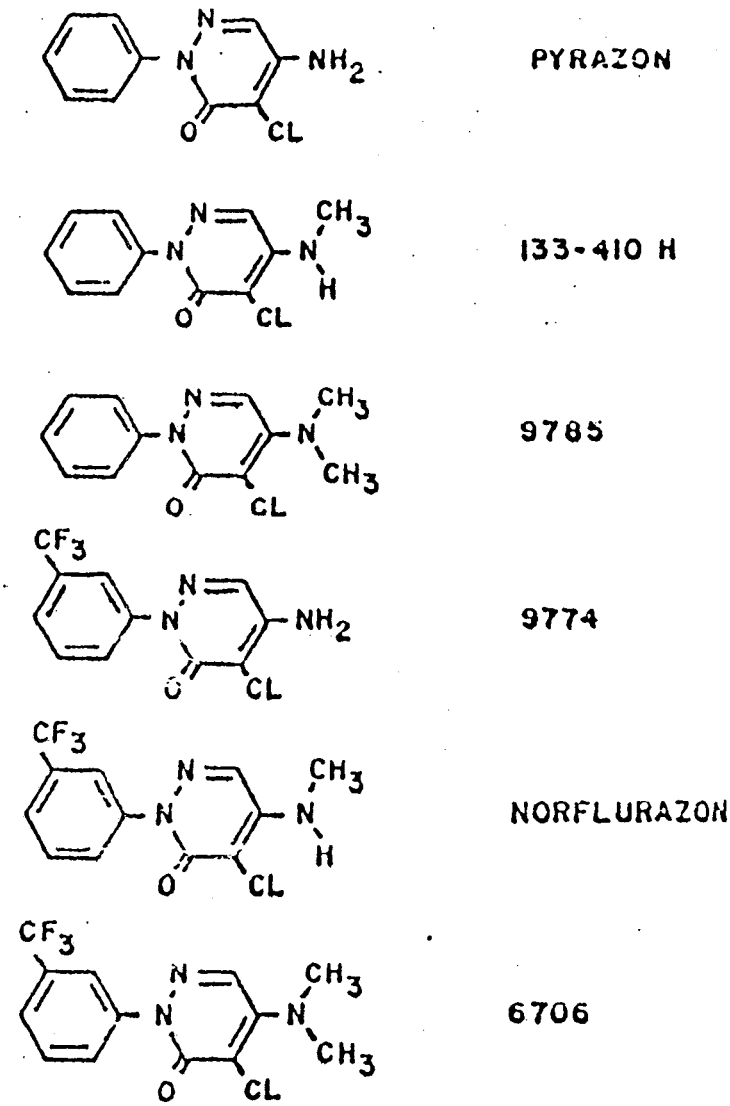


Fig. 10. Structure of six substituted pyridazinone herbicides (From John and Hilton, 1976).

and other cell components (Burn et al. 1971). Apparently the cyclization reaction (Ben-Aziz and Koren, 1974) resulting in the accumulation of intermediate products phytoene and phytofluene is inhibited.

Some others advocate a simultaneous inhibition of both carotene and chlorophyll formation (Lichtenthaler and Kleudgen, 1977; Kleudgen, 1979). Their explanation is that pyridazinone herbicides interfere at the ribosomal level leading to a reduced synthesis of carotene and chlorophyll forming enzymes. Pyridazinones may act multifunctionally and different concentrations and assay times can differentiate possible primary and secondary effects (Sandman et al. 1980). Norflurazon (San 6706) inhibits α and ß-carotene formation with concurrent accumulation of phytoene and some phytofluene (Sandman and Boger, 1982a). In fact San 6706 reduces the number of chloroplast ribosomes but leaves those of cytoplasm unaffected (Lichtenthaler and Kleudgen, 1977). It is quite interesting that the plant growth regulator glyphosine, which induces chlorosis, also acts via a reduction of the level of chloroplast ribosomes and chloroplastic RNA. Other herbicides such as amitrole, dichlormate difunon, fluridone etc. act the same way at the ribosomal level as the pyridazinones.

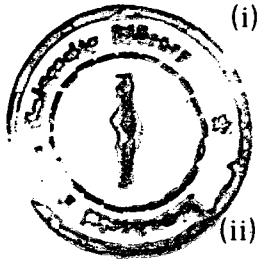
These herbicides have a pronounced effect on the chloroplast membrane lipid. The immediate effect of the herbicides on photosynthesis by isolated chloroplasts shows that BASF 13-338 (a substituted pyridazinone) enters the organelles and reaches the site of action (Khan et al. 1979) which contradicts the view of Ridley and Ridley (1979). The inhibition of linoleic acid desaturation in

discs, but not in isolated chloroplasts, suggests that for this action the herbicide has to be modified inthe cytoplasm before entering the organelles (Willemot et al. 1982). Pyridazinone herbicides (BASF 13-338) act mainly on the desaturation step of DGG from diene to triene (Harwood, 1980; Murphy et al. 1980). The only glycerolipid showing similar response to BASF 13-338 was diacyl digalactosyl glycerol (Harwood, 1980; Murphy et al. 1980; Khan et al. 1979; John, 1976).

In case of San 133-410H (4-chloro-5-(Methyl-amino)-2-Phenyl-3(2H)-pyridazinone) the amine group of the parent pyrazon molecule is substituted by monomethyl group. The application of SAN133-410H on 4 day-old wheat seedling shows 50% inhibition of pigment accumulation (John and Hilton, 1976). It alters the ratio of linoleic acid to linolenic acid and causes a shift towards a greater relative proportion of saturated fatty acids in the polar lipids (John and Hilton, 1976).

AIM OF THE WORK

To investigate the interaction of herbicides, DCMU and SAN 133-410H, with the cyanobacteria Anacystis nidulans;



(i) study of the effect on growth pattern by monitering the change in dry wt., pigment and protein contents of the cyanobacteria during growth;

(ii) study of the effect on electron transport activity at late log phase of growth;

(iii) study of the changes in the polypeptide pattern of herbicide treated cultures by SDS-PAGE analysis.

The effect of the above mentioned herbicides has been extensively studied in higher plants. The effect on cyanobacteria, grown in a medium containing specific concentrations of herbicides will contribute further in understanding the role of herbicides, during growth as well as photosynthetic electron transport. Both DCMU and San 133-410H are known to inhibit PS II activity and have overlapping binding sites on the 32 kD herbicide binding protein.

MATERIALS AND METHODS

MATERIALS AND METHODS

I. SOURCE OF THE ORGANISM

Axenic culture of the unicellular cyanobacteria, Anacystis nidulans (Strain No. ARM 336), was obtained from the National Facility for Blue-green Algae, Indian Agricultural Research Institute, New Delhi.

II. CHEMICALS

All the chemicals used were of analytical grade, obtained from Sigma Chemicals Company, U.S.A., BDH & Sisco Research Lab. Pvt. Ltd., India, unless otherwise mentioned.

III. GROWTH CONDITIONS

(i) Control

The cyanobacteria Anacystis nidulans was grown in the $BG11^a$ medium (Allen, 1968).

Composition of $BG11^a$ Medium:

Compounds	Amt. (g/liter)
$NaNO_3$	1.5
K_2HPO_4	0.04
$MgSO_4.7H_2O$	0.075
$CaCl_2.2H_2O$	0.036
Citric acid	0.006
Ferric ammonium citrate	0.006
EDTA (disodium magnesium salt)	0.001
Na_2CO_3	0.02
Trace metal mix A_5[b]	1 ml./liter

(a) After autoclaving and cooling the pH of the medium was 7.2.

A_5(b) Trace metal mix (g/l)

H_3BO_3	2.86
$MnCl_2.4H_2O$	1.81
$ZnSO_4.7H_2O$	0.222
$Na_2MoO_4.2H_2O$	0.39
$CuSO_4.5H_2O$	0.079
$Co(NO_3)_2.6H_2O$	0.0494

The cultures were kept under continuous illumination (intensity 4000 lux).

The temperature was maintained at $30^{o}C \pm 2$. For rapid growth, the culture flasks were aerated with aquarium air pumps (model WISA-100, made in India). The culture was maintained in agar slants of $BG11^{a}$ medium and subculturing was done twice a month.

Sterile conditions were maintained by washing and autoclaving the glassware before use. The medium was autoclaved at 15 lbs/sq. inch. pressure for 15 minutes. Inoculation was done in laminar flow chamber.

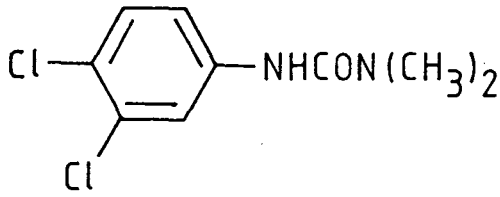
(ii) Growth in herbicide containing medium

The cells were grown in $BG11^{a}$ medium containing 10^{-7}M, 10^{-8}M and 10^{-9}M of DCMU and 20 μM, 60 μM, 100 μM, 100 μM of SAN 133-410H under identical conditions as control.

DCMU, Diuron

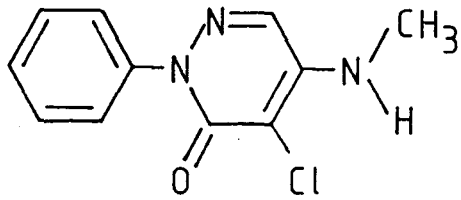
3-(3,4 Dichlorophenyl)-1, 1-dimethylurea, $C_9\ H_{10}\ Cl_2\ N_2O$, mol. wt. 233.10, crystals, mp. 158-159^{o}C, manufactured by Sigma

Chemicals Company, U.S.A.



SAN 133-410H

[4-chloro-5(methylamino)-2-phenyl-3(2H)-pyridazinone] , mol. wt. 235.52.



Manufactured by Sandoz Ltd., Switzerland.

IV. ABSORPTION SPECTRA OF INTACT CELLS

5 ml. of cells grown in BG11[a] medium was taken on the 8th day of growth and the absorption spectra was taken between 400 nm to 750 nm in a Shimadzu UV-260 double-beam spectrophotometer with the growth medium as blank.

V. DETERMINATION OF DRY WEIGHT

Whatman GF/C millipore filters of 2.5 cm size were weighed and numbered. 5 ml of culture was taken from control and treated cultures and filtered by vacuum filtration. The filter papers along with the residue were kept in a hot air oven at 40°C overnight and then weighed. The difference between the final weight and the intial weight gave the dry weight of 5 ml of culture.

VI. HARVESTING OF CELLS

(i) Preparation of harvesting buffer

PEG - 4000	7.5%
$CaCl_2.2H_2O$	1 mM
HEPES	20 mM

pH was adjusted to 7.5 with NaOH.

(ii) Preparation of reaction buffer

HEPES	25 mM
NaCl	20 mM

pH was adjusted to 7.5 using NaOH.

Anacystis nidulans cells grown in $BG11^a$ medium in presence and absence of herbicides were harvested by centrifuging the cell suspension at 9000 g for 10 minutes at 25^oC in a Sorvall RC-5 Refrigerated Centrifuge. The pellet was washed with isolation buffer and suspended in minimum volume of reaction buffer.

VII. ESTIMATION OF PIGMENT CONTENT

(i) Chlorophyll a

5 ml of culture was harvested and the pellet was washed twice with double distilled water. Then 5 ml of methanol was added to the pellet and kept at room temperature for 15 minutes. The extract was centrifuged at 5,000 rpm for 5 minutes in a table top Remi centrifuge. The absorbance A of the supernatant was taken at 665 nm to quantify Chl a using Mackinney's formula (Mackinney, 1941;

$$A_{665} = 74.5\ C(\text{in mg})\ ml^{-1}cm^{-1}.$$

where 74.5 is the extinction coefficient and C is the concentration of Chl a.

(ii) Phycocyanin

The phycobilin pigments include phycocyanin, allophycocyanin and phycoerythrin. 5 ml of culture was harvested and the pelleted cells were washed twice with saline (0.15 M NaCl) water. Then the pellet was resuspended in 5 ml of 0.15 M NaCl and sonicated in an ultrasonicator for 2.5 minutes (with 30 second pulses) in an ice bath. The suspension of broken cells was centrifuged at 15,000 g for 45 minutes at $4^{o}C$. The absorbance A of the supernatant was taken at 615 nm and 652 nm in a Shimadzu UV-260 spectrophotometer to quantify PC according to Bennet and Bogorad, 1971);

$$\text{Conc. of Phycocyanin (PC)} = \frac{A_{615} - 0.474\ (A_{652})}{5.34}$$

(iii) Carotenoids

5 ml of culture was harvested and methanol was added to the pellet. Then it was centrifuged for 5 minutes at 5,000 g at room temperature. The supernatant was diluted till negligible amounts of chlorophylls are present. Then total carotenoids was estimated by measuring the absorbancy at 451 nm, using 2500 for the $E^{1\%}_{1\ cm}$ value (Laczko and Kaiseva, 1987).

$$C = \frac{D \times V \times f \times 10}{2500}$$

C is the total amount of carotenoids in mg.; D is absorbancy in a 1.0 cm cell; V is volume of original extract in ml.; and f is the dilution factor.

VIII. ESTIMATION OF PROTEIN BY BRADFORD'S METHOD

The protein content was estimated by using Bradford's reagent

(Bradford, 1976) based on the principle of protein-dye binding. Cells harvested from 5 ml of culture were suspended in 10 ml of 10% TCA solution in test tubes. The tubes were kept in deep freezer overnight. Next day after thawing, the suspension was centrifuged at 5000 g for 10 minutes at room temperature in a table top centrifuge (Remi). The pellet was suspended in 2 ml of 1 N NaOH. 0.5 ml of this suspension was taken to estimate the protein content.

(a) Preparation of reagent

100 mg of Coomassie Brilliant Blue G-250 was dissolved in 50 ml of 95% ethanol. To it 100 ml of 85% (w/v) phosphoric acid was added and thefinal volume was made to 1 liter by adding double distilled water.

(b) Plotting of calibration curve

Bovine serum albumin protein solution containing 10-100 ug of protein in a volume upto 0.1 ml was pipetted into test tubes i.e. final volume was made 0.1 ml by adding the appropriate buffer. Then 5.0 ml of the reagent was added to the test tubes and mixed properly. The absorbance at 595 nm was measured after 2 minutes against a reagent blank containing 0.1 ml of appropriate buffer and 5 ml of the reagent. The absorbance at 595 nm was plotted against the corresponding concentrations of protein resulting in a standard curve which was used to determine the protein content of unknown samples.

IX. MEASUREMENT OF PS I AND PS II ACTIVITY

Photochemical activities of the cells were measured using

of YS I Clark type oxygen electrode, thermostated at 25°C. 100 divisions of the recorder were adjusted with 100 divisions of instrumental monitor by using aerated water. The reaction buffer was bubbled with nitrogen to remove the oxygen.

(i) Photosystem I activity

TMPD or DCPIP mediated PS I activity was measured using MV (methyl viologen) as an artificial acceptor. TMPD reduced by ascorbate acted as the donor to PS I. Since methyl viologen does not enter into intact cells, membrane fragments were prepared by sonicating the cells in an ice bath for 2.5 minutes with 30 second pulses. Then the suspension was centrifuged at 10,000 rpm for 10 minutes at 5°C to get rid of the unbroken cells. The supernatant, thus obtained was centrifuged at 20,000 rpm for 45 minutes at 5°C. The pellet containing the membrane fragments was suspended in minimum volume of reaction buffer and the chlorophyll content was estimated. 3 ml of reaction mixture containing cells equivalent to 15 ug Chl a 1 mM ascorbate, 1 mM TMPD or DCPIP 10 uM DCMU, 0.5 mM MV was taken in the cuvette for each experiment. DCMU was used to block the PS II mediated electron transport. The reaction mixture was continuously stirred and the temperature was maintained at 25°C by thermostated water bath. The reaction mixture was illuminated with $(3.6 \times 10^5 \frac{erg}{cm^2 sec})$ a projector and the consumption of oxygen was recorded.

(ii) Photosystem II activity

PS II activity was measured as parabenzoquinone (PBQ) mediated electron transport using intact cells. Since PBQ is lipophilic, it enters the cells and accepts electrons at the

plastoquinone (PQ) site. 3 ml of reaction mixture contained cells equivalent to 15 ug of Chl a and 0.5 mM PBQ. The reaction mixture was constantly stirred and illuminated with light from a projector (3.6×10^5 erg/cm^2/sec) and O_2 evolution was recorded.

Calculation of O_2 evolution/uptake

During calibration of the instrument with aerated water, 100 divisions on the chart paper were taken as 240 nano moles/ml at 25°C. The number of divisions were counted from the slopes recorded during O_2 evolution/uptake. The final expression for PS I activity is the amount of O_2 consumed per mg of Chl a per hour (O_2/mg Chl/h) and the same for PS II activity is the amount of O_2 evolved per mg Chl a per hour (O_2/mg Chl a/h).

X. POLYPEPTIDE ANALYSIS BY SDS-PAGE

(i) Preparation of stock solution

1. 30% (w/v) acrylamide - 0.8% (w/v) bisacrylamide

   Acrylamide 30 gm

   Bisacrylamide, 0.8 gm

   Final volume was made upto 100 ml with double distilled water. Filtered and kept in cold.

2. 10% (w/v) SDS

   SDS, 10 gm

   Made upto 100 ml with dist. H_2O and stored at room temperature.

3. 10% (w/v) Ammonium persulfate

   Made upto 10 ml with dist. H_2O. Prepared fresh every time.

4. **Stacking gel buffer**

6.06 g Tris dissolved in 80 ml of H_2O; used concentrated HCl to make the pH 6.8 and final volume was made upto 100 ml with dist. H_2O.

5. **Resolving gel buffer**

18.2 g Tris in 80 ml dist. H_2O, concentrated HCl was added to make the pH 8.8 and the final volume was made 100 ml with distilled water.

6. **Running gel buffer**

6 gm Tris, 28.8 gm glycine and 10 ml of 10% SDS in 1 litre of water pH was adjusted between 8.0-8.8 using Tris or glycine but not HCl.

(ii) Preparation of sample

Equal volumes of culture were taken in two tubes from each sample (treated and untreated) and broken by sonication for 2 and 1/2 minutes with 30 second pulses, in an ice bath. Then 10% TCA was added to precipitate the proteins. After centrifugation the pellets containing proteins were collected. Pellet of one tube from each sample was used for estimation of protein by Bradford's method (as described earlier). The pellets of remaining tubes were suspended in the sample buffer and heated for two minutes. Then the protein samples thus prepared were stored in deep freezer at $-20^{o}C$.

To determine the M_r of the polypeptides, standard proteins obtained from Pharmacia, Phosphorylase b M_r 94,000, Albumin M_r 67,000, Ovalbumin M_r 43,000, Carbonic anhydrase M_r 30,000, Trypsin inhibitor 20,100, α-lactalbumin 14,400, were loaded along with

the samples.

Preparation of sample buffer

Ingredients	Stock solution	Final concentration	Volume
Tris-HCl (pH 6.7)	1 M	10 mM	1 ml
SDS	10%	2%	20 ml
EDTA	0.5 M	1 mM	0.2 ml
β-mercaptoethanol	100%	5%	5 ml
Glycerol	100%	5%	5 ml

Final volume was made upto 100 ml with dist. water.

(iii) Preparation of gels

Resolving gel solutions	Dense20% acrylamide	Light 10% acrylamide
30% Acrylamide - 0.8% bisacrylamide	10 ml	5 ml
Resolving gel buffer	3.75 ml	3.75 ml
10% SDS	0.15 ml	0.15 ml
Water	1 ml	6 ml
TEMED	16 µl	19 µl
10% APS (added at the end)	80 µl	80 µl

Stacking gel solution (6% acrylamide)

30% Acrylamide - 0.8% bisacrylamide	0.025 ml
Stacking gel buffer	3.75 ml
10% SDS	0.15 ml
H_2O	9 ml
TEMED	10 µl
10% APS (added atthe end)	80 µl

The glass plates and spacers were cleaned, dried and assembled to form a gel mold. The dense (20%) and light (10%) resolving gel solutions were prepared. The linear acrylamide concentration gradient, was established by mixing the two solutions using a density gradient mixer and stirrer assembly. The volumes were determined by gel dimensions. The slightly smaller volume of the dense solution prevented its back flow into the light solution. The resolving gel solution was poured with a steady flow rate into the mold. Then it was overlaid with a few ml of water so that complete polymerization of the gel was marked by the formation of a water-resolving gel interface. The water was removed and the stacking gel was poured. The slot former was inserted with care avoiding air bubbles. Before use the mold containing the slab gel was fastened to the electrophoresis apparatus and the upper and lower reservoir buffers were poured. The slot former was removed carefully. The gel projections of the wells were straightened with a spatula before applying samples into the slots. Portein of equal concentrations from all the samples (treated and untreated) were loaded in the slots with a hamilton syringe. Electrophoresis was run at a constant voltage of 60 volts using a LKB power supply for the intial two hours till the samples reach the resolving gel; and then at 200 volts for four-and-half hours. After the completion of the electrophoretic run, the gel was removed from the plates, stained and destained.

(iv) Staining and destaining

Staining solution

2.5 gm Coomassie Brilliant Blue R250

454 ml methanol

96 ml glacial acetic acid made upto 1 litre with distilled water.

Destaining solution

75 ml glacial acetic acid

250 ml methanol

675 ml H_2O

The gel was immersed in the staining solution for four hours with moderate shaking. Then it was transferred to the destaining solution and kept for 7-8 hours with moderate shaking for complete destaining. Photographs of the destained gels were taken.

RESULTS

RESULTS

In the present work we have studied the effect of various concentrations of DCMU (10^{-7}M, 10^{-8}M, 10^{-9}M) and San 133-410H (20 µM, 60 µM, 100 µM, 140 µM) on the cyanobacteria Anacystis nidulans (Strain No. ARM 336). The effect on growth was studied till the late log phase (8th day of growth). For this we determined the dry weight, pigment content and protein content of the control and treated cultures at 48 hours intervals.

I. EFFECT ON GROWTH

(i) Effect on dry weight

The dry weight of the control and herbicide treated cultures was measured on alternate days starting from 0 hour (Figs. 1 and 2) to determine herbicide induced changes in growth curve. With DCMU (Fig. 1) it was observed that growth in cultures treated with 10^{-7}M DCMU was significantly inhibited. The lag phase of growth was extended and the exponential phase appeared only around the 6th day. At this time the control culture was already in the late log phase reaching the stationary phase (see Fig. 1). In cultures treated with low concentrations of DCMU (10^{-8}M and 10^{-9}M) a stimulation in growth (around 11% and 20% respectively) was observed around the 8th day. Presence of DCMU at a concentration of 10^{-7}M, inhibited growth as determined by the dry weight, by 33% (see Table 1) at the late log phase (8th day).

San 133-410H treated cultures showed a concentration dependent inhibition of growth (Fig. 2) i.e., the higher the concentration the higher the inhibition (Table 2). Stimulation of growth

Fig. 1 Effect of various concentrations of DCMU (10^{-7}M, 10^{-8}M and 10^{-9}M) on the dry weight of A. nidulans (For details refer to Materials and Methods).

Fig. 2 Effect of various concentrations of San 133-410H (20 μM, 60 μM, 100 μM and 140 μM) on the dry weight of A. nidulans (For details refer to Materials and Methods).

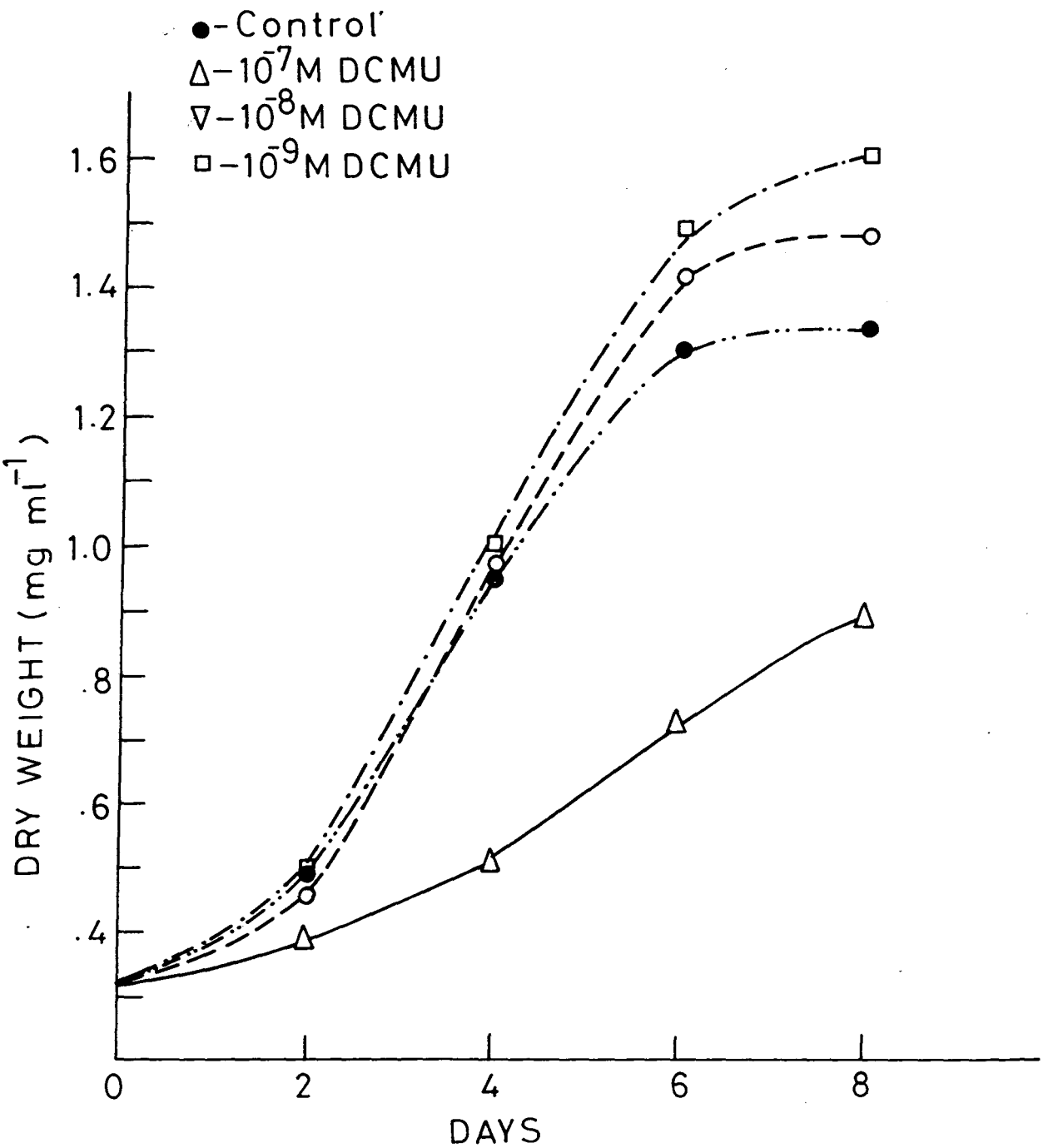

●-Control
Δ-10⁻⁷M DCMU
▽-10⁻⁸M DCMU
□-10⁻⁹M DCMU
DRY WEIGHT (mg ml⁻¹)
1.6
1.4
1.2
1.0
.8
.6
.4
0
2
4
6
8
DAYS

Fig. 1

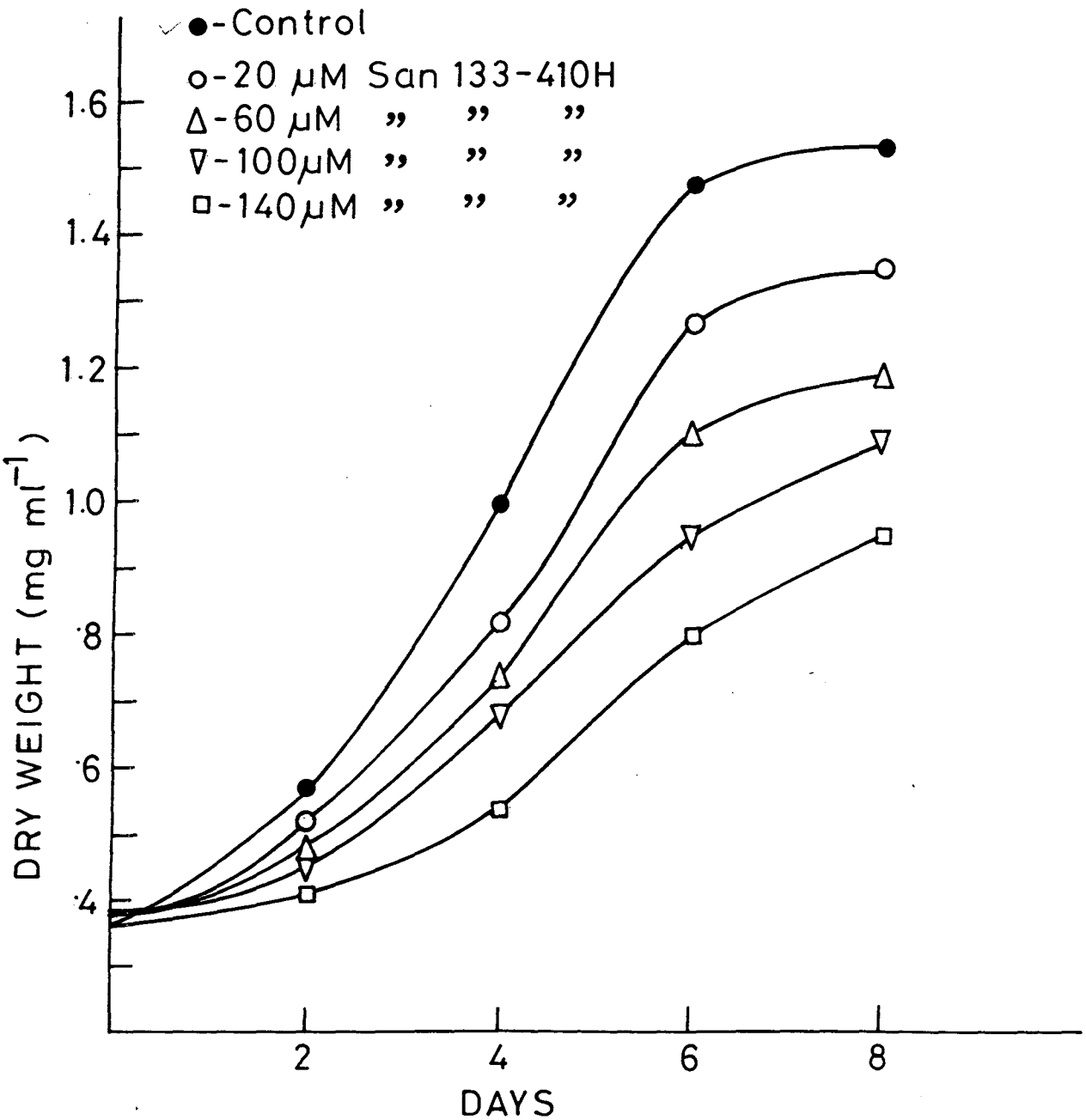

●-Control
o-20 μM San 133-410H
Δ-60 μM " " "
▽-100μM " " "
□-140μM " " "
1.6
1.4
1.2
1.0
·8
·6
·4
DRY WEIGHT (mg ml⁻¹)
2
4
6
8
DAYS

Fig. 2

Table 1. Dry weight ($mg.ml^{-1}$) of cell suspension of A. nidulans grown with various concentrations of DCMU (10^{-9}M, 10^{-8}M, and 10^{-7}M).

Days	Control/%	10^{-9}M	10^{-8}M	10^{-7}M
0	0.33 (100)	0.34 (102)	0.34 (102)	0.35 (106)
2	0.49 (100)	0.50 (102)	0.46 (94)	0.39 (80)
4	0.95 (100)	1.00 (105)	0.97 (102)	0.51 (54)
6	1.30 (100)	1.49 (115)	1.41 (108)	0.73 (56)
8	1.33 (100)	1.60 (120)	1.47 (111)	0.89 (67)

Table 2. Dry weight ($mg.ml^{-1}$) of cell suspension of A. nidulans grown with various concentrations of San 133-410H (20 μM, 60 μM, 100 μM and 140 μM).

Days	Control/%	20 μM	60 μM	100 μM	140 μM
0	.36 (100)	0.38 (105)	0.38 (105)	0.39 (108)	0.36 (100)
2	.57 (100)	0.52 (91)	0.48 (89)	0.45 (79)	0.41 (72)
4	1.00 (100)	0.82 (82)	0.74 (74)	0.68 (68)	0.54 (54)
6	1.48 (100)	1.27 (86)	1.10 (74)	0.95 (69)	0.80 (54)
8	1.52 (100)	1.35 (88)	1.19 (78)	1.09 (72)	0.95 (62)

Values in brackets give percentage of control values (For details refer to Materials and Methods)

was not observed at low concentrations as was seen with DCMU. Cultures treated with higher concentrations (100 μM and 140 μM San 133-410H) did not reach the stationary phase by the 8th day. 20 μM and 60 μM treated cultures and the control were almost in stationary phase by this time. An inhibition of 38% in dry weight content was observed in 140 μM San 133-410H treated cultures on the 8th day (see Table 2).

(ii) Effect on pigment content

(a) Chlorophyll a

Both DCMU and San 133-410H are known to inhibit chlorophyll biosynthesis. We observed a similar effect on chlorophyll content as seen earlier in the case of dry weight. With DCMU (Fig. 3) at low concentrations (10^{-8}M and 10^{-9}M) the chlorophyll a content was slightly higher than that of the control (Table 3). However, cultures grown in presence of 10^{-7}M DCMU showed a significant decrease in Chl a content. Table 3 shows the per cent inhibition observed during growth. On the 8th day there was a 40% inhibition in Chl a content.

Cultures treated with San 133-410H (Fig. 4) did not show a stimulation of Chl a content at low concentrations. The Chl a content decreased with increasing concentrations of the herbicide (Table 4). At higher concentrations, 100 μM and 140 μM, the inhibition was found to be 35% and 54% respectively on the 8th day.

(b) Carotenoids

Presence of low concentrations of DCMU (10^{-8}M and 10^{-9}M) (Fig. 5) in the growth medium did not have much effect on the carotenoid content. On the 8th day of growth a slight

Table 3. Chl a content ($\mu g.mg^{-1}$ dry weight) of DCMU treated cultures.

Days	Control/%	10^{-9}M	10^{-8}M	10^{-7}M
0	10 (100)	10 (100)	11 (110)	9 (90)
2	27 (100)	33 (122)	29 (107)	20 (74)
4	79 (100)	88 (111)	83 (105)	39 (49)
6	119 (100)	130 (109)	126 (106)	51 (43)
8	123 (100)	139 (113)	132 (107)	74 (60)

Table 4. Chl a content ($\mu g.mg^{-1}$ dry weight) of San 133-410 treated cultures.

Days	Control/%	20 μM	60 μM	100 μM	140 μM
0	12 (100)	12 (100)	12 (100)	13 (108)	12 (100)
2	38 (100)	27 (71)	22 (58)	18 (47)	16 (42)
4	85 (100)	64 (75)	51 (60)	36 (42)	24 (28)
6	137 (100)	110 (80)	99 (72)	75 (55)	47 (34)
8	145 (100)	125 (86)	112 (74)	94 (65)	66 (46)

Values in brackets give percentage of control values (For details refer to Materials and Methods)

Fig. 3 The effect of various concentrations of DCMU (10^{-7}M, 10^{-8}M and 10^{-9}M) on Chl a content of A. nidulans (For details refer to Materials and Methods).

Fig. 4 The effect of various concentrations of San 133-410H (20 μM, 60 μM, 100 μM and 140 μM) on Chl a content of A. nidulans (For details refer to Materials and Methods).

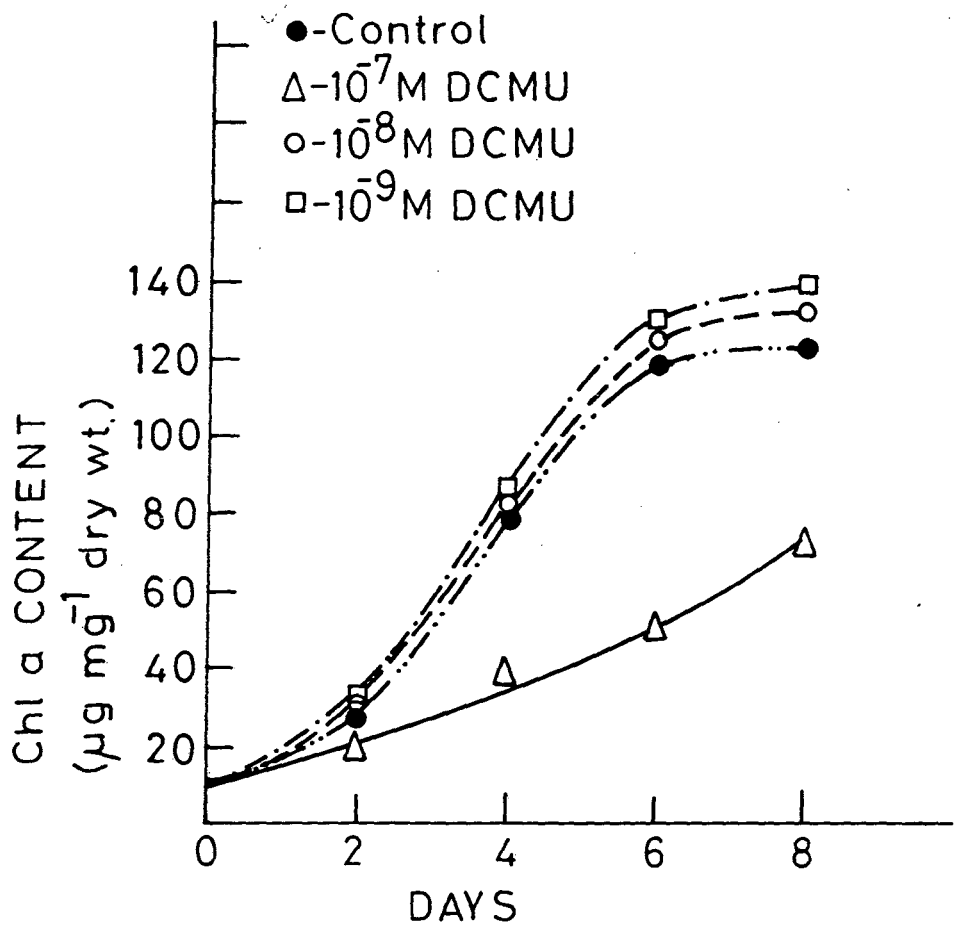

●-Control
Δ-10⁻⁷M DCMU
o-10⁻⁸M DCMU
□-10⁻⁹M DCMU
Chl a CONTENT
(μg mg⁻¹ dry wt.)
140
120
100
80
60
40
20
0
2
4
6
8
DAYS

Fig. 3

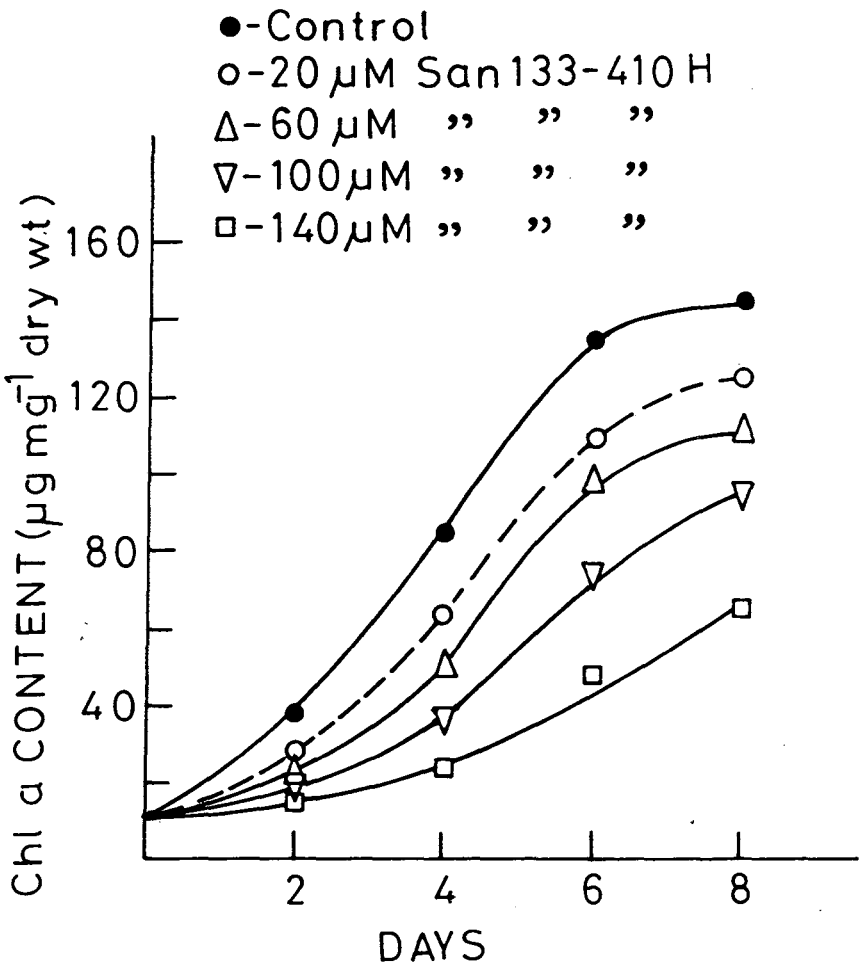

●-Control
o-20 μM San 133-410 H
Δ-60 μM " " "
▽-100μM " " "
□-140μM " " "
160
120
80
40
Chl a CONTENT (μg mg⁻¹ dry wt.)
2
4
6
8
DAYS

Fig. 4

increase (4% and 3% respectively) was observed. At 10^{-7}M DCMU the carotenoid content was significantly decreased (Table 5) and 43% inhibition was observed on the 8th day. There was a significant inhibition of carotenoid content in San 133-410H treated cultures, proportional to the concentration (Fig. 6). We observed 37%, 55%, 63% and 70% inhibition (see Table 6) in carotenoid content at 20 μM, 60 μM, 100 μM and 140 μM of San 133-410H respectively on the 8th day.

(c) Phycocyanin

The effect of different concentrations of the herbicides on the phycocyanin content of the cells (extracted in 0.15 M NaCl) was studied (Figs.7 and 8). With DCMU at 10^{-7}M the inhibition was 39% and at 10^{-8}M and 10^{-9}M the stimulation was 9% and 26% respectively on the 8th day of growth (Fig. 7 and Table 7). With San 133-410H the inhibition was 10%, 15%, 26% and 30% at 20 μM, 60 μM, 100 μM and 140 μM concentrations respectively (Fig.8 and Table 8), on the 8th day of growth.

(iii) Effect on the protein content

Protein (from control and herbicide treated cultures) was extracted and estimated at 48 hours intervals till the late log phase (Figs.9 and 10). It was observed that at 10^{-7}M DCMU in growth medium there was a significant decrease in protein content. The maximum difference between the control and 10^{-7}M DCMU treated culture being 62% on the 6th day of growth. On the 8th day the inhibition was 46%. At 10^{-8}M and 10^{-9}M DCMU there was a slight stimulation (12% on the 8th day).

The effect of various concentrations of San 133-410H on protein content is shown in Fig. 10. The decrease in protein content

Table 5. Carotenoid content ($\mu g.mg^{-1}$ dry weight) of DCMU treated cultures.

Days	Control/%	10^{-9}M	10^{-8}M	10^{-7}M
0	35 (100)	34 (97)	34 (97)	34 (97)
2	58 (100)	65 (112)	61 (105)	56 (97)
4	135 (100)	143 (106)	138 (102)	78 (58)
6	196 (100)	206 (105)	199 (102)	95 (48)
8	203 (100)	211 (104)	209 (103)	115 (57)

Table 6. Carotenoid content ($\mu g.mg^{-1}$ dry weight) of San 133-410H treated cultures.

Days	Control/%	20 μM	60 μM	100 μM	140 μM
0	36 (100)	34 (94)	37 (103)	36 (100)	35 (97)
2	73 (100)	58 (79)	45 (62)	40 (55)	38 (52)
4	149 (100)	80 (54)	63 (42)	51 (34)	43 (29)
6	216 (100)	107 (50)	85 (39)	69 (32)	52 (24)
8	222 (100)	140 (63)	100 (45)	82 (37)	66 (30)

Values in brackets give percentage of control values (For details refer to Materials and Methods)

Fig. 5 The effect of various concentrations of DCMU (10^{-7}M, 10^{-8}M and 10^{-9}M) on the carotenoid content of A. nidulans (For details refer to Materials and Methods).

Fig. 6 The effect of various concentrations of San 133-410H (20 μM, 60 μM, 100 μM and 140 μM) on the carotenoid content of A. nidulans (For details refer to Materials and Methods).

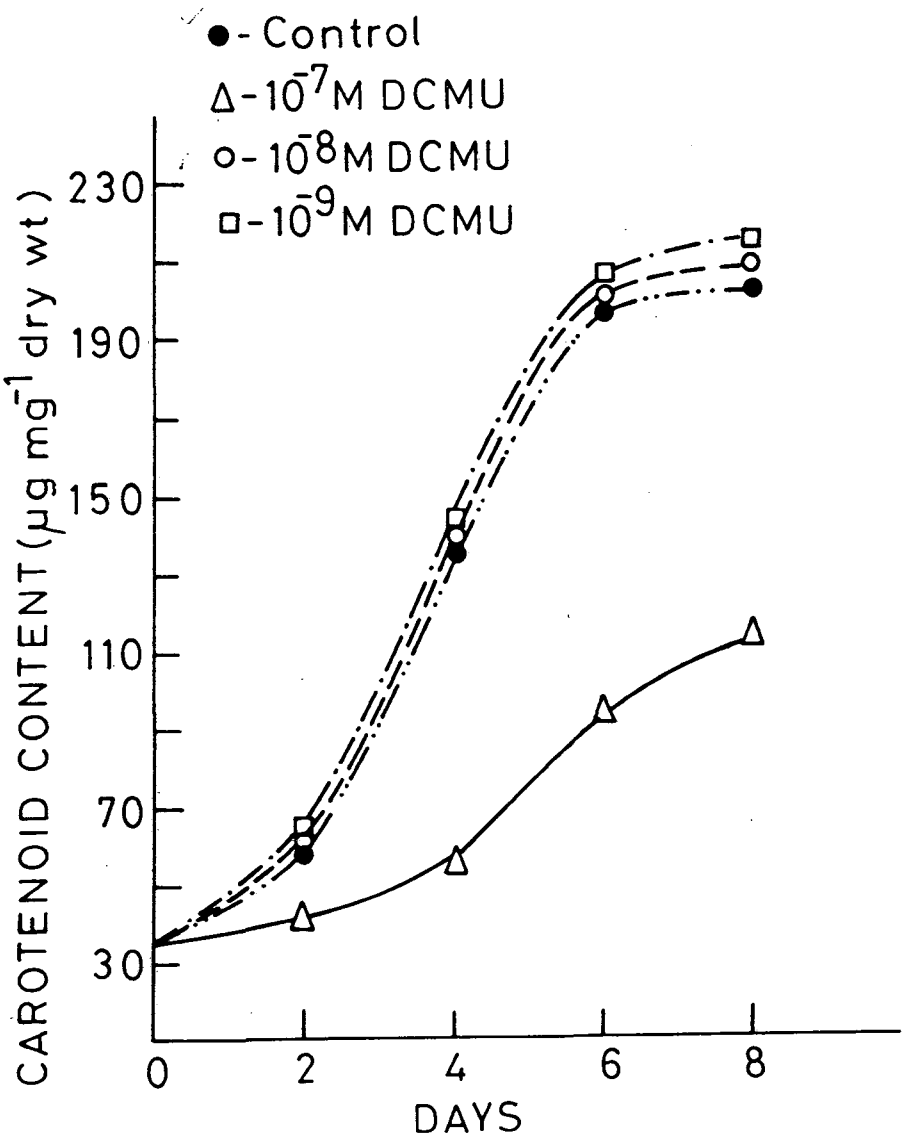

●- Control
Δ-10⁻⁷M DCMU
o-10⁻⁸M DCMU
□-10⁻⁹M DCMU
CAROTENOID CONTENT (μg mg⁻¹ dry wt)
230
190
150
110
70
30
0
2
4
6
8
DAYS

Fig. 5

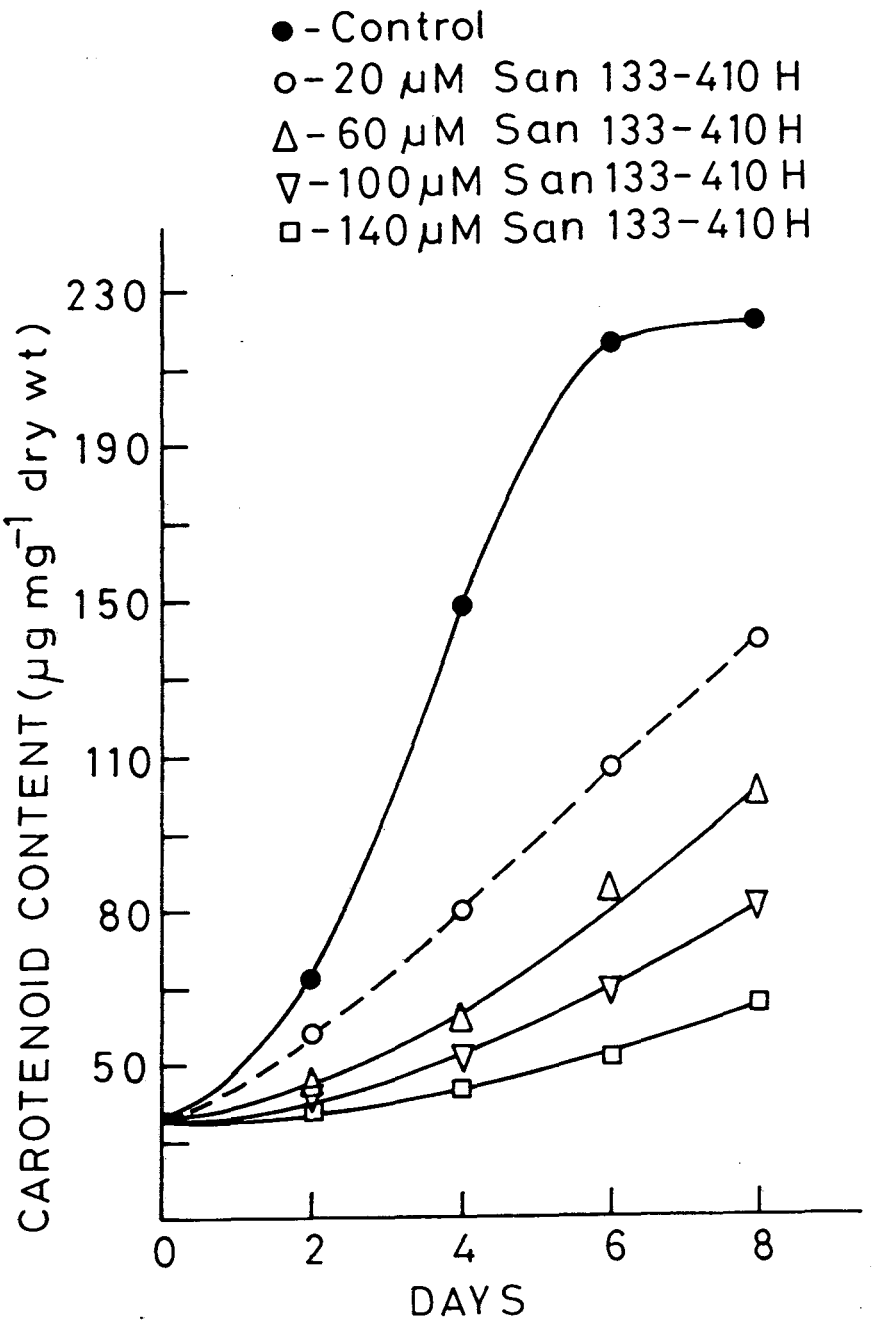

● - Control
o - 20 μM San 133-410 H
Δ - 60 μM San 133-410 H
▽ - 100 μM San 133-410 H
□ - 140 μM San 133-410 H
CAROTENOID CONTENT (μg mg⁻¹ dry wt)
230
190
150
110
80
50
0
2
4
6
8
DAYS

Fig. 6

Table 7. Phycocyanin content ($\mu g.mg^{-1}$) of DCMU treated cultures.

Days	Control/%	10^{-9}M	10^{-8}M	10^{-7}M
0	17 (100)	18 (106)	17 (100)	18 (106)
2	56 (100)	68 (121)	60 (107)	32 (57)
4	99 (100)	127 (128)	106 (107)	60 (61)
6	132(100)	168 (127)	141 (107)	72 (55)
8	139(100)	175 (126)	152 (109)	85 (61)

Table 8. Phycocyanin content ($\mu g.mg^{-1}$) of San 133-410H treated cultures.

Days	Control/%	20 μM	60 μM	100 μM	140 μM
0	18 (100)	20 (111)	20 (111)	20 (111)	19 (105)
2	39 (100)	35 (90)	30 (78)	26 (67)	22 (56)
4	87 (100)	79 (91)	62 (71)	51 (59)	36 (41)
6	148(100)	130 (88)	110 (74)	92 (62)	68 (46)
8	155(100)	139 (90)	132 (85)	115 (74)	108 (70)

Values in brackets give percentage of control values (For details refer to Materials and Methods)

Fig. 7 Effect of various concentrations of DCMU (10^{-7}M, 10^{-8}M and 10^{-9}M) on the phycocyanin content of A. nidulans (For details refer to Materials and Methods).

Fig. 8 Effect of Various concentrations of San 133-410H (20 μM, 60 μM, 100 μM and 140 μM) on phycocyanin content of A. nidulans (For details refer to Materials and Methods).

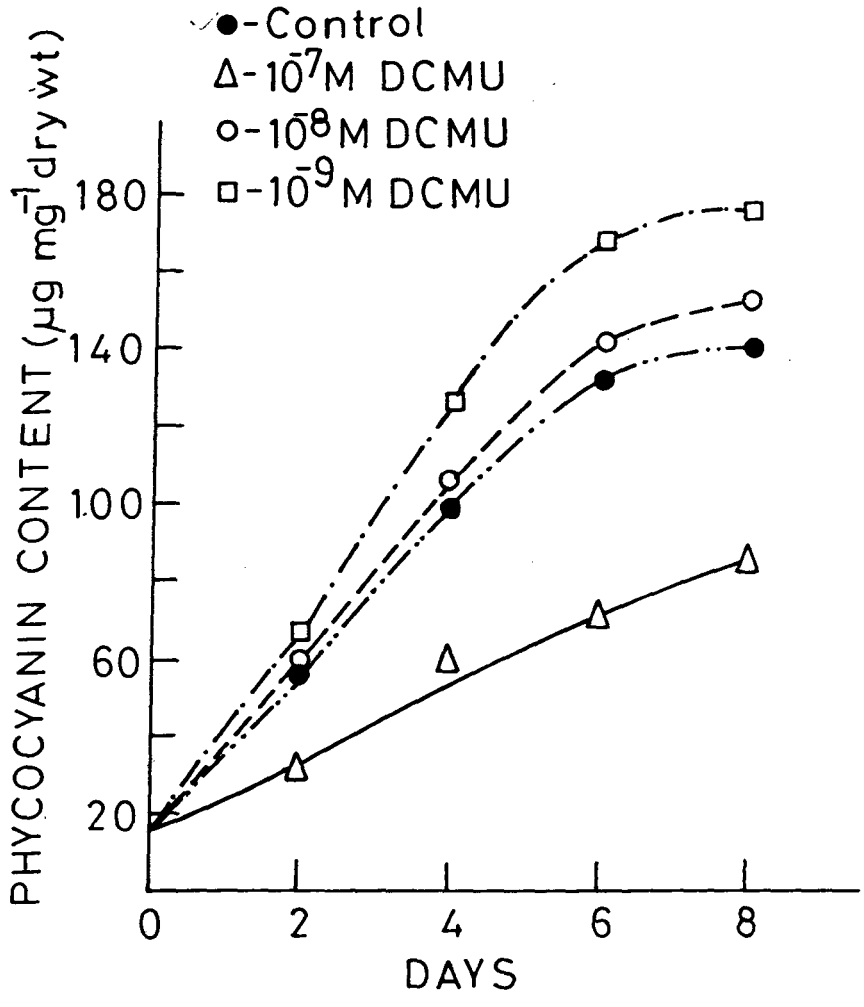

●-Control
Δ-10⁻⁷M DCMU
○-10⁻⁸M DCMU
□-10⁻⁹M DCMU
PHYCOCYANIN CONTENT (μg mg⁻¹dry wt)
180
140
100
60
20
0
2
4
6
8
DAYS

Fig. 7

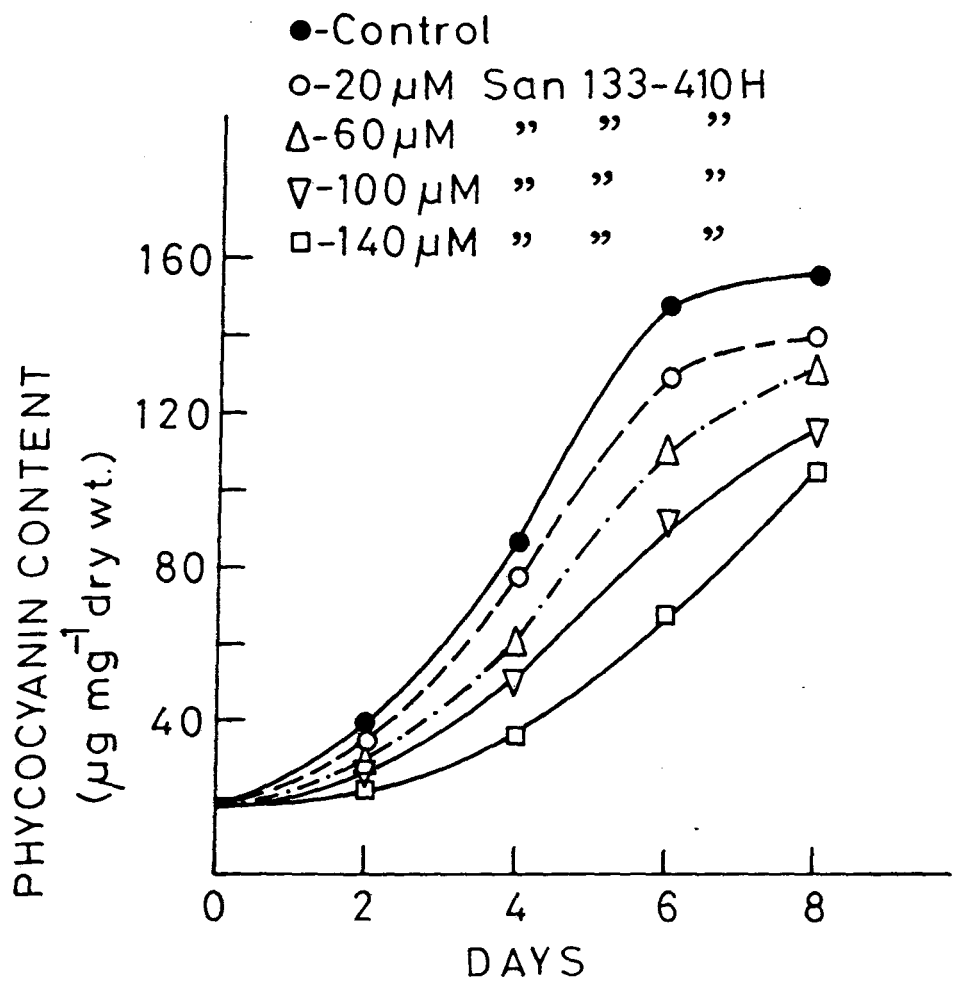

●-Control
o-20 μM San 133-410H
Δ-60 μM " " "
▽-100 μM " " "
□-140 μM " " "
160
120
80
40
PHYCOCYANIN CONTENT
(μg mg⁻¹ dry wt.)
0
2
4
6
8
DAYS

Fig. 8

Fig. 9 The effect of various concentrations of DCMU (10^{-7}M, 10^{-8}M and 10^{-9}M) on the protein content of A. nidulans (For details refer to Materials and Methods).

Fig. 10 The effect of various concentrations of San 133-410H (20 µM, 60 µM, 100 µM and 140 µM) on the protein content of A. nidulans (for details refer to Materials and Methods).

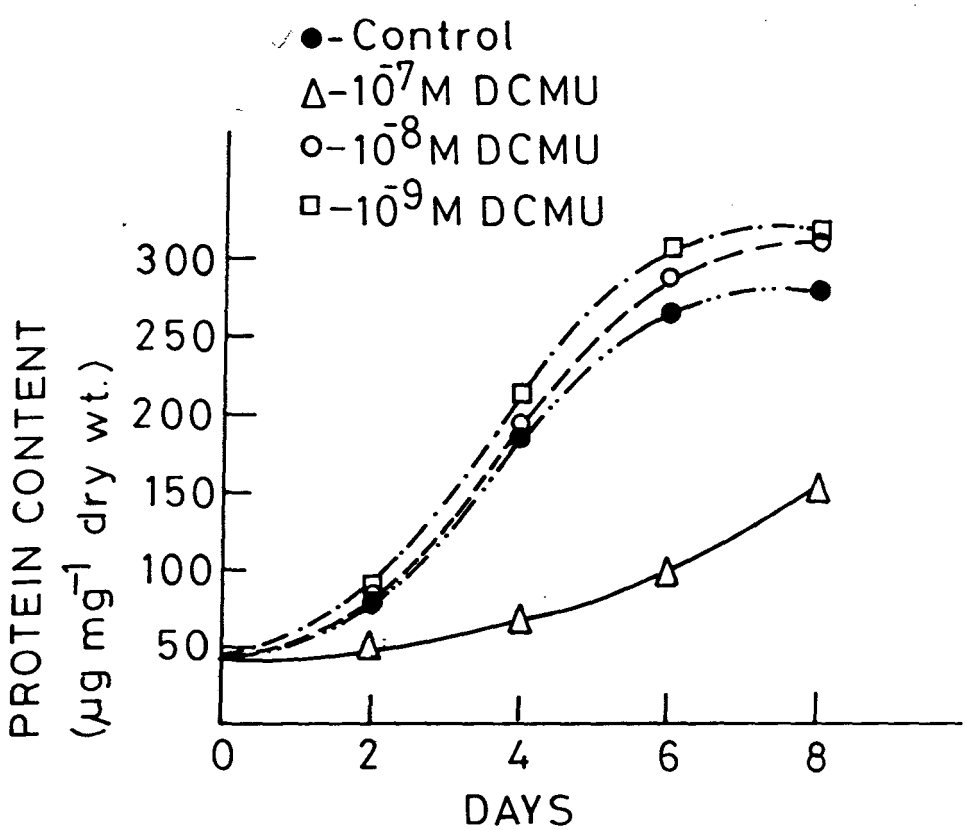

●-Control
Δ-10⁻⁷M DCMU
o-10⁻⁸M DCMU
□-10⁻⁹M DCMU
PROTEIN CONTENT
(μg mg⁻¹ dry wt.)
300
250
200
150
100
50
0
2
4
6
8
DAYS

Fig. 9

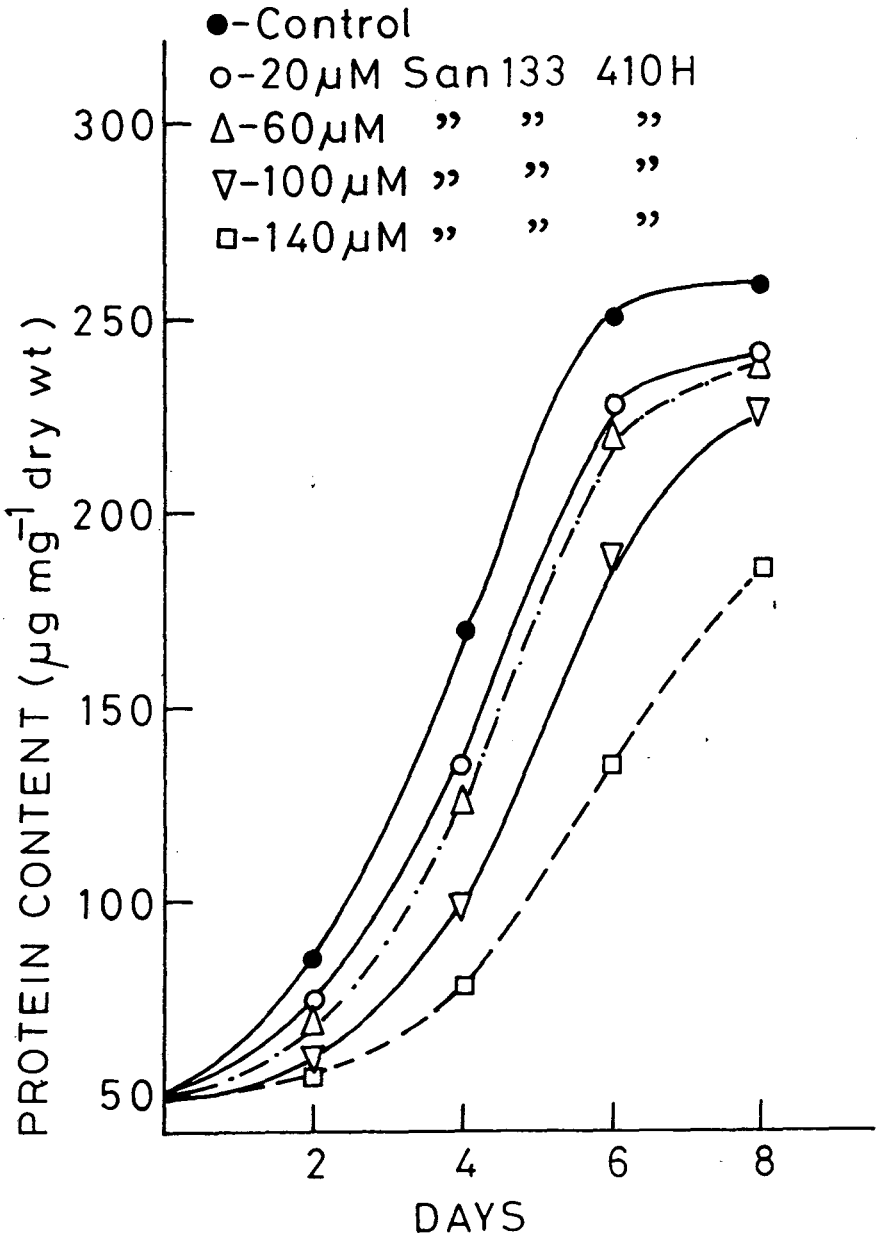

●-Control
o-20μM San 133 410H
Δ-60μM " " "
▽-100μM " " "
□-140μM " " "
PROTEIN CONTENT (μg mg⁻¹ dry wt)
300
250
200
150
100
50
2
4
6
8
DAYS

Fig. 10

Table 9. Protein content ($\mu g.mg^{-1}$) of DCMU-treated cultures.

Days	Control/%	10^{-9}M	10^{-8}M	10^{-7}M
0	48 (110)	49 (100)	48 (100)	47 (98)
2	82 (100)	91 (111)	74 (90)	50 (61)
4	187(100)	215 (115)	196 (105)	68 (36)
6	265(100)	307 (116)	287 (108)	100 (38)
8	282(100)	320 (113)	316 (112)	153 (54)

Table 10. Protein content ($\mu g.mg^{-1}$) of San 133-410H treated cultures.

Days	Control/%	20 μM	60 μM	100 μM	140 μM
0	50 (100)	51 (102)	48 (96)	50 (100)	49 (98)
2	88 (100)	75 (85)	69 (78)	60 (68)	55 (62)
4	170 (100)	135 (79)	127 (75)	99 (58)	79 (46)
6	251 (100)	228 (91)	220 (88)	187 (75)	137 (55)
8	258(100)	241 (93)	239 (93)	225 (91)	185 (72)

Values in brackets give percentage of control values (For details refer to Materials and Methods)

was proportional to the concentration of herbicide (Table 10). On the 8th day the decrease in protein content was 28% in presence of 140 μM San 133-410H. Lower concentrations of the herbicide did not affect the protein synthesis significantly. The inhibition observed was only 7-9% (see Table 10).

(iv) Absorption spectra

Absorption spectra of control and DCMU-treated cultures were taken on the 8th day of growth. We observed peaks at 680 nm, 624 nm, 496 nm and 439 nm for Chl a, phycocyanin, carotenoids and Chl a (in the blue region) respectively. With 10^{-7}M DCMU the pigment content is significantly reduced and this is seen as reduction in the absorption peaks. With 10^{-8}M and 10^{-9}M DCMU the stimulation in pigment content is observed corresponding to the stimulation observed in growth pattern (Fig. 11 and Table 11).

II. EFFECT ON PHOTOCHEMICAL ACTIVITY

The effect of DCMU and SAN 133-410H on photochemical activity (PS I and PS II activity) was determined for 8 days old Anacystis nidulans cells grown with various concentration of the herbicides.

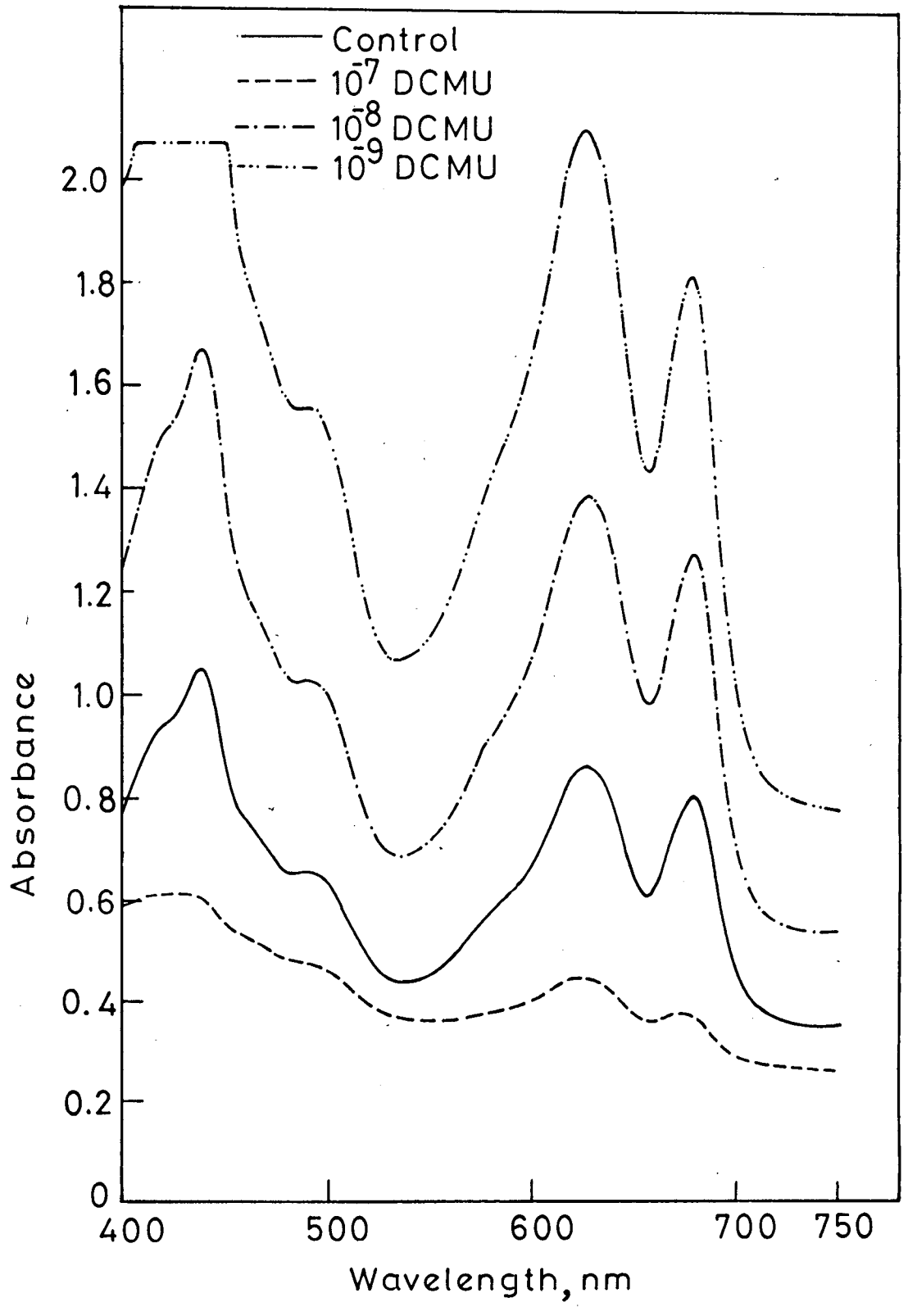
(a) PS I activity

The PS I activity was determined with thylakoid membrane fragments of 8-days old culture since MV, DCPIP and TMPD do not enter the intact cells. With ascorbate-TMPD as electron donor to PS I no significant inhibition was observed either with DCMU-treated or San 133-410H treated cultures (see Figs.12 and 13). The

Fig. 11 Absorption spectra of intact cells of A. nidulans grown with different concentrations 10^{-7}M, 10^{-8}M and 10^{-9}M) of DCMU. Equal volume (5 ml) from each culture flask was taken on the 8th day of growth. A 6 nm shift in the Chl a peak was observed with $10\text{-}7_M$ DCMU. The peak appeared at 673 nm as compared to 679 nm in control.

Table 11 Effect of DCMU (10^{-7}M, 10^{-8}M and 10^{-9}M) on the absorption at 679 nm. The peak with 10^{-7}M was shifted (by 6 nm) to 673 nm. The right hand column gives percentage of control.

	Absorbance	%
Control	0.21	100
10^{-7}M DCMU	0.045	21
10^{-8}M DCMU	0.365	173
10^{-9}M DCMU	0.51	242


Control
10⁻⁷ DCMU
10⁻⁸ DCMU
10⁻⁹ DCMU
Absorbance
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
400
500
600
700
750
Wavelength, nm

rates of O_2 uptake in case of DCMU were inhibited slightly ($\sim$10%), and in the case of SAN 133-410H the rates were almost the same as in control (Tables 12a and 13a).

With ascorbate-DCPIP as electron donor to PS I an inhibition ($\sim$ 20%) in O_2 uptake was observed in case of DCMU-treated as well as San 133-410H treated cells (see Tables 12b and 13b). Low concentrations of DCMU (10^{-9}M and 10^{-8}M) did not have much effect on the PS I activity.

(b) PS II activity

We studied the PS II activity of 8 days old intact cells of A. nidulans grown with various concentrations of the herbicides DCMU and San 133-410H. Para-benzoquinone was used as the electron acceptor. A dose-response curve for inhibition of PS II activity was observed with both DCMU and San 133-410H treated cells (see Figs. 12 and 13). The PS II activity was inhibited by 23%, 35% and 45% with 10^{-9}M, 10^{-8}M and 10^{-7}M DCMU-treated cells (see Table 12c) respectively. Similarly the inhibition of O_2 evolution was 25%, 30%, 36% and 40% with cells grown in medium containing 20 μM, 60 μM, 100 μM and 140 μM San 133-410 H respectively.

Both the herbicides, DCMU and San 133-410H inhibit the PS II activity significantly and do not seem to affect the PS I activity.

III. EFFECT ON POLYPEPTIDE PATTERN

The SDS-PAGE analysis with control cultures showed major bands around 70-90 kD corresponding to phycobilisome linker polypeptides, around 64 kD for PS I Chl binding protein, 56-60 kD for

Table 12. DCMU treated cultures

12a. PS I activity: TMPD-Ascorbate --→ MV

	μmole O_2 mg^{-1} $Chl.h^{-1}$	% activity
Control	420	100
10^{-9}M	389	93
10^{-8}M	389	93
10^{-7}M	379	89

12b. PS I activity: DCPIP Ascorbate --→ MV

	μmole O_2 mg^{-1} $Chl.h^{-1}$	% activity
Control	286	100
10^{-9}M	265	93
10^{-8}M	249	87
10^{-7}M	233	81

12c. PS II activity: H_2O --→ PBQ

	μmole O_2 mg^{-1} $Chl.h^{-1}$	% activity
Control	359	100
10^{-9}M	276	76
10^{-8}M	233	65
10^{-7}M	199	55

Table 13. San 133-410H treated cultures

13a. PS I activity: TMPD-Ascorbate --→ MV

	μmole O_2 mg^{-1} $Chl.h^{-1}$	% activity
Control	342.5	100
20 μM	342	39.9
60 μM	335	98
100 μM	327	96
140 μM	327	96

13b. PS I activity: DCPIP-Ascorbate --→ MV

	μmole O_2 mg^{-1} $Chl.h^{-1}$	% activity
Control	265	100
20 μM	233	88
60 μM	233	88
100 μM	218	82
140 μM	218	82

13c. PS II activity: H_2O --→ PBQ

	μmole O_2 mg^{-1} $Chl.h^{-1}$	% activity
Control	313	100
20 μM	235	75
60 μM	218	70
100 μM	200	64
140 μM	188	60

Fig. 12 Electron transport activities of A. nidulans (8 days old) grown with various concentrations of DCMU (10^{-7}M, 10^{-8}M and 10^{-9}M).

o---o --- Represents PS II activity (O_2 evolution) [H_2O----→ PBQ]

Δ--- Δ --- Represents PS I activity (O_2 uptake) [Asc-TMPD --→ MV]

□---□ --- Represents PS I activity (Asc-DCPIP--→ MV)

(For details of assay conditions refer to Materials and Methods)

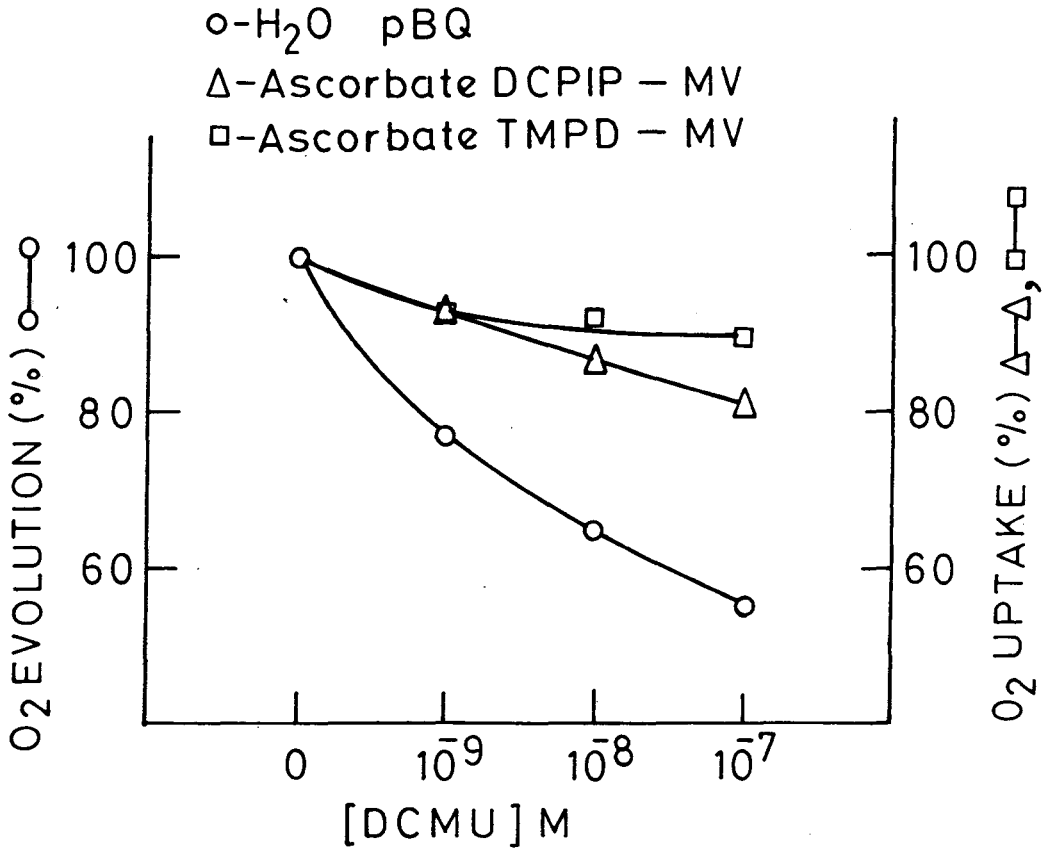

o-H2O pBQ
Δ-Ascorbate DCPIP – MV
□-Ascorbate TMPD – MV
O2 EVOLUTION (%)
100
80
60
100
80
60
O2 UPTAKE (%) Δ, □
0
10⁻⁹
10⁻⁸
10⁻⁷
[DCMU] M

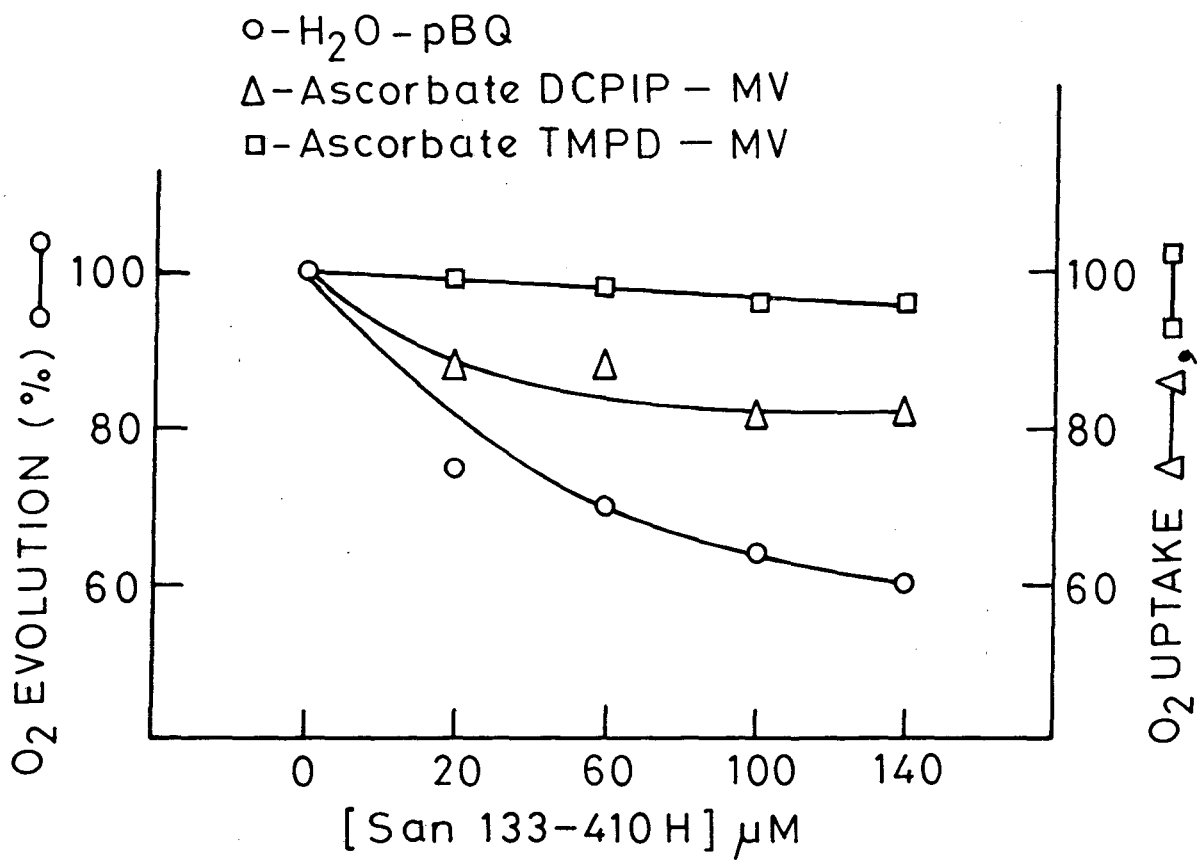
Fig. 13 Electron transport activities of A. nidulans (8 days old) grown with various concentrations of San 133-410H (20 µM, 60 µM, 100 µM and 140 µM)

o---o--- Represents PS II activity (O_2 evolution) [H_2O----→ PBQ]

Δ---Δ--- Represents PS I activity (O_2 evolution) [Asc-TMPD--→ MV]

□---□--- Represents PS I activity (O_2 evolution) [Asc-DCPIP--→ MV]

(For details of assay conditions refer to Materials and Methods)


o-H2O-pBQ
Δ-Ascorbate DCPIP – MV
□-Ascorbate TMPD – MV
O2 EVOLUTION (%)
100
80
60
0
20
60
100
140
[San 133-410 H] μM
O2 UPTAKE

F_1 complex, 48 kD and 45 kD corresponding to PS II proteins, the 32 kD herbicide binding protein, 32-34 kD proteins which have been recently reported as PS II reaction center core proteins (M_r of 29, 30, 32, 34-36 also correspond to some of the rod-linker proteins), 22-24 kD for the polypeptides involved in oxygen evolution and phycocyanin, 16-18 kD for allophycocyanin and 8-10 kD for cytochrome b-559.

With samples from DCMU-treated cultures (Fig. 14) the intensity of all the bands were relatively less. All the three concentrations of DCMU showed a pronounced decrease in intensity of the 22-24 kD proteins and proteins around 60 kD. However, with 10^{-7}M DCMU the 64 kD (PS I Chl binding protein), 32-34 kD (PS II core protein) and 29-30 (LHC proteins) were practically absent or very faint. At all the three concentrations the intensity of APC and cytochrome b-559 bands (16-18 and 8-10 kD respectively) was much less as compared to that of control.

With San 133-410H at 20 μM and 60 μM concentrations no remarkable difference was observed. However, bands around 70-90 kD of phycobilisome linker polypeptides and 22-24 kD (of O_2 evolving complex) were slightly less in intensity. But the effect of 100 μM and 140 μM was significant. At 100 μM most of the bands were missing or very faint, excepting those around 56 kD (F_1 complex) and 64 kD (PS I proteins). The intensity of bands around 32-34 kD, 36 kD and 29-30 kD (PS II proteins) and the 16-18 kD (APC proteins) was drastically decreased. The 22-24 kD bands were absent. With 140 μM San 133-410H only one prominant band was

Fig. 14 SDS-PAGE analysis of protein extract from A. nidulans (8 days old) grown with various concentration of DCMU

Std. --→ Standard proteins (Pharmacia)

a --→ 10^{-7}M DCMU treated culture

b --→ 10^{-8}M DCMU treated culture

c --→ 10^{-9}M DCMU treated culture

d --→ Control.

(For details refer to Materials and Methods)

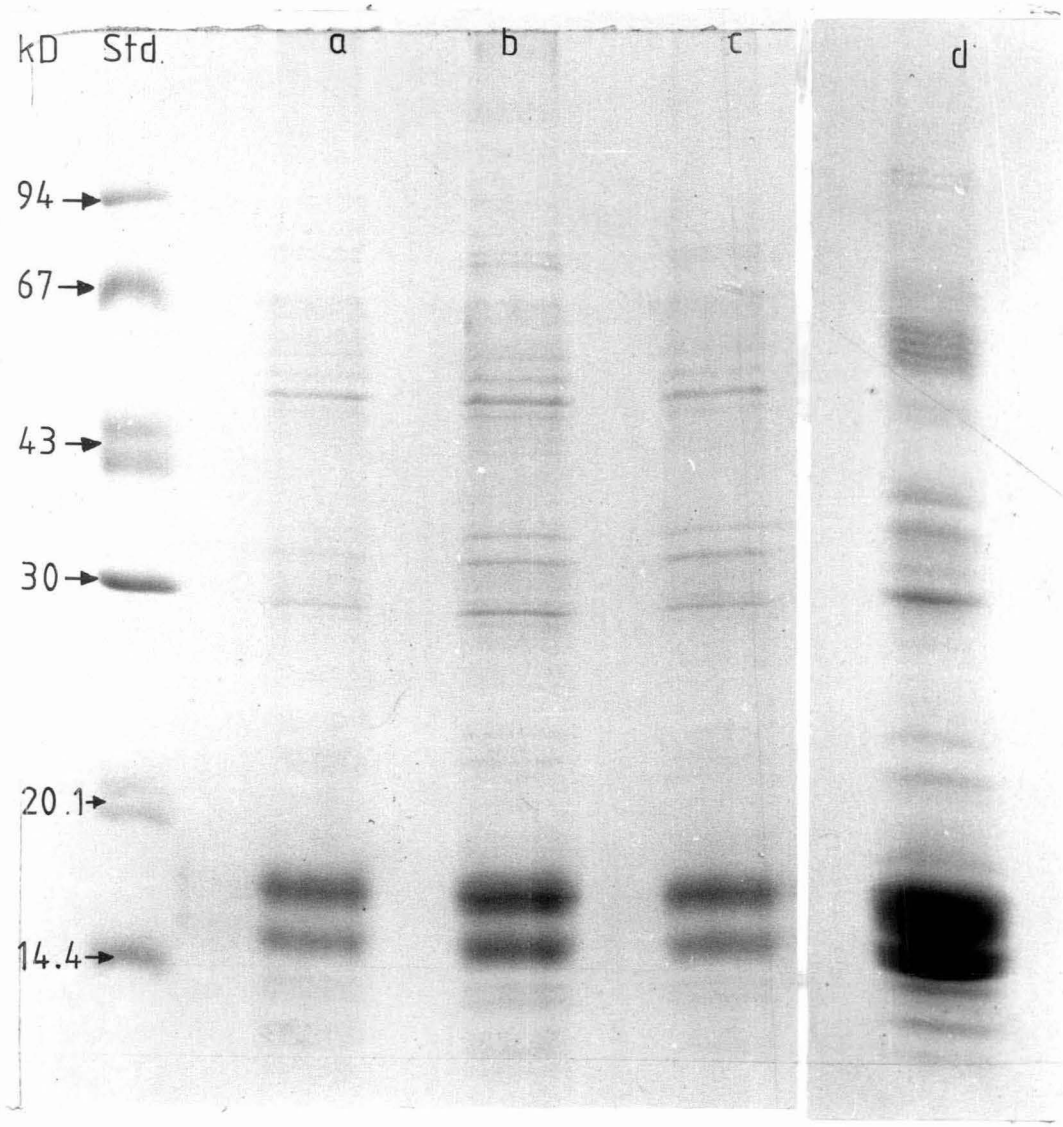

kD
Std.
a
b
c
d
94
67
43
30
20.1
14.4

Fig. 15 SDS-PAGE analysis of protein extract from A. nidulans (8 days old) grown with various concentrations San 133-410H

Std. --→ Standard proteins (Pharmacia)

a --→ Control

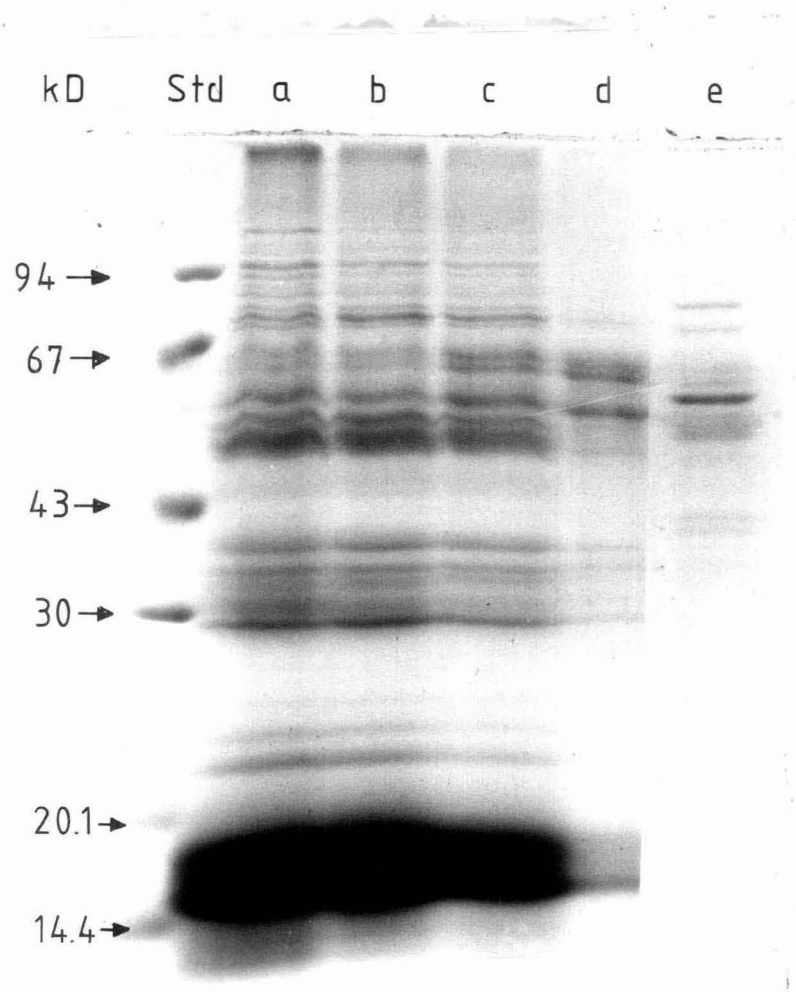
b --→ 20 µM San 133-410H treated culture

c --→ 60 µM San 133-410H treated culture

d --→ 100 µM San 133-410 H treated culture

e --→ 140 µM San 133-410H treated culture

(For details refer to Materials and Methods)


kD
Std
a
b
c
d
e
94
67
43
30
20.1
14.4

observed around 60-62 kD which is probably a degradation product of higher molecular weight polypeptides. All the other protein bands were practically absent. Smaller fragments of degradation products were visible. (Fig. 15)

DISCUSSION

DISCUSSION

Herbicides may affect the structure and function of membranes either directly or indirectly. The mechanisms and mode of action involve interference with electron transfer, carotenoid synthesis, photosynthetic electron transport and phosphorylation, oxidative phosphorylation, membrane and nuclear activity (depending on the type of herbicide) [Moreland, 1980] and their primary and secondary actions.

In the present study we have investigated the effect of various concentrations of the herbicides DCMU (10^{-9}M, 10^{-8}M and 10^{-7}M) and San 133-410H (20 μM, 60 μM, 100 μM and 140 μM) on growth, photochemical activities and polypeptide pattern of the cyanobacteria Anacystis nidulans (ARM 336) in vivo. The growth parameters were studied by determining the dry weight, pigment content (Chl a, carotenoids and phycocyanin) and protein content at 48 hours intervals till the late log phase i.e. the 8th day of growth. The most sensitive site of action of these herbicides in chloroplasts is on the acceptor side of photosystem II (Ashton and Crofts, 1981). The binding and inhibition site of DCMU or triazine-type herbicides is the Q_B protein of PS II, identified as the 32 kD polypeptide (Arntzen et al. 1982).

Our observations on growth parameters (dry weight, pigment, and protein content) of Anacystis nidulans show that presence of DCMU (10^{-7}M) or San 133-410H (20 μM-140 μM) in the growth medium inhibits cell division extending the log phase and reduces the protein content. Direct effect of some herbicides on mitosis has been reported (see Moreland, 1980). Herbicides could suppress

mechanisms to low levels of inhibitory action, resulting in growth greater than that of the control. It would suggest that increase in growth is not a minimizing effect of growth inhibitors rather it is the consequence of adaptive behaviour of rate-sensitive control mechanisms which might be occuring in case of DCMU. Non-observance of the phenomenon with San 133-410H would further suggest that hormesis is not a universal phenomenon.

Blockage of electron transport between Q and PQ prevents formation of ATP and NADPH which are required for CO_2 fixation. However, the phytotoxic symtoms of herbicides may not be only due to lack of photosynthate but also due to production of secondary phytotoxic substances, and/or photo-oxidative pigment-destructive reactions may be involved (Ashton and Crofts, 1981).

Both the herbicides used, DCMU and San 133-410H, are strong inhibitors of the Hill reaction and the PS II activity (O_2 evolution) assayed with PBQ as acceptor was significantly decreased (Figs. 12 and 13). The PS I activity (O_2 uptake) was not affected. However, the slight inhibition of O_2 uptake assayed with Ascorbate-TMPD ---→ MV in case of San 133-410H only, suggests that the site of action of SAN is after TMPD binding site in PS II system. Or that SAN preferentially alters the binding environment of TMPD and not DCPIP (see Figs. 12 and 13; Tables 12a,b and 13a,b).

SDS-PAGE analysis of protein extract from Anacystis nidulans cultures grown with various concentrations of herbicides (DCMU and San 133-410H) in the medium indicate an effect on protein biosynthesis. These synthetic processes probably are not affected by the herbicides per se but only indirectly through the

cell division by directly inhibiting protein synthesis or active transport of precursors or inhibiting ATP production which is required for these biosynthetic reactions (Linck, 1976). The primary symptom of photosynthetic inhibitors that block electron transport is chlorosis. This was distinctly visible in our experiments. It is considered to be induced by the inability of chlorophyll to dissipate the absorbed light energy, which normally leads to its photodestruction (Heath and Packer, 1968a). Carotenoids which act to protect chlorophyll from photodestruction (Anderson and Robertson, 1960) are not effective in presence of herbicides since interruption of electron flow causes inhibition of NADPH formation, which is necessary to maintain a functional carotenoid protective mechanism (Stanger and Appleby, 1972). The bleaching action of herbicides, indicated in the absorption spectrum (Fig. 11 and Table 11) with 10^{-7}M DCMU, is also a result of inhibition of carotene and chlorophyll biosynthesis or of pigment destruction (Sandmann et al. 1984a,b). The pyridazinone herbicides (like San 133-410H) are known specifically to inhibit carotenoid synthesis (Rudiger et al. 1976). Our experiments also revealed maximum inhibition in carotenoid content in presence of San 133-410H (Figs. 5 and 6) as compared to other pigments (Figs. 3, 4, 7 and 8; and Tables 3-8).

With DCMU at very low concentrations (10^{-8}M and 10^{-9}M) we observed some stimulation in all the growth parameters (dry weight $\sim$ 20%, pigment content $\sim$ 10% and protein content $\sim$ 10%) which might be due to a phenomenon known as growth hormesis reported by Stebbing (1982). The growth hormesis may be a consequence of regulatory over-corrections by biosynthetic control

inhibition of the process of photosynthesis which provides most of the ATP needed. Our analysis of polypeptides by SDS-PAGE suggests that 10^{-7}M DCMU affects the biosynthesis of both PS I and PS II polypeptides (see Fig. 14). But at lower concentrations (10^{-8}M and 10^{-9}M) the effect is relatively less.

With San 133-410H also the polypeptides of both PS I and PS II get affected (Fig. 15). The effect is quite prominent at 140 uM and somewhat less at 100 uM. Thus it may be concluded that both DCMU and San 133-410H interfere with the pathway of biosynthesis of photosystem I and II in somewhat different ways but we are unable to find out the exact mechanism involved in the process.

SUMMARY

SUMMARY

We have studied the effect of various concentrations of two herbicides, DCMU and San 133-410H, on growth and photochemical activity of the cyanobacteria Anacystis nidulans (ARM 336). For growth parameters the dry weight, pigment and protein contents were monitored at 48-hours intervals till the late log phase (8th day). It was observed that presence of 10^{-7}M DCMU and 20 μM, 60 μM, 100 μM and 140 μM of San 133-410H, was inhibitory for the above mentioned growth parameters. However, with 10^{-8}M and 10^{-9}M of DCMU, some degree of stimulation was apparent in the growth pattern leading to the conclusion that DCMU at low concentrations causes growth hormesis of the cyanobacteria Anacystis nidulans (ARM 336). Data on dry weight and protein content show that these herbicides interfere with cell division and the data on pigment content suggest that these herbicides interfere with the biosynthesis of Chl a, carotenoids, and phycocyanin. San 133-410H shows a preferential inhibition of carotenoids content as compared to other pigments. The PS II activity studies show a concentration dependent inhibition whereas PS I activity is not affected. These observations support the view held earlier that PS II is the main site of interaction of these herbicides.

Our data on SDS-PAGE analysis of protein extracts from Anacystis nidulans cultures grown with various concentrations of herbicides indicate that these herbicides inhibit the pathway of biosynthesis of both photosystem I and II, though differently. Further investigation in this aspect would reveal the correlation

between the effect of different herbicides on biosynthesis and photochemical activity of the organism.

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