STUDIES ON ALUMINIUM TOXICITY : ANALYSIS OF GROWTH CHARACTERISTICS OF A RED ALGA—*Cyanidium caldarium*.

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Dissertation submitted to the Jawaharlal Nehru University
in partial fulfilment of the requirements for
the award of the Degree of
MASTER OF PHILOSOPHY

117p

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CERTIFICATE

The research work embodied in this dissertation has been carried out in the School of Life Sciences, Jawaharlal Nehru University, New Delhi under my supervision. This work is original and has not been submitted in part or in full for any other degree or diploma of any other Institution/University.

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ACKNOWLEDGEMENTS

I express my sincere gratitude to my teachers, Prof. Jaweed Ashraf and Prof. Prasanna Mohanty for their valuable guidance, encouragement and all other assistance and personal affection to me, without which this work could not have been completed. I especially want to thank Prof. Prasanna Mohanty for providing laboratory facilities in his lab to facilitate a number of experiments during the course of my investigations and helping with literature as well as proper formulation of conclusion.

I sincerely thank Mr. S.D.S. Murthy, Mr. Narendra Mohanty, Dr. S.C. Sabat, Mrs. Shubhra, Miss Mahima Jauhari, Miss Nilima, Mr. Praveen Gurg, Mr. N. Chakrabarty, Md. Azam, Mr. Ravindra Pratap Singh and Mr. Chaman Lal for their help and assistance to me in my work.

I thank Mr. Saini and Mr. Manoharan for helping with photography and drawings incorporated in this dissertation.

I am grateful to the University Grants Commission for the grant of financial assistance through the award of Junior/Senior Research Fellowship/Assistance since 1986.

HARI RAM RAO

ABBREVIATIONS

ADP	Adenosine 5'-diphosphate
$AlCl_3$	Aluminium Chloride
ATP	Adenosine 5-triphosphate
A^o	Angstrom Unit
pBQ	Para-Benzoquinone
Chl	Chlorophyll
CF_0	Coupling factor 0 of ATP synthetase
CF_1	Coupling factor 1 of ATP synthetase
$Cytb_6f$	Cytochrome b_6-f complex
^oC	Degree celcius
DAPI	4,6 diamidino-2 phenylindole
DCCD	Dicyclohexyl car bodimide
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethyl urea
DCPIP	2,6-dichlorophenol indophenol
DPC	Diphenyl carbazide
Fd	Ferredoxin
h	Hour
H^+	Proton
HEPES	N-2-Hydroxyethyl piperzine-N-2-ethane sulfonic acid
H_2O	Water
KD	Kilo dalton
LHCP	Light harvesting chlorophyll protein

μg/ml	Microgram per mililitre
min	Minute
mM	Milimolar
nm	Nanometer
NaCl	Sodium chloride
NADP	Nicotinamide Adenine Dinucleotide Phosphate
OD	Optical density
OES	Oxygen evolving complex
PC	Phycocyanin
PEG	Polyethylene glycol
PQ	Plastoquinone
PSI	Photosystem I
PSII	Photosystem II
$PSII_{\alpha}$ $PSII_{\beta}$	Photosystem II alpha, Photosystem II bita,
Pi	Inorganic phosphate
Q	Primary electron acceptor
S_0, S_1, S_2 S_3, S_4	Different oxidation states involved in oxygen evolution.
Wm^{-2}	Watts per meter square.

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B. Thin section of intact cells. Outer parts of the cell wall were removed, the low electron dense granules denoted as starch-like granules may be cyanophycean or floridean starch (Isao, et al., 1975b).

OW, Outer layer of the cell wall
MW, Middle layer of the cell wall
IN, Inner layer of the cell wall
M, Cell membrane
Ch, Chloroplasts
N, Nucleus
S, Starch-like Granules

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INTRODUCTION

Photosynthesis is that fundamental process by which green plants and cyanobacteria convert solar energy into chemically rich compounds like $NADPH_2$ and ATP. These energy rich compounds are necessary for the formation of carbohydrates, fats and other substances required for the maintenance of life on our planet.

PHOTOSYNTHESIS

Photosynthetic process occurs in green parts of the plants viz. leaf. Chloroplasts in green plants and thylakoid membranes in cyanobacteria are the centres for this process. The chloroplast is a complicated, semi-autonomous body which is the seat of photosynthesis and its DNA replication. On the photosynthetic membrane (chloroplast membrane) two different pigment systems are known to constitute and carry out two independent photochemical reactions namely PSI and PSII. The two photosystems are connected by a series of electron carrier proteins which are arranged according to their redox potential for down hill electron flow. Starting with PSII, light of 680 nm, or of shorter wavelength, excites the

chlorophyll antenna of PSII and oxidizes the reaction centre, the P680, and reduces the stable electron acceptor Q, which, in turn, transfers electrons to the Plastoquinone (PQ Pool). The electron hole left on the P680 is filled ultimately by the electron coming from the oxidation of water. Photo-oxidation of water occurs to evolve oxygen, along with the release of protons to the inside of thylakoid membrane in chloroplasts, and PQ shuttles to PSI complexes located primarily in non-appressed region of the thylakoid membrane. Electrons are transferred from carrier to carrier down, or according to electrochemical gradient, to the PSI reaction centre P-700, which gets oxidized mainly in the far red light. PSI, already with an electron hole created by light absorption of pigments belonging to PSI, reduces NADP to $NADPH_2$ by protons pumped from outside to the inside of the thylakoid membrane vesicles during the oxidation of water. PQ shuttle largely accounts for a proton motive force (PMF) needed for the synthesis of ATP from ADP and Pi. The products of light reaction, namely $NADPH_2$ and ATP, are used for the reduction of CO_2 to carbohydrates through the catalysis of a number of enzymes present in the chloroplast stromal region (See Fig. 1).

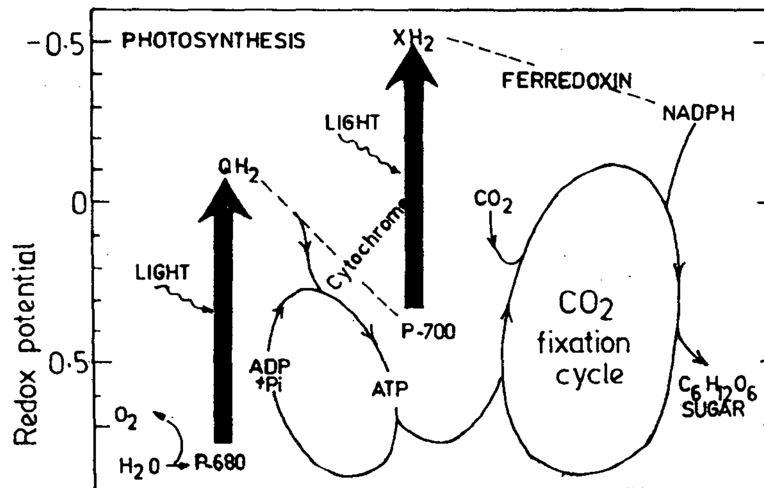


Fig. 1 Skelton scheme of photosynthesis indicating light and dark reaction (from Avron, 1981).

STRUCTURE AND FUNCTIONS OF COMPONENTS OF PHOTOSYNTHETIC MEMBRANE

Chloroplast thylakoids are centres for trapping of the solar energy and its conversion to chemical energy. The stromal matrix houses a host of enzymes responsible for carbon dioxide reduction to carbohydrates. Chloroplasts are double membranous structures resting on a fluid matrix of the cells. No other biological membrane is so sophisticated in its architecture, and specialised in function, as the thylakoids of photosynthetic apparatus. The specialised functions of thylakoids are attributed to the architecture of its membranes. Some specialised and specific polypeptides are responsible for the functioning of thylakoid membranes. A number of polypeptides are organised into supra molecular complexes responsible for the light driven electron transport from H_2O to NADP and the associated synthesis of ATP from ADP and Pi, embedded or associated in the bulk of the thylakoid membranes (Fig. 2).

THYLAKOID MEMBRANE

The thylakoids are the complexly folded membrane systems consisting of stacked (granal) and unstacked (stomal) lamellae in higher plant chloroplasts (Coombs and Greenwood 1976). The thylakoid membrane has proteins

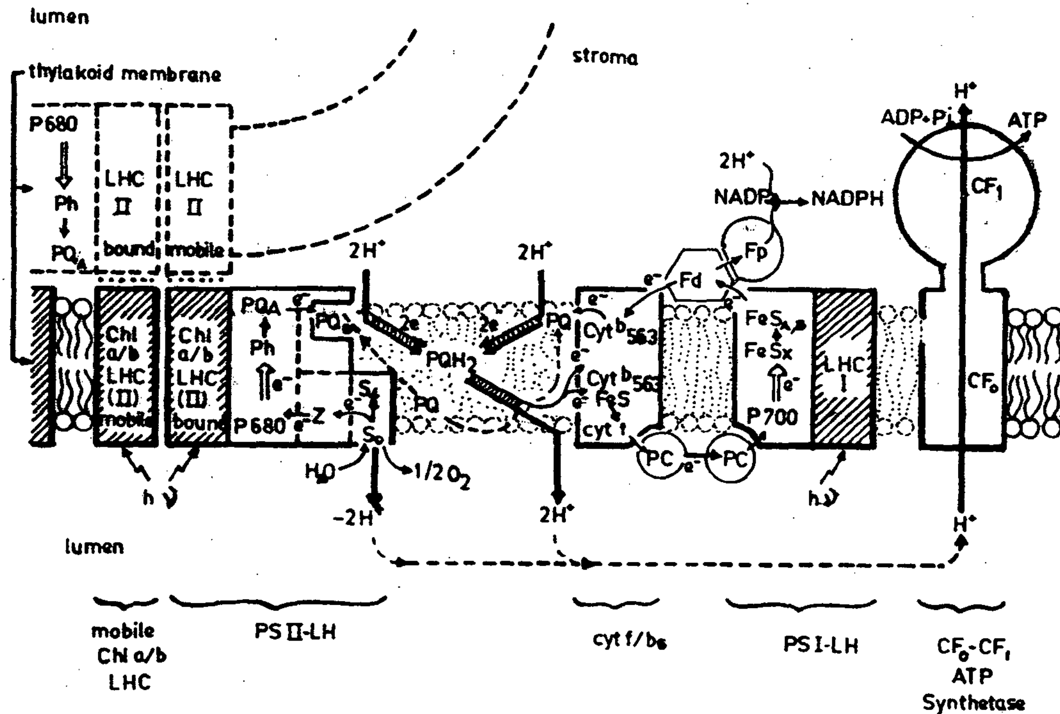


Fig. 2 The organization of chloroplast membrane components participating in the electron coupling reactions of photosynthetic electron transport. Solid, thin arrows indicate electron transfer reactions; open arrows, chemical transitions; thick arrows, proton movements; dashed arrows, recycling routes for PQ. Three .structurally distinct protein complexes participate in the linear electron transport pathway from water to NADP: a PSII complex, which is linked by a lipid soluble pool of plastoquinone molecules to a cytochrome f/b_6 complex, and a PS I complex, which receives electrons from the cytochrome complex via the water-soluble protein, plastocyanin. Also indicated is a quinone electron transport cycle, or Q cycle, which causs the translocation of one additional proton for every two electrons passing through the linear electron transport chain. Protons deposited within the thylakoid lumen exit through the proton-translocating CF_0-CF_1 ATP synthetase complex. Two types of light-harvesting pigment-protein complexes (LHC) serve both photosystem; these preferentially associate with either PSI or PSII and are designated LHC I or LHC II respectively. Abbreviations: LHC, light-harvesting complex; S and Z, water-splitting and O_2-evolving enzymes that donate electrons to P680; P680, reaction center of PSII; Ph, bound phenophytin (a primary electron acceptor of PSII); PQ_A and PQ_B, special bound plastoquinone molecules; PQ and PQH_2, plastoquinone and reduced plastoquinone; FeS, Rieske Iron sulfur protein; cyt, f and cyt b_{563}, cytochromes f and b_{563} (b_6); FeS_x, special FeS center that serves as the primary acceptor of PSI; $FeS_{A,B}$, two special FeS centers; Fp, flavoprotein (ferrodoxin-NADP reductase); CF_0 and CF_1, basepiece and headpiece of ATP synthetase; hv, light energy. (from: Staehelin and Arnstzen, 1983).

associated with its intrinsic and extrinsic structures. In thylakoid membranes, the acyl lipids play an important role as a support matrix as well as a fluid medium which allows diffusion process to occur. 75 per cent of acyl lipids are monogalactosyl diacyl glycerol (MGDG) and digalactosyl diacyl-glycerol (DGDG). 15 per cent are phospholipids and the remaining 10 per cent phosphotidyl glycerol.

(a) Intrinsic Proteins:

Photosystem (PS) I complex:

Plants have about 30 per cent of their total chlorophylls grown under normal light (Thornber *et al*., 1979). Each complex contain, 150 to 200 Chla molecules; one reaction centre P-700 and the associated primary electron acceptors, A_1, A_2 as well as two iron sulphur centres (Bengis and Nelson, 1977). Detergent treatment strips away the outer chlorophyll containing polypeptides but inner reaction centre core complex, having 40 Chla absorbing centres, mostly in the region of 680-690 nm, does not get released by detergent treatment. These chlorophylls are bound non covalently, together with carotenoid (B-carotene) to a polypeptide chain of about 68 kD, one of which contains P-700, the reaction centre of PSI, while the other polypeptides are associated with the constitution of the

complete reaction centre (Bengis and Nelson, 1977; Anderson, 1980b).

Photosystem II complex

It contains 10-15 per cent of the total chlorophyll with 60 Chla per P680 (Anderson, 1980a). PSII in higher plants contains more than 10 polypeptides. A 40-50 kD polypeptide constitutes the reaction centre complex. Approximately, 40-50 Chla molecules are bound to this polypeptide. A 32 kD polypeptide has been shown to be the apoprotein of the secondary PSII acceptor B (QB) polypeptides. 10 kD cytochrome b_{559} is also the bound constituent of PSII complex (see Fig. 3).

LIGHT HARVESTING CHLOROPHYLL a/b PROTEIN COMPLEX (LHCP-2):

Light harvesting chlorophyll-protein complex of PSII (LHCP-2) contains 40-60 per cent of the total chlorophyll and is closely associated with the PSII complex (Anderson, 1980a; Thornber, 1975). It contains two polypeptides of molecular weight 26 kD and 28 kD and high content of Xanthophyll (Thornber, 1975). It assists in light harvesting. Chlorophyll is distributed in four separate LHCP complexes which aggregate with a single PSI complex to form an overall particle of about 160 A^{o} (Armond and

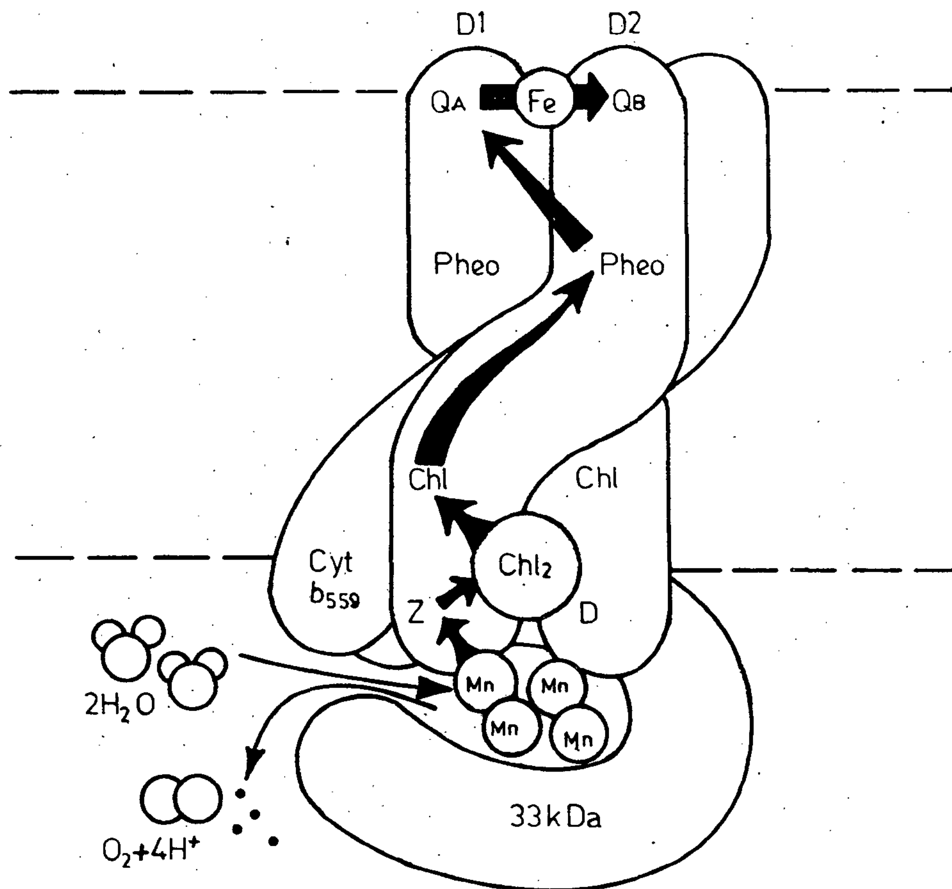


Fig. 3 A schematic representation of the polypeptides of PSII reaction centre and the associated water splitting apparatus. Q_A and Q_B are plastoquinone molecules which act in series as electron acceptors and are associated with a ferrous ions, Fe. Pheo are phyeophytin molecules, one of which (probably on the D_1 polypeptide) acts as a primary acceptor. Chl_2 is speculated to be a special pair acting as the primary donor P680. Chl represents each of the monomeric chlorophyll molcules, one or both may help to facilitate electron transfer to pheo. Z is an electron donor to P680 and the 33 KD a protein is responsible for stabilizing the manganese cluster necessary for water oxidation. D is a component similar to Z which does not seem to be involved in electron trasfer from water to $P680^+$. Cyt b559 is cytochrome b559 of unknown function. Arrows show possible routes for electron transport (from Barber, J. TIBS, 1987).

Arntzen, 1977; Mullet and Arntzen, 1980). LHCP-2 complex contain 60 chlorophylls and has a molecular weight in the range of 300 kD (Hiller and Goodchild 1981).

Cytochrome b_6-f (Cytb_6-f) Complex:

This serves as an intermediate carrier betwen PSII and PSI. It is a physiological acceptor of electrons coming from Plastoquinone (PQ Pool). It acts as a plastoquinone $\rightleftharpoons$ Plastocyanin oxidoreductase. Its location and association with PSI polypeptides is important as it is involved in the regulation of electron flow between two photosystems. Five polypeptides have been isolated from this complex having a molecular weight- 34, 33, 23, 20, 17.5 kD and containing one cytochrome f, two cytochrome b-563, the Rieske-Fe-S centre and some bound plastoquinone-9 (Hurt et al. 1981).

ATP Synthetase (CFo Complex)

This complex has never been isolated as a complete entity. Its three polypeptides have a molecular weight of 15, 12 and 8 kD. The third polypeptide, which binds to dicyclohexyl carbodimide (DCCD), constitutes a protein conducting channel across the membrane. Second polypeptide helps in the structural maintenance, and the third polypeptide acts as a binding protein between CF_1 and CF_o channel.

(b) Extrinsic Proteins - External Surface

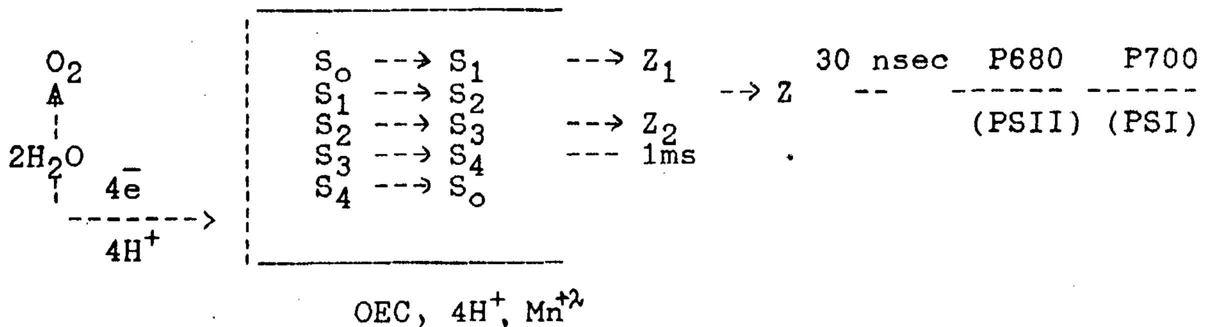
Ferredoxin is an easily removable extrinsic protein present on the surface of higher plant thylakoids. Its molecular weight is 11 kD. It contains a 2Fe-2S centre and has 95-100 aminoacid residues (Hall and Rao, 1977). Ferredoxin-NADP oxidoreductase is a flavonoid protein with a molecular weight of 40 kD which is firmly arranged on the thylakoid outer surface. It reduces NADP to NADPH, H^+ ATP synthetase (CF_1 complex). Its native molecular weight is 32.5 kD. It can be resolved into five subunits, α, β, γ, δ and ε with molecular weights 59, 54, 37, 17.5 and 13 kD. These subunits take part in ATP synthesis/ATP hydrolysis activity (Nelson, 1982).

Extrinsic Proteins - Inside the Membrane

Plastocyanin (PC) is a one-copper-atom containing protein, loosely bound to the inner thylakoid surface (Haehnel *et al*. 1981). It appears blue in its oxidized state and has a molecular weight of about 10.5 kD. The core of the molecule is hydrophobic and largely aromatic, while the polar surface has negative charges at physiological pH that are clustered near one end of the molecule. The copper-atoms are located near the other end of the molecule (Colman *et al*. 1978).

Water Oxidation Complex:

Kok *et al*. (1970) formulated the generally accepted "S-state scheme" for photosynthetic oxygen evolution i.e., linked with PSII activity. Each individual oxygen-evolving complex undergoes a successive series of increasing oxidation states from S_o to S_4 on excitation of its associated PSII reaction centre. After the enzyme reaches S_4 state, it releases oxygen and returns to the S_o state. Each of the light induced transitions from S_1 to S_2 and from S_2 to S_3 results in release of one proton; that from S_3 to S_o of two protons. 'Z' is the primary physiological electron donar to P-680. The fast reaction between P-680 and Z is assumed to be 30 nano seconds. Water soluble polypeptides and cofactors, such as Mn^{+2}, Cl^- and Ca^{+2} are involved in the oxygen evolution system.



OEC, $4H^+$, Mn^{+2}

Scheme of the reactions at donor site of PSII. S_0, S_1, S_2, S_3 and S_4 are states of oxygen evolving complex (Kok, 1980).

Polypeptides:

Three water soluble polypeptides have the molecular weights of 33, 23, and 17 kD respectively. They are coded in the nucleus (Jansson et al., 1983; Kuwabara and Murata, 1979, 82). These are peripheral proteins situated at the luminal side of the thylakoid membrane (Akerlund et al., 1982). A 23 kD polypeptide is associated with the physiological donor to P-680. Since Z is linked to metal clusters (Mn^{+2}). This polypeptide links PSII to oxygen evolving system (OES).

33 kD polypeptide is peripheral in nature and distinct from the 34 kD polypeptide which is absent in such algal mutants that lacked oxygen evolution capacity. These two polypeptides are involved in clustering of Mn^{+2}-ions. 16 kD polypeptide helps in the Ca^{+2} binding as well as in Cl^- association in oxygen-evolving system.

Manganese (Mn^{+2}) undergoes oxidation in oxygen evolution and 4 manganese atoms are necessary per P-680 in PSII oxygen evolution.

Chloride (Cl^-) binding to Manganese cluster is necessary for $S_1 \rightarrow S_2$ transition. This requirement of chloride may be invoilved in plant adaptation to various light intensities.

Calcium (Ca^{+2}) is necessary for tight binding of manganese and photoreaction of OES. In cyanobacteria the Ca^{+2} is necessary for retention of a polypeptides in PSII. The 16 kD polypeptide stimulates the oxygen evolution by the addition of Ca^{+2}.

Organisation of Membrane Components:

Lateral Heterogenity:

Biochemical and ultrastructural studies suggest extreme lateral heterogenity in the distribution of PSII and PSI in the appressed and non-appressed region respectively. This has considerable significance for the concept of electron transport as well as for energy transfer between the two photosystems. Along with PSI the ATP synthetase and ferredoxin-$NADP^+$ oxido-reducatase are also located in the non-appressed regions of the chloroplast stroma. PSI is almost excluded from appressed region to non-appressed stroma lamellae (Akerlund et al., 1979; Anderson and Anderson, 1980). While the major pool of PSII is located in the appressed membrane of grana, a small fraction of PSII is also found in non-appressed membrane (Anderson and Anderson 1980). PSII present in appressed and non-appressed regions is termed as $PS\text{-}II_\alpha$ and $PS\text{-}II_\beta$ respectively (Anderson and Melis 1983). The cyt b_6-f complex is probably localized at

the border between appressed and non-appressed regions (Barber, 1983). The other two electron carrier proteins, namely, plastoquinone and plastocyanin are mobile and possibly shuttles between the appressed and non-appressed regions, serving as a mobile link for linear electron transfer from PSII of appressed region to PSI of non-appressed regions.

Lateral heterogenity distribution of thylakoid membrane proteins makes the membrane more dyanamic. Secondly the diffusion efficiency of mobile carrier (PQ and PC) under different environmental conditions can control the electron transport and may also influence the energy transfer between the two photosystems.

Photosynthetic Electron Transport System

In chloroplasts of higher plants, electron transfer from PSII complex of appressed region to the PSI complex of non-appressed region occurs through mobile electron transfer between PSII and PSI. In the *in vitro* system, partial photochemical reactions are catalysed with the help of artificial exogenously added electron donors and acceptors, and by the use of electron transport inhibitors. These exogenous donors and acceptors are used to evaluate the photochemical activities of thylakoid membranes as well as

photochemical reactions catalysed by PSI and PSII either separately or together. These partial reactions are valuable tools in assessing the photochemical potential of chloroplasts and of intact cells.

Cyanobacteria and Red Algae:

Blue Green Algae are oxygenic photosynthesizing prokaryotes. Cyanobacteria are significant contributors to aquatic photosynthetic system. Some cyanobacteria are capable of nitrogen fixation and offer several advantages for studying the mechanism of photosynthesis. Results thus obtained can be compared to photosynthetic system of higher plants. Cyanobacteria have unique capability to adapt easily and quickly to fluctuations in the environmental conditions such as variation in light colour and intensity, temperature, salinity and metal toxicity. They offer great promise for the study of compositional alterations arising due to changes in environmental conditions. The use of cyanobacterial system for elucidation of the mechanism of photosynthesis will be of great help in the study of topography and architecture of thylakoid membranes.

The red algae which are mostly confined to sea have fresh and salt water forms. They show various colourations

from bright red and purple to dark brownish red, brownish green, blue green and black. Each cells of algae contains one or many chromatophores of various shape. The chromatophores contain Chla and d, α and β-carotenoid, lutin, taraxanthin, allophycocyanin, or a special water soluble red pigment α-phycoerythrin and a blue pigment. Cyanobacteria show certain similarities with red algae that may be put as follows:

(i) Both lack flagella throughout the life-cycle.

(ii) Both contain water soluble, phycobilio-proteins phycocyanin and phycoerythin.

(iii) Both have chemicals as reserve food.

<u>Photosynthesis in Cyanobacteria and Red Algae</u>:

Photosynthesis depends upon the efficient capture of light energy by pigment protein complexes present in photosynthetic system. This process is initiated through the absorption of light and this energy being utilised for the reduction of CO_2. The most abundant of these pigments is chlorophyll. However in addition to Chla they contain carotenoids and phycobilin containing protein as accessary pigments involved in photosynthesis.

Chlorophyll (Chl) a:

All photosynthetic organisms that evolve oxygen possess Chla as the major pigment. It is believed that Chla occurs *in vivo* in several spectroscopic forms: Chla 660, Chla 670, Chla 680; Chla 685; Chla 690 and Chla 700-720. This large number indicates their red light absorption maxima in each of the spectral forms (French, 1966: Robinowitch and Govindjee, 1961). These strongly fluorescing short wavelength forms of Chla are mainly present in PSII. The weakly fluorescing long wavelength forms are mostly present in PSI. In all the higher plants Chlb is present but in cyanobacteria and red algae it is absent.

Carotenoids and Xanthophylls:

Almost all the photosynthetic organism contain the yellow and orange pigment called carotenoid and xanthophyll, which act as accessory pigments in photosynthesis. The action spectra of photosynthesis demonstrates that light absorbed by carotenoids is utilised with varying degree of efficiency in photosynthesis. The light energy absorbed by the carotenoids is not used directly but transferred to Chla, where it is efficiently used in the photosynthetic process (Claylon, 1962).

Phycobilins:

In cyanobacterial system, the PSII contains only small fraction of Chla. The major light harvesting pigments are phycobilins. These pigments are assembled in granular aggregates - (phycobilisomes) that are bound to the cytoplasmic surface of the thylakoid. Phycobilisomes act as light harvesting antenna, as do the light harvesting chlorophyll protein complexes (LHCP) for photosystem reaction centre in chloroplasts present in higher plants. Light energy trapped by phycobilisomes is channelled to photosystem II reaction centre either directly or through a stepwise energy transfer process known as radiation-less resonance transfer from phycocyanin (PC) to Chla. The light absorbing components, or chromophore, of phycobilisomes are open chain tetra pyrrole called phycobilins. The phycobilins are attached to apoproteins of phycobiliproteins. There are different kinds of phycocyanobilin in the chromophore are phycocyanin, phycoerythrin and allophycocyanin. Phycocyanobilin is the chromophore of phycocyanin and allophycocyanin. Phycoerythrin is the chromophore of phycoerythrin. In R-phycoerythrins, in addition to phycoerythrin, a second chromophore group is present i.e., known as phycourobilin.

Phycobiliproteins:

The phycobilins are linked to the polypeptide chain of apoprotein by thioester linkage to form phycobiliproteins. There are six forms of biliproteins present in light harvesting systems of cyanobacteria namely, allophycocyanin β, allophycocyanin, C-phycocyanin, phycoerythrin, C-phycoerythrin and R-phycoerythrin.

The Molecular Structure of Cyanobacterial Phycobilisome:

Phycobilisome is made up of two distinct domains; a core made up of two or three cylindrical objects which contain allophycocyanin from which six rods, made up of stalked discs, extend in a hemidiscoidal array containing phycoerythrin. Each phycocyanin disc in the rod structure is associated with one copy of a specific uncoloured polypeptide which is known as "linker polypeptide". Linker polypeptide mediates three types of functions. Firstly it mediates the assembly of the biliprotein to the appropriate aggregate. Secondly it modifies the spectroscopic properties of the phycocyanin. Thirdly it determines the location of particular aggregate within the rod structure so that spectroscopic properties of the rod favour the flow of energy towards the core (Glazer, 1983) (See Fig. 4).

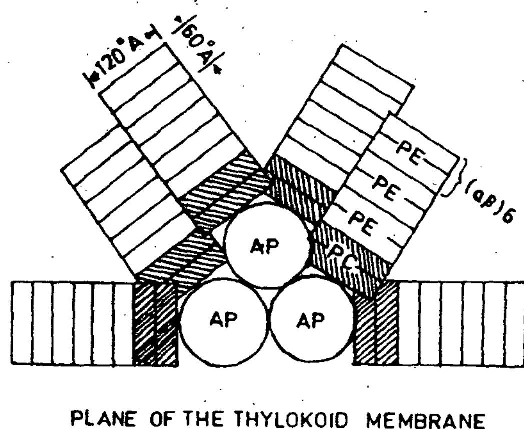


Fig. 4 Schematic representation of side view of a phycobilisome which contains Phycoerythrin (PE), Phycocyanin (PC) and allophycocyanin (APC) showing the location of biliproteins in the structure (Glazer, 1981).

Energy Transfeer in Phycobilisomes (PBSomes):

Phycobilisomes are the light harvesting antenna in cyanobacteria in the same way as the light harvesting chlorophyll protein complexes are for photosystem reaction centres in higher plants. Radiation-less resonance tranfer is the most probable mechanism of energy transfer from phycocyanin to Chl*a*. The unique arrangement of pigments in phycobilisome structure provides for a down-hill cascade of excitation energy through the phycobilins to Chl*a*. According to Glazer (1983) the energy transfer from phycocyanin to chlorophyll occur in the following steps.

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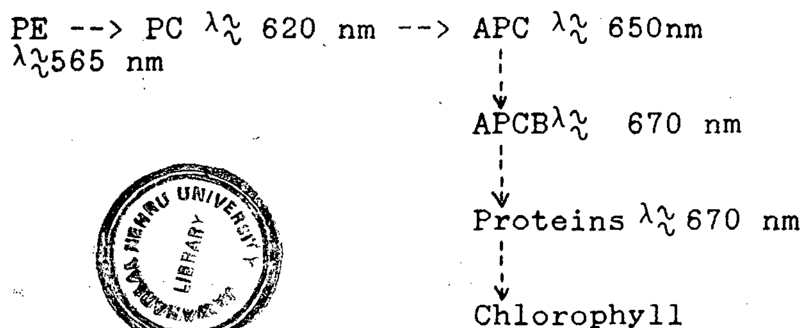

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Energy transfer scheme in phycobilisomes (Glazer, 1983).

In addition to energy transfer phycobilisomes are also helpful in chromatic adaptation (Fritsch, 1945). They are capable of adapting to different light intensities and colour qualities. The sum total is known as chromatic adaptation. This property of chromatic adaptation makes the

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bacteria compete with other photosynthetic organisms and run their photosynthetic apparatus efficiently.

Phycobilisomes, and their constituent pigments, are responsible for bacterial adaptation. Phycobilisomes number increases with decreasing light intensities. Cyanobacteria, grown in average light, show synthesis of relatively more phycocyanin. Other types of endogenous controls may also exist, illustrating the economical nature of structural adaptations possible by the molecular nature of the phycobilisome.

REVIEW OF LITERATURE

METAL IONS

Introduction: Availability of inorganic nutrients determines plant growth and development. Depending upon their necessity, these nutrients are classified into two main types, i.e. macronutrients which are required in higher quantities like K^+, Ca^{+2}, Mg^{+2}, and micronutrients, which are required in small amounts like Cu^{+2}, Mn^{+2}, Fe^{+2}, Co^{+2}. Any change in the level of these elements in soil, or in aquatic environment, results in manifestation of either difficiency symptoms, or toxic effects, that finally leads to reduced plant growth and development. Other metals like Pb^{+2}, Cd^{+2}, Ni^{+2}, Co^{+2}, Al^{+3} are toxic even in very small amounts and eventually leads to reduced growth, impaired metabolism and poor dry matter production (Hewitt, 1958).

Photosynthesis, the fundamental process by which green plants synthesize their food, is influenced by the above-mentioned cations. The effects of monovalent and divalent cations on primary process of photosynthesis have been studied in some details (Sponcer and Possingham, 1960; Jagendrof and Smith, 1961; Haberman, 1969; Gross and Hess, 1973; Shioi et al., 1978; Troxel et al., 1981).

Metal Ions and Their Role:

A large number of metal ions are involved in light as well as in dark reaction. K^+ is the major intra-chloroplastic cation. Mg^{+2} is the major cation of intact pea chloroplast (Nakatani *et al*., 1979). Mg^{+2}, Mn^{+2}, Cu^{+2}, and Fe^{+2} form integral part of several photosynthetic components. Cu^{+2} is found to be a constituent of photosystem II (Droppa *et al*., 1984). Fe^{+3} atoms are in association with sulphur atoms or with prosthetic group of ferredoxin and Reiske iron-sulphur centre. Metal ions serve not only as integral constituents but also have a functional role in the regulation of primary photosynthetic process.

Photosynthetic Electron Transport and Metal Ions

Iron is present in the form of iron-sulphur protein which acts as electron carrier in the thylakoid membrane. Cytochromes contain the heme iron-porphyrin complex as a prosthetic-group which acts as an electron carrier in electron transport. Plastocyanin, the most prominent blue copper protein functions as electron donor to P-700 in the PSI region of photosynthetic electron transport system of higher plants and of some green and blue green algae. One Mn^{+2} ion per 50-100 Chl*a* is assumed for the pool associated with PSII and it is needed for maximum oxygen evolution.

Sources of Toxic Elements:

Toxic metals naturally occur in soil because parent material of soil contained the metals. Metal ions are deposited on the surface of the earth due to human interaction with the environment. Metal smelters, foundaries, thermal power plants are the major source of heavy metals in air, water and soil. Many heavy metals used in agriculture (fertilizers and pesticides) contain the high concentration of heavy metal as compared to its concentration in the normal soil. Burning of fuel including coal, petroleum products also emit substantial amount of heavy metals. Thus the continued accumulation of heavy metal ions result in metal toxicity of the environment.

Problem of Toxicity:

Any change in the level of essential elements in the soil, air, in aquatic environment, results in the creation of either deficiency effects, or inhibitory effects on plants. Heavy metal ions pollution, resulting from unmindful industrialization and excessive use of fertilizers, herbicides and pesticides, are some of the reasons for heavy metal toxicity in atmosphere and water bodies. The level of toxic heavy metals in water bodies effect the photosynthetic

efficiency. Therefore, it is essential to study the effect of heavy metal ions on photosynthetic processes.

Toxic Elements and Photoelectron Transport:

The effect of heavy metal ions on photoelectron transport has been studied in some detail (Maldonado *et al.*, 1972; Bazzaz and Govindjee, 1974; Samuelsson and Oquist, 1980, 1980; Tripathy *et al.*, 1981; 1982; Backer *et al.*, 1982; Wavare and Mohanty 1982; Droppa *et al.*, 1984). Some of the electron transfer carrier proteins are specially affected by heavy metal ions. It is observed that the oxidising site of PSII is susceptible to metal ion inhibition (Mohanty and Mohanty, 1986).

Copper and Electron Transport:

Copper is an essential micronutrient which influences PSII electron transport either directly as constituent of an electron transport carrier (Plastocyanin), or indirectly via regulating the polypeptide composition of the membrane in PSII complex (Droppa *et al.*, 1984). The sensitive site for Cu^{+2} inhibition is on the oxidising side of PSII. In light, Cu^{+2} binds preferably at this inhibitory site of PSII, than in dark (Cedeno-Maldonado *et al.*, 1972). Inhibition of DCPIP supported Hill reaction could not be restored by

supply of DPC at the reaction centre of PSII (Shioi _et al_., 1978). In presence of ascorbate, Cu^{+2} causes rapid inactivation of PSI reaction and concomitant destruction of chlorophyll protein molecules (Samuelson and Oquist, 1980).

Lead and Electron Transport:

At low concentration, 1 mM, lead salts inhibit PSII catalysed DCPIP Hill activity in isolated chloroplasts (Miles _et al_., 1972). Electron flow from H_2O -> MV is also inhibited by lead salts. At 1 mM concentration, PSI mediated reactions remain unaffected. Only at relatively high concentration, lead ions can inhibit PSI activity (Wong and Govindjee, 1976).

Cadmium and Electron Transport:

Cadmium nitrate at concentration of 0.5 mM strongly inhibits PSII activity in maize mesophyll chloroplasts (Bazzaz and Govindjee 1974; Li and Miles, 1975). Addition of DPC to the medium restores the PSII catalysed activity. However at this level cadmium inhibits oxygen evolution and interrupts electron flow at the oxidising site of PSII. However, Li and Miles (1975) observed that electron donors like DPC do not restore the PSII catalysed activity. They suggest that the site for Cd^{+2} action is PSII reaction centre. Cd^{+2} has no effect on PSI reaction.

Zinc and Electron Transport:

Zinc is an essential micronutrient and its deficiency leads to poor dry matter production in plants (Agrawal *et al*., 1977; Shrotri *et al*., 1981). Zinc salts show maximum inhibitory effect on PSII catalysed reactions at relatively low concentrations (0.5-3 mM). Whole chain electron transport activity, monitored by MV photoreduction is inhibited by 0.5 mM $ZnSO_4$. At 4 mM concentration of $ZnSo_4$, both whole chain and PSII mediated electron transport is fully inhibited. At 3 mM concentration zinc inhibits PSI mediated eletron transport reaction (Tripathy, 1980; Tripathy *et al*., 1982). On washing, zinc treated chloroplasts partially restores the oxygen evolving activity (Tripathy and Mohanty, 1982). Backer *et al*., (1982) suggested that zinc acts on the photosynthetic electron transport system at two sites. Site I lies on the oxidising side of PSII and site 2 is located in between the PSII and PSI. During the zinc inhibition, manganese is released from the OES (Miller and Cox 1983) into restricted aquous compartment of the non-vesicular thylakoid lumen preparations (Miller and Cox 1984).

Cobalt and Electron Transport:

Both, the PSII reaction and whole chain electron transport activity are markedly inhibited in 3 mM to 8 mM of

reaction is different from that of Zn^{+2} and Pb^{+2} (Tripathy *et al*., 1982).

Mercury and Electron Transport:

Mercury is a potent inhibitor of electron transport chain of both isolated chloroplasts and cyanobacterial cells. Kimimura (1972) has reported that incubation of spinach chloroplast with $HgCl_2$ at molar ratio of $HgCl_2$ to chlorophyll ($HgCl_2$/Chl) of unity induces a complete inhibition of MV Hill reaction and also MV photoreduction with reduced DCPIP as electron donor. Photooxidation of cytochrome is similarly sensitive towards $HgCl_2$, whereas P-700 photo-oxidation is resistant to this element. $HgCl_2$ probably inhibits PSI activity by inactivating plastocyanin. There are other compounds like $HgCl_2$, Phenyl mercuric acetate which inhibit electron transport reaction. $HgCl_2$ causes the removal of protein bound copper from plastocyanin (Katoh, 1964). In spinach chloroplasts Hg^{+2} ions abolish the communication of PSII electron pool with PSI (Radmer and Kok, 1974) and selectively inhibits phosphorylation associated with PSI (Bradeen and Winget, 1974).

Aluminium Effects:

Aluminium toxicity is an important growth limiting factor for plants in many soils below pH 5. However, this

can also occur at pH 5.5. This problem is particularly serious in strongly acidic subsoils. This process is being intensified by heavy application of acid forming nitrogenous fertilizers. Al^{+3} toxicity reduces plant rooting depth, increases susceptibility to drought and decreases the utilization of subsoil nutrients.

Physiological Effect of Aluminium:

Physiological effect of Al^{+3} is as follows: Al^{+3} has been shown to interfere with cell-division in plant roots; fix phosphorus in less available forms in the soil and in/on plant roots; decrease root respiration; interfere with certain enzymes governing the deposition of polysaccharides in cell walls; increase cell wall rigidity (by cross linking pectins) and interfere with the uptake and transport as well as use of several elements (Cu, Mg, P, K and H_2O) by plants. Metal ions, such as Al^{+3} are known to form strong complexes and to precipitate nucleic acids. In fact metal ions are used to complex and isolate polynucleotides from leaves.

Aluminium Effect on Calcium/Phosphate Uptake:

Aluminium toxicity appears either as an induced Ca^{+2} deficiency or as reduction in Ca^{+2} transport. Elution

experiments showed that polyvalent cations reduce the amount of calcium held in the waterfree space (WFS) and Donnan free space (DFS) but increase both, the exchangeable and absorbed chloride content of the root. Al^{+3} treated roots transported much less calcium to the root system than untreated plants. Autoradiographs showed that this difference was reflected in a greatly reduced labelled calcium concentration in the tissue of the stele. In contrast, the non-exchangeable fraction of labelled Ca^{+2} in the cortex was similar in both treatments (Clarkson and Sanderson, 1971).

The influence of Al^{+3} on Ca^{+2} uptake would be explained equally either by decreased influx or increased efflux of Ca^{+2} across the plasmalemma. Interactions between the plasma membrane and polyvalent cations have been reported but it is not known how permeability of the membrane to ions is affected (Fluri, 1909; Scarth, 1923; Hofler, 1958; Bohm-Tuchy, 1960).

The location of the sites from which polyvalent cations control uptake and translocation in barley roots is indicated in autoradiographic studies (Clarkson and Sanderson, 1969). $AlCl_3$ and scandium distribution is restricted to the epidermis and outer layers of cortical

cells. The influence that this peripheral accumulation might have on Ca^{+2} held in the free space, is envisaged as a system of pores (20 nm in diameter) carrying fixed negative charges. The entry of trivalent aluminium ions into these pores would neutralize these negative charges and displace cations of lower affinity already held by them. Further, the electrical sign of the pore might be reversed by precipitates of aluminium hydroxide which are usually positively charged (Hsu and Bates, 1964; Clarkson, 1967). If the above supposition is correct, Al^{+3} should increase the binding of anions in that part of the free space which it occupies and perhaps, increases their absorption with the cells. It has been shown that both predictions are correct. Rather small increase in Donnan exchangeable chloride (25 percent) is a reflection of the limited penetration of Al^{+3} into the free space. Evidence from other sources indicates that the mechanical rigidity of the plasmalemma is increased by Al^{+3} (Hoffer, 1958) and that the stability and structural order of lipid bilayer is greatly incresed in the presence of trivalent Lanthanum.

In many plants Al^{+3} tolerance appears to be closely associated with phosphorus-use-efficiency, e.g., in certain cultivars of wheat and tomato and inbred lines of corn, Al^{+3} tolerance coincides with the ability to tolerate low-

phosphorus levels in nutrient solutions, either in the presence or absence of Al^{+3} (Clark and Brown, 1974; Foy, 1974; Foy et al., 1973). Al^{+3} tolerance in certain pea cultivars has been associated with higher phosphorus concentration in plant roots (Klimaschevskii and Dedov, 1975).

Andrew and Vandenberg (1973) found that Al^{+3} increases phosphorus concentrations in the top of Al^{+3}-tolerant legume species but decreases in Al^{+3}-sensitive species. Al^{+3} markedly increased the redox potential of root tissues, decreased content of high bond energy P, and increased contents of mineral phosphate in the root of peas (Dedov and Klimashevskii, 1976).

Al^{+3} toxicity was attributed to inhibition of cell division and immobilization of phosphorus within plant roots. Subsequent studies with an electron microscope confirmed that an Al^{+3}-PO_4^{-3} precipitated in meristematic zone of primary and lateral roots, but some Al^{+3} penetrated to the stele (Kesar et al., 1977).

Al^{+3}-phosphorus interaction appeared to be located in the cell wall and cytoplasmic membrane. Results also suggested that the Al^{+3} absorbed by root surfaces or that in intracellular free spaces may immobilize the phosphorus

present in root tissue or external substrate. Stryker *et al*. (1974) reported that Al^{+3} in roots may extract phosphorus from adjacent root cells, tissues and thus serve as a sink for plant phosphorus absorbed from other sites. Al^{+3} was bound to esteric phosphorus in nucleic acids and to membrane lipids and that it reduced or inhibited cell division by alternation of nucleic acid molecules (Naidoo *et al*., 1976).

Clarkson and Sanderson, Jr. (1969) found that most of the Phosphorus-32 absorbed by Al^{+3} injured barley root was inorganic and was exchangeable with nonlabelled phosphorus. Andrew and Vandenberg (1973) found that Al^{+3} injured legume root lost phosphorus to the solution. Al^{+3} is reported to decrease hexose phosphorylation (Clarkson, 1969; Clarkson and Sanderson, Jr. (1969) showed further that Aluminium citrate reduced the activity of a purified yeast hexokinase. It reduces the rate of sugar phosphorylation by crude mitochondrial extracts from barley roots and increases concentrations of ATP in barley roots. Clarkson (1969) concluded that Al^{+3} acts directly or indirectly to prevent the use of ATP in glucose phosphorylation.

In contrast, Klimashevskii and associates (1973) and Bernatskaya (1973) reported the activities of ATPase and acid phosphotases in pea roots. They concluded that Al^{+3}

injured the cell walls by activating polygalacturonase, which hydrolyzes pea roots and thereby promotes faster Al^{+3} penetration. The increased activity of acid phosphatase was interpreted as an induction of damage to membranes which had lead to liberation of enzymes from lysozomes, mitochondria and other PO_4^{-3} containing cell structures.

Aluminium Uptake and Translocation:

There are certain plants that are Al^{+3} tolerant. Al^{+3} tolerant plants may be divided into 3 groups with respect to Al^{+3} concentration in plant tops.

(i) Al^{+3} concentration in tops are not consistently different from those in Al^{+3} sensitive plants. However, the roots of such tolerant plants often contain less Al^{+3} than those of sensitive plants. In such cases tolerance is apparently due to an Al^{+3} exclusion mechanism e.g. in wheat, soyabean, barley, etc.

(ii) Al^{+3} tolerance is associated with lower Al^{+3} levels in tops and intrapment of excess Al^{+3} in roots e.g. in azalea, cranberry, rice, triticale rye, alfalfa clones, etc.

(iii) Al^{+3} tolerance is directly associated with Al^{+3} accumulation by the tops, e.g. in tea, certain Hawaiian grasses, *Atriplex hastates*, pine tree, and Mangrove.

From respiration inhibitor studies with DNP, Rhue (1976) concluded that Al^{+3} uptake was not an active process but rather the result of passive diffusion across the plasmalemma. He suggested that the role of metabolism in Al^{+3} uptake is in the maintenance of root membrane structure and integrity.

Matsumoto <u>et</u> <u>al</u>. (1977) speculated that plant differences in Al^{+3} tolerance may depend upon differences in structural properties of cell walls, particularly in the extent of methylation of galactoronic acid; however, he found no positive interaction between Al^{+3} on the rootcell surface was associated with the polymerization of the absorbed monomeric Al^{+3} due to pH increase in the root.

<u>Beneficial Effect of Aluminium</u>:

Al^{+3} is not generally regarded as an essential element for plant growth. Nevertheless under certain conditions, low concentration of Al^{+3} can stimulate growth or produce other desirable effects. Possible explanation of this effect includes:

(a) Increasing iron solubility and availability in calcareous soils in slightly acid, neutral or alkaline nutrient-solutions by hydrolysis of Al^{+3} and a pH-decrease.

(b) Correcting or preventing internal iron deficiency by displacing iron from bound metabolically inactive sites in calcicolous plants.

(c) Correcting or preventing phosphorus toxicity (Al^{+3}-tolerant corn-inbreds) etc.

(d) Blocking negative charges on active sites and thereby promoting phosphorus uptake (Eucalyptus).

These positive effects may be related to

(i) increased uptake of K^{+1} and Mg^{+2} (Lee 1971)

(ii) Displacement of iron by Al^{+3} from bound cites within the plants, thus enabling better iron-transportation and iron-utilization (Grime and Hogson, 1969).

Using Electron Paramagnetic Resonance spectroscopy (EPR) Al^{+3} was shown to produce a dramatic decrease in membrane lipid fluidity in case of microoganisms, eg. <u>Thermoplasm acidophilum</u>, at pH 2. The ability of Al^{+3} to alter lipid fludity was enhanced with increase in pH from 3-5. At pH 4, 10^{-2}M Al^{+3} increased the lower lipid layer phase transition by 39°C. A detectable change was observed with $AlCl_3$ at concentration as low as 10^{-5}M, the ability of Al^{+3} to increase the lower lipid layer phase transition

temperature of Thermoplasm acidophilum is the largest of any cation/lipid interaction yet reported (Haug and Vierstra, 1978).

The influence of Al^{+3} on the cell size distribution of two green algae, i.e. Monographidium and Stichococcus is chosen to be as follows: The cell size of Monographidium was more differentiated than that of Stichococcus. This distribution changed some what during the growth period but continued to manifest itself in the two algal species. Monographidium showed bimodel cell division whereas Stichococcus showed a single peak distribution pattern. Changes in mean cell size for the two algal populations were similar during growth in batch culture. The cells grew larger before division, mean-cell size increased during the logarithmic growth phase; during the stationary growth phase a slight increase reappeared. Both species displayed cell decomposition when exposed to Al^{+3}. The surviving cells grew larger than the control cells when Al^{+3} was added to the medium. The effect of Al^{+3} concentration on cell size differed during the growth phase for two algal cultures. The algal populations seemed to be most sensitive during the lag and the early logarithmic growth phases.

Aluminium Effect on Photosynthesis Process

Wavare and Mohanty (1983) reported that in isolated chloroplasts of cyanobacterium Synechococcus cedrorum, Al^{+3} at a low concentration stimulates the PSII catalysed electron flow and at high concentration inactivates it. The stimulation of Hill activity with a low concentration Al^{+3} may be due to coupling of the electron transport as a result of a change in membrane organisation while the loss of Hill activity at high Al^{+3} concentration results from inactivation of oxygen evolution, they induce the expression of the PSI mediated electron transport both in the isolated beat-spinach chloroplasts and Synechoccus spheroplasts. The extent of stimulation of PSII mediated function by Al^{+3} ion is more than the extent of inhibition of PSI activity (Wavare et al., 1983).

The effect of intracellular ATP prevails on the light induced H^+-efflux from intact cells of Cyanidium caldarium is reported to be as follows: light induced H^+- efflux, observed at acidic pH in case of Cyanidium cells, was shown to be an active H^+ transport depending on the intracellular ATP produced by cyclic-photophosphorylation. Triton X-100 was found to act as an effective uncoupler in intact Cyanidium cells without collapsing the pH gradient across the plasma membrane. Triton X-100 at 0.015 percent

significantly reduced the intracellular ATP levels, stimulate the pBQ Hill reaction and completely inhibited the light induced H^{+}- effluex. Inhibition of H^{+}-efflux by triton X-100 correlated well with the depression of apparent rate of light induced ATP synthesis as well as with the decrease in the intracellular ATP level in light. The light-induced H^{+}-efflux was completely inhibited by diethyl-stiblestrol, a specific inhibitor of Plasma membrane ATPase, without any change in intracellular ATP level, thereby suggesting the participation of the plasma membrane ATPase in the light induced H^{+}- efflux.

Respiration-dependent H^{+} efflex from intact cells of Cyanidium caldarium, reported by Hotta and Enami (1984) is as follows: An active H^{+}-efflux depending on respiration was found in an acidophilic unicellular alga Cyanidium. Alkalization of the medium due to passive H^{+} transport into the cell was observed when the respiratory activity was inhibited by adding respiratory poisons, viz. rotenone, or antimycin A, or by introducing pure nitrogen into the cell-suspension. The extent of H^{+}-influx increased as the pH of the medium was lowered to 2.9, indicating that H^{+} leaks into cells according to the pH - gradient across the plasma membrane. The medium pH, that had increased under anaerobic conditions, returned to the original level with aeration of

the cell suspensions. These observations suggest that an active H^+ transport related to respiration, pumps out the excess H^+-accumulated in the cells during anaerobic preincubation. The pH changes in the cell suspension were found to be related to the intracellular ATP level. From these results it was concluded that active H^+ efflux dependent upon oxidation phosphorylation, therefore, functions in the dark to maintain a constant intracellular pH against passive H^+-leakage through the plasma membrane.

Cyanidium caldarium M-8 type, grown in the dark, was illuminated for three days, and changes in its cell and cell-organelle structures and of photosynthetic activity, were observed quantitatively. Dark grown cells showed no photosynthetic activity and no phycocyanin synthesis. During three days illumination, they fully recovered their photosynthetic activities, as measured by Hill reaction, also synthesized Chla and phycocyanins. Size of cells, their number, chloroplast morphology and its nucleoid structure, which was observed by flourescence microscopy after staining it with DAPI, increased simultaneously after illumination. Concomitant with the recovery of Hill activity, the chloroplast size, with their ring shaped nucleoid, increased especially rapidly. In fully recovered cells, after three days, a good correlation was found between the size of cells, chloroplast and chloroplast nucleoids.

The Organism Cyanidium:

Structure of Cyanidium caldarium:

The acido and thermophilic uni-cellular alga, *Cyanidium caldarim* is a common member of the microflora in acid hot springs throughout the world. The alga has the following remarkable physiological and structural characteristics:

It grows optimally at pH 3.0 and at 45° - $50^{o}c$. It has a thick cell wall, about 0.05 u, which is markedly rich in protein. The relationship between the structure and function of the alga is of particular interest. The alga has thick cell wall, a single well defined chloroplast, a nucleus and a large vacuole. Bailer and Staehelin (1968) reported that the cell wall of the alga is unusually rich in protein and suggested that the higher protein content of cellwall is related to the acid and heat-tolerance. Kleinschmidt and McMohan (1970) studied the effect of growth temperature on the lipid composition and indicated that heat tolerance is attributable to the high degree of saturation of fatty acids contained in the membrane. The thick cellwall consists of two electron dense layer (each ca 5 nm thick) and one less dense layer (ca 30 nm thick) and a well defined lamellar structure of a single chloroplast. No pyrenoid was discovered. The double membrane formation of

the chloroplast envelop was rather indistinct, mostly it consisted of only one layer. There was no vacuole in the cell.

Physiological Role of the Cellwall:

1) A partially disintegrated cell preparation of *Cyanidum caldarium* (i.e. the 100 G fraction; cells from which the outer parts of the cell wall had been removed) was obtained by differential centrifugation of a cell suspension treated with a french press.

2) The 100 G fraction cells had completely lost the outer parts of their cell walls, but retained their sub cellular structure unchanged and had hill activity that was equally as high as that of the intact cells.

3) The Hill activity of the 100 G fraction showed an optimum at pH 7.0 and at 35°c. This activity was lost under acid and high temperature conditions (e.g. pH 3.0, 10 min) or pre-illumination ($3x10^5$ lux, 30 min) though intact cells were not inactivated by these treatments. However, no remarkable difference in sensitivity towards inhibitors was found between the 100 G fraction and intact cells.

5) We thus conclude that the cellwall plays an important role in the acido- and thermophily of *Cyanidium caldarium* cells (see Fig.5).

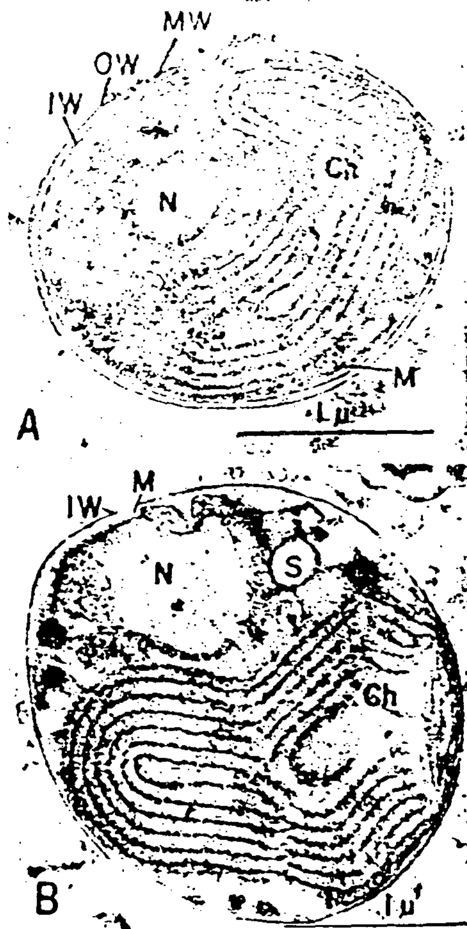


Fig. 5 Electron micrograph of intact cells of Cyanidium caldarium.

A. Thin section of an intact cell. The cell shows a thick cell wall with atleast three layers and a well defined lamillar structure of a single chloroplast. No pyrenoid was discovered. The double membrane formation of the chloroplast envelop was rather indistinct, mostly it considered of any one layer. There was no vacuole in the cell.

B. Thin section of intact cells. Outer parts of the cell wall were removed, the low electron dense granules denoted as starch-like granules may be cyanophycean or floridean starch (Isao, et al., 1975b).

OW, Outer layer of the cell wall
MW, Middle layer of the cell wall
IN, Inner layer of the cell wall
M, Cell membrane
Ch, Chloroplasts
N, Nucleus
S, Starch-like Granules

Mechanism of the Acido- and Thermophily of *Cyanidium caldarium*

Photosynthetic oxygen evolution in an acido- and thermo-philic unicellular alga, *Cyanidium caldarium*, was measured under various conditions (i.e. temp. pH, light intensity) using a clark-type oxygen electrode.

1) Maximum Hill reaction activity with pBQ as the Hill oxidant was obtained at 45°C in a wide pH range from 1.0 to 7.0.

2) The pH activity curve showed two peaks at pH 3.0 and 7.0. The Hill activity had an optimum at pH 3.0 in cells preilluminated under strong light (300,000 lux, 30 min, 40°C). Sonication of algal cells abolished the pH 3.0 component of the Hill reaction producing an activity maximum at pH 7.0.

3) Endogenous oxygen evolution in the absence of the Hill oxidant which lasted for several minutes after illumination, had a maximum at pH 7.0.

4) This endogenous O_2 evolution was abolished by sonication.

5) KCN inhibited endogenous O_2 evolution, but not the Hill reaction in the presence of p-benzoquizone.

The Plan of Work

The following parameters are to be studied in the presence and absence of $AlCl_3$ in white light, red light and green light in red alga Cyanidium caldarium.

(i) Rate of growth (as optical density/unit area).

(ii) Chlorophyll a estimation

(iii) Protein estimation.

(iv) Absorption spectra properties.

(v) Photosynthetic Electron Transport activity (O_2 evolution, H_2O -> pBQ).

(vi) Fluorescence characteristics.

Culture medium pH 3.5, Temperature 30±1 or 2°C

EXPERIMENTAL MATERIALS AND METHODOLOGY

Cyanidium caldarium is a thermophilic unicellular red alga which grows at very low pH ranging from 1 to 5.5. This alga was brought from Japan (see Fig. 6).

Culture Conditions

The composition of the liquid broth used in the present study to culture *Cyanidium caldarium* is given below :

MACRONUTRIENTS	QUANTITY g/l
Ammonium Sulphate - $(NH_4)_2SO_4$	0.35
Potassium Dihydrogen Orthophosphate - (KH_2PO_4) Monobasic	0.183
Calcium Chloride - $CaCl_2.2H_2O$	0.183
Magnesium Chloride - $MgCl_2.6H_2O$	0.183
MICRONUTRIENTS (A_5)	
Ferrous Sulphate - $FeSO_4.7H_2O$	0.0026
Manganese Chloride - $MnCl_2.4H_2O$	0.0026
Cobalt Chloride - $CoCl_2.6H_2O$	0.0026
Sodium Molybdate - $Na_2MoO_4.2H_2O$	0.0026
Cupric Chloride - $CuCl_2.2H_2O$	0.0026

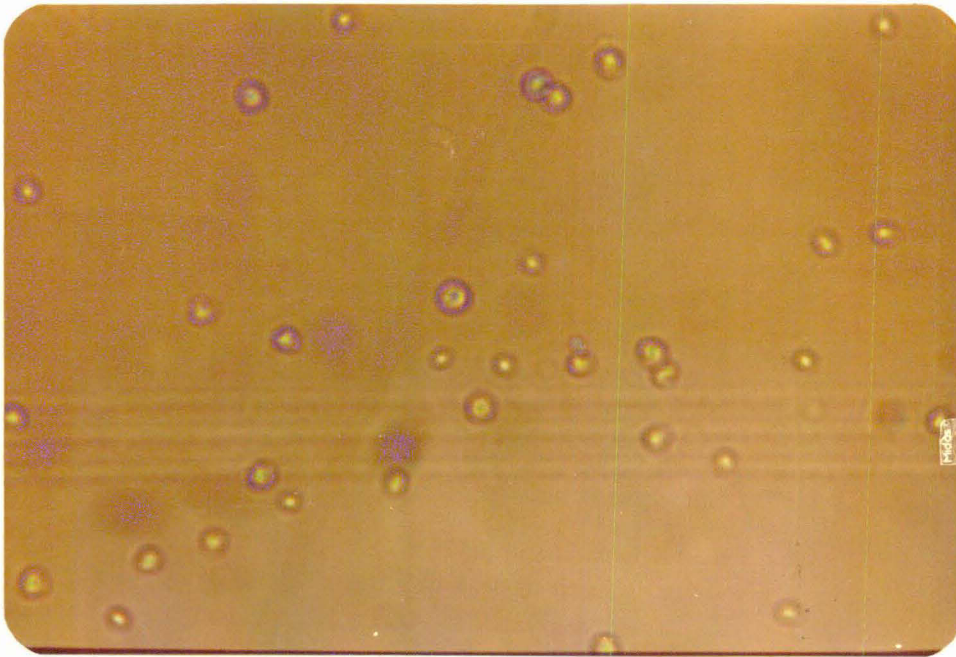


Fig. 6 Intact cells of *Cyanidium caldarium*.

Culture was kept at pH 3.5; temperature at which culture was maintained was 30$\pm$1 or 2°C.

Culture Vessels and Sterilization:

Superior corning glass conical flasks were used for the growth and maintenance of culture. Culture medium was prepared in conical flask.

pH of the culture medium was determined by pH meter and adjusted to 3.5. This culture was autoclaved at 15 lb/inch^2 for 15 min. The pipettes used for transfer of culture were also autoclaved in a pipette container covered with tinfoil. Plastic tubes, rubber corks, glass bending tubes were also autoclaved. Distilled water was also autoclaved before using for bubbling.

Inoculation and Maintenance of Culture

Cyanidium caldarium was inoculated in sterilized culture medium. The stock mother culture was maintained in culture room under continuous illumination by fluorescent tubes. Light intensities of fluorescent tubes were as follows: White light, 0.75 WM^{-2}; red light, 0.55 WM^{-2} and green light, 0.70 WM^{-2}.

Culture media with or without $AlCl_3$ were maintained at the same temperature as mentioned above. White, red and green lights were provided to culture in culture room to study the effect of white, red and green lights on the alga. All the culture flasks were continuously bubbled with steriled air. The cultures were maintained in a bacteria free state by regularly transferring the exponentially growing culture to fresh sterile medium. These cultures were periodically examined to check contamination (Fig. 7). The following parameters were studied :

Extraction and Estimation of Pigments:

1. Optical Density Measurement:

Five ml of cell suspension was drawn from control and treated cell culture after thoroughly shaking the flasks and the optical density of the cell suspension was measured in Shimadzu UV-260 dual beam spectrophotometer at 550, 630 and 678 nm wavelengths at room temperature.

2. Chlorophyll *a*:

Five ml of *Cyanidium caldarium* cell suspension was drawn from control and treated cultures after thoroughly shaking the flask, and cells were collected by centrifuging at 5,000

Fig. 7 Growth condition of the *Cyanidium caldarium* in presence and absence of $AlCl_3$. Culture was bubbled by sterile air.

rpm for 10 min at room temperature, in a table top Remi centrifuge. Then the pellet was suspended into 0.5 ml of reaction buffer. Then 20 µl cells were drawn and 2.98 ml methanol was added to it. These were then properly mixed in vortex mixer and centrifuged again in the above mentioned centrifuge. The supernatant was taken for Chl*a* estimation and optical density was taken at 665 nm in Shimadzu UV-260 dual beam spectrophotometer.

The chlorophyll *a* was estimated using the extinction coefficients E_{665} = 74.5 mg ml^{-1} cm^{-1} (methanol) (MacKinney, 1941).

3. Estimation of the Total Protein Content :

The total cell protein content was determined with folin ciocalteu reagent according to Lowry *et al*. (1951). The reagents used were as follows:

1. 2% of sodium carbonate in 0.1N NaOH.
2. 0.5% copper sulphate in 1% potassium sodium tartarate.
3. To 50 ml of reagent 1, one ml of reagent 2 was added.
4. Dilute folin-ciocalteu reagent (1:1 vol/vol).

Five ml of cell suspension was drawn from control and treated cell culture after thoroughly shaking the flask and

the cells were collected by centrifuging at 5,000 rpm for 10 min at room temperature. The pellets were washed with 5 ml of saline (0.1% NaCl). The washed pellets were suspended in 5 ml of 10% TCA and allowed to stay at -20°C for overnight and after thawing centrifuged at 10000 rpm for 15 min. The pellet was suspended in 2 ml of 1N NaOH and thoroughly mixed. To 50 μl of test solution 0.95 ml of 1N NaOH was added. To this 1 ml of protein solution, 5 ml of alkaline copper sulphate solution was added and allowed it to stand for 10 min at room temperature. To this solution0.5 ml of diluted folin-ciocalteu reagent was added rapidly and thoroughly mixed and allowed to stand for 30 min for full colour development. The optical density was measured at 750 nm in Shimadzu UV-260 spectrophotometer against a reagent blank. A set of standard BSA solution (10-100 μg) was taken for standard curve preparation.

Standard curve was drawn by plotting optical density against μg/ml of standard protein (see Protein Standard Curve, Fig. 8).

4. Absorption Spectra of Intact Cells:

After harvesting the cells, the pellets were suspended in 3 ml of reaction buffer. The reaction mixture contained reaction buffer 0.25 mM HEPES-NaOH (pH 7.5), 20 mM NaCl and

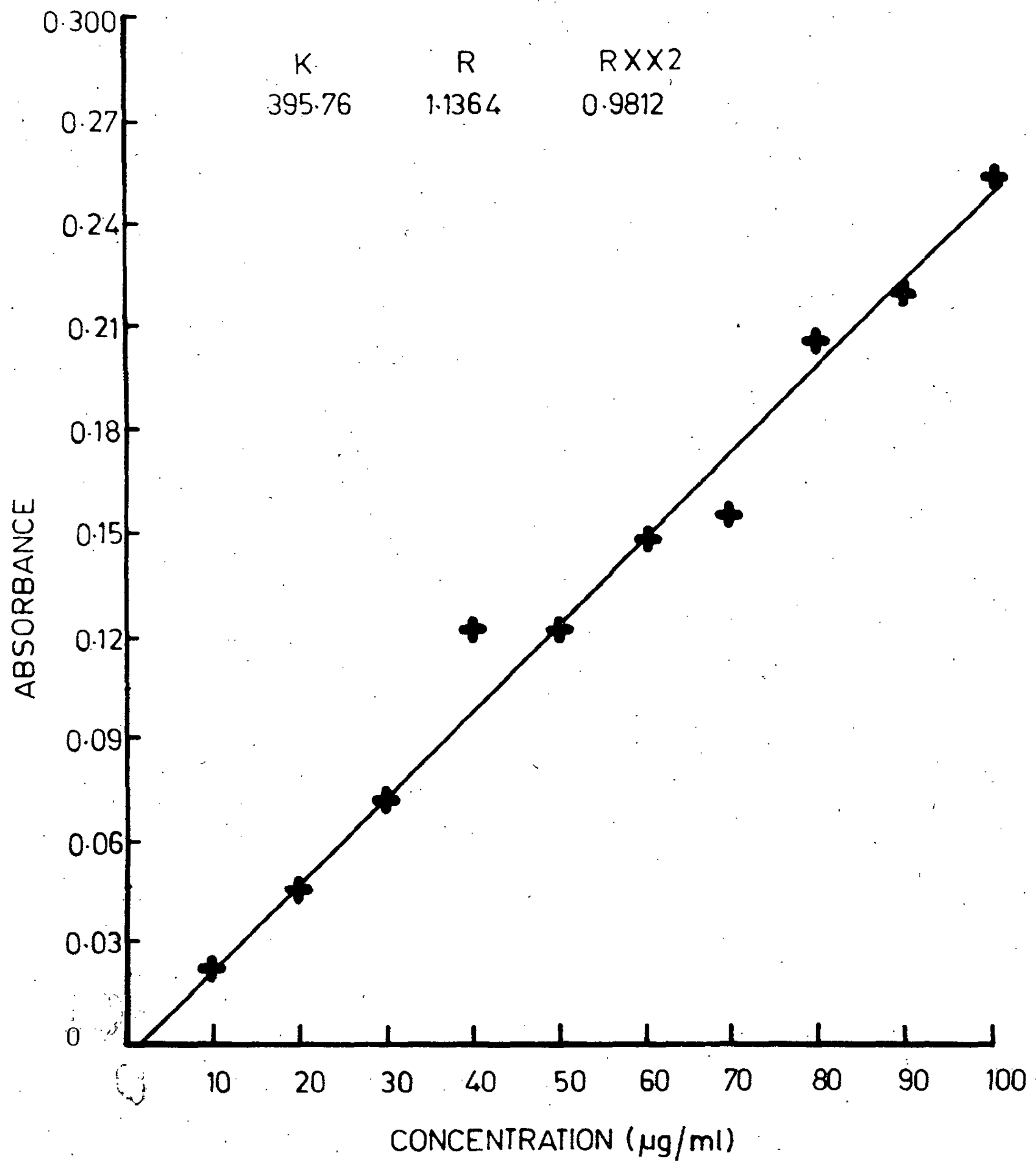


Fig. 8 Estimation of Protein by Lowry Method.

the intact cells equivalent to 10-15 µg Chl*a* from control and treated cell culture. This cell suspension was taken for scanning the absorption spectra from 400 nm to 750 nm. The absorption spectra of intact cells suspension were taken in UV-260 double beam spectrophotometer. At 750 nm the absorption of the cell suspension was adjusted to give approximately the same reading.

5. Fluorescence Emission Spectra of Intact Cells:

After harvesting the cells, the pellets were suspended in 3 ml of reaction buffer. The reaction mixture contained reaction buffer 0.25 mM HEPES-NaOH (pH 7.5), 20 mM NaCl and the intact cells equivalent to 12-15 µg Chl*a* from control and treated cell culture.

In intact cells the phycocyanin was excited at 545 nm to take the emission spectra of intact cells. The emission spectra was scanned from 600 nm to 750 nm in Shimadzu (RF-540) spectrofluorophotometer.

6. Photosystem II Assay

Para Benzoquinone (pBQ) mediated electron transport of PSII (H_2O -> pBQ) was conducted in intact cells. Being a lipophilic compound pBQ enters into the intact cells and

accepts electrons at plastoquinone (PQ) position (Warburg, 1941; Tribst, 1974). The reaction mixture contained reaction buffer 0.25 mM HEPES-NaOH (pH 7.5), 20 mM NaCl, 0.5 mM pBQ and the intact cells equivalent to 12 to 15 µg Chla.

CHEMICALS

HEPES : NaOH, PEG, $CaCl_2$, pBQ - Sigma Chemical Company; DCMU from Dupont Chemical Company; $AlCl_3$ from B.D.H.; Citric acid, sodium phosphate. All the other chemicals are of analytical quality.

RESULTS

Cyanidium caldarium is a thermophilic and acidophilic alga which grows at very low pH (1-5). It is a eucaryotic, unicellular alga consisting of double membrane organelles such as chloroplast, prominent nucleus, mitochondria etc. It also contains of phycobilin pigments (phycobilisomes) like cyanobacteria.

Cyanidium caldarium was grown in different concentrations (2 mM, 3 mM, 5 mM and 10 mM) of $AlCl_3$. It was observed that in 2 mM and 3 mM concentration of $AlCl_3$ there was a slight stimulation in cell growth in treated cells than in control cells. It was also observed that in 5 mM $AlCl_3$ there was a significant stimulation in cell growth. But in 10 mM $AlCl_3$ treated culture there was inhibition in growth (data not shown). Hence 5 mM $AlCl_3$ was used in our studies. It was also observed that in green and red light there was no significant change in $AlCl_3$ treated cells as compared to control cells. So white light was used for our studies.

We have considered optical density as a growth parameter and monitored the growth in presence and absence of $AlCl_3$.

1a. Effect of $AlCl_3$ on Growth Pattern in Intact Cells of *Cyanidium caldarium* (Optical density at 550 nm)

Cyanidium caldarium was grown in presence and absence of 5 mM of $AlCl_3$. The Optical densities were measured in control and treated cells in Shimadzu UV-260 dual beam spectrophotometer at 550 nm. It was observed that in the beginning cell growth was slow in both control and treated cells. But the cell growth was less in treated cells than in control cells upto 6 days. The optical density measured at first day was 0.084 in both control and treated cells. On 6th day, it was 0.22 and 0.16 in control and treated cells respectively. After 6 days, it was observed that cell growth in treated samples showed the gradual increase in optical density. The optical density measured at 7th day was 0.324 and 0.34 in control and treated cells respectively. At 9th day, optical density measured was 0.62 and 0.72 in control and treated cells respectively. On 12th day, the increase in growth rate was maximum showing optical density a value of 1.18 and 1.36 in control and treated cells respectively. After 12th day, there was a gradual decrease in growth rate in control cells. But, this decrease in growth rate was less in treated cell culture. The 12th day was considered as the late log phase of the *Cyanidium caldarium* (Table 1a and Fig. 9a).

Table 1a: $AlCl_3$ - induced stimulation of growth in terms of optical density at 550 nm in intact cells of Cyanidium caldarium

Days	Control cells	Treated cells (5 mM $AlCl_3$)
1	0.084 ± 0.0004	0.084 ± 0.0007
2	0.090 ± 0.0004	0.080 ± 0.0004
3	0.102 ± 0.0004	0.078 ± 0.0004
4	0.142 ± 0.0004	0.075 ± 0.0004
5	0.160 ± 0.0004	0.083 ± 0.0004
6	0.220 ± 0.0003	0.160 ± 0.0004
7	0.324 ± 0.0004	0.340 ± 0.0003
8	0.395 ± 0.0003	0.490 ± 0.0001
9	0.620 ± 0.0007	0.720 ± 0.0007
10	0.800 ± 0.0004	0.962 ± 0.0004
11	1.042 ± 0.0004	1.195 ± 0.0004
12	1.180 ± 0.0001	1.360 ± 0.0001
13	1.178 ± 0.0001	1.356 ± 0.0001
14	1.170 ± 0.0001	1.350 ± 0.0001

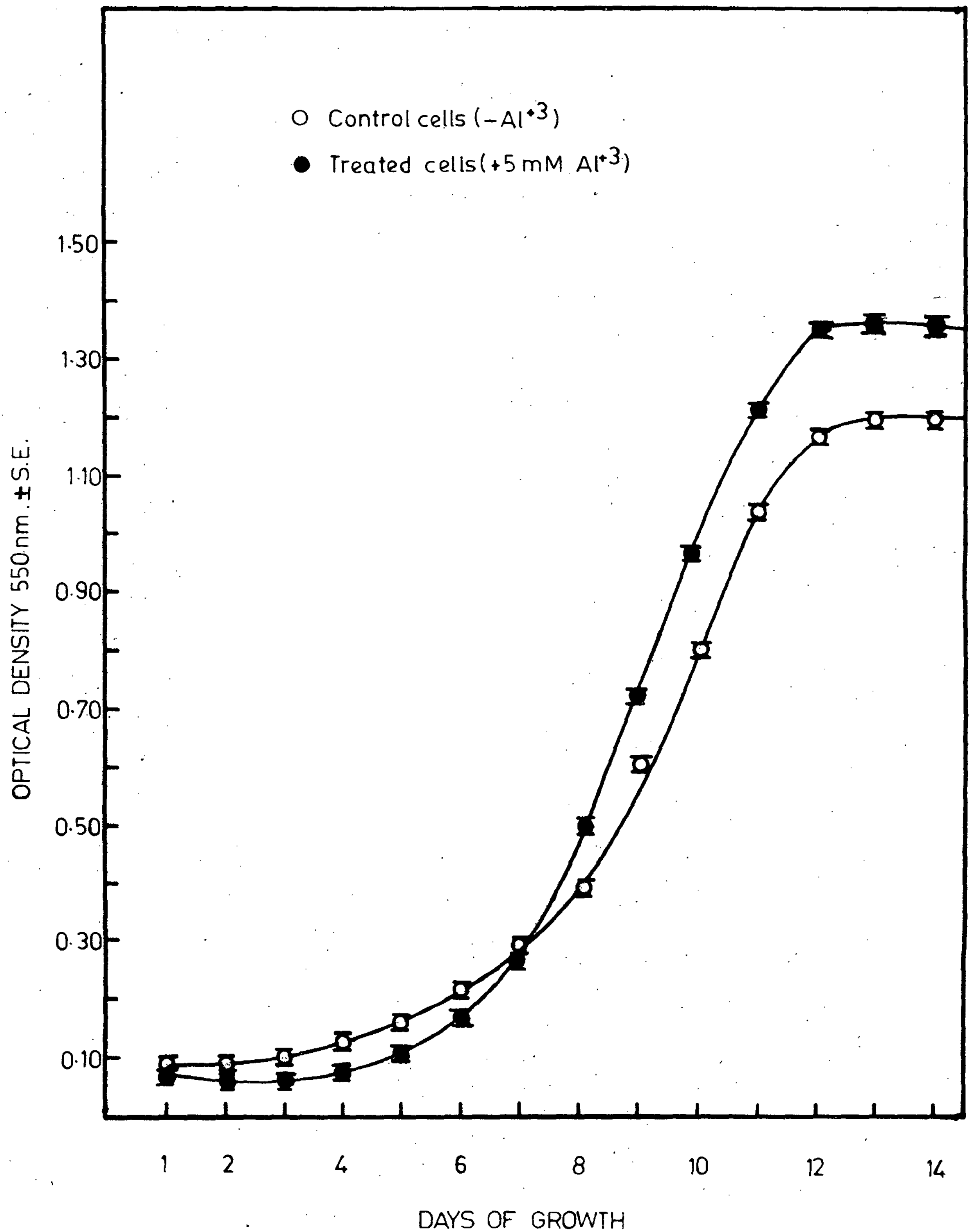


Fig. 9a $AlCl_3$ induced stimulation of cell growth in terms of OD at 550 nm in intact cells.

1b. **Effect of $AlCl_3$ on Phycocyanin Absorption in Intact Cells of *Cyanidium caldarium***

The optical density measured at 630 nm was assumed as the light absorption by phycocyanin pigment. The optical density measured at 1st day was 0.086 and 0.087 in control and treated cells respectively. It was observed that optical density measured upto 6th day was less in treated cells than in control cells. At 6th day, in control cells OD was 0.25 while in treated cells OD was 0.20. But after sixth day, optical density increased in treated cells than in control cells. The optical density at 7th day was 0.32 and 0.35 in control and treated cells respectively. It was clear that on 1st day there was no difference in optical density at 630 nm of phycocyanin. After six days, there was increase in optical density of phycocyanin in treated cells. On 9th day, optical density was 0.68 and 0.84 in control and treated cells respectively. But on the 12th day, it was maximum, i.e., 1.35 and 1.53 in control and treated cells respectively, which could be possible due to stimulatory effect of $AlCl_3$ in phycocyanin content in treated cell culture. After 12th day, stationary phase of the alga started and decrease in optical density was observed (Table 1b and Fig. 9b).

Table 1b: Effect of $AlCl_3$ on phycocyanin absorption in intact cells of <u>Cyanidium caldarium</u> (OD at 630 nm)

Days	Control cells	Treated cells (5 mM $AlCl_3$)
1	0.086 $\pm$ 0.0004	0.087 $\pm$ 0.0004
2	0.100 $\pm$ 0.0004	0.078 $\pm$ 0.0004
3	0.120 $\pm$ 0.0001	0.080 $\pm$ 0.0004
4	0.160 $\pm$ 0.0007	0.085 $\pm$ 0.0007
5	0.170 $\pm$ 0.0004	0.110 $\pm$ 0.0007
6	0.240 $\pm$ 0.0007	0.200 $\pm$ 0.0007
7	0.320 $\pm$ 0.0004	0.350 $\pm$ 0.0007
8	0.485 $\pm$ 0.0004	0.580 $\pm$ 0.0007
9	0.680 $\pm$ 0.0004	0.840 $\pm$ 0.0004
10	0.942 $\pm$ 0.0004	1.122 $\pm$ 0.0004
11	1.190 $\pm$ 0.0004	1.380 $\pm$ 0.0004
12	1.350 $\pm$ 0.0004	1.530 $\pm$ 0.0004
13	1.340 $\pm$ 0.0004	1.500 $\pm$ 0.0007
14	1.300 $\pm$ 0.0004	1.400 $\pm$ 0.0004

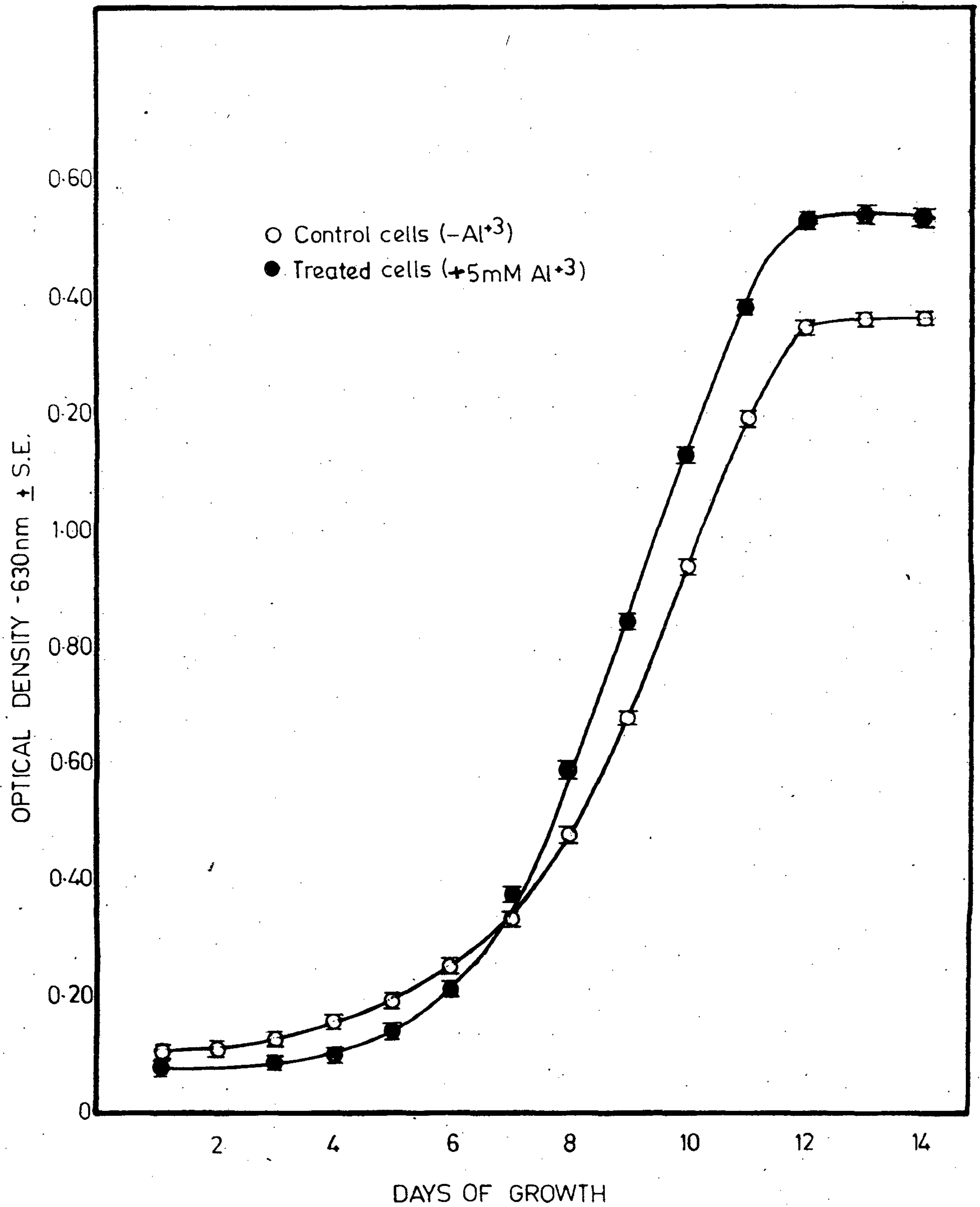


Fig. 9b Effect of $AlCl_3$ on phycocyanin absorption at 630 nm in intact cells.

1c **Effect of $AlCl_3$ on Chlorophyll (Chl) *a* Absorption in Intact Cells of *Cyanidium caldarium***

The optical density measured at 678 nm was assumed as the light absorption by Chl*a*. The OD measured on 1st day was 0.087 in both control and treated cells. It was observed that optical density measured upto 6th day was less in treated than in control cells. At 6th day in control cells, OD was 0.25, while it was 0.22 in treated cells. But after 6th day, OD increased in treated cells as compared to control cells. It was clear that on 1st day there was no change in optical density at 678 nm in control and treated cells. But after 6th day, there was stimulatory effect of $AlCl_3$ on OD. This stimulatory effect was more clear on 9th day showing OD value of 0.77 and 0.88 in control and treated cells respectively. On 12th day optical density was maximum in treated cells than in control cells, shwoing optical density 1.40 and 1.78 in control and treated cells respectively.

This indicated that the stimulatory efect of $AlCl_3$ on optical density of Chl*a* was maximum in treated cells as compared to control cells. After 12th day the culture reached a stationary phase showing decrease in optical density as shown in table 1c and Fig. 9c.

Table 1c: Effect of $AlCl_3$ on chlorophyll absorption in intact cells of <u>Cyanidium</u> <u>caldarium</u> (OD at 678 nm)

Days	Control cells	Treated cells ($5\ mM\ AlCl_3$)
1	0.087 ± 0.0004	0.087 ± 0.0004
2	0.100 ± 0.0007	0.080 ± 0.0007
3	0.120 ± 0.0004	0.078 ± 0.0004
4	0.162 ± 0.0004	0.095 ± 0.0007
5	0.175 ± 0.0001	0.100 ± 0.0007
6	0.250 ± 0.0007	0.220 ± 0.0007
7	0.330 ± 0.0007	0.380 ± 0.0007
8	0.530 ± 0.0007	0.590 ± 0.0007
9	0.770 ± 0.0007	0.882 ± 0.0007
10	0.991 ± 0.0001	1.144 ± 0.0004
11	1.220 ± 0.0004	1.420 ± 0.0004
12	1.400 ± 0.0004	1.780 ± 0.0004
13	1.350 ± 0.0004	1.580 ± 0.0004
14	1.300 ± 0.0004	1.500 ± 0.0004

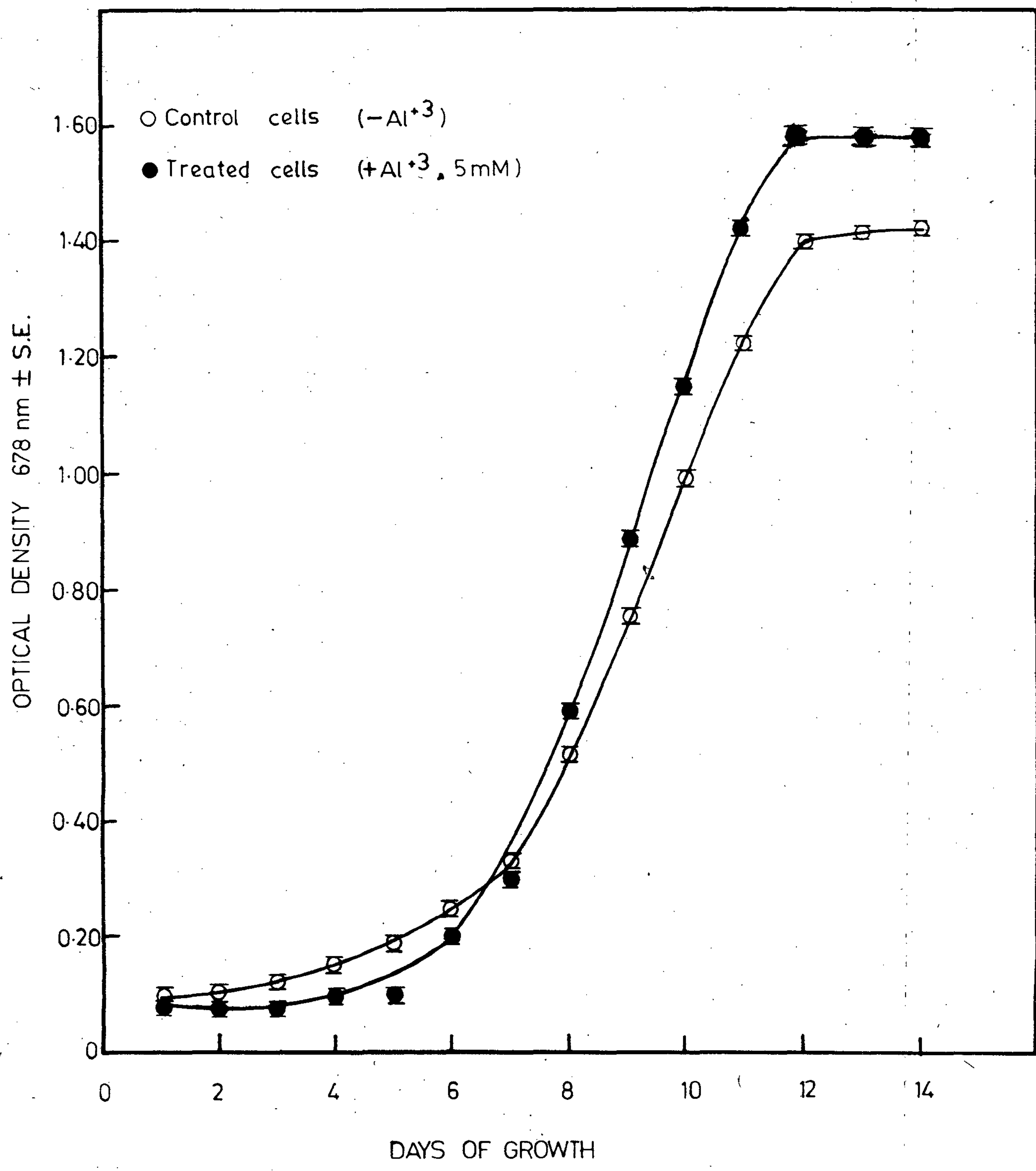


Fig. 9c Effect of $AlCl_3$ on chlorophyll absorption at 678 nm in intact cells.

2. Effect of $AlCl_3$ nn Chlorophyll a Content in Intact Cells:

Five ml of Cynidium caldarium cell suspension was drawn from control and treated cultures after thoroughly shaking the flasks and cells were collected by centrifuging at 5,000 RPM for 10 min, using table top Remi centrifuge. The pellets were suspended in 0.5 ml of buffer consisting of 25 mM HEPES-NaOH (pH 7.5) 1 mM $CaCl_2$ and 7.5% PEG.

Twenty µl cells were drawn and 2.98 ml methanol was added to it. Sample was mixed properly and centrifuged. The supernatant was taken and optical density was measured at 665 nm in Shimadzu UV-260 dual beam spectrophotometer. The concentration of Chla was estimated by using extrinction coefficient E_{665} = 74.5 mg ml^{-1} cm^{-1} (MacKinney, 1941).

The Chla content was estimated at three different days, i.e., at 6th, 9th and 12th day from control and treated cell culture. At 6th the Chla content estimated was 1.23 µg/ml and 0.91 µg/ml in control and treated cells respectively. It was observed that Chla content was less in treated cells as compared to control cells at 6th day. This inhibition might be due to inhibition in cell division and cell number in culture in presence of 5 mM $AlCl_3$, but it was observed that this inhibition was marginal. After 6th day, it was observed that there was increase in synthesis of Chla

content in culture in presence of 5 mM $AlCl_3$. This might be due to the stimulatory effect of $AlCl_3$. At 9th day Chl*a* content estimated was 1.41 µg/ml and 1.86 µg/ml in control and treated cells respectively. At 9th day the Chl*a* content was more in control cells than in treated cells. On 12th day it was increased in treated cells as compared to control cells. The Chl*a* content estimated on 12th day was 2.23 µg/ml and 2.71 µg/ml in control and treated cells respectively (see Table 2 and Histogram 1).

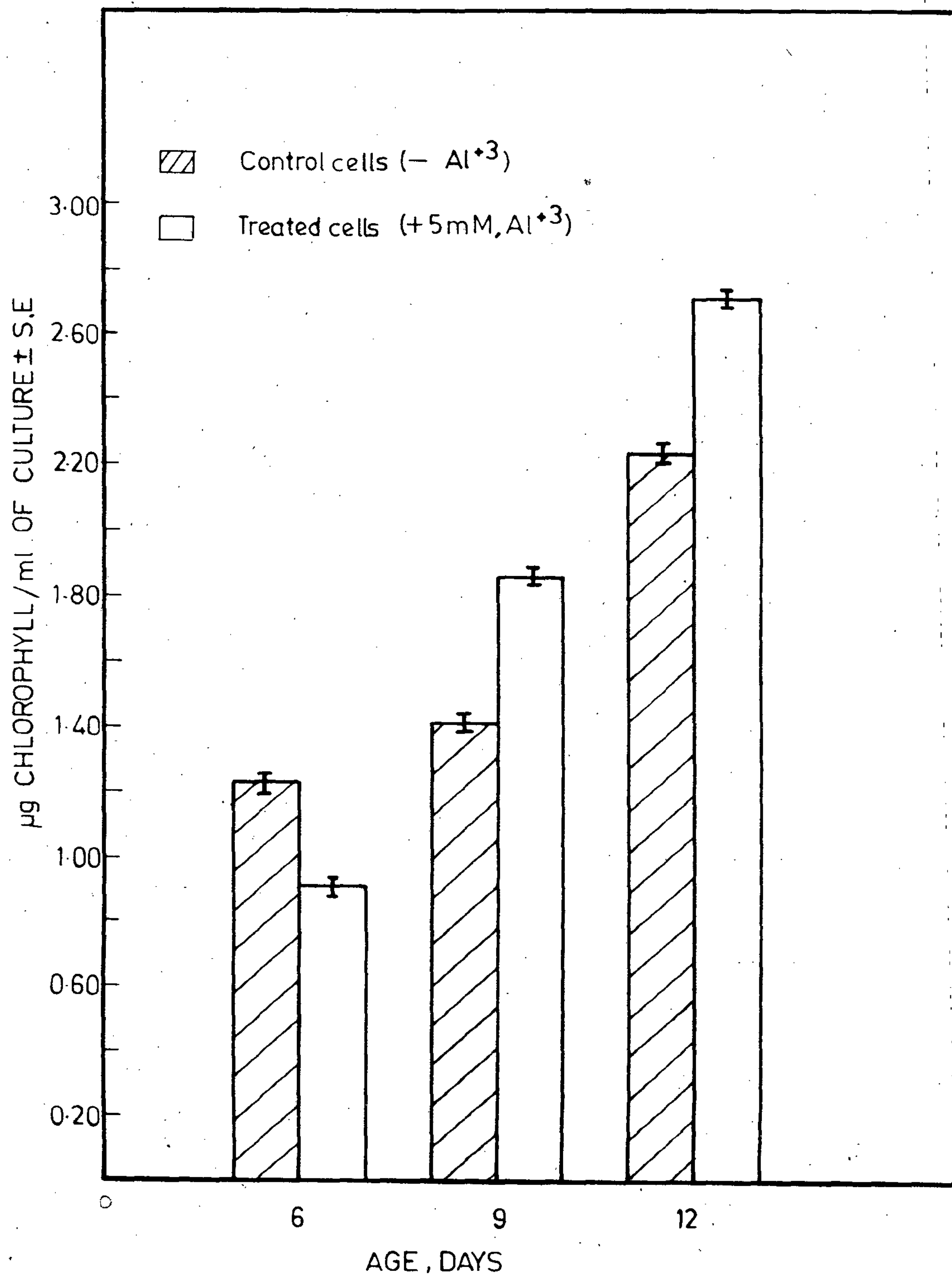
3. $AlCl_3$-Induced Changes in the Protein Content of Intact Cells

Protein content was estimated from intact cell of control and treated cell culture at 3 different days i.e., at 6th, 9th and 12th days. Protein content estimated at 6th day was 40 µg/ml and 24 µg/ml cell culture in control and treated cells respectively. It was observed that in beginning there was inhibition in synthesis of protein content in presence of 5 mM $AlCl_3$ in treated cells upto 6th day. But it was observed that this inhibition was marginal. And after 6th day, there was gradual increase in synthesis of protein in presence of 5 mM $AlCl_3$ as compared to control cells. Protein content estimated at 9th day was 92 µg/ml and 108 µg/ml in control and treated cells respectively. The

Table 2 . Effect of $AlCl_3$ on Chlorophyll a Content in Intact Cells of *Cyanidium caldarium*.

Days	Control Cells ($-Al^{+3}$) (µg/ml)		Treated Cells ($+Al^{+3}$) (µg/ml)	
1.	-	-	-	-
6.	1.20 1.21 1.30	1.23 ± 0.038	0.91 0.90 0.92	0.91 ± 0.004
9.	1.41 1.41 1.42	1.41 ± 0.004	1.86 1.86 1.87	1.86 ± 0.004
12.	2.20 2.20 2.30	2.23 ± 0.04	2.65 2.65 2.84	2.71 ± 0.08

Chlorophyll content was estimated by using extinction coefficient E_{665} = 74.5 mg ml^{-1} cm^{-1} (MacKinney, 1941).



Histogram 1 Histogram showing variation in Chla content in cultures with and without 5 mM $AlCl_3$.

protein content estimated at 9th day was more in treated cells than in control cells and protein content at 12 days old culture was further increased. It was 144 µg/ml and 176 µg/ml cell culture in control and treated cells respectively. The increase in protein content might be due to the increase in cell division and cell number of culture. On the whole it was found that 5 mM $AlCl_3$ had stimulatory effect on the protein content (see Table 3 and Histogram 2).

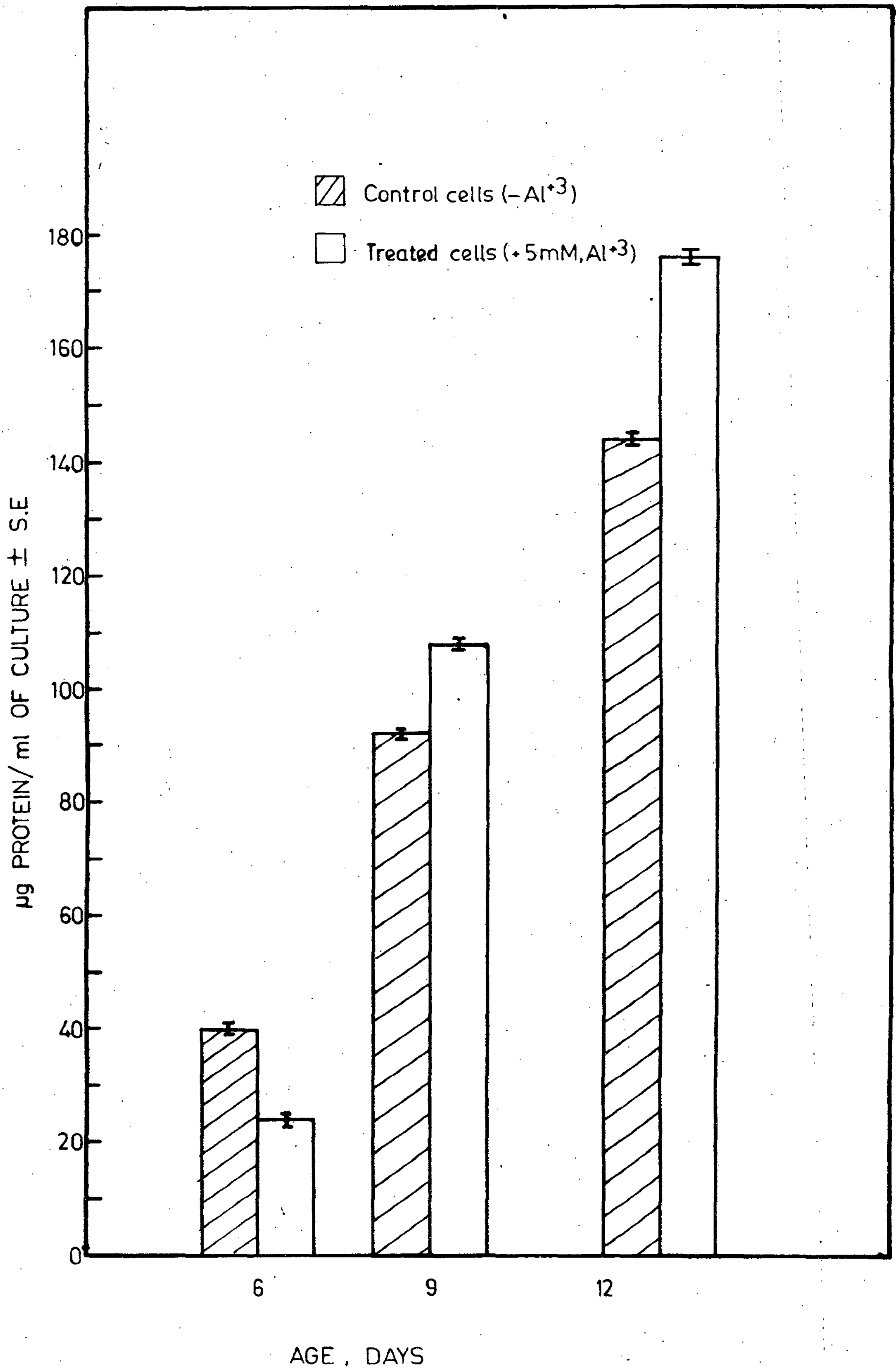
4. Absorption Spectra of Intact Cells

After harvesting the cells, drawn from control and treated cell culture the pellets were suspended in 3 ml of reaction buffer. The reaction mixture contained reaction buffer 25 mM HEPES-NaOH (pH 7.5), 20M NaCl and cells equivalent to 15-18 µg Chl$\underline{a}$. This cell suspension was taken for scanning the absorption specta from 400-750 nm. These absorption spectra were taken in UV-260 dual beam spectrophotometer. The absorption spectrum of cell culture gave four peaks. These peaks were due to light absorption by pigments at 3 different wavelengths, i.e., 678 nm (Chl$\underline{a}$), 630 nm (PC), 480 nm (carotenoids) and 438 nm (Chl$\underline{a}$ soret band).

Table 3: Effect of $AlCl_3$ on Protein Content in Control and Treated Cells in Cyanidium caldarium.

Days	Control cells ($\mu g/ml$)	Treated cells (5 mM $AlCl_3$) ($\mu g/ml$)
1	-	-
6	40 ± 1.1	24 ± 0.41
9	92 ± 0.71	108 ± 0.41
12	144 ± 0.71	176 ± 0.71

Lowry Method (1951) was used for protein content estimation in intact cells of Cyanidium caldarium.



Histogram 2 Histogram showing variation in protein content in cultures with and without 5 mM $AlCl_3$

4a. $AlCl_3$ - Induced Changes in Absorption Properties of Intact Cells :

The absorption spectra were taken from control and treated cell cultures at 3 different days i.e., 6th 9th and 12th day. From 6 days old control cell culture the absorption spectra were taken. This gave four peaks at 678 nm (Chla), 630 nm (PC), 480 nm (Chla soret band). On the 6th day it was found that the ratio of absorbance at 630 nm (PC) and at 678 nm (Chla) was 0.61 and 1.36 in control and treated cells respectively. On 9th day absorption spectra were taken from control and treated cells. The ratio of absorbance at 630 nm (PC) and at 678 nm (Chla) was 0.96 and 0.95 in control and treated cells respectively. In 12 days old culture the ratio of absorbance at 630 nm (PC) and at 678 nm (Chla) was 0.93 and 0.82 is control and treated cells respectively. The absorption spectra were taken from 24th days old control and treated cell culture. The ratio of absorbance at 630 nm (PC) and at 678 nm (Chla) was 0.75 and 0.52 in control and treated cells respectively. This decreases in ratio was due to degradation of phycobilisomes (PBSomes) it was observed that the peak ratios decreased after 6th days old culture in control and treated cells (see Table 4). It was also found that the treated cell culture seemed to be deeper green in colour than in control cells

Table 4. $AlCl_3$-Induced Changes in Absorption Spectra of 12 Days Old Cells

Days	Optical Density at				OD at 630 / OD at 678	
	678 nm		630 nm			
	Control cells	Treated cells	Control cells	Treated cells	Control cells	Treated cells
1	-	-	-	-	-	-
6	0.074	0.050	0.045	0.068	0.61	1.36
9	0.064	0.070	0.061	0.067	0.96	0.95
12	0.116	0.142	0.108	0.116	0.93	0.82
24	0.132	0.210	0.099	0.109	0.75	0.52

which was clearly shown from photographs taken from control and treated cell culture (Fig.10a-e).

5. **Parabenzoquinone (pBQ) Mediated Electron Transport of Photosystem-II (H_2O -> PBQ) in Intact Cells of *Cyanidium caldarium* Grown in Presence and Absence of 5 mM $AlCl_3$**

Photosystem-II assay was conducted in intact cells. Being a lipophilic compound pBQ enters into the intact cells and accepts electrons at plastoquinone (PQ) position (Warburg, 1941; Tribst 1974). The reaction mixture contained reaction buffer (HEPES-NaOH, pH=75), 20 mM NaCl, 0.5 mM pBQ and the intact cells equivalent to 12-15 μg Chl*a*.

In 12 days old cell culture the Hill activity of (PS II assay (H_2O - pBQ) was measured in two buffers. Firstly in citrate-phosphate buffer (pH 2.6-7.0) and Secondly reaction buffer (pH 7.5).

The Hill activity was measured in citrate-phosphate buffer at different pH ranges, pH 4.0, 5.0, 5.6, 6.5, and 7.0. The Hill activity measured in the same buffer at pH 4 was 248.82 and 298.82 μmoles oxygen evolved mg $Chl^{-1} \cdot h^{-1}$ in control and treated cells respectively. It was observed that the Hill activity was more in treated cells than in

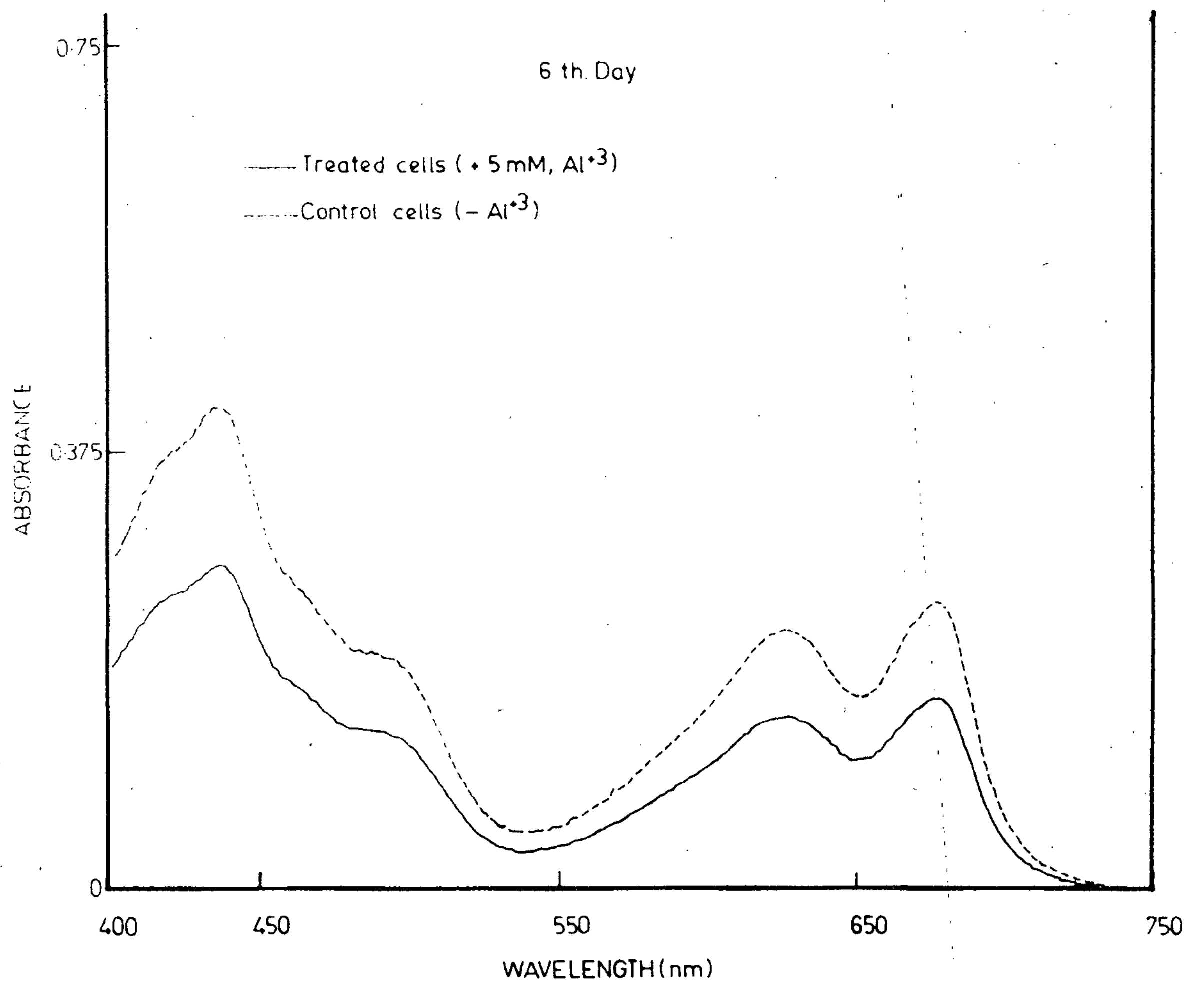


Fig. 10a $AlCl_3$ induced changes in absorption spectra in intact cells (6th day).

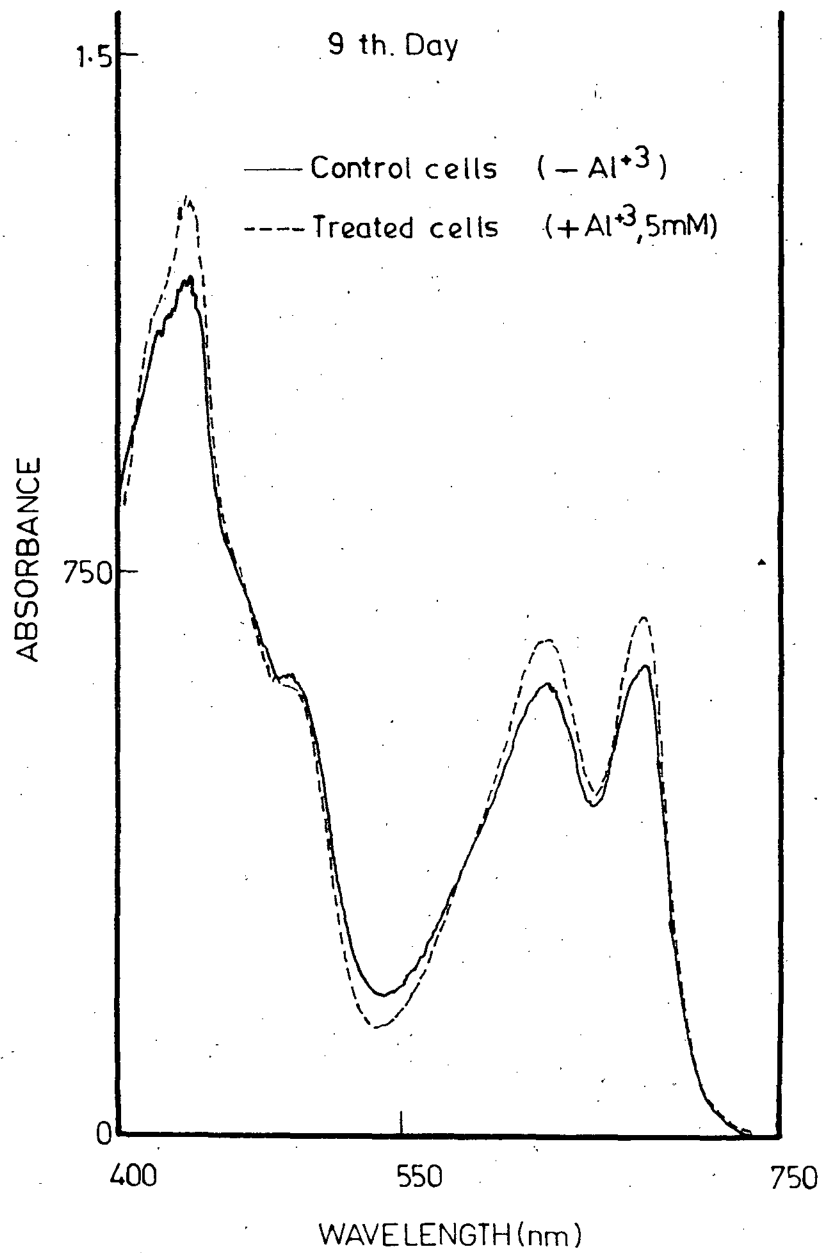


Fig. 10b $AlCl_3$ induced changes in absorption spectra in intact cells (9th day).

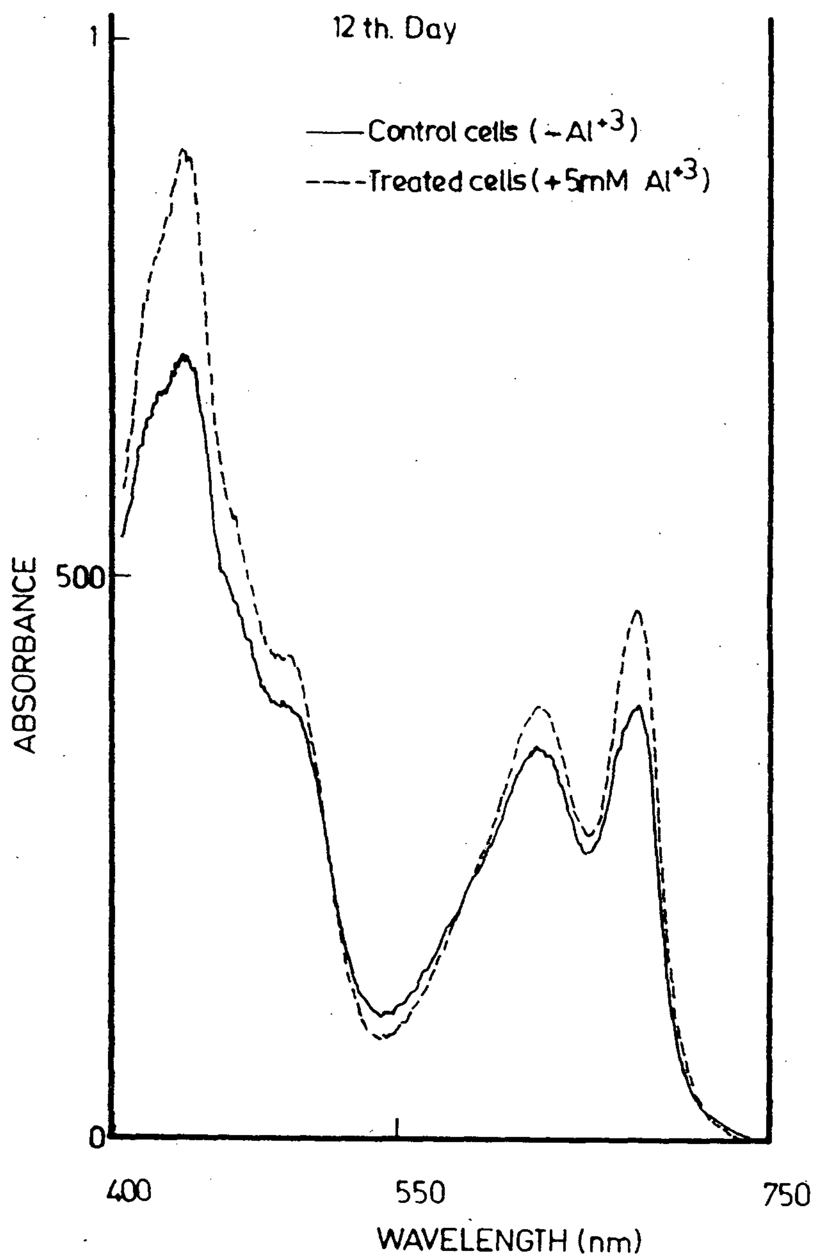


Fig. 10c $AlCl_3$ induced changes in absorption spectra in intact cells (12th day).

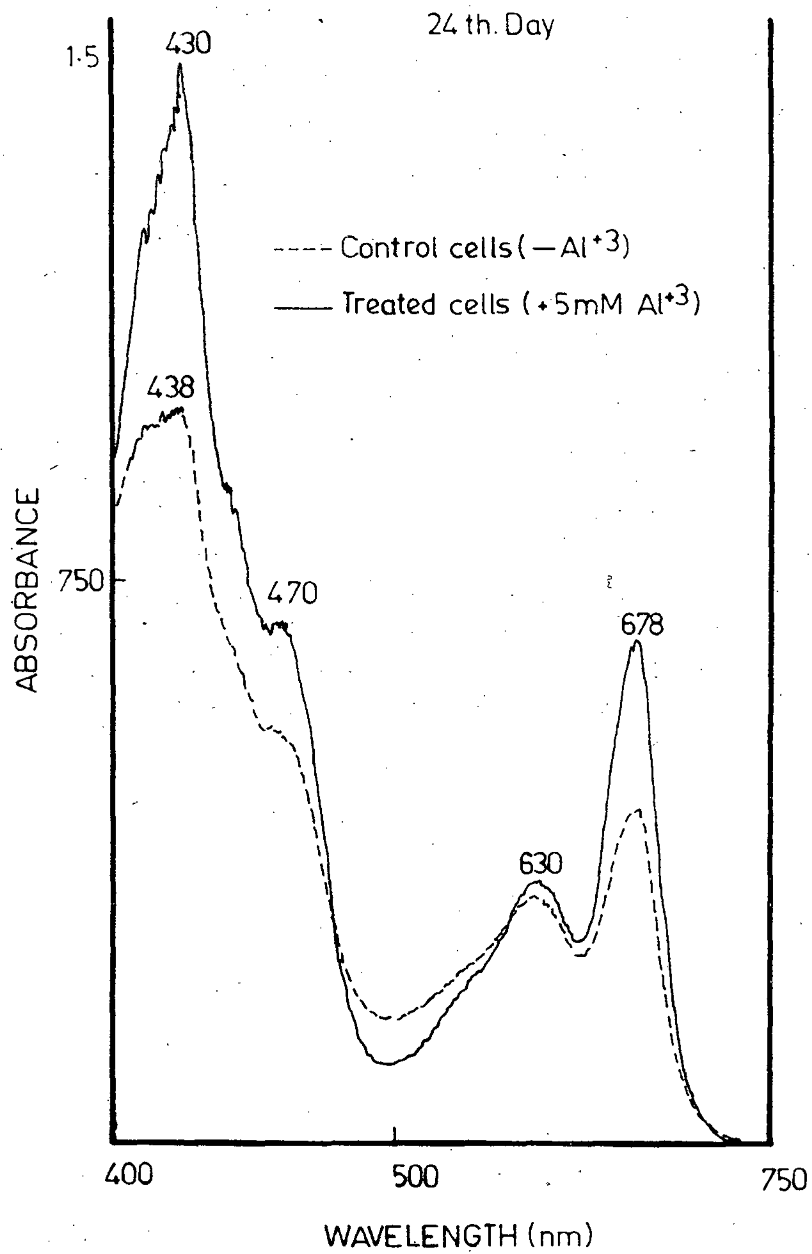


Fig. 10d $AlCl_3$ induced changes in absorption spectra in intact cells of Cyanidium caldarium (24th day).

Fig. 10e $AlCl_3$ induced visual changes in control and treated cell culture.

control cells. In the same buffer at pH 5.0 the hill activity was 398.12 and 447.88 µmoles in control and treated cells respectively. Treated cells showed more Hill activity as compared to control cells at this above pH. At pH 5.6 the Hill activity was measured at 348.35 and 423 µmoles in control and treated cells respectively. The treated cells showed more Hill activity as compared to control cells in this pH range. In pH 6.5 in the same buffer, the Hill activity was 447.88 and 522.53 µmoles in control and treated cells respectively. The Hill activity was observed maximum in this pH 6.5 in treated cells as compared to control cells. In pH 7.0 in the same buffer the Hill activity was 423 and 472.76 µmoles O_2 evolved mg chl^{-1} h^{-1} in control and treated cells respectively.

It was observed that there was a gradual increase in the Hill activity in the citrate-phosphate buffer from pH 4-5.0 in treated and control cells. Later on this activity declined in pH 5.6 and after that in 6.5 this activity increased and in pH 7.0 it declined again in control and treated cells. At pH 5 and 6.5, there were two peaks in control and treated cells (Table 5a).

Another experiment was conducted of pBQ mediated Hill activity in the same buffer. The Hill activity in pH 4.0 was 316.25 and 352.50 µmoles O_2 mg $chl^{-1}h^{-1}$ in control and

Table 5a: Parabenzoquinone Mediated Hill Activity in Control and Treated Cells at Different pH (H_2O -> pBQ) - Experiment I.

pH	Activity (μmoles O_2 evolved mg $Chl^{-1}h^{-1}$)	
	CONTROL	TREATMENT
4.0	248.82	298.82
5.0	398.12	447.88
5.6	348.35	423.00
6.5	447.88	522.53
7.0	423.00	472.76

Chlorophyll contents = 17 μg Chl<u>a</u>/3ml butter, 12 days cells were used. Citrate phosphate buffer pH (4.0, 5.6, 6.5 and 7.0).

treated cells. In pH 5, it was 352.5 and 385.75 µmoles in control and treated cells respectively. In pH 5.6, the Hill activity was 324.3 and 352.5 µmoles in control and treated cells respectively. In pH 6.5, it was 380.50 and 423.00 umoles in control and treated cells respectively. In pH 7.0, Hill activity was 338.40 and 380.70 µmoles in control and treated cells respectively. It was observed that the Hill activity was more in pH 6.5 in control and treated cells and it decreased in pH 7.0. This indicated that the Hill activity was maximum in pH 6.5 (Table 5b).

From these two experiments, it was observed that Hill activity was maximum in citrate-phosphate buffer (pH 6.5).

In another experiment in reaction buffer (pH 7.5), pBQ mediated Hill activity was measured. The activity was found to be 367 and 560 µmoles O_2 mg $chl^{-1}h^{-1}$ in control and treated cells respectively. In treated cells, Hill activity increased as compared to control cell culture (Table 5c).

In another experiment Hill activity was measured in reaction buffer (pH 7.5). It was 116 and 169.55 µmoles O_2 mg $chl^{-1}h^{-1}$ in control and treated cells. Hill activity was increased in treated cells as compared to control cells (Table 5d).

Table 5b: Parabenzoquinone Supported Hill activity in control and treated cells at different pH (H_2O -> pBQ) - Experiment II.

S.No.	pH range	Activity (μmoles O_2 evolved mg $Chl^{-1}h^{-1}$)	
		Control	Treated
1.	4.0	316.25	352.50
2.	5.0	352.50	387.75
3.	5.6	324.3	352.50
4.	6.5	380.50	423.00
5.	7.0	338.40	380.70

12 days old culture was used. Chlorophyll content was 15µg/3ml buffer. Citrate-phosphate buffer consists of 0.1 M solution of citric acid and 0.2 M solution of sodium phosphate pH ranging from 2.6 to 7.0.

Table 5c: Relative O_2 Evolution in control and treated cells - Experiment III.

S.No.	Control cells	Average	Treated cells	Average
1.	347		543	
2.	363	367	535	560
3.	393		604	

Reaction buffer : pH 7.5 consist of 25 mM HEPES-NaOH; 20 mM NaCl. Chl*a* 14 µg/3 ml buffer. 12 days old culture was used.

Table 5d: Relative O_2 Evolution in control and treated cells - Experiment IV.

S.No.	Activity (umole O_2 evolved mg $chl^{-1}h^{-1}$).			
	Control cells	Average	Treated cells	Average
1.	112		174.50	
2.	112	116	163.37	165.55
3.	124		170.79	

Reaction Buffer pH 7.5. 12 days old culture was used. Chl*a* content was kept same in both sets. Chl*a*, 25µg/3ml buffer.

From all the above observations, it was clear that in citrate buffer the activity was maximum in pH 6.5 and in the reaction buffer the activity was maximum in pH 7.5. But the Hill activity was more in reaction buffer (pH 7.5) than in citrate phosphate buffer (2.6 - 7.0).

It was observed that there was stimulation in O_2 evolution rate in $AlCl_3$ treated cells as compared to control cells.

6. Fluorescence Emmission Spectra of Intact Cells of Cyanidium caldarium Grown in Presence and Absence of 5 mM $AlCl_3$

After harvesting the cells from control and treated culture, the pellets were suspended in 3ml of reaction buffer. The reaction mixture contained reaction buffer (pH 7.5) and the intact cells equivalent to 12-15 µg/ml Chla.

Fluorescence emmission spectra of intact cells were measured by exciting the cells at 545 nm. The fluorescence emission spectra were scanned from 600 nm to 750 nm in Shimadzu (RF-540) spectrofluoro-photometer.

6a. Effect of DCMU on Fluorescence Intensity in Intact Cells of Cyanidium caldarium Grown in Presence and Absence of 5mM $AlCl_3$ at 440 nm.

Chlorophyll a were excited with 440 nm light beam for collecting emission spectra in control and treated cells. The fluorescence intensity ratio was found to be 1.5 in control cells in presence and absence of DCMU as an electron inhibitor. The peak position was at 683 nm. The fluoresence intensity ratio in $AlCl_3$ treated cells was found to be 1.7 in presence and absence of DCMU and the peak position was at 683 nm (Table 6a; Figs. 11a and b).

6b. Changes in Fluorescence Intensity at Room Temperature in Intact Cells of Cyanidium caldarium Grown in Presence and Absence of 5mM $AlCl_3$

Cells were excited at 440 nm (chla). Peak position was at 682 nm. The ratio of the fluorescence intensity (control/treated) was 1.06. Cells were excited at 545 nm (PC). Peak position was at 654 nm. The fluorescence intensity ratio (control/treated) was found to be 1.16 (Table 6b; Fig. 11c and d).

Table 6a: Effect of DCMU on Fluorescence Intensity in Intact Cells of <u>Cyanidium</u> <u>caldarium</u> Grown in Presence and Absence of 5 mM $AlCl_3$ Excited at 440 nm.

Sample	DCMU	Peak positions (nm)	Fluorescense intensity (relative units)	ratio
Control cells	-DCMU	683	41	1.5
	+DCMU	683	61	
Treated cells (5 mM)	-DCMU	683	38	1.7
	+DCMU	683	65	

12 days old culture was used, excited with 440 nm light beam for collecting Chl<u>a</u> emission spectrum. Slit width : 5, 5 nm.

Table 6b: Changes in Fluorescence Intensity at Room Temperature in Intact Cells of Cyanidium caldarium - Grown in Presence and Absence of 5 mM $AlCl_3$ Excited at 440 and 545 nm.

Sample	440 nm Excitation (Chla)			545 nm Excitation (PC)		
	Peak position (nm)	Fluorescence intensity (relative units)	Ratio	Peak position (nm)	Fluorescence intensity (relative units)	Ratio
Control cells	682	10.5	1.06	654	73	1.16
Treated cells (5 mM)	682	11.2		654	65	

12 days old culture was used, excited with 440 and 545 nm light beam for collecting Chla and PC emission spectra respectively. Slit width : 5, 5 nm.

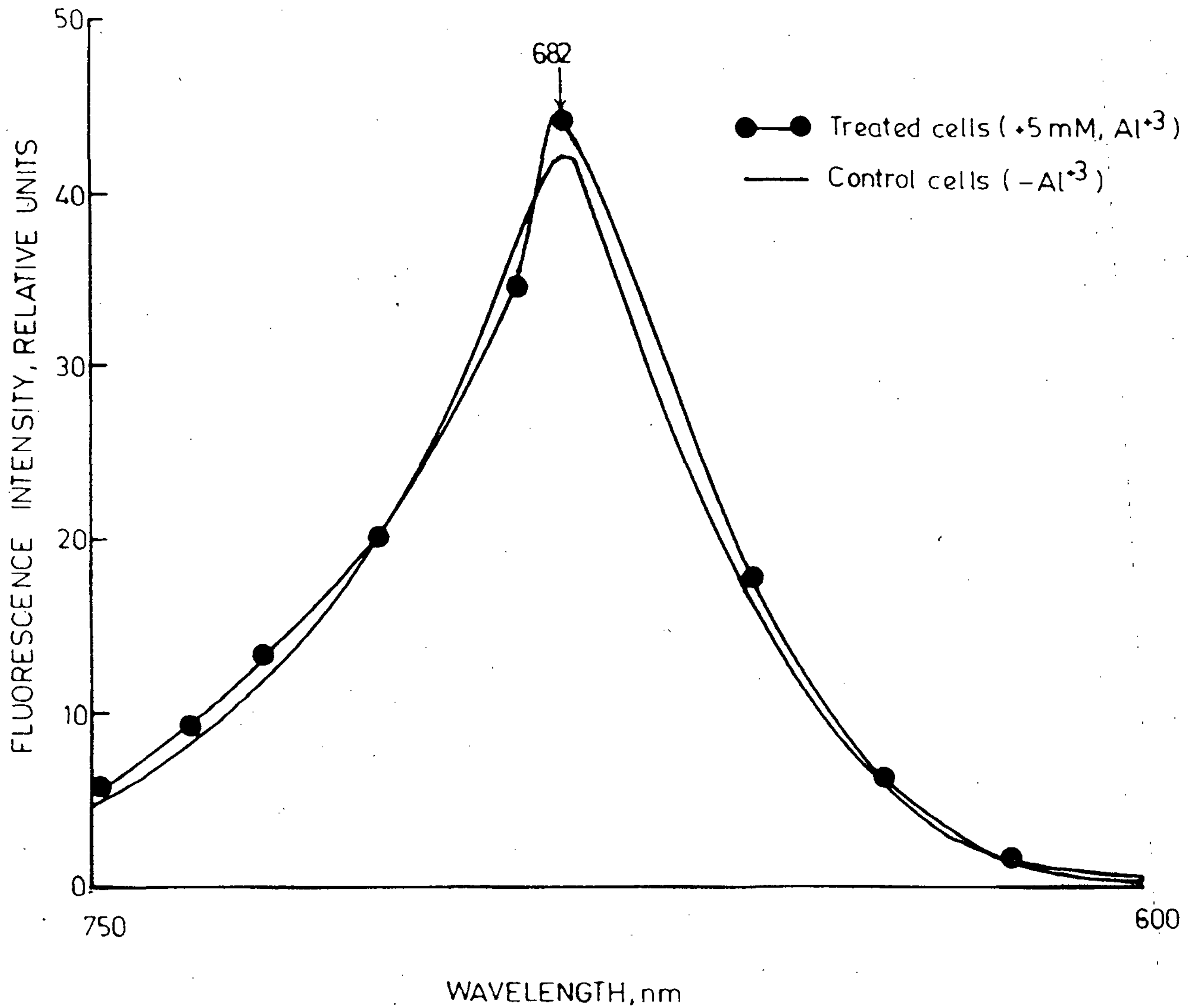


Fig. 11a $AlCl_3$ induced changes in Chla fluorescence emission spectra in control and treated cells. Cells were excited with 440 nm light beam. Slit-width - 5, 5 nm; Fix Scale, 200; Ex/Em - 5; Rec. speed, 20 nm/cm; Scan, 240 nm/min.

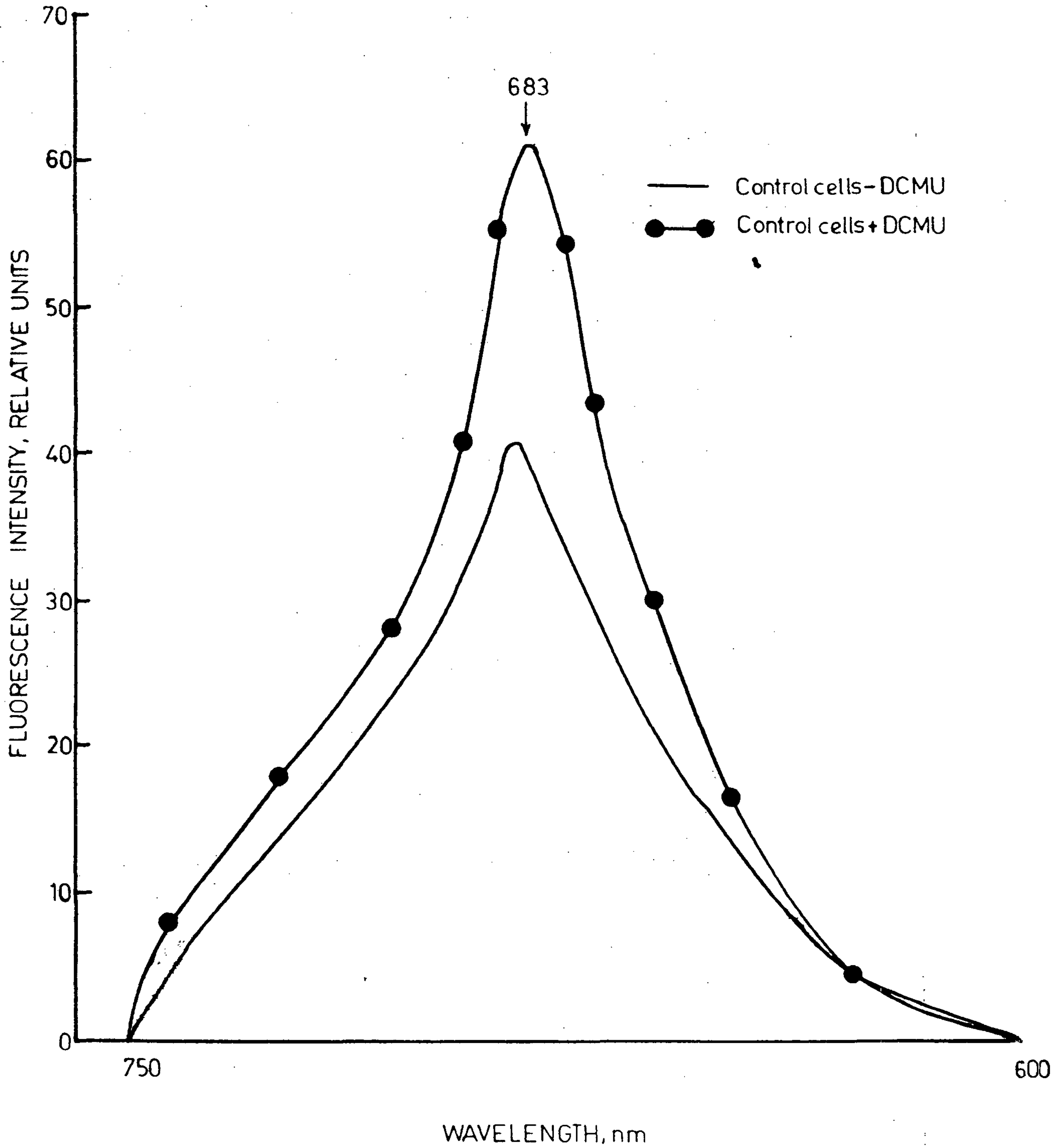


Fig. 11b Changes in Chla fluorescence intensity in presence and absence of DCMU in control cells. Cells were excited with 440 nm light beam. Slit-width - 5, 5 nm; Fix Scale, 200; Ex/Em - 5; Rec. speed, 20 nm/cm; Scan, 240 nm/min.

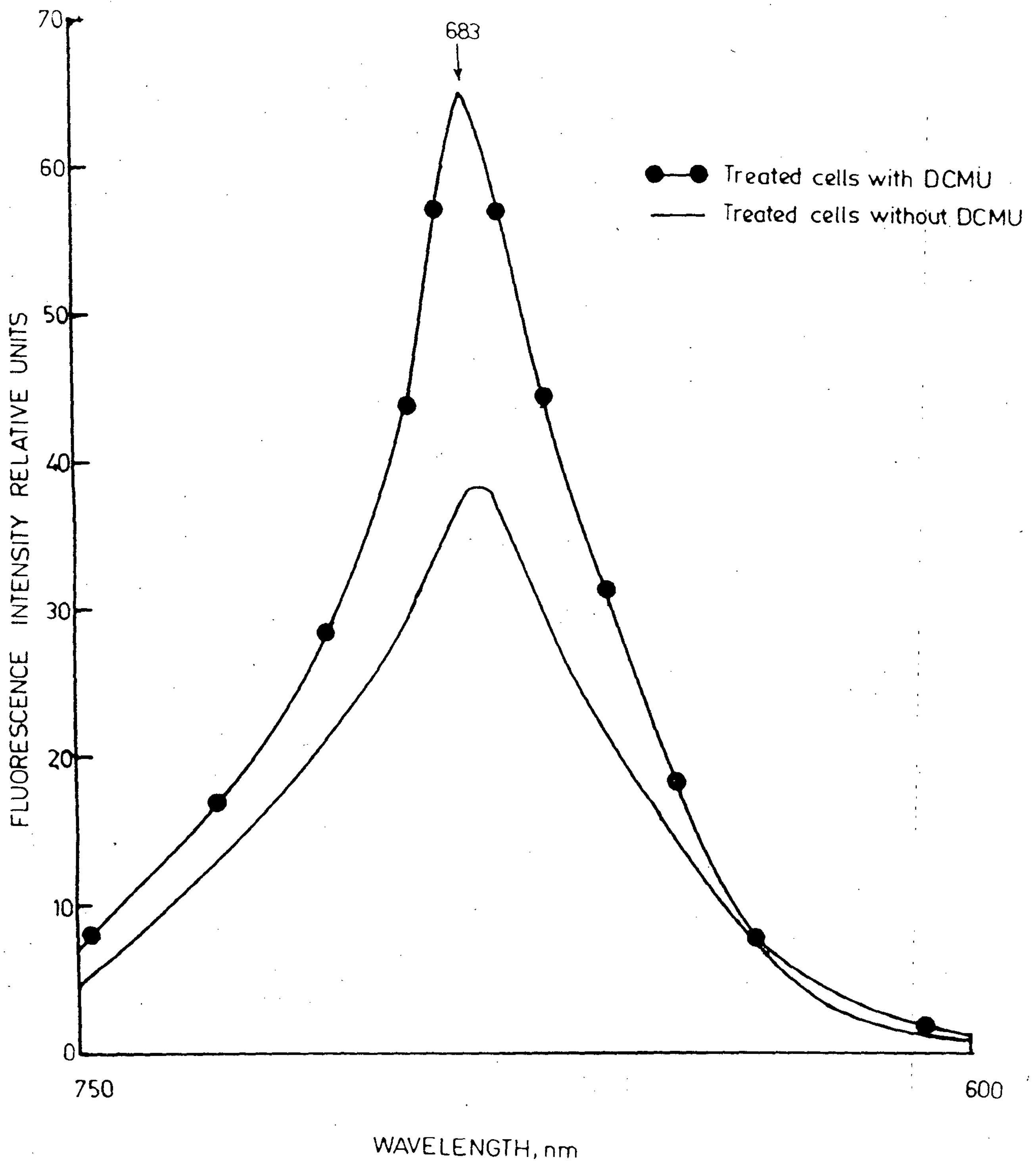


Fig. 11c $AlCl_3$ induced changes in Chla fluorescence intensity presence and absence of DCMU in treated cells. Cells were excited with 440 nm light beam. Slit-width - 5, 5 nm; Fix Scale, 200; Ex/Em - 5; Rec. speed, 20 nm/cm; Scan, 240 nm/min

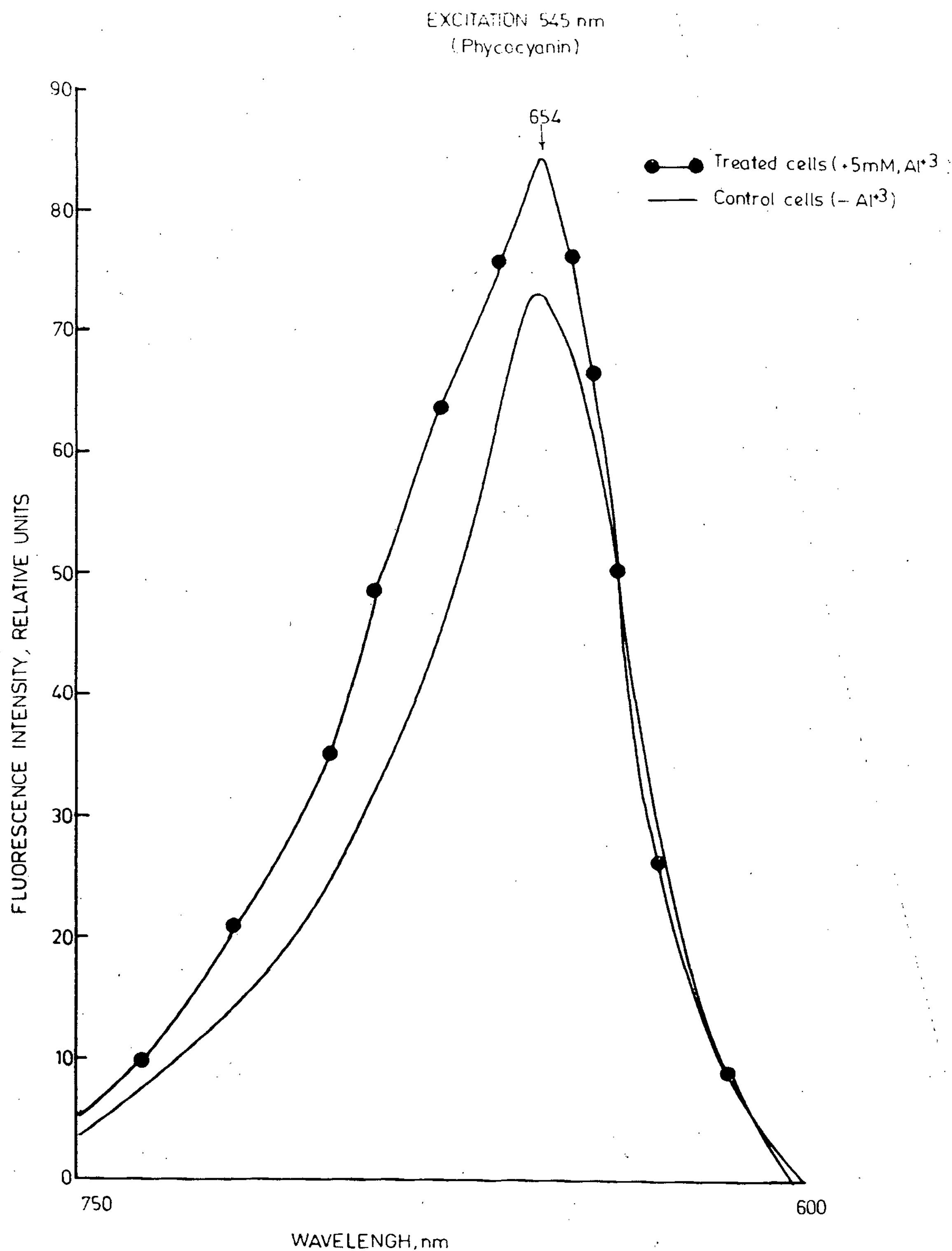


Fig. 11d $AlCl_3$ induced changes in phycocyanin fluorescence intensity in intact cells. Cells were excited with 545 nm light beam. Slit-width - 5, 5 nm; Fix Scale, 200; Ex/Em - 5; Rec. speed, 20 nm/cm; Scan, 240 nm/min

DISCUSSION

Because of a variety of uses of aluminium, there has been the necessity to increase in the number of aluminium based industries in country. As a result of the increase in the number of aluminium industries, there has been an elevation in the level of aluminium toxicity in the cultivable lands. The level of aluminium toxicity increases in acidic soil which occur abundantly in coastal regions of India. This increased level of aluminium toxicity hampers real productivity in our agriculture.

Solubilized Al^{+3} ions in acidic soil strongly inhibit plant growth, especially the development of their root system (Foy et al. 1978). Several mechanisms have been proposed to explain aluminium toxicity :

(1) Disturbance of the metabolism of mineral nutrition (Rorison 1958, Foy et al. 1974, Matsumoto and Yamaya 1986).

(2) Inhibition of cell division (Aimi and Murakami 1964, Morimura et al. 1978). The inhibition of cell division might be due to nuclear activity disturbance which could be one of the primary metabolic disorders in tissue treated with aluminium ions.

(3) Structural chromatin changes in pea by aluminium might be due to chromatin condensation and/or aggregation. It was observed that this condensation disturbed nuclear activity by altering the structure, i.e., through condensation and/or aggregation of the chromatin material (Hideaki Matsumoto 1988).

(4) Binding of aluminium to plasma membrane (Haug 1984) and calmodulin (Suhyda and Haug 1984) which thereby inhibited their function.

(5) Aluminium is antagonist of calcium ion (Ca^{+2}) and effects the phosphate metabolism (Foy et al. 1978).

Wavare and Mohanty (1983) have studied the stimulatory effect of exogenously added aluminium ion (Al^{+3}) on Synechococcus cedrorum spheroplasts in relation to photosynthetic activity. In blue-green algae pH of cell-culture increases during growth and aluminium precipitates. It is, thus, desirable to study aluminium ions effects on culture medium in the red alga Cyanidium caldarium, which grows at very low pH (pH - 3).

The major objectives of these research efforts lies in the nature of stimulation induced by $AlCl_3$ in cell growth

rate, Chl*a*, protein content, absorption characteristics, Hill activities and fluorescence properties.

Aluminium was not generally regarded as an essential element for plant growth, but under some conditions, low concentration could increase growth or produce other desirable effects (Foy *et al*. 1978). This argument could favour our experimental results. During the course of an investigation of the nutrition of a calcifuge, *Deschampsia fluxuosa* (L) Trin; experiments were carried out with different levels of aluminium in water-culture solutions. The results strongly suggests that aluminium could be of benefit to plant growth (Hackett, 1962). This argument could favour our experimental results. It was observed that $AlCl_3$ stimulated the growth pattern of the cell culture in treated cells as compared to control cells. Absorbance by phycocyanin (PC) and Chl*a* was also enhanced by $AlCl_3$ (Fig. 9a-c).

It was observed that $AlCl_3$ increased Chl*a* content in treated cells as compared to control cells. This increase in Chl*a* content might be due to increase in rate of cell division and cell numbers. $AlCl_3$ might be enhancing the synthesis of pigments. Our results do not clearly suggest whether the Chl*a* content increased is per cell or not, as this po was not especially studied in *Cyanidium caldarium* (Table 2).

It was observed that $AlCl_3$ had stimulatory effect on protein content similar to an increase in Chl*a* content in treated cells as compared to the control cells (Table 3). The results on absorption characteristics of _Cyanidium caldarium_ suggested that there was stimulation in $AlCl_3$ treated cells as compared to control cells. The ratio of PC at 630 nm and Chla at 678 nm decreased continuously in successive days. It was observed that decrease was due to degradation of phycobilisomes (PBSomes) in cell culture with and without $AlCl_3$ (Table 4).

Our studies on Hill activity suggested that $AlCl_3$ had stimulatory effect in treated cells (Table 5). It might be possible that $AlCl_3$ changed the fluidity of membranes (Vierstra and Haug 1978) and thus they induced a stimulation of electron flow. The presence of Al^{+3} ions might have screened the membrane negative charges and induced structural changes leading to stimulation of PSII activity (Barber and Mills, 1978; Barber _et al_. 1977, Olser and Cox 1980, Wollama and Ding 1980).

It was also shown that low concentration of Al^{+3}ions did not impair the photosynthetic electron flow. However, exogenously added Al^{+3}ions induced stimulation of the PSII reaction at low concentrations and high enough concentration

of Al^{+3} inactivated the PSII dependent photosynthetic activity of algal spheroplasts (Wavare 1982). Our results on fluorescence characteristics of Cyanidium caldarium suggested that $AlCl_3$ had stimulatory effect in the energy transfer among the constituents of phycobilisomes (Table 6).

All these results make it clear that 5 mM $AlCl_3$ had stimulatory effect in cell culture, as a result of which, there was a stimulation induced by $AlCl_3$ in growth characteristics (cell growth, Chl*a*, protein contents, absorption spectra), Hill activities and fluorescence properties of Cyanidium caldarium.

CONCLUSION

Cyanidium caldarium was grown in 5 mM $AlCl_3$. It was observed that upto six days there was lag period of alga showing marginal inhibition in treated cell culture as compared to control cells. Later on 12th day alga reached in late log phase and after 12th day stationary phase of alga started and decrease in absorbance by pegments was observed. It was concluded that $AlCl_3$ had stimulatory effect in treated culture as compared to control cells in the following characteristics of intact cells in *Cyanidium caldarium* :

(a) Cell growth and absorbance by pigment (Chl*a* and PC)

(b) Enhancement of Chl*a* and protein content

(c) Stimulation in absorption properties

(d) Enhancement in photosynthetic electron transport activity i.e. pBQ mediated Hill activity (H_2O - pBQ)

(e) Stimulation in fluorescence characteristics.

SUMMARY

Our results suggested that $AlCl_3$ had stimulatory effect in the increase of cell growth and absorbance by pigments; increase in Chl*a* and protein content; $AlCl_3$-induced increase in absorption characteristics by PC and increasing energy transfer from PC to Chl*a*; increase in Hill activity and fluorescence characteristics in treated cells as compared to control cells in intact cells of *Cyanidium caldarium*.

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