REGULATION OF AEROBIC HYDROGEN METABOLISM IN DIAZOTROPHIC CYANOBACTERIA

A THESIS SUBMITTED TO THE UNIVERSITY OF HYDERABAD
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

BY

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1988

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TO MY PARENTS

CERTIFICATE

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Date:

This is to certify that I, A. Pratap Kumar, have carried out the research embodied in the present thesis for the full period prescribed under Ph.D. Ordinances of the University under the supervision of Professor H.N. Singh.

I declare to **the** best of my knowledge that no part of this thesis was earlier submitted for the award of Research Degree of any other university.

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ACKNOWLEDGEMENTS

I thank Dr. H.N. Singh, Professor of Life Sciences for suggesting me the problem and guiding me throughout the course of this investigation.

I owe a special debt of gratitude to late Dr. R.S. Rana, with whom 1 started my research career.

I wish to express my gratitude to Prof. K. Subba Rao, Dean, School of Life Sciences for providing me with the necessary facilities to conduct this study.

1 am highly thankful to Prof. Tauro and Dr. Dadarwal of Haryana Agricultural University, Hissar; Prof. Prasanna Mohanty of Jawaharla! Nehru University, New Delhi; Dr. S.P. Wani and Mr. N.T. Lu of ICRISAT, Hyderabad; Dr.(Mrs) Renuka Rao and Dr. Vinayakumar of Osmania University, Hyderabad for generously extending their facilities and helping me during my research work.

With gratitude 1 acknowledge the help and cooperation of Dr. T. Suryanarayana and Dr. K.V.A. Ramaiah.

My thanks are due to Dr. K.S. Ramaiah and Mr. K. Subrahmanyam for their help and assistance in the preparation of the thesis. I indicate my cordial appreciation for the courtesy shown by my friends, Ms. Rita, Mrs. Sangeetha and Ms. Uma.

I rejoice in acknowledging the help of Prof. A.N. Rai, Dr. S.N. Bagchi and Dr. D.T. Singh with whom I shared my research career in this laboratory.

My words fail to express my great fulness to my friends, Messrs. Krishnamurthy, Prabhakar Reddy, K.V.S. Rao, J. Venugopal, Pasha, Anna, Kishan, Modi and Sri Pattabhi whose unbound enthusiasm in leading a helping hand in times of need played no small part during critical stages of my research work.

I owe a special debt of gratitude to my uncle, Dr. A.S. Murthy, 1CR1SAT for his constant encouragement and support throughout my academic career.

I thank my sisters and brother-in-law for their constant encouragement during the period.

I wish to thank Sri Venkata Rao for typing the thesis.

1 sincerely acknowledge the CSIR For financial assistance in the form of Senior Research Fellowship.

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C O N T E N T S

ABBREVIATIONS USED

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
DCIP	2,6-Dichlorophenol indophenol
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DMSO	Dimethyl sulfoxide
EDTA	Ethylene diamine tetraacetic acid
Fe Mo-Co	Iron-molybdenum-Cofactor
x g	multiples of gravitational constant
GSH	Reduced glutathione
HEPES	N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid
MA	Methylammonium
Mpa	Mega pascal
MSX	L-methionine-DL-sulfoximine
Mtn	Metronidazole (2-methyl-5-nitroimidazole, 1-ethanol)
MV	Methylviologen
nif	Genes Coding for 'nitrogen fixation'
ntr	Genes Coding for overall 'nitrogen regulatory system'
PS1	Photosystem 1
PS1I Photos	**ystem** 11
PVP	Polyvinylpyrrolidine
RUBISCO	Ribulose bisphosphate Carboxylase
Tricine	N-tris[hydroxymethyl]-methyl glycine
Tris	Tris-(hydroxymethyl)aminomethane
v/v	Percent 'volume in volume' number of cubic centimeters of a constituent in 100 cm of solution
w/v	Percent 'weight in volume' number of grams of constituent in 100 Cm^3 of solution.

CHAPTER I

GENERAL INTRODUCTION

Cyanobacteria popularly **known** as Blue-green algae are a group of photosynthetic prokaryotes with oxygenic mode of photosynthesis and carbon nutrition similar to chloroplasts of eukaryotic algae and higher plants. The photosynthetic apparatus, the genetic apparatus and the respiratory apparatus of cyanobacteria arc not organised into membrane-bound organelles characteristic of eukaryotic cells. The principal photosynthetic pigment of the group is undoubtedly is Chlorophyll a but the accessory photosynthetic pigment phycobilins is characteristic of the group (Cohen-Bazire and Bryant, 1982). A decade back, a new member of prokaryote, with oxygenic mode of nutrition like cyanobacteria but containing chlorophyll b instead of phycobilins, as accessory photosynthetic pigment was discovered and was named Prochloran didemni (Levin, R.A., 1976). The Prochloran didemni assigned **to** a new phyla prochlorophyta is an obligate symbiont of didemnid **ascidians** and **is** difficult to grow in the absence of the host (Patterson and Withers, 1982). In 1986 a second prochlorophyte of filamentous free-living nature was isolated and on characterization was found to be similar to Prochloran didemni in cellular structure and photosynthetic organization (Burger-Wiersma et al, **1986). The** new isolate prochlorophyte still unnamed has opened up itself for detailed analysis of its mode of nutrition, photosynthesis, reproduction and genetic organization and thereby for a comparison with cyanobacteria. While prochlorophyte and cyanobacteria are similar in photoautotrophic mode of nutrition and basic pattern of ultrastructural organization they differ at the moment in the mode of their nutrition such as diazotrophy, chemoheterotrophy **etc.** characteristic of a majority of cyanobacteria (Smith, 1982).

The attention to study cyancbacteria as a biological system of

considerable interest arose because of their diazotrophic mode of nutrition at the simple expense of light energy, water and air and because of the ease with which their photosynthesis can be studied by the modern techniques of genetic engineering thereby, providing a model system for genetic manipulation of higher plant chloroplast system (Golden et al, 1986). Heterocystous cyanobacteria are the only group of photosynthetic prokaryotes capable of fixing N_2 under aerobic oxygenic photosynthesis and this ability has been the result of the production of a specialized structure called heterocyst, the site of nitrogenase synthesis and activity. The reason for the localization of nitrogenase syntnesis and activity within the heterocyst has been guided by the anaerobic nature of heterocyst metabolism conducive to the activity and production of oxygen-sensitive nitrogenase (Haselkorn, 1978; Stewart, 1980). The applied significance of heterocystous nitrogen-fixing cyanobacteria was realized with the observation that wet-land rice fields of tropical countries like India are the ideal habitats for these forms during rainy season and that they are the biological agents of sustaining the nitrogen fertility of rice-fields years after years without addition of synthetic nitrogen fertilizers (Singh, R.N. 1961). Over the subsequent years, this aspect of cyanobacteriology was developed along two lines - one related to agriculture started developing technical know-how to use them as biofertilizers in rice cultivation and the recent results suggest a decisive role of diazotrophic cyanobacteria in regulating the supply of fixed nitrogen to the paddy crop for better productivity and yield (Venkataraman, 1972). The other line of approach has been to understand the molecular mechanism of heterocyst differentiation and its role in regulation of nitrogen fixation (Haselkorn et al, 1987). The research over the last twenty years clearly indicates that the heterocyst differentiation and the nitrogenase activity do not occur in NO_3^- or NH_4^+-assimilating cultures.

They are differentiated in the growth medium lacking fixed source of nitrogen (Haselkorn et al, 1987). The heterocyst has been found to lack RUBISCO, the key enzyme of calvin cycle (Codd et al, 1980), PSII activity (Fay et al, 1968; Tel-or and Stewart, 1977 and Peterson et al, 1981) and nitrate reductase activity (Kumar et al, 1985).

Nitrogenase activity (Stewart et al, 1969) and synthesis (Flemming and Haselkorn, 1973) both are localized in heterocysts under aerobic growth conditions. Glutamine synthetase-Glutamate synthase (GS-GOGAT) pathway is the main primary route of ammonia assimilation in cyanobacteria. In heterocystous cyanobacteria, while GS activity is located within heterocyst and vegetative cells, the GOGAT activity remains mainly confined to vegetative cells (Thomas et al, 1977). Evidences indicate operation of more efficient oxidative pentose phosphate pathway in heterocysts than in vegetative cells (Apte et al, 1978). In heterocysts while oxidative pentose phosphate pathway serves to provide reductant and a pool of ATP for nitrogenase activity, PS-I appears to supply major part of ATP-requirement for nitrogenase, although it may also mediate generation of reductant for the enzyme (Donge et al, 1974; Tel-or and Stewart, 1976; Wolk, 1982). Uptake-hydrogenase, the enzyme for conserving nitrogenase catalysed H_2-evolution is shown to be present in heterocysts under nitrogen-fixing conditions (Peterson and Wolk, 1978). The pattern of distribution of primary enzymes of CO_2-assimilation, N_2-fixation and NH_4^+-assimilation in heterocystous cyanobacteria suggest that heterocyst is an anaerobic, N_2-fixing, non CO_2-fixing cell and vegetative cell is an aerobic photosynthetic, CO_2-fixing, non nitrogen-fixing cell and that heterocyst is the source of fixed nitrogen for growth of vegetative cells. This compartmentalization of N_2-fixation in heterocyst and CO_2-fixation in vegetative cells suggests a kind of close metabolic symbiosis between the two cell types.

Studies of organization of **nif** genes in cyanobacteria has shown a kind of nif gene rearrangement peculiar to heterocyst differentiation. N_2-fixing non-heterocystous forms show a contiguous **nifHDK** gene cluster, whereas all the heterocystous, forms show a separation of **nif**K from the nifDH gene by a DNA sequence of nearly 11 kbp. The separated **nif**KDH gene in vegetative cells of heterocystous forms are brought closer together by heterocyst differentiation by a mechanism which eliminates the intervening 11 kbp DNA. The significance of such a rearrangement of **nif** genes during N_2-fixation in heterocystous forms is far from clear (Haselkorn et al, 1987).

A Vanadium - nitrogenase alternative to Molybdenum-nitrogenase has been demonstrated to work as a nitrogen fixing enzyme in Azotobacter vinelandii (Bishop et al, 1980; Bishop et al, 1982; Robson et al, 1986) W-resistant strains of Nostoc muscorum capable of diazotrophic growth **in** W-medium (Singh et al, 1978) has been interpreted to result from working of alternate Vanadium-nitrogenase under such conditions (Robson et al, 1986). Recently, more definitive evidence for functional operation of Vanadium-nitrogenase has been provided by the work of Kentemich et al (1988) in the cyanobacterium Anabaena variabilis. However, this new emerging area of diazotrophy requires more concerted and detailed study in various cyanobacterial forms. Stewart (1973) has classified on physiological grounds diazotrophic cyanobacteria into three groups.

1. Nonheterocystous diazotrophic cyanobacteria: This group of organisms perform N_2-fixation under anaerobic or microaerobic conditions. eg: Plectonema boryanum. In all the non-heterocystous forms effective devices to protect nitrogenase from damage by O_2 have apparently not been developed.
2. Nonheterocystous forms which express nitrogenase activity under aerobic conditions. eg. unicellular forms Gloeocapsa, Aphanothece etc. These aerobic

cyanobacteria must protect their nitrogenase against O_2 present in air and that formed by photosynthesis. Gloeocapsa copes with the problem by separating N_2-fixation and photosynthetic O_2 production temporarily. A role of Ca^{++} has been proposed for aerobic N_2-fixation by Gloeothece (Hamadi and Gallon, 1981; Gallon and Hamadi, 1984).

3. Filamentous heterocystous forms: Organisms of this group solve the problem of O_2-inactivation of nitrogenase by separating photosynthetic O_2-evolution and nitrogen fixation spatially. The former is performed in the vegetative cells and the latter in the heterocysts which do not possess PS 11 activity and RUBISCO.

H_2 production and NH_3 production by nitrogenase are concurrent reactions and under normal conditions 20-30% of the total electrons reaching nitrogenase are used for production of H_2 from protons and the remaining electrons are utilized for production of NH_3 from N_2. The mechanism which permits and regulates the two concurrent reactions of the enzyme are far from clear and their understanding at molecular and genetic level is a must for manipulation of nitrogenase activity exclusively for NH_3 production from N_2 or H_2-production from protons. Nitrogenases from cyanobacteria and other diazotrophic systems have been shown to carry out exclusive production of H_2 at the expense of reductant and ATP in anaerobic atmosphere devoid of molecular N_2. Such a limited success at laboratory scale would require its translation at a industrial scale which has not been possible, obviously because of existing gaps in our knowledge of nitrogenase enzyme in respect of its biotechnology and molecular genetics (Evans et al, 1987).

Heterocystous cyanobacteria have been considered to hold great potential for photosynthetic production of molecular H_2 from H_2O and this realization is a result of compartmentalization of oxygenic photosynthesis

related to CO -assimilation in vegetative cells and anoxygenic photosynthesis (P5-I) related to nitrogen-fixation in heterocysts by nitrogenase which receives the required reductant from photosynthetically fixed carbon from vegetative cells through active transport systems (Houchins, 1984). In other words, operation of nitrogenase-catalysed H_2-production in heterocysts and photosynthetically-catalysed O_2 production in vegetative cells under oxygenic photoautotrophic growth conditions has tempted the cyanobacteriologist to examine the photosynthetic H_2-producing potential of such forms from water. Heterocystous cyanobacteria offers an ideal opportunity to study at molecular level the mechanism of coupling between photosynthesis, nitrogen-fixation and H_2 metabolism. Such studies obviously require an understanding of functional and regulatory aspects of photosynthetic metabolism, N_2-metabolism and H_2 metabolism. Studies along these lines with special emphasis on photobiological production of H_2 has so far been limited with very little encouraging results (Bothe, 1982).

Historically, a relationship between H_2-metabolism and N_2-metabolism was recognized by Phelps and Wilson (1941), Wilson et al, (1942), Lee and Wilson, (1943), Green and Wilson, (1953), Hyndman et al, (1953) when they demonstrated the operation of uptake hydrogenase activity under N_2-fixing conditions in Azotobacter and Rhizobium bacteroids. Since that time until today the research done on H_2 and N_2 metabolism in various N_2-fixing organisms have led to the discovery of three kinds of H_2-metabolizing enzymes (Bothe and Eisbrenner, 1981). Reversible hydrogenase found in Clostridium pasteurianum, an obligate anaerobic diazotroph carries out the function of H_2-uptake and H_2-evolution. The reversible hydrogenase activity of H_2-uptake or H_2-evolution is not dependent on ATP but is sensitive to CO (carbon monoxide). The second H_2 metabolizing enzyme is nitrogenase itself which while

carrying out nitrogen-fixation also carries out H_2 evolution driven by reduced ferredoxin and ATP. This nitrogenase catalysed H_2-evolution activity is CO-insensitive in contrast to that catalysed by reversible hydrogenase which is CO-sensitive. In fact, the ATP-dependence and CO-insensitivity are the parameters normally used to differentiate between reversible hydrogenase catalysed H_2-evolution and nitrogenase catalyzed H_2-evolution. Theoretically, one molecule of H_2 is produced per molecule of N_2 reduced to NH_3 in a reaction of nitrogenase consuming 8 electrons and around 24 molecules of ATP. The third H_2-metabolizing enzyme is uptake hydrogenase which is a membrane-bound enzyme. While reversible hydrogenase and nitrogenase activities are O_2-sensitive reactions operating under anaerobic conditions, uptake hydrogenase is O_2-resistant reaction catalyzing H_2-utilization for various reductive biochemical reactions associated with primary metabolism of the organism (Houchins, 1984). Uptake hydrogenase activity is not restricted to only N_2-fixing organisms as it has been demonstrated to be active in a number of nonnitrogen-fixing cyanobacteria (Howarth and Codd, 1985) and is also present in H_2-oxidizing bacteria (Schlegel and Schneider, 1978). Evolution of molecular H_2 readily occurs in anaerobic N_2-fixing bacteria like C. pasteurianum but it fails to occur at significant detectable levels in aerobic N_2-fixing organisms. However, general consensus is that the H_2-evolution in aerobic N_2-fixers is generated by the activity of nitrogenase because the H_2-evolution occurs only under N_2-fixing conditions and not under conditions of combined nitrogen for growth (Houchins, 1984). Inhibitors of ATP formation are known to abolish H_2-evolution by aerobic diazotrophs thereby, further emphasizing the involvement of aerobic nitrogenase in production of H_2 under N_2-fixing conditions. There are a number of factors regulating the quantity of H_2 produced by nitrogenase and such conditions include the nature of the strain used, the temperature, the composition of growth medium, the presence of

fixed nitrogen sources etc. (Lambert and Smith, 1981).

Role of uptake hydrogenase has received much attention for study in view of its proposed four-kinds of functions in nitrogen-fixation (Dixon, 1972). (1) The uptake hydrogenase can mediate oxy-hydrogen reaction leading to the production of ATP which can meet **the** energy requirement of nitrogen-fixation otherwise limited by ATP inavailability. Such a function of uptake hydrogenase has been demonstrated in Azotobacter (Walker and Yates, 1978) and Anabaena (Bothe et al, 1978). (2) Uptake hydrogenase can also functions as O_2-protective mechanism for O_2-sensitive nitrogenase activity through oxy-hydrogen reaction as mentioned above. Thus N_2-fixing cells with uptake hydrogenase activity should show more O_2-resistant N_2-fixing ability than corresponding strains deficient in uptake hydrogenase activity. O_2-resistant acetylene reduction as a function of uptake hydrogenase activity has been demonstrated in Azotobacter (Walker and Yates, 1978), Anabaena cylindrica (Bothe et al, 1977, 1978). (3) H_2 is an inhibitor of N_2-fixation by nitrogenase and since H_2 is produced by nitrogenase during N_2-fixation, the optimization of N_2-fixation could be possible if nitrogenase-produced H_2 is prevented from accumulation at the site of enzyme activity. Presence of uptake hydrogenase activity is suggested to prevent inhibition of N_2-fixation by nitrogenase-generated H_2 through oxy-hydrogen reaction. (4) Uptake hydrogenase can recycle the nitrogenase evolved H_2 for fresh generation of reductant for nitrogenase activity and experimental evidences for such a role of uptake hydrogenase are there in literature (Peterson and Wolk, 1978; Eisbrenner and Bothe, 1979; Houchins and Hind, 1982). Thus, it is apparent that there is a close physiological interdependence between uptake hydrogenase activity and nitrogenase activity in aerobic diazotrophic forms.

Nitrogenase-catalyzed H_2-evolution in photosynthetic prokaryotes

like heterocystous cyanobacteria requires knowledge about nitrogen regulatory genes regulating nitrogenase production, nitrogenase activity, uptake-hydrogenase activity and heterocyst production and heterocyst metabolic activities. In enteric diazotrophic bacteria nitrogen regulatory genes abbreviated as **ntr** genes have been isolated and characterized in a great detail. **ntr** A and **ntr** C are known to function in activation of **nif** genes under N_2-fixing conditions and **ntr** A and **ntr B** genes are known to function in deactivation of nif genes under NH_4^+-assimilatory conditions (Kennedy and Toukdarian, 1987). Differentiation of heterocysts and nitrogenase activity under nitrogen limited growth conditions thus suggests the operation of nitrogen-regulatory genetic system in production of aerobic N_2-fixing heterocysts in cyanobacteria. In symbiotic associations involving cyanobacteria and other photosynthetic partners of higher plant type like fern Azolla, or Cycas, the heterocyst frequency and nitrogenase activity both increase many-fold over the level in free-living state thereby, further suggesting the operation of nitrogen-regulatory system in control of heterocyst and nitrogenase specific to symbiotic systems. Nothing is known about the structure, organization and distribution of such proposed nitrogen-regulatory genes in any cyanobacteria symbiotic or free-living. A knowledge of such nitrogen-regulatory genetic systems in heterocystous diazotrophic cyanobacteria is a prerequisite for manipulation of nitrogenase activity with a view to augmenting photoproduction of H_2 from H_2O. Such a knowledge could also be helpful in producing mutant strains derepressed for heterocyst and nitrogenase. In free-living cyanobacteria inhibitors of aromatic aminoacids like 7-azatryptophan is known to derepress heterocyst formation without causing derepression of nitrogenase (Bottomley et al, 1980). More search for inhibitors may finally result in discovering one inhibitor which coordinately can derepress both heterocyst and nitrogenase in free-living forms as well.

In addition, more detailed physiological and biochemical knowledge is required to understand the physiological interdependence of N_2-fixing heterocysts and CO_2-fixing vegetative cells in order to successfully alter the ratio of vegetative cells to heterocysts as it happens in symbiotic associations in favour of increased nitrogenase activity.

Participation of nitrogenase activity exclusively in H_2-production under N_2-fixing conditions would be of limited significance in biotechnological programmes of photoproduction of H_2 by cyanobacteria because under such conditions cyanobacteria will suffer for nitrogen nutrient and will therefore fail to grow. A solution to this problem could be production of derepressed mutants for both heterocyst and nitrogenase so that the fixed nitrogen-sources like NH_4^+-can serve the function of nitrogen-source without causing repression of heterocyst and nitrogenase, and such derepressed mutant strains would continue generating molecular H_2 in an anaerobic atmosphere devoid of molecular N_2. Success of such approaches depends greatly on our knowledge of biochemical and genetical mechanism of repression and derepression control of heterocyst and nitrogenase. First attempt in this direction was made by Stewart and Rowell (1975) who, by using irreversible inhibitor of GS activity L-Methionine-DL-sulfoximine showed derepression of heterocyst and nitrogenase in NH_4^+-medium. This led them to conclude that not NH_4^+, but a product of GS activity is the repressor of heterocyst and nitrogenase in the cyanobacterium Anabaena cylindrica. According to this view GS deficient mutant should be derepressed for heterocyst and nitrogenase activity in NH_4^+-medium which however, was not found occurring in GS-deficient mutant of Anabaena cycadeae by Singh et al (1983) who concluded that NH_4^+ is the initial repressor signal of heterocyst and nitrogenase. A recent study in anaerobic cultures of A. variabilis on regulation of nitrogenase gene expression has shown that neither NH_4^+ nor glutamine or glutamate is the repressor signal of **nif** genes

in A. variabilis (Helber et al, 1988). This contradicting conclusions only go to show that our knowledge is far from clear about the mechanism of regulation of heterocyst and nitrogenase by ammonia in cyanobacteria.

Regulation of uptake hydrogenase activity in N_2-fixing cyanobacteria in respect of carbon nutrition is very little studied. A knowledge about the role of carbon nutrition in regulating uptake hydrogenase activity and thereby of nitrogenase activity and vice versa is necessary in order to manipulate H_2 metabolism by uptake hydrogenase activity in favour of photoproduction of H_2 by nitrogenase. The application of genetic, biochemical and physiological approaches together will go a long way in clarifying the role of uptake hydrogenase in N_2-fixation and H_2-metabolism.

A knowledge of interrelationship of photosynthesis, respiration and nitrogen fixation at molecular level in cyanobacteria is required in order to understand the relative contribution and efficiency of photosynthesis and respiration in N_2-fixation. It is known, that aerobic respiration is inhibited in light and the recent research seems to suggest that aerobic respiration and photosynthesis share a common electron transport path and that this sharing of the electron transport pathway is the most probable reason for occurrence of either photosynthesis or respiration at a time in cyanobacteria (Scherer et al, 1988). The present available knowledge suggest, that respiratory electron transport except for cytochrome oxidase is a part of photosynthetic electron transport in respect of the identity. May be this was the reason for evolutionary process to have resulted in causing development of specialized structures like heterocysts for its exclusive function of N_2-fixation. However, many more studies are required to substantiate this view firmly.

In the present work we have concentrated on the study of aerobic H_2-metabolism relating to nitrogenase catalysed H_2-production and uptake

hydrogenase catalysed H_2-consumption and to nitrogenase-uptake hydrogenase physiological and genetical interdependence. We also studied the nitrogen and carbon regulation of H_2-production and H_2-consumption in two heterocystous cyanobacteria Nostoc muscorum and Anabaena cycadeae and one unicellular aerobic diazotrophic form Gloeocapsa sp. Mutants resistant to photosynthetic inhibitors like DCMU [(3,4-dichlorophenyl)-1,1-dimethyl-urea)] and Atrazine were isolated and examined for their efficiency in photoharvesting of H_2. Atrazine-resistant mutants lacked uptake hydrogenase activity and all such strains showed H_2-sensitive nitrogenase activity.

CHAPTER II

MATERIALS AND METHODS

Organisms and Maintenance

Parent strains

The parental strain of Nostoc muscorum and Anabaena cycadeae are unbranched, filamentous cyanobacteria capable of growing at the expense of N_2 as nitrogen source under photoautotrophic growth conditions, in modified Chu-10 medium as described by Gerloff et al (1950). Under these conditions, 5-6% of the vegetative cells get differentiated into regularly spaced heterocysts alone the filament. The combined nitrogen-free medium is represented as N_2-medium and when N_2-medium is supplemented **with** a final concentration of 5 mM KNO_3, 5 mM $NaNO_2$ and 1 mM NH_4Cl (buffered with 50 mM HEPES-NaOH, pH 8.5), represented as NO_3^-, NO_2^- and NH_4^+-media respectively. Addition of NO_3^-, NO_2^- or NH_4^+ inhibits heterocyst formation and nitrogen-fixation under diazotrophic growth conditions (Stewart and Singh, 1975). Gloeocapsa sp. is a unicellular aerobic diazotrophic cyanobacterium and is a natural isolate from paddy fields. It was found to be inherently resistant to herbicides like Machete and Basalin (Singh et al, 1986).

Axenic batch cultures of these strains were grown in liquid medium in air-conditioned illuminated culture room at a photon fluence rate of 50 u mol. m^{-2} s^{-1} and temperature of 28 ± 2°C. Clonal cultures were maintained on Agar slants which were prepared by adding 1% (v/v) Agar-Agar to the liquid medium before autoclaving.

Culture medium:

The modified Chu-10 medium as described by Gerloff et al (1950), having the following composition was used for growing the cyanobacteria!

strains, Nostoc muscorum and Anabaena cycadeae.

Concentration of Stock solutions ($g.l^{-}$)

Macronutrients

1. Calcium chloride	11.0
2. Dipotassium hydrogen phosphate	2.0
3. Magnesium sulfate	5.0
4. Sodium carbonate	4.0
5. Sodium silicate	8.8
6. a) citric acid	0.6
b) Ferric citrate	0.6

Micronutrients

1. Boric acid	2.86
2. Copper sulfate	0.08
3. Manganous chloride	1.81
4. Sodium molybdate	0.02
5. Zinc sulfate	0.22

Preparation of Final medium for use:

5 ml each of the macronutrient and 1 ml of micronutrient solution was taken in a final volume of 1 1 of double distilled water and pH was adjusted to 9.0 before autoclaving. NO_3, NO_2 and NH_4 were added to the above medium when required.

Growth of Escherichia coli:

Escherichia coli strain HB101 was grown under aerobic conditions in minimal medium composed of the following in (g/l) : Na_2HPO_4 7.0g; KH_2PO_4 3.0 g; NaCl 0.5 g; NH_4Cl 1.0 g and casamino acids 0.02%. The following components were autoclaved separately and added aseptically (per 1) : 1 M $MgSO_4$, 0.2 ml; 20% Glucose, 1.0 ml; 1M $CaCl_2$, 0.01 ml. The pH of the medium was adjusted to 7.4. The above minimal medium was supplemented with 1 mM Na_2MoO_4. Cells were grown at 37°C with vigorous shaking on a rotary shaker. Bacteria were harvested during exponential growth phase

and used for isolation of Mo-cofactor.

Metronidazole-resistant mutant isolation:

The metronidazole-resistant mutant strain (Mtn-R) of Nostoc muscorum was isolated by following the method of Singh and Singh (1978). A thick suspension of N_2-grown parental Nostoc muscorum culture was spread on N_2-plates containing 0.5 mM of the metronidazole. These plates were incubated for a fortnight at 28 + 2°C and light intensity of 2.5- 3 K lux in culture room. These plates were observed periodically for developing colonies and a few of those individual colonies were transferred into sterile medium in tubes. These were further purified by repeated subculturing on N_2-plates with metronidazole and maintained on NH_4-slants containing metronidazole (0.5 mM).

Growth measurements:

Since cyanobacterial cultures are homogenous in liquid medium, a direct absorbance change at 665 nm is proportional to its multiplication and is used for growth measurement.

Estimation of Chlorophyll:

To determine the chlorophyll content, 3.0 ml samples were extracted in equal volume of 100% Methanol for 10 min. The chlorophyll concentration was calculated from the absorbance of the centrifuged extract at 665 nm spectrophotometrically, using an extinction coefficient of 74.5 $g^{-1}.Cm^{-1}$ (Mackinney, 1941).

Estimation of cellular protein:

Protein was estimated according to the procedure of Lowry et al (1951). The protein in the crude extracts was estimated after precipitating

with an equal volume of 10% TCA (Bailey **et** al, 1967). The mixture was left overnight at room temperature and then centrifuged at 3000 xg for 10 minutes. The precipitate was dissolved in 1N NaOH and an aliquot of it was withdrawn for estimating the protein content. A standard curve for the protein estimation was prepared by using BSA as standard.

Measurement of Heterocyst frequency:

Heterocyst frequency was calculated as percentage of total cells by light microscopic observation of the filaments of the cyanobacterium used.

Estimation of Glycogen content:

Glycogen content of the cyanobacterial cells was determined according to the method of Lehmann and Wober (1976). Cyanobacterial cells in the exponential phase of growth were harvested, washed and resuspcnded **in** 10 ml of 10 mM Sodium acetate buffer, pH .5.0. Then, the cells were sonicated at 4°C for 5-6 min in 30 sec. burst period punctuated by 30 sec. rest period. The broken cells were centrifuged at 10,000 xg for 15 min. The 10,000 xg supernatant was used for estimation of glycogen content. 1.0 ml of the clear supernatant was incubated for 1.5 hr with 2.0 ml of a specific glucoamylase/glucose - oxidase/peroxidase reagent (Marshall and Whelan, 1970). The reaction mixture was prepared by dissolving 10 mg of Amyloglucosidase; 40 mg of Glucose oxidase; 4 mg of peroxidase and 15 mg of O-dianisidine dihydrochloride in 100 ml of 0.1M citric acid-glycerol buffer, pH 5.5. The reaction was terminated after 1.5 hr by adding 4 ml of 5N hydrochloric acid and the absorbance was determined at 540 nm.

Isolation of heterocysts:

Heterocysts were isolated using a combination of the methods

described by Peterson and Burris (1976) and Thomas et al (1977). All manipulations were performed at 0-4°C, unless stated otherwise. Cyanobacterial cells from 5 1 of a log phase culture was harvested by centrifugation at 2,500 xg for 15 rnin and resuspended to a density of 80-100 µg Chl a.ml⁻ in 10 mM HEPES-NaOH buffer, pH 7.5 containing lmM EDTA and *0AM* Mannitol. The suspension was incubated with Lysozyme (1 mg. ml) at 37°C for 60 min on a reciprocating shaker at 60 cycles min . Lysozyme disrupted the filaments and produced detached heterocysts, vegetative cells and filament fragments and it made vegetative cells more susceptible to sonication. The resulting suspension was suspended in 10 mM HEPES-NaOH buffer, pH 7.5 containing Mannitol (0.4 M) but no EDTA and then subjected to ultrasonication for 6 min in 30 sec. bursts punctuated by 30 sec. rest periods. The suspension was then centrifuged at 250 xg for 10 min to provide a blue-green supernatant consisting of vegetative cells extract and fragments, but devoid of heterocystous material. The pellet containing heterocysts and few vegetative cells was washed repeatedly with HEPES-Mannitol buffer (no EDTA) by centrifugation at 250 xg for 10 min. Microscopic examination showed such suspensions were composed entirely of heterocysts with less than 5% of vegetative cell contamination.

Preparation of Mo-cofactor from heterocysts:

Heterocysts isolated from Mo-containing medium were washed and resuspended in Tris buffer (50 mM, pH 7.5) containing 100 mM NaCl, 300 mM sucrose, lmM EDTA and 5 mM $MgCl_2$. The cell-free extract was then prepared by passing through a French pressure cell at 110 MPa followed by centrifugation at 30,000 xg for 20 min at 4°C. The supernatant was used as a source of Mo-cofactor for complementation analysis.

Preparation of Mo-cofactor from E. Coli:

The Mo-cofactor was prepared from E. Coli by following the method of Miller and Amy (1983). E. Coli cells were harvested by centrifugation, washed and resuspended in 100 mM potassium phosphate buffer, pH 7.4 containing 0.5 mM EDTA and 1 mM β-mercaptoethanol. These cells were passed through French pressure cell at 110 MPa to break the cells. After centrifugation of the broken cells at 3,000 xg for 15 min, the resulting supernatant was used for Mo-cofactor assay.

Preparation of Nitrate reductase apoprotein (Cofactor-free nitrate reductase)

The N_2-grown and NO_3^--grown parent N. muscorum cells were harvested, washed and resuspended in 50 mM Tris-HCl buffer, pH 7.5 containing 100 mM NaCl, 300 mM sucrose, 1mM EDTA and 5 mM $MgCl_2$. This suspension was sonicated for 10 min at 4°C with 30 sec. bursts punctuated by 30 sec. rest periods, followed by centrifugation at 30,000 xg for 20 min at 4°C. The supernatant containing nitrate reductase made free of Mo-cofactor by acid and subsequent neutral treatments: 0.02 ml of cell-free extract was incubated with 0.02 ml of molybdate-GSH solution (10 mM GSH and 5 mM sodium molybdate in 100 mM sodium acetate buffer, pH V.5). This brought the pH of the reaction mixture to 4.8. After 30 sec-60 sec. incubation at 30°C (acid incubation) the pH was adjusted to 7.2 by adding 200 mM K_2HPO_4 and incubated for 15-20 min at 30°C (neutral incubation).

Complementation assay procedure:

Reconstituted nitrate reductase activity was measured by mixing equal volumes of the nitrate reductase apoprotein preparation from N_2-grown and NO_3^--grown cells and the Mo-cofactor prepared from heterocysts. This

mixture was incubated for 10 min at 30°C to achieve complementation (Miller and Amy, 1983) and nitrate reductase activity was estimated.

Estimation of Ammonium (methylammonium) transport activity:

The method of Rai et al (1984) was used for measurement of CH, NH, uptake. The cyanobacterial cells were centrifuged, washed and resuspended in 10 mM HEPES-NaOH buffer, pH 7.0 and equilibriated for 30 min at 25"C at a Photon fluence rate of 50 μ mol m^{-2} s^{-1}. ^{14}C-labelled CH, NH, was then added to the incubation mixture to a final concentration of 30uM (specific activity 10 KBQ cm). After 60 sec., the cells were separated from their bathing medium by microcentrifugation through silicon oil/dinonylphthalate (40:60, v/v) into perchloric acid/water (15:85, v/v) (Scott and Nicholls, 1980). In long term experiments, i.e. upto 24 min, samples were incubated with ^{14}CH,NH, for varying periods of time and then subjected to silicon oil microcentrifugation. Samples of the bathing medium and perchloric acid fractions were withdrawn for the estimation of ^{14}C incorporation. ^{14}C-labelling was determined by liquid scintillation spectrometry using a toluene-based scintillant.

Measurement of electron transport activities:

Cyanobacterial cells were harvested and washed twice with harvesting buffer consisting of 20 mM HEPES-NaOH, pH 7.5; 1 mM $CaCl_2$ and 7.5% PEG-4000 and resuspended in the same buffer. The cell suspension was centrifuged at 10,000 xg for 10 min. Then, the cells were suspended in reaction buffer consisting of 25 mM HEPES-NaOH, pH 7.5, 20 mM NaCl. These cells were used for measuring PSI1 activity.

Assay of electron transport activities:

The photochemical activities **(PS11** and PSI) were measured by following the method of Robinson et al **(1982).** Assays of electron transport activities were carried out using the thermostated (25 ± 1°C) glass cuvette, fitted within a dark-type O_2 electrode (Yellow Spring Instrument Co., USA). The cells were illuminated with white saturating light (450 w m) at the surface of the vessel obtained from a projector. Thermostated water bath was used to maintain the temperature (25°C) of the reaction mixture constant during the illumination. The changes in the oxygen concentration were recorded.

Photosystem II assay:

p-Benzoquinone (pBQ) mediated electron transport activity of **PS1I** (H_2O → pBQ) was used for intact cells. Being a lipophilic compound BQ enters into the intact cells and accepts electrons at the plastoquinone position (Trebst, 1974). The reaction mixture in a final volume of 3.0 ml consisted of reaction buffer, 0.5 mM pBQ and the intact cells equivalent to 12-15ug Chl a. The samples were illuminated with the help of a 300 w projector lamp which provided a saturating incandescent light (450 wm) on the surface of the sample cuvette.

Partial electron transport activity of spheroplasts mediated by PSII resulting in oxygen evolution was also measured polarographically using H_2O to potassium ferricyanide [$K_3Fe(CN)_6$] and phenylene diamine (PD) assay. FeCN cannot enter into intact cells. O_2-evolution due to H_2O to FeCN assay is not an exclusively PSII mediated measurement since, FeCN accepts at both **the PSI** and PSII reducing sites constituting mostly whole chain electron transport, depending upon the type of preparation (Trebst, 1974). Saha et al **(1971)** have shown that oxidized phenylene diamines **are excellent lipophilic**

PSII electron acceptors. PD , when used in combination with FeCN mediates electrons effectively to FeCN, thus constituting a reliable PSII assay. We have, therefore, used H_2O to FeCN + PD as an assay system for the estimation of O_2-evolution for PSII catalyzed Hill activity.

Three ml. of the reaction mixture contained the above reaction buffer, 1 mM $[K_3Fe(CN)_6]$, 100 u M PD . Spheroplasts equivalent to 15-20 ug Chl a was taken for each estimation.

Preparation of spheroplasts:

Spheroplasts for measurement of Absorption spectra and PSI activity of Nostoc muscorum and herbicide-resistant mutant strains were prepared by following the method of Peter and Sherman (197S). The cyanobacterial cells in exponential growth phase were harvested and washed with 20 mM Tricine-KOH buffer (pH 7.5) containing 400 mM sucrose, 10 mM Kcl and 10 mM E.DTA. The cells were suspended in the same buffer and incubated with 0.5 mg. ml of Lysozyme for 15-20 min at 37°C. After Lysozyme treatment, the cyanobacterial cells were centrifuged at 5,000 xg for 15 min and used for measurement of photosystem I activity.

Photosystem 1 assay:

Photosystem I (PSI) mediated electron transport activity was estimated as a measure of the oxygen uptake due to MV photoreduction and its subsequent autooxidation with 2,6-dichlorophenol indophenol (DCIP) and sodium ascorbate donor couple. The experimental set up was the same as described for PSII assay. Three ml of the reaction mixture consists of 25 mM HEPES-NaOH, pH 7.5; 100 µ M DCIP, 2 mM sodium ascorbate; 0.5 mM MV; 1 mM sodium azide and 10 µ M DCMU. Spheroplasts equivalent to 15-20 ug Chl a was used for each estimation (Robinson et al, 1982).

Photochemical activity estimated in terms of O_2 exchange has been expressed as µ mol O_2 evolved or consumed mg Chl a^{-1} **hr"** . The amount of O_2 exchanged was calculated using the dissolved oxygen concentration of 235 n mol. ml at 25°C (assay temperature).

Measurement of O_2 evolution:

CL evolution was measured with a dark-type CL electrode. The cyanobacterial cells were deposited on a flat platinum cathode that was polarized at 0.6V with reference to a large Ag/AgCl electrode. The electrodes were immersed **in** an electrolyte (consisting of 0.05 M phosphate buffer at pH 7.8, 0.1 M KQ). The electrode separated from the **magnetically** stirred assay medium by a Teflon membrane. The difference between the output of the electrode in water in equilibrium with air and water in equilibrium with pure nitrogen was considered to represent 0.235 µ mol.ml^{-1} in the assay medium. After injection of the sample into the assay medium, the medium was illuminated from opposite sides with projector lamps. The rate of O_2 evolution was determined from the initial slope of electrode output as a function of time.

Measurement of chlorophyll a fluorescence:

Room temperature Chl a fluorescence originates mostly from PSII and is measured at 680-685 nm (Papageorgiou, 1975). Since the Chl a fluorescence intensity at 680-685 nm is a measure of PSII photochemistry and is sensitive to the electron flow through the reaction centres of **PSII,** it provides an additional method to monitor the **PSII** activity. We measured the relative intensity of Chl a fluorescence at 680 nm in the presence (F_{+DCMU}) and absence of 5 µM DCMU (F_{-DCMU}). Addition of DCMU to the cells closes

all the PSI1 traps and raises the fluorescence intensity to the maximum level. Hence, the relative enhancement in room temperature fluorescence intensity, measured at 680 nm with and without the addition of DCMU was recorded using Perkin-Elmer LS-5 luminescence spectrometer. Intact cells and spheroplasts equivalent to 5 u g Chl a suspended in 3.0 ml of the reaction buffer (same as PS11 assay) were excited at 440 nm (5 nm slit width was selected for both the excitation and emission monochromators) to take the emission spectra. The emission spectra was scanned from 600-750 nm. 5 u M DCMU was added to the sample cuvette directly after completing the measurement without the herbicide. Fluorescence yield was recorded after the excitation light was put on. The data was recorded as relative ratios of F_{+DCMU}/ F_{-DCMU}.

Assay procedures for enzyme activities:

Nitrogenase:

Nitrogenase activity was measured using whole cells by estimating the acetylene reduction activity as described by Stewart et al (1967). Assay was done in test tubes fitted with serum stoppers. Air was removed from the tubes containing cyanobacterial cells (10-15 µg. Chl a) by evacuation and replaced by the desired gas phase which contained 10% (v/v) acetylene. These tubes were incubated with intermittent shaking at 28 + 2°C and a photon fluence rate of 50 u mol m s . After 1 hr of incubation, ethylene production was assayed gas chroma tographicall y using a Perkin-Elmer Sigma 3B **Gas** Chromatograph fitted with a hydrogen flame ionization detector and a column of **Porapak** R. **The** nitrogenase activity was expressed in terms of u mol C_2H_4 formed mg Chl a . hr .

Uptake-Hydrogenase:

Uptake hydrogenase activity was measured using whole cells by the method of Tel-or et al (1977). Cyanobacterial cells were harvested, washed and resuspended in 2 ml of fresh N_2-medium in 15.0 ml capacity test tubes scaled with rubber stoppers. H_2 gas was injected (2% v/v) with a gastight syringe. The tubes were then incubated in an incubator under a photon flux of about 50 u mol m s and temperature of 28 *t* 2°C. 0.5 ml of the gas samples were withdrawn from the tubes at regular intervals and injected into a Perkin-Elmer Sigma 3B dual FID chromatograph provided with a molecular sieve column 5A and thermal conductivity detector with Argon as the carrier gas. Tubes containing fresh N_2-medium and H_2 served as control. Enzyme activity was expressed as µ mol H_2 consumed. mg Chl a . hr .

Nitrogenase mediated H_2-evolution:

H_2-evolution mediated by nitrogenase was measured under Argon atmosphere. Cyanobacterial cells were harvested, washed and resuspended in fresh N_2-medium and flushed with Argon for 30 minutes-60 minutes. Gas samples were withdrawn from the tubes at regular intervals and H_2 concentration was measured gas chromatographically.

Glucose-6-phosphate dehydrogenase:

Cyanobacterial cells were harvested by low-speed centrifugation and washed twice with Tris-Maleate 0.05 M buffer, supplemented with 0.1% (v/v) β-mercaptoethanol, pH 6.5. The cells were then disrupted by passage through a French pressure cell operated at 110 MPa. The extract thus obtained was centrifuged at 19,000 xg for 30 min and the supernatant was used as crude enzyme.

The glucose-6-phosphate dehydrogenase activity was estimated by following the method of Schaeffer and Stanier (1978). Aliquots of the enzyme solution were added to Tris- maleate (0.05 M) buffer, pH 7.4 containing 10 mM $MgCl_2$ and 0.5 mM $NADP^+$ and 5 mM glucose-6-phosphate in a final volume of 1.0 ml. Rates of NADPH formation were measured at 340 nm in a Gilford spectrophotometer at room temperature. Enzyme activity was estimated by using the molar extinction coefficient of NADFH at 340 nm as 6.22×10^6.

Clutamine synthetase:

Cyanobacterial cells were harvested and washed twice with 40 mM Tris-HCl buffer, pH 7.5 and resuspended in the washing buffer supplemented with 5 mM $MgCl_2$, 10 mM sodium glutamate, 5 mM ß-mercaptoethanol and I mM EDTA. This suspension was passed through a French pressure cell at 110 MPa. Cell-free extract was prepared by centrifuging the broken cells at 35,000 xg for 30 mm.

Glutamine synthetase activity was estimated using the γ-glutamyl transferase reaction in which the formation of γ-glutamyl hydroxamate from glutamine and hydroxylamine was measured (Shapiro and Stadtman, 1970). The reaction mixture contained in addition to enzyme, in 1.0 ml : 40 mM Tris-Hcl buffer, pH 7.5; 3 mM $MnCl_2$; 20 mM potassium arsenate; 0.4 mM Na-ADP and 60 mM hydroxylamine (neutralized with 2 N NaOH) and 30 mM glutamine. This was incubated at 37°C for 10 min and the reaction was terminated by adding 2.0 ml of stop-mixture (10% $FeCl_3$; 24% TCA & 6N HC1) and the Y-glutamyl hydroxamate formed was estimated by measuring the absorbance at 540 nm and the amount of Y-glutamyl hydroxamate formed was determined from the standard curve. One unit of enzyme is defined as

the enzyme required to catalyse **the** synthesis of 1µ mol oi γ-glutamyl hydroxamate per minute under standard transferase assay conditions. Specific activity is defined as units mg protein . mir.

NADPH : Fd oxido **reductase:**

Cyanobacterial cells were harvested by centrifugation, washed twice with 50 mM Tris-HCl buffer, pH *7.5* and resuspended in the same butler. Cells were disrupted by passage through a **French** pressure cell **at** 110 MPa and the broken cell material was centrifuged at 35,000 xg for 30 ruin. The 35,000 xg supernatant was used as crude enzyme.

The enzyme activity was measured by following the method of Rowell et al (1981). The reaction mixture contained, in addition to the enzyme, **in** 1.0 ml: 100 mM Tris-HCl buffer (pH 7.5); 100 **n** mol DCIP and 60 **n** mol NADPH. The rate of DCIP reduction was measured at 600 nm at 30°C. The molar extinction coefficient of DCIP of 20.6 was used for the estimation of the enzyme activity.

Nitrate reductase:

Cyanobacterial cells in the exponential phase of growth were harvested by low-speed centrifugation washed twice with 50 mM Tris-HCl, pH 7.5 buffer containing 0.1 M NaCl; 0.3 M sucrose and 1 mM EDTA and resuspended in the same buffer. This suspension was sonicated at 4°C for 6-8 min. The broken material was centrifuged at *30,000* xg for 30 min and the resulting supernatant was used as crude enzyme.

The method of Manzano et al (1976) was followed for estimating nitrate reductase activity. The enzyme activity was measured colorimetrically

following the appearance of nitrite using methyl viologen chemically reduced by dithionite as electron donor. The reaction mixture contained in a final vol. of 1.0 ml: Glycine-KOH buffer, pH 10.5 100 u moles; 20 u moles KNO_3 · 4 u moles MV; 2 mg $Na_2S_2O_4$ in 0.1 **ml** of 0.23 M $NaHCO_3$ and an appropriate amount of enzyme. This was incubated at 30°C for 10 min and the reaction was terminated by the addition of 0.2 **ml** of 1.0M zinc acetate and **the** nitrite thus formed was estimated by following the method of Snell and Snell (1966).

Chemicals:

Metronidazole and DCMU were purchased from Sigma Chemical Company, St. Louis, USA. All other chemicals used were of analytical grade. DCMU is only sparingly soluble in water, and a stock solution of 1 mM is made up in ethanol, taking care to keep the final ethanol concentration **in** the reaction mixture below 0.1%. Similarly Atrazine stock solution was prepared **in** Dimethyl sulfoxide (DMSO). C-methylammonium with a specific activity of 56 mci. mmol was obtained from Amersham, England.

C H A P T E R III

FIXED NITROGEN-SOURCE AND REGULATION OF AEROBIC: HYDROGEN METABOLISM

INTRODUCTION

H_2 formation by intact cyanobacteria occurs only under N_2-fixing conditions and their NO_3 and NH_4^+-assimilating cultures show absence of both N_2-fixation and H_2-formation in parallel way. Such H -production by cyanobacteria is oxygen-sensitive, ATP-dependent and sensitive to uncouplers (Bothe, et al, 1980). An interesting cyanobacterium Oscillatoria limnetica capable of H_2S and H_2-dependent anoxygenic photosynthesis is known to produce sufficient amount of H_2 in a hydrogenase dependent reaction under anaerobic conditions. Thus, this organism is like Clostridium pasteurianum capable of removing excess reductant by hydrogenase and not by nitrogenase under anaerobic growth conditions.

H_2-evolution by obligate photoautotrophic cyanobacteria has in recent years attracted special consideration as a system lor harvesting H_2 in solar energy conversion projects. The idea of using cyanobacteria to photoproduce H_2 under aerobic photoautotrophic growth conditions as an alternative energy source arose with the work of Benemann and Weare (1974) and Weissman and Benemann (1977) on Anabaena cylindrica. Work of Bothe group on this aspect of cyanobacterial biology is not very encouraging for variety of reasons (Bothe and Eisbrenner, 1981). Nitrogenase-catalysed H_2-evolution is not always a loss to cyanobacteria, because of the presence of uptake hydrogenase in N_2-fixing cells. The uptake hydrogenase catalysed consumption of H_2 can follow two different pathways, one called oxy-hydrogen reaction involves aerobic respiratory pathway in the presence of O_2 leading to ATP production and the other by PS-I mediated pathway under strictly anaerobic conditions in

the presence of photosynthetic light to generate reduced ferredoxin as a source of reductant for nitrogenase activity (Bothe et al, 1977; 1978; Eisbrenner et al, 1978; Peterson and Burris, 1978; Peterson and Wolk, 1978; Tel-or et al, 1978; Tetley and Bishop, 1979).

Reactions of aerobic H_2-production and aerobic H utilization in heterocystous cyanobacteria occur in specially differentiated cells called heterocysts and therefore all the factors that affect heterocyst differentiation are bound to affect aerobic H_2-metabolism. In free-living cyanobacteria, NO, and NH.-nitrogen sources are found to repress coordinately heterocyst formation and nitrogenase activity (Flemming and Haselkorn, 1973; Singh et al, 1977; Haselkorn, 1978; Stewart, 1980; and Houchins, **1984).** However, Anabaena sp. CA has been reported to show NO_3-repression and not NH_4^+ repression of heterocyst and nitrogenase activity, a finding which has not yet been reported in any other heterocystous cyanobacterium and for which there is no as yet clear cut biochemical explanation (Bottomley et al, 1979). Nitrate reductase enzyme has been found to play a role in NO_3-repression control (Bagchi and Singh, 1984). Mutational loss of glutamine synthetase activity does not result in the abolition of NH_4^+-repression control **in** Anabaena cycadeae (Singh et al, 1983). O_2-evolution seems to be the repressor of nitrogen-fixation **in** Klebsiella pneumoniae (Brill, 1975). A role of O_2 as a repressor exclusively of nitrogenase has recently been implied in a study of **nif** gene expression in anaerobic cultures of A. variabilis (Helber et al, 1988). Glutamine is known to repress heterocyst and nitrogenase in A. cycadeae (Singh et al, 1983). The first report of genetic uncoupling of heterocyst and nitrogenase regulation was provided by the work of Singh and Singh (1978), where heterocyst and nitrogenase both showed derepression in *NO'* and NH_4^+-medium but not in glutamine medium where nitrogenase activity was found repressed without repression of heterocyst.

The mechanism of nitrogen regulation of aerobic nitrogen fixation in cyanobacteria at molecular level is not yet as clear as reported for Klebsiella and Azotobacter (Kennedy and Toukdarian, 1987).

Ammonium nitrogen not only shows NH_4^+-repression of heterocyst and nitrogenase but also shows NH_4^+-repression of nitrate reductase activity **(Bagchi** and Singh, 1984). The cyanobacterial nitrate reductase has been shown to be NO_3^--inducible in Anabaena cylindrica (Hattori, 1962; Ohmori and Hattori, 1970); Anabaena 7119 and Nostoc 6719 (Herrero et al, 1981); NH_4^+-repressible in Anacystis nidulans (Herrero et al, 1981); and NO_3^--inducible and NH_4^+-repressible in Augmnellum quadruplicatum (Stevens Jr and Van Baalen, 1974). The understanding of nitrate reductase regulation **is** important in the context of nitrogenase because both are molybdo-enzymes. No previous study has been attempted to analyse **the** mechanism by which molybdenum requirement of the two molybdo-enzymes is met efficiently.

Nitrate reductase in Nostoc muscorum is NH_4^+-repressible and **derepressible** under N_2-fixing conditions. Recently Bagchi et al, (1985) has demonstrated a method of reconstituting active nitrate reductase with cyanobacterial apoprotein and Escherichia coli Mo-cofactor. Using this method they have shown that NH_4^+-regulation of nitrate reductase activity functions only in respect of apoprotein repression and that Mo-cofactor production is constitutive occurring in vegetative cells of N_2-fixing heterocystous filaments and in NO, and NH_4^+-grown nonheterocystous non N_2-fixing filaments.

We have used reconstitution method of nitrate reductase as described above in order to locate the presence of nitrate reductase activity in heterocysts to understand the role of nitrate metabolism in regulation of aerobic metabolism of H_2, that occurs exclusively in heterocysts under oxygenic photosynthetic conditions. We also describe the results on the behaviour of

metronidazole-resistant mutants of cyanobacterium Nostoc muscorum derepressed for heterocyst **formation** and uptake-hydrogenase activity without showing any alteration in **NH** $_4^+$-repression **of nitrogenase** activity. The special physiological and biochemical properties of this mutant are described **here** with **special emphasis on physiological** and **regulatory** interdependence of **nitrogenase** activity and uptake-hydrogenase activity.

EXPERIMENTAL PROCEDURE

Organism

The parent Nostoc muscorum was grown **axenically in** N_2-medium **(diazotrophic** growth medium), **in** NO_3-medium or **in** NH_4^+-medium as described **in Chapter** II. Metronidazole (0.5 mM) was included in **the** growth medium whenever required.

Metronidazole-resistant mutant (Mtn-R) was isolated on combined nitrogen-free medium (N_2-medium) containing metronidazole by lollowing the method of Singh and Singh (1978) as described in Chapter II.

Growth, chlorophyll and protein content were estimated by following the methods of Mackinney (1941), and Lowry **et** al **(1951)** respectively as described earlier (Chapter II).

The method and materials for isolation of heterocysts, measurement of nitrate reductase activity (NR), nitrogenase activity, uptake-hydrogenase activity, Glucose-6-phosphate dehydrogenase activity, NADPH:Fd oxidoreductase activity, Glutamine synthetase activity, Reserve polymer glycogen content and ammonium transport activities were same as described in Chapter 11.

RESULTS

Differentiation of vegetative cells into heterocyst is accompanied by synthesis of envelope compounds, rearrangement of photosynthetic apparatus, destruction of O_2-generating PSII activity and suppression of a system concerned with RUBISCO production (Haselkorn **et** al, 1987). Along with **these** changes occur the synthesis of nitrogenase, operation of PSI activity associated with ATP production (Thomas et al, 1977) and activation of oxidative pentose phosphate pathway for the generation of reductant required for N_2-fixation (Stewart, 1980).

Nitrate reductase activity distribution in vegetative cells and heterocysts: **We** measured nitrate reductase activity in heterocyst **extract** and N_2-grown heterocystous filament extract and NO_3-grown nonheterocystous filament extract and in heterocyst extracts supplemented svitn apoprotein from N_2-grown heterocystous filaments as well as with apoprotein preparation **from** NO_3-grown nonheterocystous filaments. These results are given in Table 1. Nitrate reductase activity was missing in heterocyst extract but when this heterocyst extract was supplemented with apoprotein from N_2-grown filaments or NO_3-grown filaments, nitrate reductase activity appeared and the level of recovered nitrate reductasc activity under the two given reconstitution conditions was found almost to be of a similar level. This finding indicates that the cyanobacterium heterocyst is deficient in nitrate reductase activity and that **the** loss of nitrate reductase activity from heterocyst is due to the absence of apoprotein component and not due to Mo-cofactor component. The level of nitrate reductase activity in N_2-grown N. muscorum extract was nearly half of that found in NO_3^--grown filaments (Table 1). This apparent high level of nitrate reductase activity in NO_3^--assimilating filaments appear to indicate a role for NO_3 in

Table I : Nitrate reductase activity in cell-free extracts of isolated heterocysts and whole filaments of Nostoc muscorum grown in nitrogen-free medium, nitrate medium and ammonium medium.

Enzyme source	Activity
N_2-grown N. muscorum extract	3.25
NO_3^--grown N. muscorum extract	7.14
NH_4^+-grown N. muscorum extract	not detectable
Isolated heterocyst extract	not detectable
Apoprotein from N_2-grown N. muscorum	not detectable
Apoprotein from NO_3^--grown N. muscorum	not detectable
Isolated heterocyst extract (Mo-cofactor) supplemented with apoprotein preparation from N_2-grown N. muscorum	6.25[a]
Isolated heterocyst extract (Mo-cofactor) supplemented with apoprotein preparation from NO_3^--grown N. muscorum	6.39[a]

[a]Specific activity expressed per mg protein of the heterocyst extract. The amount of apoprotein preparations used were similar in both cases (protein concentration 2 $mg.cm^{-3}$).

Enzyme activity expressed as nmol NO_2^- formed. min^{-1}. mg $protein^{-1}$.

For other details please see text.

maintaining a higher level **of** reductase activity in **cyanobacteria** but a similar level of nitrate reductase activity shown by reconstituted extracts comprising heterocyst Mo-cofactor and **apoprotein** preparation from N_2-grown and NO_3^--grown filaments suggests that, lower level of nitrate reductase activity in N_2-fixing cultures is because of limited availability ot **Mo-cofactor** and not because of limitation of apoprotein level. NH_4^+-grown cultures cannot reconstitute nitrate reductase activity with Mo-containing **cofactor** extract **from** heterocyst and can reconstitute with apoprotein preparations from NO_3-grown filaments or N_2-grown filaments (data not shown). This indicates that NH_4^+-grown cultures of the cyanobacterium while containing Mo-cofactor does not contain apoprotein of nitrate reductase and that, the inability of NH_4^+-grown cultures to show nitrate reductase activity is due to their inability to produce apoprotein.

Growth response of the cyanobacterium Nostoc muscorum to different nitrogen-sources:

The cyanobacterium Nostoc muscorum grew well with N_2, NO_3^- or NH_4^+ as nitrogen source although NO_3^- was the best source of nitrogen than NH_4^+ or N_2 (Fig I). The N_2-fixing cultures of the cyanobacterium differentiated nearly 5-6% of the vegetative cells into heterocysts with a characteristic spacing along the filament. On transfer of such cultures to fresh NO_3^- or NH_4^+-medium, growth was accompanied by a gradual decrease in nitrogenase activity (Fig.II), heterocyst frequency and such 4-6 days old cultures contained **filaments** devoid of heterocysts and nitrogenase activity. The transfer of such non-heterocystous **filaments** from NO_3^--assimilating or NH_4^+-assimilating cultures to fresh **No-medium** showed heterocyst differentiation and nitrogenase activity after nearly 24-30 hr of such transfer and reached their optimum level by 48-56 hr (Fig.III). We examined the growth response ol N_2-assimilating, NO_3^--assimilating and NH_4^+-assimilating cultures of the cyanobacterium to dilferent

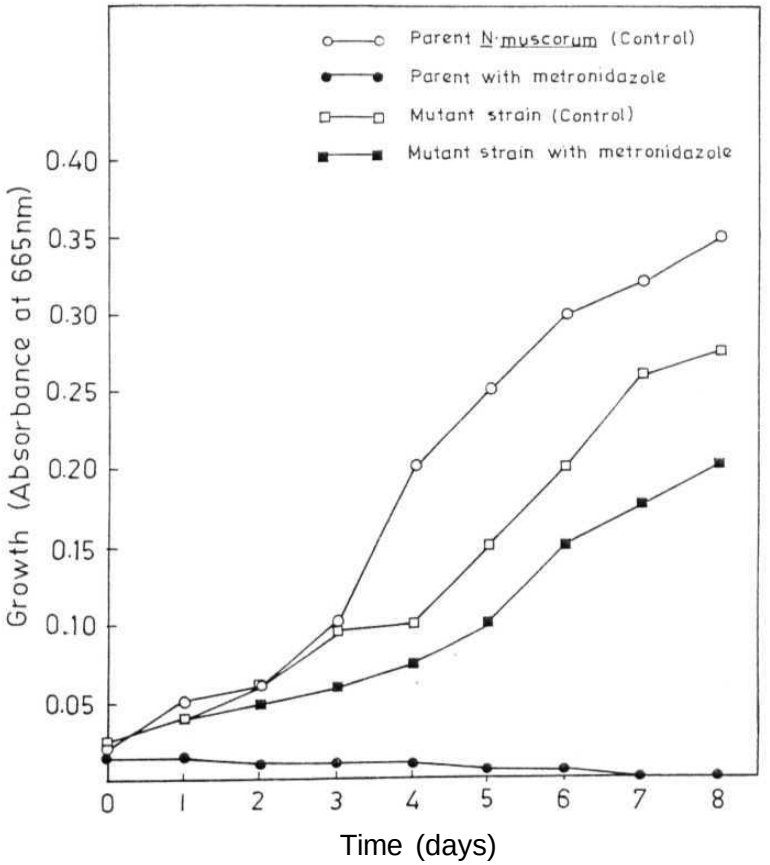


Fig. 1 A. Growth response of parent Nostoc muscorum and its metronidazole-resistant mutant strain under various growth conditions.

A. N_2-assimilating conditions.

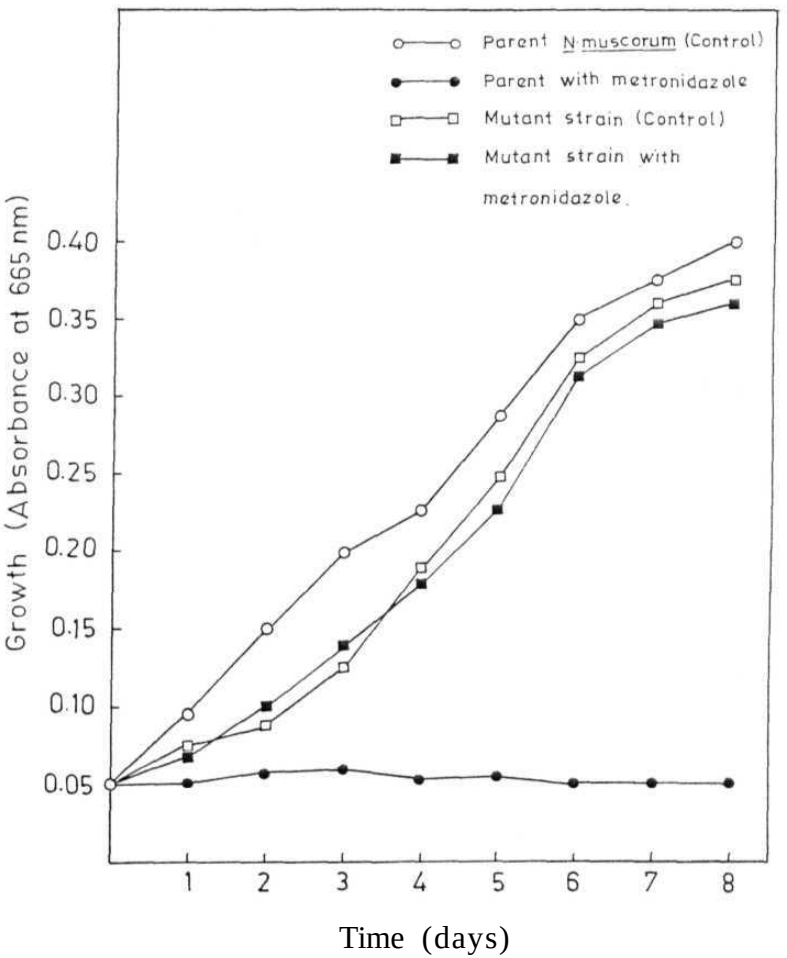


Fig. I B. Growth response of parent Nostoc muscorum and its metronidazole-resistant mutant strain under various growth conditions.

B. NO_3^--assimilating conditions.

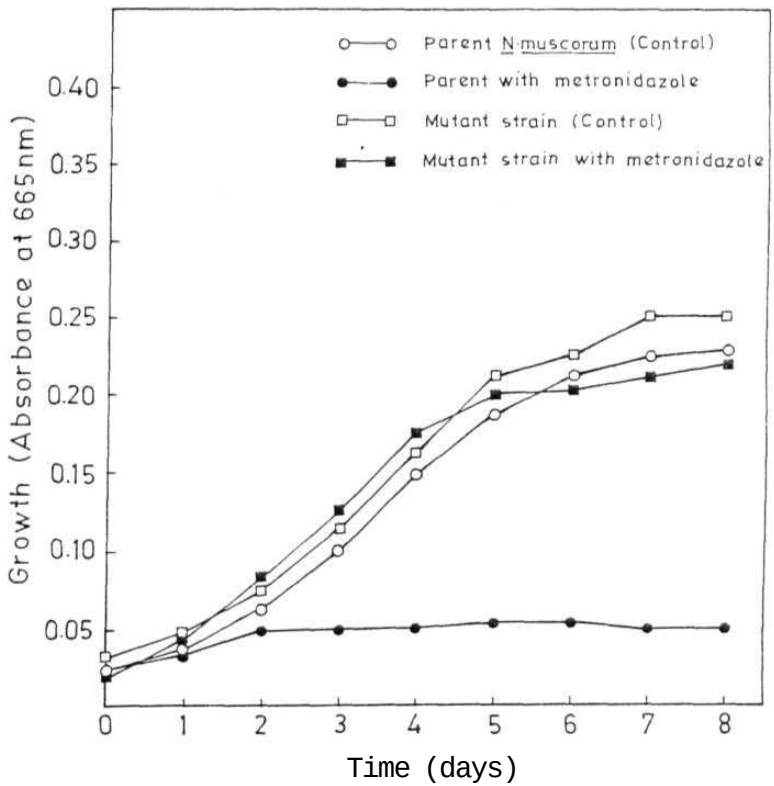


Fig. I C. Growth response of parent Nostoc muscorum and its metronidazole-resistant mutant strain under various growth conditions.

C. NH_4-assimilating conditions.

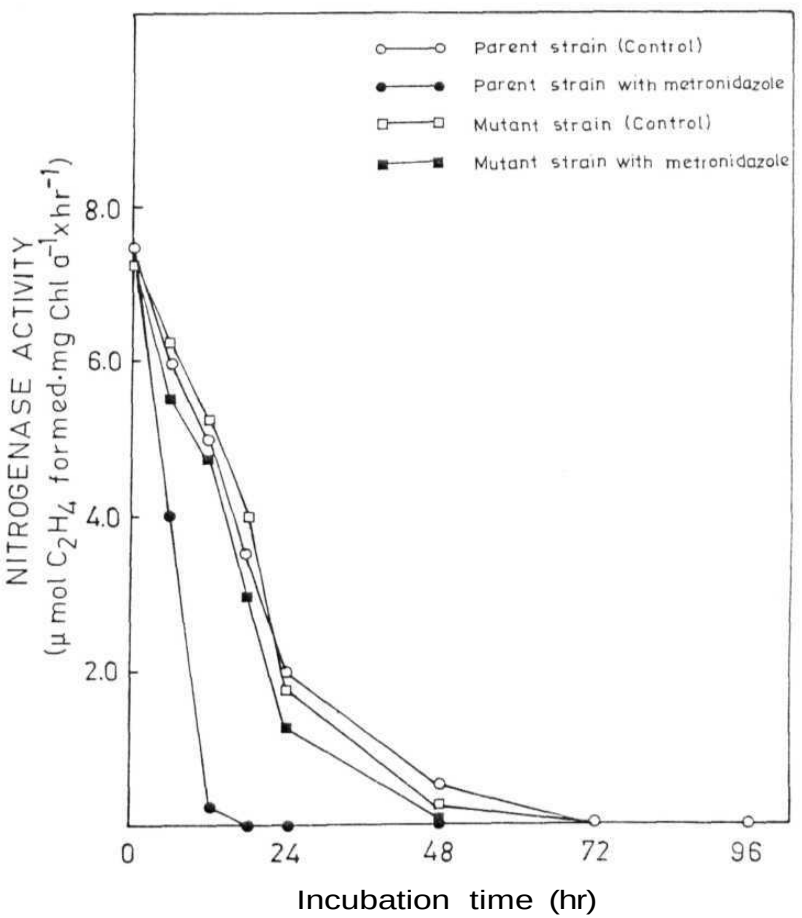
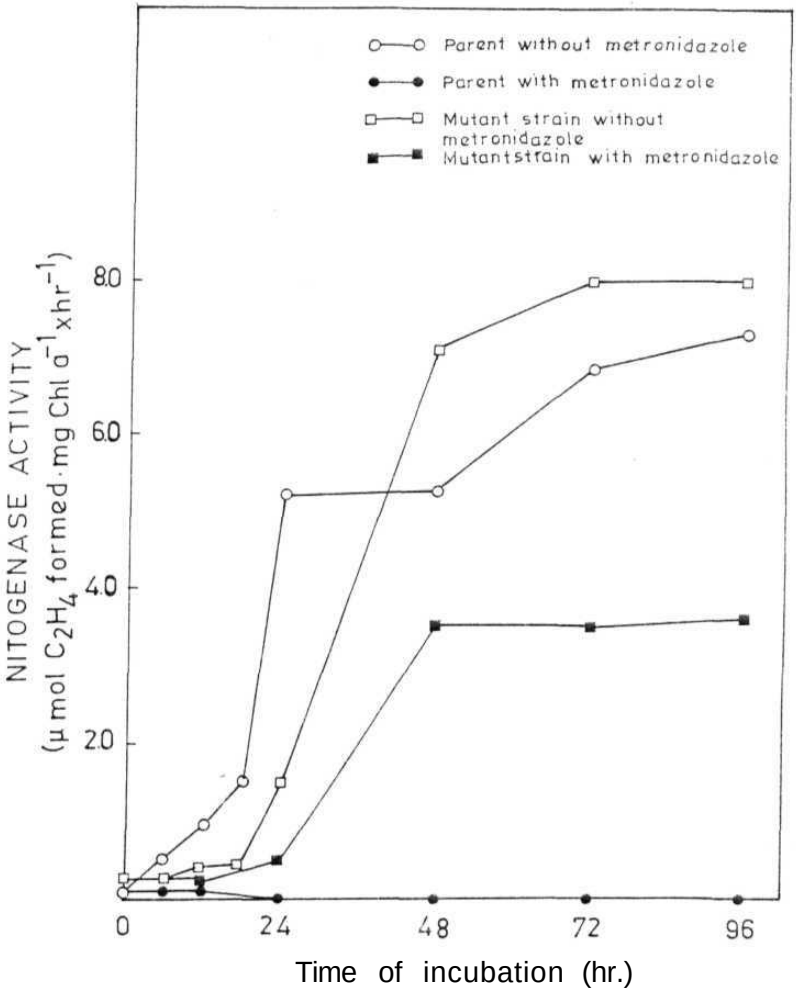


Fig. II. Kinetics of repression of nitrogenase activity in parent Nostoc mu scorum and its metronidazole-resistant mutant strain.

Exponentially growing cells under No-fixing conditions, were washed and re suspended in NH_4^+-medium and nitrogenase activity was estimated at different intervals of time.



Tig. III. Kinetics of derepression of nitrogenase activity in parent Nostoc muscorum and its metronidazole-resistant mutant strain.

Exponentially growing cells, under NH_4^+-assimilating conditions, were washed and resuspended in fresh N_2^- medium and nitrogenase activity was estimated at different intervals of time.

concentrations **of** metronidazole. As shown in the results, parent strain failed to grow in N_2-medium, NO_3-medium or NH_4-medium containing 0.25 mM of the drug, metronidazole. This indicated that the drug is growth toxic to the cyanobacterium under photoautotrophic conditions and that the degree oi **toxicity** is not **a function** of **the** nitrogen-source **in the growth medium** (Fig. 1).

Since **the cyanobacterial** cultures died **on** or in the growth medium containing 0.25 mM of **the** drug, we were tempted to select out spontaneous mutants capable of growth on the N_2-medium containing 0.5 mM of **the** drug. The colonies that appeared on such plates alter a growth incubation period of **nearly** a month arose with a irequency of nearly 10^{-} . We tested **individually** the stability of such colonies on fresh nutrient medium containing 0.25 mM oi the drug. All the colonies started growing **immediate!)** on such drug-containing plates, thereby suggesting their genetic stability. **Thereafter,** they were translerred to the drug-containing NH_4- medium to check lor the presence ol heterocysts in the presence oi NH. as nitrogen-source. We found them differentiating heterocysts even in NH_4^+-medium. This became a basis tor our subsequent work of NH_4^+-regulation of heterocyst, nitrogenase and uptake hydrogenase along with NH_4^+-regulation ol the activity ol **NADPH:Fd** oxidoreducta.se (FNR), glucose-6-phosphate dehydrogena.se activity. We also measured the ammonium regulation of glycogen content in the mutant and the parent strains.

Heterocyst frequency and nitrogenase activity in various inorganic and organic nitrogen media in the parent Nostoc muscorum and its metronidazole-resistant mutant strain:

Parent strain and mutant strain were compared **with** respect to their heterocyst frequency and nitrogenase activity in various inorganic and

organic nitrogen media (Table II). Both the parent and the drug-resistant mutant produced heterocysts and nitrogenase activity in drug-free N_2-medium. However, the addition oi the drug to the N_2-medium of both the parent and the mutant caused progressive decline in nitrogenase activity ol **the** parent which reached a zero value by 12 hr. In **comparison,** the drug caused decline of nitrogenase **activity** in the mutant strain by a level ol 50% only (Fig.IV). However, the mutant strain grew **diazotrophically** with the drug as well as **without the** drug, thereby suggesting that 50% **difference** in nitrogenase activity of the cyanobacterium is not relevant to diazotrophic growth. Pareni **differentiated** heterocysts with a **frequency** of 5-6% **while** its mutant **differentiated** heterocysts with a frequency of 8-9%. In addition, while parent (ailed to differentiate heterocysts in NH.-medium, and glutamine medium its mutant strain diflerentiated heterocysts with a frequency in NH.-medium which was the same as that in N_2-medium and **with** a frequency of 4-5% in **glutamine-medium.** Interestingly, both **the** parent and **the** mutant sixains **fail** to **differentiate** heterocysts during their growth **in** NO, or **in** NO_3-medium, thereby suggesting the mutant to be **specifically** defective in NH.-generated repressor signal lor heterocyst. N_2-fixing cultures **of** the parent and mutant strain showed almost similar level ol nitrogenase activity in N_2-medium. The mutant strain while differentiating heterocysts **in** NH_4^+-medium did not show nitrogenase activity thereby suggesting the mutant strain to be specifically altered in NH_4^+-regulation of heterocyst diflerentiation.

Uptake hydrogenase activity in parent Nostoc muscorum and its metronidazole-resistant mutant strain in various inorganic nitrogen media:

We measured uptake hydrogenase activity of the parent and mutant in N_2-medium and as results indicated (Table III), the activity oi the uptake

Table II : Nitrogenase activity and Heterocyst frequency in parent Nostoc muscorum and its metronidazole-resistant mutant strain under various growth conditions.

Growth Condition	PARENT STRAIN				MUTANT STRAIN			
	Nitrogenase activity		Heterocyst frequency (%)		Nitrogenase activity		Heterocyst frequency (%)	
	-Mtn	+Mtn	-Mtn	+Mtn	-Mtn	+Mtn	-Mtn	+Mtn
N_2-medium	7.84	0.0	5-6	0.0	8.25	4.21	8-9	8-9
NO_3^--medium	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
NH_4^+-medium	0.0	0.0	0.0	0.0	0.0	0.0	8-9	8-9
Glutamine-medium	0.0	0.0	0.0	0.0	0.0	0.0	4-5	4-5

Nitrogenase activity expressed as μ mol C_2H_4 formed mg Chl $\underline{a}^{-1}$. hr^{-1}.

Heterocyst frequency expressed as number of heterocysts per 100 vegetative cells.

-Mtn indicates cultures growing without metronidazole.

+Mtn indicates cultures growing with metronidazole.

All the above results are an average of three or four independent experiments which did not vary by more than 10%.

For other details please see text.

Table III : Uptake hydrogenase activity in parent Nostoc muscorum and its metronidazole-resistant mutant strain under various growth conditions.

Condition	Uptake-hydrogenase activity	
	Parent Strain	Mutant Strain
N_2-medium	6.16	5.18
NO_3^--medium	*0.0*	*0.0*
NH_4^+-medium	*0.0*	5.36

Uptake hydrogcnasc activity expressed as μ mol H_2 consumed. mg Chl *a* -hr . All the above results are an average of three independent experiments which did not vary by more than 10% under the experimental conditions. For other details please sec text.

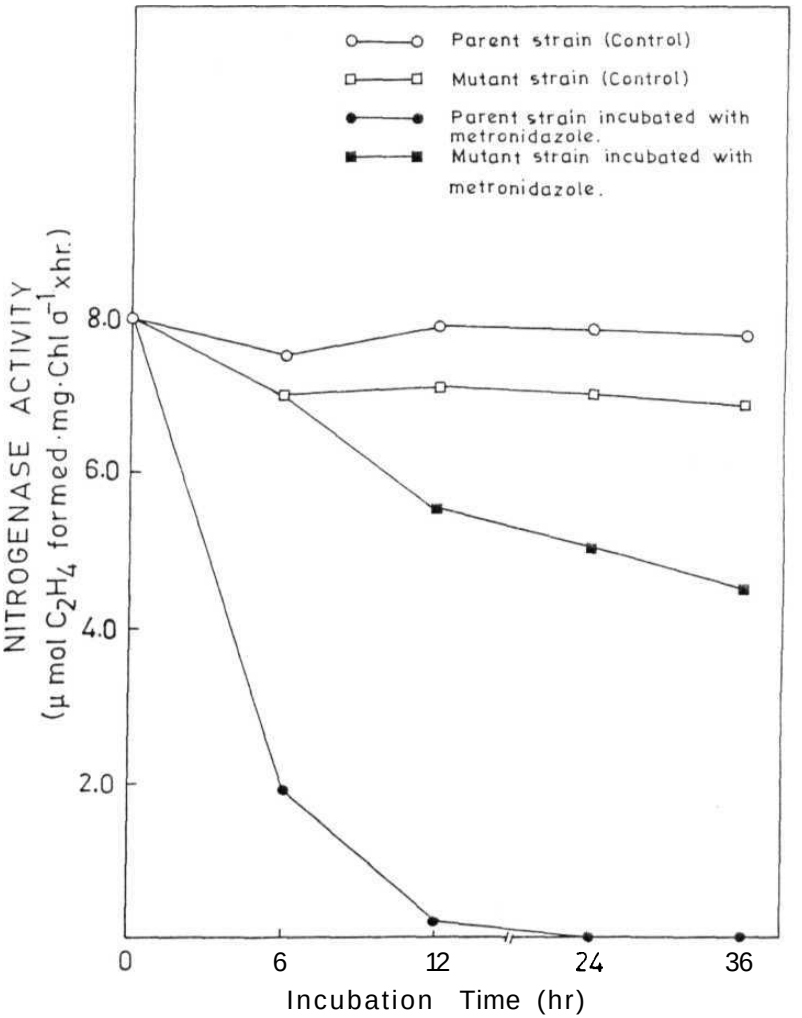


Fig. IV. Effect of adding metronidazole on nitrogenase activity in Nostoc muscorum and its metronidazole-resistant mutant. Exponentially growing cells, under N_2-fixing conditions, were washed and resuspended in fresh N_2-medium to a final chlorophyll concentration of 15 $g.ml^{-1}$. Metronidazole was added at zero time and nitrogenase activity was estimated at different time intervals.

hydrogenase was almost similar in the two strains. When NH.-grown cultures of both the strains were compared for uptake hydrogenase activity, the mutant strain showed uptake hydrogenase activity. In comparison, parent strain in NH_4^+-medium showed complete lack of uptake hydrogenase activity.

Levels of Glucose-6-phosphate dehydrogenase and NADPH:Fd oxidoreductase in parent N. muscorum and its metronidazole-resistant mutant strain:

The oxidative pentose phosphate pathway operates in heterocysts for the generation of reduced ferredoxin, the reductant required for nitrogenase activity. We analysed the level of glucose-6-phosphate-dehydrogenase and NADPH:Pd oxidoreductase (FNR) in isolated population oi heterocysts and whole filaments of the parent and mutant strains (Table IVA). The activity of glucose-6-phosphate-dehydrogenase varied with the nitrogen source in the growth medium. The activity being highest in NO_3-assimilating cultures, lowest in NH.-assimilating cultures and intermediate level under N_2-fixing conditions in the parent strain. This enzyme activity in the mutant strain was found nearly 3 times lower under N_2-assimilating, 6 times lower under NO_3-assimilating, and 3 times lower under NH_4^+-assimilating conditions than the parent under similar conditions. The significant point was that in mutant strain, the dehydrogenase enzyme was less sensitive to the drug inhibitory action than the enzyme from the parent strain which showed no activity in the presence of the drug. The activity of FNR varied with the nitrogen source in the parent as well as in the mutant strains, although the parent strain produced more activity than the mutant strain under any given nitrogen source condition. The FNR activity of the parent was completely inhibited by the drug, while this activity was resistant to the drug in the mutant strain (Table IVB).

Table IV A : Glucose-6-phosphate dehydrogenase (G6PDH) and NADPH:Fd oxidoreductase (FNR) activities in cell-free extract from whole filaments and heterocysts isolated from Nostoc muscorum and its metronidazole-resistant mutant strain.

	Whole Filaments				Isolated heterocysts			
	Parent		Mutant		Parent		Mutant	
	-Mtn	+Mtn	-Mtn	+Mtn	-Mtn	+Mtn	-Mtn	+Mtn
G6PDH	19.89	0.0	5.59	3.21	80.75	0.0	26.79	15.75
FNR	870.0	0.0	253.96	269.16	622.78	38.12	180.16	199.16

-Mtn : indicates cell-free extract treated without metronidazole.

+Mtn : indicates cell-free extract treated with metronidazole.

G6PDH activity expressed as nmol NADPH formed. mg protein^{-1}.min^{-1} and FNR activity expressed as nmol DCIP reduced. mg protein^{-1}.min^{-1}. All the values are an average of three independent experiments which did not vary by more than 10%. For other details see text.

Table IV B: Glucose-6-phosphate-dehydrogenase (G6PDH) and NADPH : Fd oxido-reductase (FNR) activities in parent Nostoc muscorum and its metronidazole-resistant mutant strain under various growth conditions.

Growth Condition	PARENT STRAIN				MUTANT STRAIN			
	G6PDH		FNR		G6PDH		FNR	
	-Mtn	+Mtn	-Mtn	+Mtn	-Mtn	+Mtn	-Mtn	+Mtn
N_2-medium	19.89	0.0	870.0	0.0	5.59	3.21	253.96	269.16
NO_3^--medium	30.94	0.0	452.64	0.0	4.45	3.15	158.5	171.0
NH_4^+-medium	6.21	0.0	220.0	0.0	2.10	2.08	102.0	107.0

Glucose-6-phosphate-dehydrogenase activity expressed as nmol NADPH formed. mg protein^{-1}.min^{-1}.

NADPH : Fd oxido-reductase activity was expressed as nmol DCIP reduced. mg protein^{-1}.min^{-1}.

-Mtn indicates cultures growing without metronidazole.

+Mtn indicates cultures growing with metronidazole.

All the results are an average of three independent experiments which did not vary by more than 10% under the experimental conditions.

For other details please see text.

Glutamine synthetase (GS-transferase) activity in parent Nostoc muscorum and its metronidazole-resistant mutant strain in various inorganic nitrogen media:

We also measured the glutamine synthetase activity in extracts derived from N_2-fixing, NO_3-assimilating and NH_4-assimilating cultures of both the parent and **mutant** strains **in** the presence and absence of the drug (Table V). The enzyme activity **in** both the strains varied with the nitrogen source, being more than double under N_2-fixing conditions and nearly double in NO_3-assimilating conditions in comparison to the activity in NH_4-assimilating conditions. In the mutant strain, the activity did not differ as much as that found in the parental strain. Metronidazole did not inhibit the enzyme activity to any significant extent, thereby suggesting that the drug is not an inhibitor of NH_4-assimilating activity of GS in the cyanobacterium.

Nitrate reductase activity in parent N. muscorum and its metronidazole-resistant mutant strain:

We also examined the nitrate reductase activity of the parent and the mutant grown with different nitrogen sources in the presence and absence of the drug (Table VI). In vitro activity of the enzyme from parent strain growing with N_2 as nitrogen source was nearly half of its culture grown with NO_3 as nitrogen source. Its NH_4-grown cultures did not show any nitrate reductase activity. The in vitro enzyme activity was completely inhibited by the drug in the parent strain. The mutant strain produced enzyme activity in both N_2 and NO_3-assimilating conditions with a pattern broadly similar to the parental culture. The mutant strain extract did not show inhibition of in vitro NR activity by the drug. This indicates that this enzyme activity has become resistant to the drug as a result of mutation to the drug-resistance in the cyanobacterium Nostoc muscorum.

Table V : Glutamine synthetase activity (transferase) in parent Nostoc muscorum and its metronidazole-resistant mutant strain under various growth conditions.

Growth condition	Glutamine synthetase activity			
	Parent strain		Mutant strain	
	-Mtn	+Mtn	-Mtn	+Mtn
N -medium	**1.60**	*0.0*	1.000	0.968
NO_3^--medium	**1.15**	*0.0*	0.773	0.723
NH_4^+-medium	0.46	*0.0*	0.761	0.626

GS transferase activity was expressed as µ mol product formed. mg protein min

-Mtn indicates cultures growing without metronidazole.
+Mtn indicates cultures growing with metronidazole.
All the results are **an** average of three independent experiments which did not vary by more than 10%.

For other details please see text.

Table VI : Cell-free nitrate reductase activity in parent Nostoc muscorum and its metronidazole-resistant mutant strain under various growth conditions.

Growth condition	Nitrate reductase activity			
	Parent Strain		Mutant Strain	
	-Mtn	+Mtn	-Mtn	+ Mtn
N_2-medium	3.25	*0.0*	1.50	1.21
NO_3-medium	6.82	*0.0*	2.59	2.58
NH_4-medium	*0.0*	0.0	*0.0*	0.0

Nitrate reductase activity expressed as nmol NO_2 formed. mg protein $^{-1}$. min $^{-1}$.

-Mtn indicates activity in the absence of metronidazole.

+Mtn indicates activity in the presence of metronidazole.

All the results are an average of four independent experiments which did not vary by more than 10%.

For other details please see text.

Glycogen accumulation in parent Nostoc muscorum and its metronidazole-resistant mutant strain grown with different nitrogen sources:

The carbon reserve glycogen was also investigated in the two strains grown with different nitrogen sources. Glycogen production by the parent was minimum in NH_4^+-assimilating cultures and was 10 times higher than this level in N_2-fixing cultures of the parent strain. NO_2-assimilating cultures of the parent produced nearly three times higher level of Glycogen in quantity than the level found in NH.-assimilating cultures. Mutant strain produced nearly two fold higher glycogen content than parental strain under N_2-fixing conditions. It's NO_2-assimilating cultures also gave higher level, however, its NH_4^+-assimilating cultures gave nearly 2-fold lower level **of** glycogen content than similar cultures of parental strain. The significant point to note here is that the regulation of glycogen content is mediated by the nature of nitrogen-source and higher production of this carbon reserve by the mutant under N_2 and NO_2-assimilating conditions (Table VII).

[C] Methylammonium uptake by N_2-grown N. muscorum and its metronidazole-resistant mutant strains:

We measured the NH.-uptake activity by measuring the [C] Methylammonium uptake in N_2-fixing cultures of both the strains against the level accumulated in toluene treated cells which served as control (Fig. V). Both the strains showed biphasic nature of NH_4^+-transport activity with a more rapid first phase activity. Overall pattern of ammonium transport activity in these two strains was found almost similar. These results indicate that mutation to drug resistance has not altered the ammonium transport activity of the mutant strain.

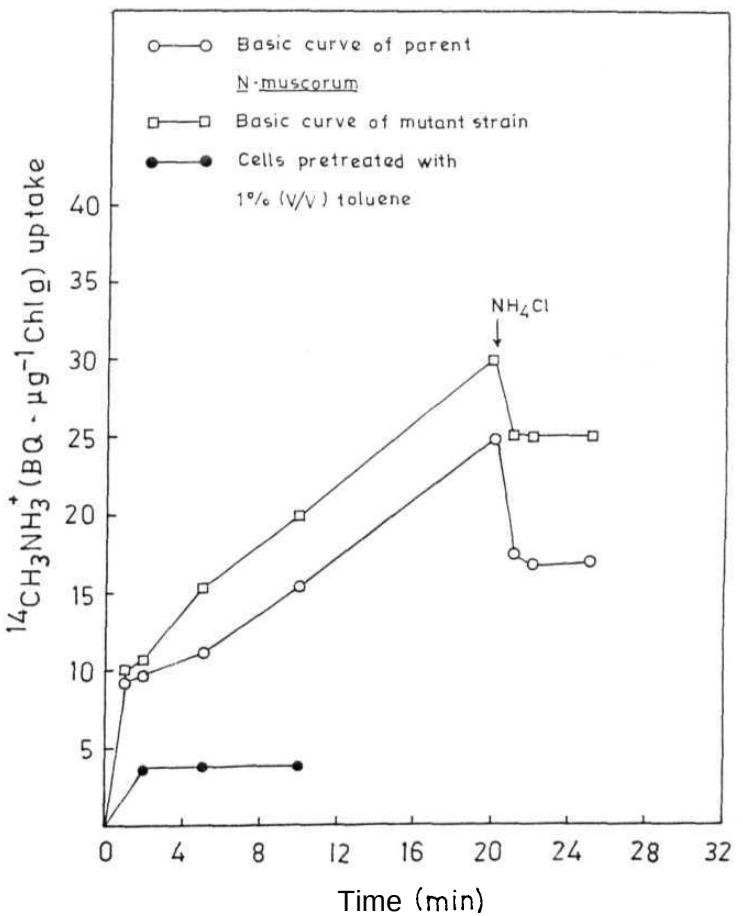
Table VII : Glycogen accumulation in parent *Nostoc muscorum* and its metronidazole-resistant mutant strains under various growth conditions.

Growth condition	Glycogen accumulation	
	Parent Strain	Mutant Strain
N_2-medium	179.70	407.20
NO_3^--medium	48.25	86.87
NH_4^+-medium	17.0	6.95

Glycogen accumulation expressed as µg glucose accumulated mg protein

All the results are an average of three independent experiments which did not vary by more than 10%.

For other details please see text.



Fiq V r 14C]Methylammonium uptake by N_2-grown Nostoc mu scorum and its metronidazole-resistant mutant strain.
[14C]Methylammonium was added at zero time and C incorporation into cells measured over a period of 10 rnin. For other details please see text.

DISCUSSION

Hydrogenase catalysed reactions occur in a diverse group of prokaryotic and eukaryotic organisms. The studies on the complexity of cyanobacterial H_2 metabolism during last decade has revealed the participation of atleast three distinct types of enzymes in this process. They comprise, nitrogenase, which catalyses H_2 evolution unidirectionally; a membrane-bound uptake hydrogenase, which functions in providing reductant to the photosynthetic and respiratory electron transport chain and a soluble hydrogenase which catalyses either H_2-evolution or H_2-uptake when provided with suitable electron donors or acceptors (Houchins, 1984). The distribution of the three enzymes in cyanobacteria depends a great deal on the organism and the growth conditions, thus further complicating the interpretation of the experimental results. The majority of the work on H_2-metabolism of cyanobacteria has been performed in heterocystous forms. For a clear understanding of the role of H_2 metabolism in these organisms, the metabolic differences between vegetative cells and heterocysts along with the cellular localization of H_2 metabolizing enzymes must be considered.

Heterocyst is the site of nitrogenase synthesis and activity in heterocystous cyanobacteria and the evolution of H_2 by cyanobacterial heterocysts always occur during N_2-fixation and a minimum of about 25-30% of the total electron flux through nitrogenase is diverted for the reduction of protons to H_2. These reactions are ATP-consuming and if the rate of nitrogenase turnover slows down owing to the supply of ATP or reductant, the relative proportion of electrons diverted to H_2-production increases, thus suggesting a role for ATP and reductant supply in regulating the rate of H_2-production during N_2-fixation by cyanobacterial nitrogenase (Hageman and Burris, 1980). The function of nitrogenase produced H_2, in cyanobacterial N_2-metabolism, if any, is not

yet understood. However, molecular H_2 is a competetive inhibitor of N_2-fixation and the function of nitrogenase catalysed H_2-evolution if any, could be inhibition of nitrogen-fixing activity of nitrogenase. Association of uptake hydrogenase activity with nitrogenase activity under N_2-fixing conditions might be the result of biological strategy to prevent H_2-inhibition of nitrogenase activity during N_2-fixation.

Heterocystous cyanobacteria are capable of aerobic diazotrophic growth because of the role of heterocyst in oxygen-protection of nitrogenase synthesis and activity under such conditions. In these organisms the ultimate electron donor for supporting compulsory side reaction of nitrogenase activity of H_2-evolution is generated in vegetative cells by photosynthetic oxidation of H_2O. These organisms therefore, constitute a biophotolytic system in which H_2 and O_2 are generated respectively in heterocysts and vegetative cells from H_2O during oxygenic process of photosynthesis. The observed rates of nitrogenase catalysed H_2-evolution is not entirely dependent on the level of nitrogenase activity because H_2-consuming reactions mediated by uptake hydrogenase activity occur side by side. The H_2 captured by uptake-hydrogenase can support ATP synthesis or provide electrons for biosynthetic **reactions,** thereby allowing the organisms to recover some of the energy that would have otherwise been lost upon diffusion of the H_2 from the cell. It is therefore natural to study the nature of factors influencing the relative rates of nitrogenase-dependent H_2-evolution and uptake hydrogenase-dependent H_2-consumption in considerable detail in the hope that this will permit development of techniques for selection of strains for photoproduction of H_2 from H_2O at a level with commercial utility (Lambert and Smith, 1981).

The uptake hydrogenase activity appears along with the heterocyst differentiation and nitrogenase activity in cultures incubated for growth in

combined nitrogen-free medium (N_2-medium) under photoautotrophic growth conditions (Peterson and Wolk, 1978; Eisbrenner et al, 1978; Daday **et** al, 1979; H ouch **ins** and fturris, 19i>l). Such a coordinated regulation of heterocyst, nitrogenase and uptake-hydrogenase indicates involvement of nitrogen regulatory genes **in** the process and suggest a physiological linkage between N_2-fixation and H_2-consumption.

Heterocysts carryout anaerobic metabolism and **the** reductant and energy available during this metabolism appears to be exclusively used for N_2-fixation reactions, **in** view of the lack of RUBISCO activity (Winkenbach **and** Wolk, 1973; Codd and Stewart, 1977) and nitrate reductase activity (Kumar **et** al, 1985). Obviously, the genes for the production of these active enzymes must get switched off during heterocyst differentiation. The absense of these enzyme **activities** from heterocyst would mean the involvement of genetic control systems in partitioning N_2-fixation in heterocyst and CO_2 and NO_3-assimilation in vegetative cells. Considering an average heterocyst frequency of about 5%, it would mean that **in** a filament about every 20th cell will become a heterocyst. Such an even distribution ot heterocyst is evidently desirable and one will conclude that a single heterocyst is capable of providing about 20 vegetative cells with fixed nitrogen. Such a role of heterocyst would certainly impose on itself a metabolic strategy exclusively geared for N_2-fixation. We have found **evidence** to show that heterocyst is deficient in nitrate reductase activity because of the lack of apoprotein component of nitrate reductase. Since heterocyst contains Mo-cofactor at a level **much** higher than vegetative cell on protein basis, the presence of Mo-cofactor in heterocyst might be a source of Mo for Fe Mo-Co of nitrogenase and earlier studies along this line do implicate such a role of Mo-cofactor in the activity regulation of Mo-nitrogenase in heterocysts (Bagchi and Singh, 1984; Bagchi et al, 1985).

Studies on the Mo-nutrition of nitrogenase synthesis and activity in heterocystous cyanobacteria is a must in order to examine the possibility of Mo-metabolism in regulating the H_2-producing activity of **nitrogenase. In this** connection, it is important to mention that Vanadium-nitrogenase catalyses more efficiently H_2-production than N_2-fixation (Bishop et al, 1980; 1982). Since Mo is a requirement for the activity of nitrogenase as well as nitrate reductase **in** the cyanobacterium Nostoc muscorum and since both the enzyme activities occur during diazotrophic growth of the cyanobacterium with nitrogenase compartmentalized in heterocyst and nitrate reductase compartmentalized in vegetative cells, we believe that heterocyst differentiation is also a mechanism for avoiding **competition** for Mo-nutrient between nitrate reductase and nitrogenase under N_2-fixing conditions. However, this view has to be substantiated with detailed study of the genetics and biochemistry of Mo-metabolism in heterocysts and vegetative cells in heterocystous filaments of the cyanobacteria.

Heterocystous cyanobacteria in symbiotic associations with Azolla or liver-worts or Cycas increase their heterocyst frequency from 5% found in tree-living forms to nearly 30% found in symbiotic association. This rise in heterocyst frequency is in parallel with rise in nitrogenase activity. This demonstrates the possibility of increasing heterocyst frequency and thereby of nitrogenase activity, the desirable features for using heterocystous cyanobacteria as a biophotolytic system for producing H_2 from H_2O during the process of photosynthesis. But the problem here is the presence of uptake hydrogenase in N_2-fixing heterocysts. It must be rendered nonfunctional in order to harvest the nitrogenase generated molecular H_2. Along with this desirability is also the requirement for growing the cyanobacterial strains in an anaerobic atmosphere free from N_2 in order to maximize H_2 production by nitrogenase since

molecular N_2 is also an inhibitor of H_2 production. This could be possible in genetically engineered strains which could differentiate heterocysts and nitrogenase without uptake hydrogenase activity in NO, or NH. -medium. This situation would allow the cyanobacterial strains under anaerobic photoautotrophic growth conditions, to generate H_2 uninhibited by uptake **hydrogenase.**

The metronidazole-resistant mutant showed increased heterocyst frequency and derepression of heterocyst and uptake hydrogenase activity in NH.-medium where NH.-repression of nitrogenase activity functioned normally. This finding does indicate the possibility of increasing heterocyst frequency with derepression property by mutational manipulation. The uncoupling of NH,-repression control of heterocyst and nitrogenase suggests that mutant is a result of the mutation in NH_4^+-sensitive regulatory unit specific for heterocyst formation and that uptake hydrogenase activity is under the regulatory control of heterocyst differentiation. That this mutant is not the result of defective ammonium transport system is borne by normal operation of ammonium transport activity in the mutant strain like that in the parent strain. Also, we rule out the possibility of alteration in the NH -assimilating activity of GS as a reason for the heterocyst, uptake hydrogenase derepression in view of the normal drug insensitive activity of the enzyme in both the parent and the mutant strains. We do find mutation causing production of drug-resistant *in vitro* activity of NADPH:Fd oxidoreductase, glucose-6-phosphate dehydrogenase and nitrate reductase. The relation between the mutational alteration in NH_4^+-sensitive heterocyst regulatory systems and the resistant activity of these enzymes in the mutant strain is difficult to visualize at the moment. The mutant strain uptake hydrogenase activity was only slightly lower than that of it's parental strain. The fact, that the *in vivo* activity of uptake hydrogenase remains insensitive to the drug suggests that some in *vivo* factor operates

in protecting uptake hydrogenase activity from inhibitory action of the drug. Also, **mutant** strain in vivo nitrogenase activity was inhibited by 50% by the drug **in** comparison to 100% inhibition of the enzyme activity by the drug in **the** parental strain. This is an evidence suggesting **that** mutant strain is not a permeability barrier for the drug and that there might be some in vivo factor produced as a result of **mutation** involved **in** diluting **the** inhibitory action **of** the drug on the drug-sensitive enzyme systems. We have also **noticed** significant difference **in** the **glycogen** content, protein content between the two strains and their regulation by different nitrogen sources. The direct relevance of such differences in diazotrophic growth and activity of nitrogenase and uptake hydrogenase is difficult to assess at the moment.

It is interesting that while the mutant strain is derepressed for heterocyst and uptake hydrogenase in NH_4^+-medium this is not so in cultures assimilating NO_3^-. In other words, NO_3^- is a represser signal for both heterocyst and uptake hydrogenase in addition to that of nitrogenase in the mutant strain. It is generally believed that NO_3^--repression of **heterocyst** operates through its reduction to Ammonia (Bagchi and Singh, 1984). Present results of NO_3^--repression of heterocyst differentiation in the absence of NH_4^+-repression of heterocyst differentiation in the mutant strain of N. muscorum question the above view and suggests that NO_3^--repression control mechanism is operationally different from NH_4^+-repression control mechanism. This finding supports the observation in Anabaena strain CA which shosvs operation of NO_3^--repression control and not of NH_4^+-repression control in **heterocysts** and nitrogenase (Bottomely **et** al, 1979).

CHAPTER IV

CARBON NUTRITION AND REGULATION OF AEROBIC HYDROGEN METABOLISM

INTRODUCTION

Some Cyanobacteria or blue-green algae belonging to heterocystous group are normally in symbiotic association with other photosynthetic partners like liverworts Blasia, Anthoceros and Cavicularia or fern Azolla or gymnosperm Cycas. The cyanobionts in all these symbiotic associations work as a source of nitrogen supply to the other photosynthetic partner which in turn supply to the N_2-fixing cyanobiont photosynthate of organic carbon nature. In fact) in such association, cyanobiont's photosynthetic CO_2-fixing ability is inhibited followed by many fold increase in its heterocyst frequency and nitrogenase activity. In such associations the cyanobiont's nutrition is heterotrophic and N_2-fixation is also heterotrophic (Peters et al, 1980; Rai et al, 1981; Rai et al, 1983; Lindblad et al, 1985). The rise in heterocyst frequency and nitrogenase activity along with the inhibition of photoassimilation of CO_2, appears to suggest a specific role of the host in affecting the observed biochemical modifications during the development of effective symbiosis. In such symbiotic associations much attention has been paid to the N_2-fixing aspect and not to the nature of H_2-metabolism.

Regulation of uptake-hydrogenase activity in Rhizobium has been studied with reference to organic carbon nutrition and O_2 atmosphere. H_2 uptake by nodules or bacteroids of Hup Rhizobium japonicum is easily demonstrable but, heterotrophically grown such strain as R. japonicum does not show significant level of uptake-hydrogenase activity (Maier et al, 1979). In fact, organic carbon source has been demonstrated to cause repression

of uptake-hydrogenase activity and Ribulose bisphosphate carboxylase activity, the two key enzymes required for sustaining chemoautotrophic growth of R. japonicum (O' Brain and Maier, 1988). The Hup^+ Rhizobial strains derepress uptake-hydrogenase activity without concomitant derepression of RUBISCO activity in bacteroids while in free-living conditions, these strains are known to derepress both the enzyme activities under low O_2 and organic carbon-limited growth conditions. (O'Brain and Maier, 1988). These observations do indicate a role of uptake hydrogenase activity in regulating N_2-fixation in bacteroids and supporting autotrophic growth in free-living condition through providing energy and reductant for assimilation of CO_2 into organic carbon.

The study on uptake hydrogenase activity in cyanobacteria has been done mainly with reference to nitrogen regulation of its activity and localization (Houchins, 1984). Study has not been done with regard to organic carbon nutrition for reasons not clear. May be, the organisms originally involved in studies of H_2-metabolism were obligate photoautotrophs not sensitive enough to respond to organic carbon sources. In fact, as we understand at the moment with respect to carbon nutrition, cyanobacteria has been divided into three nutritional groups, namely:

(1) Obligate photoautotrophs, meeting their energy requirement and carbon requirement solely from photosynthesis and CO_2;

(2) Facultative photoheterotrophs, which utilize organic carbon along with CO_2 to meet the cellular carbon requirements and photosynthesis to meet energy requirements and

(3) Facultative chemoheterotrophs, capable of using organic carbon substrates as a source of energy and cellular carbon for growth and multiplicity. Such chemoheterotrophic forms are the only one's which can be utilized profusely

in understanding the role of carbon-nutrition in regulating aerobic H_2-metabolism mediated by nitrogenase activity and uptake hydrogenase activity within the heterocyst. This obviously would require doing experiments with a facultative chemoheterotroph in order to understand the organic carbon regulation of heterocyst frequency during diazotrophic growth. Anabaena cycadeae, the cyanobiont from coralloid roots of Cycas has already been shown to be a facultative chemoheterotroph (Singh and Singh, 1964). We isolated this strain and carried out the study of uptake-hydrogenase activity and nitrogenase activity in free-living photoautotrophic growth conditions, in free-living chemoheterotrophic growth conditions and in symbiotic associations.

EXPERIMENTAL PROCEDURE

Organisms:

The cyanobacterium from Cycas circinalis coralloid roots (root nodules) was isolated, purified and raised in pure culture as described earlier (Singh and Singh, 1964; Singh et al, 1983). This free-living strain is referred to as Anabaena cycadeae. Symbiotic cyanobacterium (referred to as Cyanobiont) was freshly isolated from root nodules of C. circinalis grown in a nursery. Free-living A. cycadeae cultures were grown in modified Chu-10 medium free of combined nitrogen as described in Chapter II.

Isolation of the Cyanobiont:

Root nodules were washed in distilled water, cut into small pieces and then gently crushed, using a mortar and pestle, in modified Chu-10 medium (Gerloff et al, 1950) containing 1% PVP, to release the cyanobiont. Bulk of the host tissue was removed by passing the suspension through 2,4 and 8 layers

of muslin cloth. The filtrate was then repeatedly centrifuged at 500 xg to remove remaining host tissue debris.

Growth:

Cultures were grown in N_2-medium under photoautotrophic growth conditions. Photoheterotrophic cultures were grown in N_2-medium containing 2% glucose or sucrose or fructose (w/v) and incubated in light. Chemoheterotrophic conditions for cyanobacterial growth were obtained by wrapping the flasks with Aluminium foil. Growth rate was determined Dy measuring absorbance change at 665 nm as described in Chapter **II.**

Measurement of heterocyst frequency, nitrogenase activity and uptake hydrogenase activity:

Heterocyst frequency, nitrogenase activity and uptake-hydrogenase activities were estimated as described in Chapter **II.**

RESULTS

Characterization of Growth, heterocyst frequency, nitrogenase activity and uptake hydrogenase activity of Anabaena cycadeae under photoautotrophic and chemoheterotrophic conditions:

Table VIII compares growth, heterocyst frequency, nitrogenase activity and uptake-hydrogenase activity of the cyanobacterium grown photoautotrophically and chemoheterotrophically with glucose as carbon source. The photoautotrophic cultures growing with N_2 as sole nitrogen source grew slowly in comparison to such growth of N. muscorum under similar conditions. Chemoheterotrophic cultures growing diazotrophically showed a generation

Table V1l1 : Heterocyst frequency, nitrogenase activity and uptake hydrogenase activities of Anabaena cycadeae grown photoautotrophically and chemoheterotrophically.

Cl	Photoautotrophic growth conditions	Chemoheterotrophic growth conditions
Generation time (hr)	23	224
Heterocyst frequency	5-6%	4-5%
Nitrogenase activity	5.5	2.9
Uptake-hydrogenase activity	28.3	*0.0*

Nitrogenase activity expressed as μ mol C_2H_4 formed. mg Chl a .hr . Uptake-hydrogenase activity expressed as μ mol H_2 consumed. mg Chl a^{-1}. hr^{-1}.

All the above results are an average of three independent experiments which did not vary by more than 10%.

For other details please see text.

time nearly eight-fold higher than that of similar photoautotrophic cultures. This evidently shows chemoheterotrophy to be much inferior mode of nutrition for growth to photoautotrophy. The heterocyst frequency of photoautotrophic culture was 5-6% and that of chemoheterotrophic culture was 4-5% thereby suggesting slight decrease in heterocyst frequency as a result of change from photoautotrophic mode of nutrition to chemoheterotrophic mode of nutrition. The nitrogenase activity in the two nutritionally different cultures was also different in that, the nitrogenase activity of chemoheterotrophic cultures was nearly half of that of photoautotrophic cultures. The lower level of nitrogenase activity in chemoheterotrophic cultures might be one of the reasons for its slower growth under chemoheterotrophic growth conditions. Uptake-hydrogenase activity of the culture was evident only under photoautotrophic growth conditions as chemoheterotrophic cultures showed zero level of this activity. On comparative basis, uptake-hydrogenase activity in A. cycadeae was more than that **in** N. muscorum. The absence of uptake hydrogenase activity in chemoheterotrophic cultures growing with glucose as carbon source suggests that, the chemoheterotrophic growth condition is an inhibitor of uptake-hydrogenase activity and that organic carbon source like glucose in the growth medium appears to be the cause of such inhibition.

Growth kinetics of the cyanobacterium Anabaena cycadeae under photoautotrophic, photoheterotrophic, chemoautotrophic and chemoheterotrophic conditions:

We also studied the growth kinetics of the cyanobacterium A. cycadeae in light without glucose, and in light with glucose, in dark without glucose and in dark with glucose. The source of N_2 under such conditions was atomospheric N_2. As shown in Fig. VI cultures with glucose under photosynthetic conditions (photoheterotrophic growth condition) grew better than

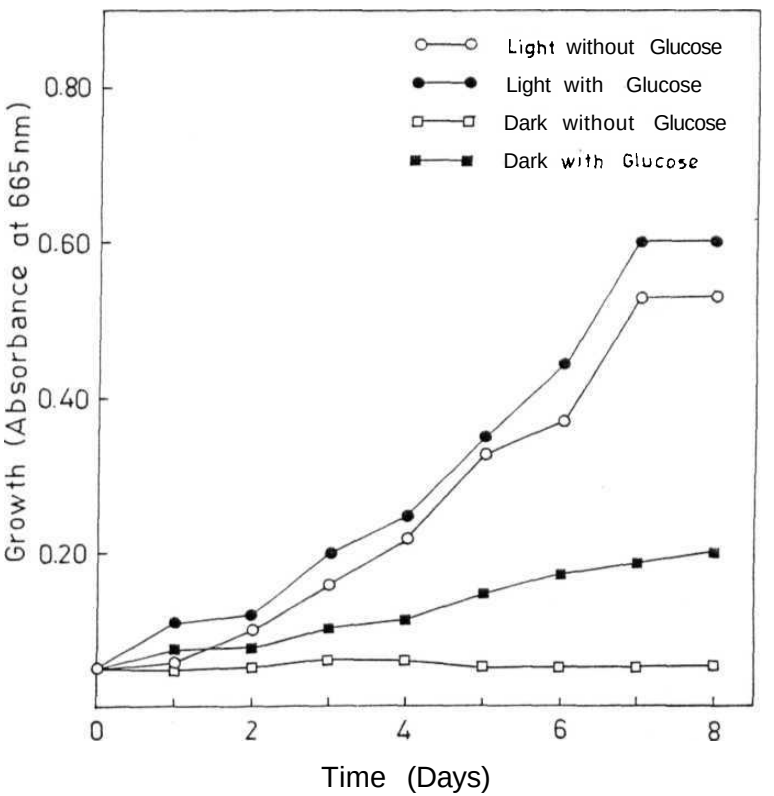


Fig. VI. Growth kinetics of Anabaena cycadeae under photoautotrophic, photoheterotrophic, chemoautotrophic and chemoheterotrophic growth conditions.

cultures without glucose under parallel condition (photoautotrophic growth condition). Culture in the dark with glucose in the growth medium grew but very slowly with the passage of time while glucose-free dark control cultures showed no growth at all. These results also suggest that the cyanobacterium is a facultative chemoheterotroph and that light is a better source of energy for the cyanobacterial growth than glucose.

Effect of carbon sources like glucose, sucrose and fructose on in vivo nitrogenase activity of the cyanobacterium A. cycadeae under diazotrophic growth conditions:

We also studied the time course effect of glucose, sucrose and fructose on the in vivo nitrogenase activity of the cyanobacterium under phototrophic conditions. The results of such studies are shown in Fig. **VII.** The control culture was organic carbon source-free photoautotrophic culture. Initial level of nitrogenase activity was lower in photoautotrophic culture and glucose-containing photoheterotrophic culture in comparison to that containing sucrose. However, with the passage of time nitrogenase activity of photoautotrophic cultures and of glucose-containing photoheterotrophic cultures increased progressively with a rate slightly higher in the former. Nitrogenase activity of phototrophic culture was highest followed by that in glucose-containing, sucrose-containing and fructose-containing photoheterotrophic cultures. These results again emphasize photoautotrophic growth condition to be the best for nitrogenase activity in the Facultative chemoheterotroph.

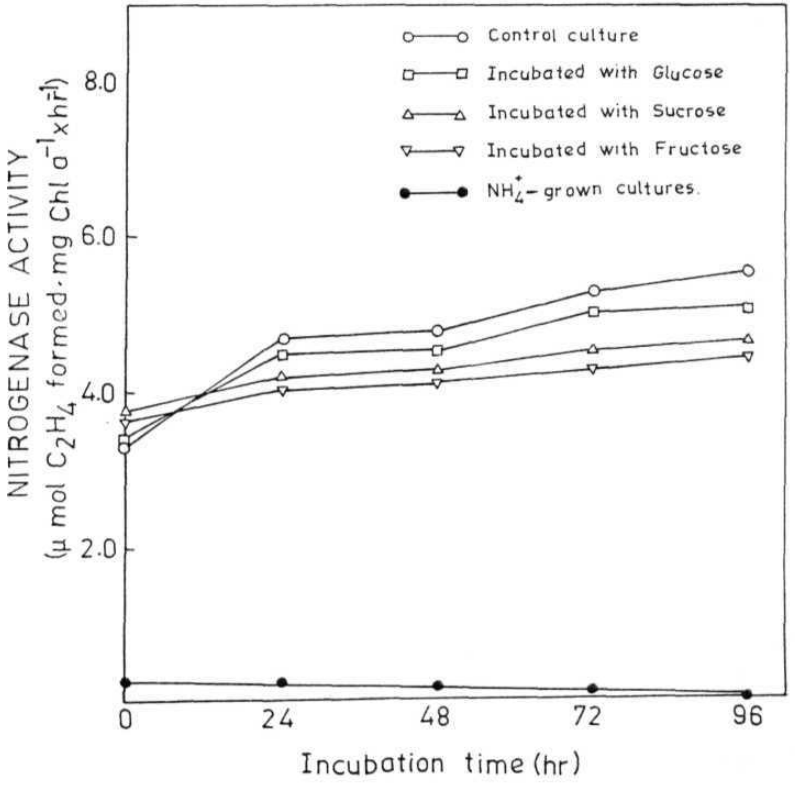


Fig. VII. Effect of different carbon sources on nitrogenase activity in Anabaena cycadeae. Glucose, sucrose and fructose were added at zero time and nitrogenase activity was estimated over a period of 96 hrs.

Effect of carbon sources like glucose, sucrose and fructose on uptake hydrogenase activity in the cyanobacterium Anabaena cycadeae under diazotrophic growth conditions:

We also studied time course effect of glucose, sucrose and fructose on uptake-hydrogenase activity. The source of inoculum for these experiments was N_2-grown cultures. NH_4^+-grown cultures under photoautotrophic conditions served as one control and N_2-grown cultures under photoautotrophic growth conditions served as another control. As shown in Fig. V111 uptake-hydrogenase activity like nitrogenase activity and heterocyst is missing from NH_4^+-grown cultures under photoautotrophic growth conditions. The parallel cultures in N_2-autotrophic medium showed highest level of activity. Addition of glucose, sucrose or fructose caused progressive decline in the uptake-hydrogenase activity which was much higher **in** glucose-containing culture followed by that in sucrose and fructose-containing cultures. This itself indicates time-bound increase in inhibitory effect of organic carbon source on the uptake-hydrogenase activity. Such an effect of organic carbon on uptake-hydrogenase activity may be because of organic carbon-repression control of uptake-hydrogenase activity in the cyanobacterium, A. cycadeae.

Effect of pre-incubation under H_2-atomosphere on nitrogenase activity of the cyanobacterium A. cycadeae under photoautotrophic, chemoautotrophic and chemoheterotrophic growth conditions:

We examined the effect of H_2 atmosphere on nitrogenase activity of A. cycadeae in the light under autotrophic conditions, in the dark under autotrophic conditions and in the dark under heterotrophic conditions (Table IX). Light autotrophic cultures showed nitrogenase activity which increased around 2-3 fold under H_2 atmosphere thereby suggesting a role of H_2 atmosphere

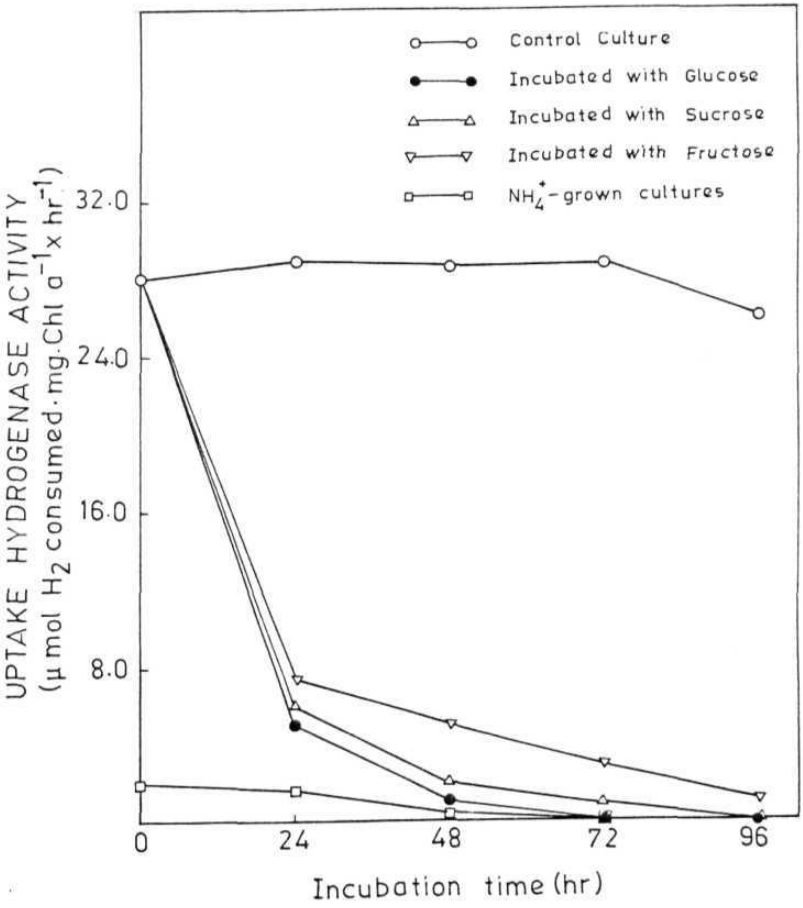


Fig. VIII. Effect of different carbon sources on uptake hydrogenase activity in Anabaena cycadeae. Glucose, sucrose and fructose were added at zero time and uptake hydrogenase activity was estimated over a period of 96 hrs.

Table IX : Influence of H_2 on nitrogenase activity in free-living Anabaena cycadeae under photoautotrophic, chemoautotrophic and chemoheterotrophic conditions.

Growth condition	Nitrogenase activity			
	Photoautotrophic conditions		Chemoautotrophic conditions	Chemoheterotrophic conditions
$-H_2$	5.8	5	*0.0*	2.90
$+H_2$	15.20		**2.04**	**2.78**

Nitrogenase activity expressed as μ mol C_2H_4 formed. mg Chl a .hr .

$-H_2$ indicates cultures incubated in the absence of H_2-atmosphere.

$+H_2$ indicates cultures incubated in the presence of H_2-atmosphere.

All **the** above results are an average of three independent experiments which did not vary by more than 10%.

For other details please see text.

Table X : Effect of preincubation for *2k* hrs in light or dark on heterocyst frequency, nitrogenase activity and uptake-hydrogenase activity of symbiotic Anabaena cycadeae in coralloid roots of Cycas circinalis.

Characters	Light	Dark
Heterocyst frequency	24-27%	24-27%
Nitrogenase activity	40.0	38.7
Uptake-hydrogenase activity	*0.0*	*0.0*

Nitrogenase activity expressed as u mol C_2H_4 formed. mg Chl a^{-1} .hr^{-1}.

Uptake hydrogenase activity expressed as p mol H_2 consumed. mg Chl a^{-1} .hr^{-1}

All the above results are an average of three independent experiments which did not vary by more than 10%.

For other details please see text.

in regulating nitrogenase activity. Dark-incubated autotrophic cultures did not show nitrogenase activity in the absence of H_2. But their parallel H_2-incubated cultures showed nitrogenase activity which was nearly as good as shown by chemoheterotrophic cultures. This is very interesting observation **in** the sense that molecular H_2 can serve as a source of reductant and or ATP lor supporting nitrogenase activity in the dark possibly involving uptake-hydrogenase activity. This also suggests that dark cultures of A. cycadeae to be active in uptake-hydrogenase activity.

Heterocyst frequency, nitrogenase activity and uptake-hydrogenase activity of symbiotic A. cycadeae in coralloid roots of Cycas circinalis:

We also studied the heterocyst frequency, nitrogenase activity and uptake-hydrogenase activity of the cyanobiont in symbiotic association in light and dark (Table X). Heterocyst frequency was nearly 5-6 fold higher in symbiotic association than in free-living condition. Similar rise in nitrogenase activity was evident under symbiotic association. The dark nitrogenase activity was not very much different from light nitrogenase activity of the cyanobiont from symbiotic association, thus suggesting no role **of** light in regulation of nitrogenase activity in coralloid roots. Interestingly uptake-hydrogenase activity was completely absent **in** symbiotic association. The rise in heterocyst frequency, nitrogenase activity and the absence of uptake-hydrogenase activity in coralloid roots suggests a role of host in regulating these biochemical and morphological modifications in the cyanobiont.

DISCUSSION

Chemoheterotrophic mode of growth and nutrition in cyanobacteria is known to operate through oxidative pentose phosphate pathway. Glucose-

supported heterotrophic growth has been firmly established to involve the oxidative pentose phosphate pathway in the presence of oxygen (Pelroy et al, 1976; Bottomley and Van Baalen, 1978). Obligate photoautotrophs and facultative chemoheterotrophs have been shown to contain the same complement of enzymes of oxidative pentose phosphate pathway, but the inability of obligate photoautotrophs to assimilate exogenous organic carbon is due to permeability barrier (Smith, 1982). Photosynthetic CO_2-fixation occurs via the calvin cycle and the oxidative pentose phosphate pathway of heterotrophic nutrition operates through the activity of two key enzymes glucose-6-phosphate-dehydrogenase and 6-phosphogluconate dehydrogenase and a role of thioredoxins has been shown to mediate the light and dark nutrition of the cyanobacterium under given condition (Rowell et al, 1987). Glucose-6-phosphate dehydrogenase is the key regulatory enzyme of the oxidative pentose phosphate pathway and is considered to be important in electron transfer to nitrogenase in cyanobacteria and in heterotrophic nutrition (Apte et al, 1978). This enzyme activity is rapidly deactivated by low concentration of reduced thioredoxin and such a reduced thioredoxin mediated activation control of calvin cycle enzymes and deactivation control of oxidative pentose phosphate pathway enzyme glucose-6-phosphate dehydrogenase might be the key mechanism regulating change in mode of nutrition from autotrophic to heterotrophic **(Cossar** et al, 1984; Udvardy et al, 1984).

The chemoheterotrophic growth is always lower than photoautotrophic growth of a cyanobacterium and different facultative chemoheterotrophs show different rate of chemoheterotrophic growth primarily because of inherent variation in the rate of ATP supply under such conditions. The rate of ATP production and supply for growth is always higher under phototrophic conditions than under chemotrophic conditions. This difference is

suggested to be the cause of difference in cyanobacterial photoautotrophic and chemoheterotrophic growth (Smith, 1982). We have not estimated the production o[ATP in the cyanobacterium under chemotrophic and phototrophic growth conditions but since chemoheterotrophic growth of the cyanobacterium was slow as expected according to the ATP supply concept, we believe that limitation of ATP availability under chemotrophy limits the cyanobacterial chemoheterotrophic growth.

It has been observed that C/N ratio rises from 4.5 to 8.0 during heterocyst differentiation in the free-living cyanobacterium and this has led to the suggestions that higher C/N ratio seems to be the internal stimulus which by some way triggers off the process of heterocyst differentiation accompanying nitrogenase synthesis (Fogg, et al, 1973; Adams and Carr, 1981). By the same logic, it could be suggested that this is also the signal for uptake-hydrogenase production. Symbiotic cyanobacterium like A. cycadeae shows nearly 5-6 told increase in its heterocyst frequency and nitrogenase activity in symbiotic association over that in free-living condition. The nature of biochemical signal for such a rise in heterocyst frequency and nitrogenase activity in symbiotic state is not known. Maybe here as well C/N ratio could be a factor in the rise of heterocyst frequency and nitrogenase activity.

Unicellular cyanobacteria both diazotrophic and nondiazotrophic have been shown to contain uptake-hydrogenase activity (Howarth and Codd, 1985). Relation between uptake-hydrogenase activity and CO_2-reduction in non N_2-fixing unicellular cyanobacteria and between uptake-hydrogenase activity and nitrogenase activity in N_2-fixing unicellular cyanobacteria have not been studied in detail and therefore there is a lack of any definitive conclusion about the role of uptake hydrogenase activity in such forms. But, a background

information from Rhizobium could be helpful to put this problem in proper perspective. The laboratory cultures of Hup strains of Bradyrhizobium japonicum require decreased O_2 tension, low level carbon substrate in the medium and H_2 in the atmosphere for development of uptake-hydrogenase activity and Ribulose bisphosphate carboxylase activity which supported chemolithotrophic growth of such cultures, in which H_2 functions as sole energy source and CO as sole source of carbon (Hanus et al, 1979; Lepo et al, 1980). These studies have indicated that the oxygen-limitation and organic carbon-limitation to be the primary cause for development of uptake-hydrogenase in Rhizobium in free-living growth conditions. In bacteroids, uptake-hydrogenase activity is expressed without concomitant production of Ribulose bisphosphate carboxylase activity and such bacteroids are active nitrogen-fixers and such a regulatory mechanism for decoupling **the** expression of uptake-hydrogenase activity from RUBISCO activity might be induced by host in favour of a role of uptake--hydrogenase activity in nitrogenase activity regulation.

Heterocyst is the site of both uptake-hydrogenase activity and nitrogenase activity and the reason for localization of nitrogenase synthesis and activity within heterocyst has been the lack of CL atmosphere within the heterocyst (Haselkorn, 1978; Houchins, 1984). N_2-fixing heterocyst lacks RUBISCO activity as well thereby making heterocyst, a cell suffering from limitation in carbon substrate. We therefore suggest O_2-limitation and organic carbon-limitation to be the reason for localization of uptake-hydrogenase activity within heterocyst.

This conclusion is supported by the findings reported here with a free-living strain of A. cycadeae that can grow with atmospheric N_2 either in light ($+CO_2$) or in darkness with glucose. The presence of glucose, allowed

N_2-fixation to occur; nitrogenase was active in both cultures albeit that its activity, on a chlorophyll basis, in the dark-grown cultures was only about half of that in the light-grown ones. The rate of growth in darkness on glucose was **much** slower than in the light, possibly because the rate of ATP formation was slower (Smith, 1982). But the most striking effect of the presence of glucose was on uptake-hydrogenase activity. Such activity was readily detectable in cells grown in light in the absence of glucose but the activity **fell** by 80 to 90% in 24 h following glucose addition and the fall occured whether cultures were illuminated or not. This finding is consistent with those from studies on Rhizobium (Maier et al, 1979) and Azotobacter, (Patridge et al, 1980) which show that uptake hydrogenase activity in these organisms is repressed in the presence of glucose. Thus, it appears that the carbon-limitation control of uptake-hydrogenase which operates in Rhizobium and Azotobacter also operates in the heterocysts of cyanobacteria.

We can also conclude that, when in symbiotic association with cycad roots, A. cycadeae is not subject to carbon-limitation. Table X shows that the symbiotic organism possesses many heterocysts and have high nitrogenase activity, as Lindblad et al, (1985) have also shown with the cyanobiont in coralloid roots of Zamia. In contrast, uptake-hydrogenase activity is completely absent from the symbiotic A. cycadeae. Although other interpretations are possible, the simplest one is that the absence of uptake-hydrogenase activity is the consequence of an ample supply of carbohydrate from the surrounding cycad root tissue.

We conclude that the rate of supply of available carbon to the heterocyst exerts an important control on the development of uptake- hydrogenase activity. These results also indicate a physiological mechanism for inactivation of uptake-hydrogenase activity to permit unhindered generation of H_2 **from** nitrogenase activity.

CHAPTER V

PHOTOSYNTHESIS AND REGULATION OF H_2-PRODUCTION AND H_2-UTILIZATION IN DIAZOTROPHIC CYANOBACTERIA

INTRODUCTION

Solar energy projects for alternative energy resources aim **at** photosynthetic production oi H_2 from water by using heterocystous cyanobacteria, as O_2-evolving reactions from H_2O occur in them during photosynthesis in vegetative cells and H_2-producing reactions by **nitrogenase** at the expense of photosynthetically-generated reductant in heterocysts. Cyanobacterial photosynthesis like chloroplast photosynthesis gets saturated at low light intensity. Of the many factors, the number of reaction centres in relation to light intensity are likely to contribute towards low-light saturation process of photosynthesis. The ratio of PS1 **to** PS1I or vice versa are also likely to influence to a large extent the rate of oxygenic photosynthesis coupled to the production of O_2 **and** H_2. In tact, the activity of PS11 alone seems to determine the overall **efficiency** ol oxygenic photosynthesis. There are a number ol nutritional factors like calcium, manganese, chloride, nitrate and carbon nutrients; and environmental factors like light intensity, light quality playing a great role in regulating the cyanobacterial oxygenic photosynthetic process (Cohen-Bazire and Bryant, 1982; Ho and Krogmann, 1982).

In recent years, molecular genetic approaches involving recombinant-DNA technology has entered into the domain of cyanobacterial photosynthesis in order to understand the structure-function relationship between various components oi PSII and PS1 and in this regard the present concerted effort primarily aims at understanding the organization and function of various proteins of P5I1 alone (Satoh, 1985; Arntzen and Pakrasi, 1986). A knowledge resulting from such studies is likely to provide an understanding for the manipulation of cyanobacterial photosynthesis. One of the approaches on this line

has made use of locating and characterizing the herbicide-resistance gene for DCMU and Atrazine. Such a study has been started with a view to understand the role of psbA gene product in photosynthetic function of PSII (Golden et al, 1986). It is therefore, clear that an approach involving determination of the role of photosynthesis in any biological process of interest like N_2-fixation, H_2-production and H_2-utilization could be studied profitably by examining the efficiency of above processes in naturally occurring or genetically engineered strains of cyanobacteria showing resistance to one or the other herbicides of photosynthesis and it is because of this reason we have chosen in the present study a naturally occurring strain of Gloeocapsa showing resistance to DCMU, Machete and Basalin and spontaneously occurring mutants of Nostoc muscorum showing resistance to DCMU and Atrazine.

The production of nitrogenase catalysed H_2-evolution could be assessed in mutant strains specifically deficient in production of nitrogenase activity. In addition to such a genetic approach, the physiological approach of inactivating nitrogenase activity by growing the culture in tungsten-medium could also be a reliable index of assessing the quantitative significance of nitrogenase activity in photoproduction of H_2 in heterocystous cyanobacteria. We have used the second approach in the present study.

In heterocystous cyanobacteria, oxygenic photosynthesis, N_2-fixation and H_2-production are separated in space. The implication of such a spatial separation of the two processes could be analysed profitably in unicellular aerobic diazotrophic cyanobacterial forms capable of carrying out the process of oxygenic photosynthesis, N_2-fixation and H_2-production within the same cell and this has been the reason for studying the process of H_2-production by nitrogenase in the unicellular aerobic diazotrophic cyanobacterium Gloeocapsa sp.

Uptake-hydrogenase activity in heterocystous cyanobacteria is located within the heterocyst. It's role in N_2-fixation and regulation of H_2-production by nitrogenase could be analysed precisely in the strains showing genetic loss of the same. We have used herbicide-resistant strains of cyanobacterium N. muscorum to examine the functional interrelationship between PSII activity and uptake-hydrogenase activity and the influence of H_2 on nitrogenase activity in the mutant strains lacking uptake-hydrogenase activity. In N. muscorum uptake-hydrogenase activity is spatially separated from the process of oxygenic photosynthesis. Use of a unicellular aerobic diazotrophic cyanobacterium in studying the uptake-hydrogenase activity under diazotrophic conditions is expected to provide the biological significance of spatial separation of uptake--hydrogenase activity and oxygenic photosynthesis. We have used a naturally occurring herbicide-resistant strain of Gloeocapsa in the present study mainly because it fixes N_2 aerobically and it shows uptake-hydrogenase activity under such a condition. We have also used organic carbon compounds (Glucose) to see the differential regulation of nitrogenase activity and uptake-hydrogenase activity by organic carbon compound.

EXPERIMENTAL PROCEDURE

Organisms

Parent Nostoc muscorum, its DCMU -mutant and Atrazine -mutant strains were grown axenically in nitrogen-free modified Chu-10 medium as described **in** Chapter 11. The BG-11 medium of Stanier et al (1971) was used **for** growing the unicellular diazotrophic cyanobacterium Gloeocapsa. Growth conditions for growing Gloeocapsa were essentially same as described for other cyanobacterial strains. **The** composition of the BG-11 medium is as follows:

Compound	$mg.l^{-1}$	Compound	$mg.l^{-1}$
$MgSO_4.7H_20$	75	H_3BO_3	2.86
$CaCl_2.2H_20$	36	$MnCl_2.4H_20$	1.81
$K_2HPO_4.3H_20$	'(0	$Na_2Mo0_4.2H_20$	0.39
FeEDTA	I	$ZnSO_4.7H_20$	0.22
$C_6H_80_7.H_20$	6	$CuSO_4.5H_20$	0.88
$Fe[(NH_4)_2C_6H_50_7]_3$	6	$CO(NO_3)_2.6H_20$	0.05
Na_2CO_3	20	Distilled water	1000 ml
		pH after autoclaving	

DC.MU -mutant and Atrazine -mutant strains of parent N. muscorum were isolated by following the method of Singh and Singh (1978) under diazotrophic growth conditions as described in Chapter 11.

Photochemical activities (PS1I and PSI), nitrogenase activity, uptake-hydrogenase activity, nitrogenase catalysed H_2-evolution and fluorescence emission characteristics were determined as described in Chapter 11.

RESULTS

Growth kinetics of parent Nostoc muscorum and its DCMU -mutant strain and Atrazine^r-mutant strain under diazotrophic growth conditions:

Parent Nostoc muscorum did not grow even in 1 u M DCMU, whereas the mutant was isolated from nutrient plates containing 5 µM DCMU. This strain was tested for its stability by repeated subculturing in 5 µM DCMU-containing medium. Fig. IX shows the growth kinetics of parent and DCMU -mutant in N_2-medium containing and lacking 5 µM DCMU. Parent as expected failed to show any growth in DCMU-containing medium. Diazotrophic growth of the parent and its DCMU -mutant when compared in the absence of the herbicide **the** parent showed a better rate of growth. In DCMU-free

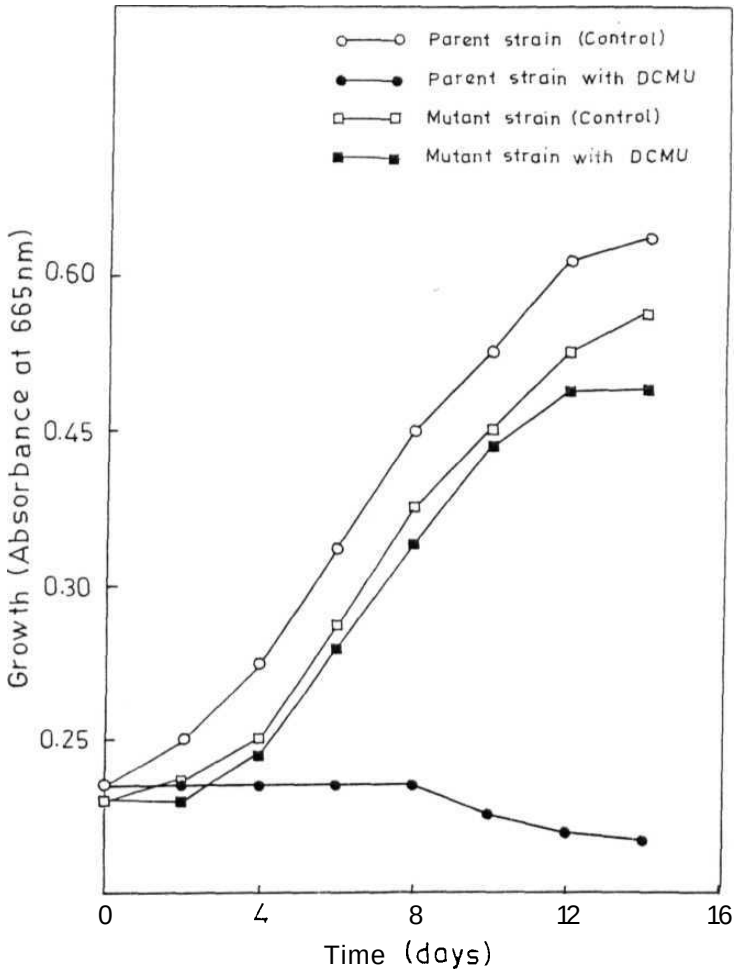


Fig. IX. Growth kinetics of Nostoc muscorum and its $DCMU^r$-mutant strain under diazotrophic growth conditions.

medium the presence of 5 uM DCMU slowed the diazotrophic growth of the mutant a little bit. Thus, mutation to DCMU-resistance appears to have been accompanied by a slight decrease in the diazotrophic growth. In addition, the mutant's diazotrophic growth was not completely resistant to DCMU inhibition. Atrazine, like DCMU in 1 μM inhibited the growth of parental strain in N_2-medium. Growth kinetics of an Atrazine^r-mutant and its parent were examined in N_2-medium containing 5 μM Atrazine (Fig. X). The parent as usual did not grow at this concentration of Atrazine. Growth of the parent and mutant in Atrazine-free medium was almost similar. Mutant diazotrophic growth with Atrazine did not seem to differ significantly from its diazotrophic growth without Atrazine. Here, the mutation to Atrazine resistance does not seem to be accompanied by any adverse alteration in the cyanobacterial diazotrophic growth.

Absorption characteristics of intact cells and spheroplasts prepared from parent Nostoc muscorum and its DCMU^r-mutant and Atrazine^r-mutant strains under diazotrophic growth conditions:

We compared the absorption spectra of intact cells and spheroplasts of parent N. muscorum, its DCMU -mutant and Atrazine -mutant. This was done to find out whether spheroplast preparation can alter the absorption properties of the parent and mutant strain. As shown in Fig. XI (a, b and c), the spheroplast preparation was not accompanied by any basic alteration of absorption characteristics of photosynthetic apparatus in vivo. Therefore, we can use spheroplasts or intact filaments in monitoring the photochemical activities of the parent and the mutant strains with and without the herbicides.

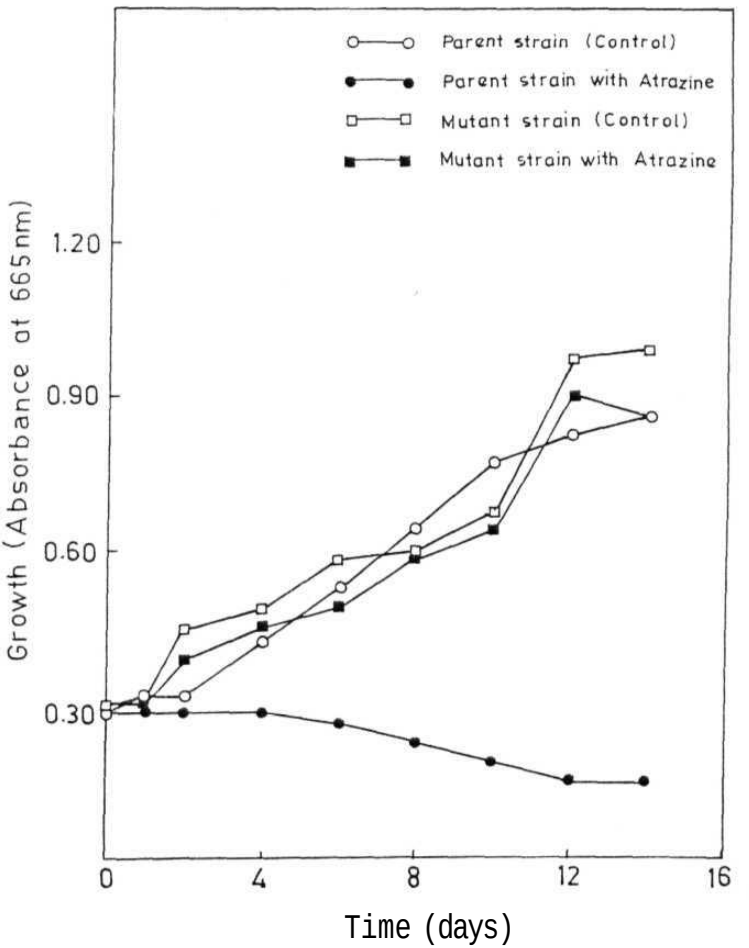


Fig. X. Growth kinetics of Nostoc muscorum and its Atrazine^{r}-mutant strain under diazotrophic growth conditions.

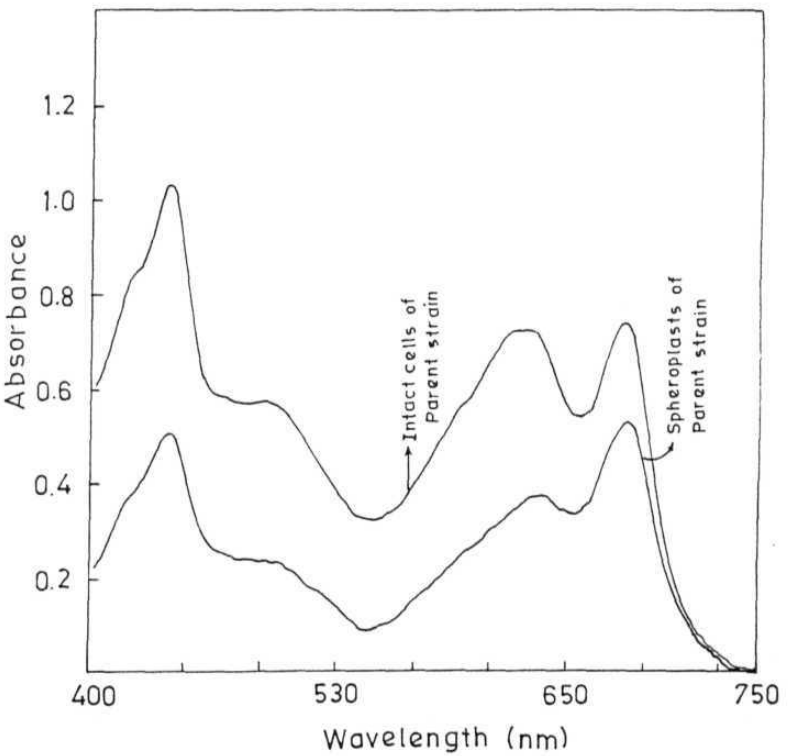


Fig. XI a. Absorption spectra of intact cells and spheroplasts prepared from *Nostoc muscorum*.

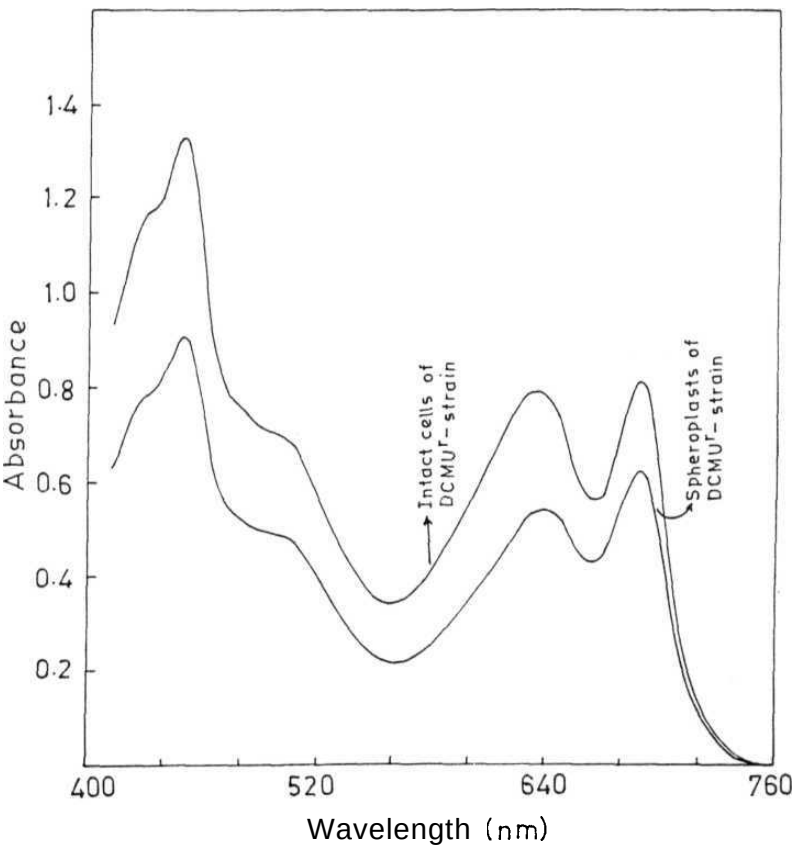


Fig. XI **b.** Absorption spectra of intact cells and spheroplasts prepared from DCMU^r-mutant strain of Nostoc muscorum.

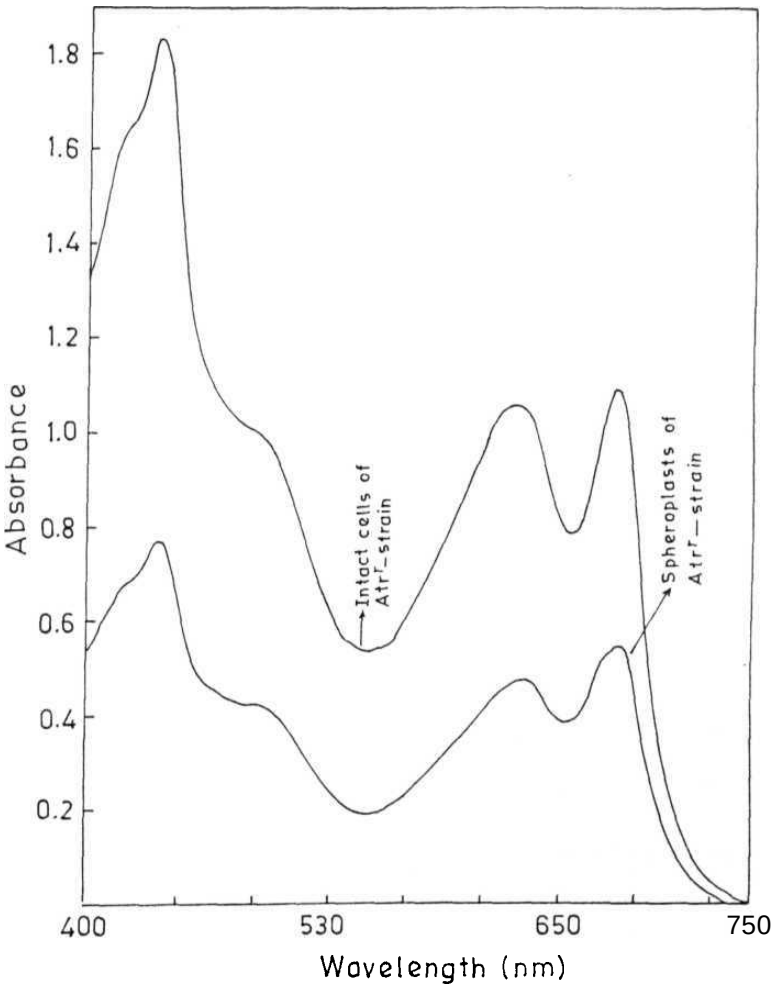


Fig. XI c. Absorption spectra of intact cells and spheroplasts prepared from Atrazine^r-mutant strain of Nostoc muscorum.

Effect of varying concentrations of DCMU on PSII activity ($H_2O \rightarrow pBQ$) in parent Nostoc muscorum and its DCMU -mutant strain under diazotrophic growth conditions:

Mutation to DCMU-resistance was found to be accompanied by nearly 50% decrease in its PSII activity in comparison to the parental strain. We checked the action of increasing concentrations of DCMU on PSII activity of the parent and its mutant (Fig. XII). The mutant strain did not show significant variation in its PSII activity with DCMU concentration upto 5 u M. However, **the** activity declined slightly when the DCMU concentration was raised to 10 uM. In contrast, PSII activity of the parent strain was rapidly inhibited with the rise **in** DCMU concentration and reached zero value **in** 1 µ M DCMU. These results indicate that mutation to DCMU- resistance is a result of development **of** PSII activity resistant to DCMU-inhibition. This mutational production of DCMU-resistant PSII activity appears to be the reason for the mutant strain to grow in the presence of DCMU. The diazotrophic growth of the mutant strain was found to be lower than that of its parent and the observed lower growth **of** the mutant correlates with the observed decrease in photochemical activity of its PSII.

Effect of varying concentrations of Atrazine on PSII activity in parent Nostoc muscorum and its DCMU -mutant strain under diazotrophic growth conditions:

We have studied the effect of different concentrations of Atrazine on PSII activity in the parent and DCMU -mutant strains. The parent PSII activity showed a linear decline with Atrazine concentration. PSII activity of the mutant strain did not show much decrease with Atrazine concentration upto 5 µM. These results suggest that PSII activity in the mutant strain (DCMU) is not only resistant to DCMU but is also resistant to the inhibitory action of Atrazine (Fig. XIII).

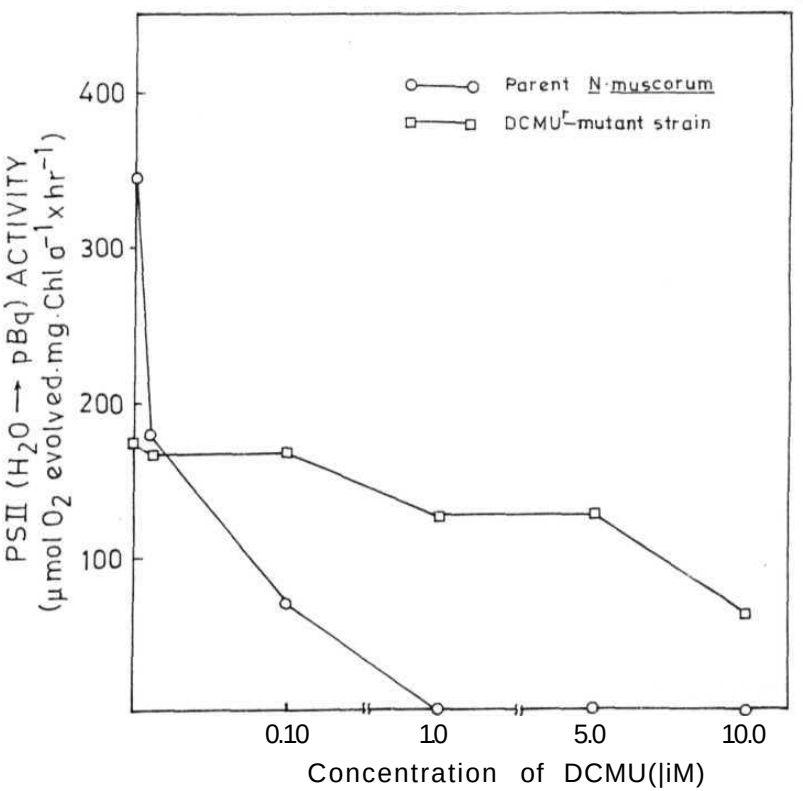


Fig. XII. PSII electron transport activity in Nostoc muscorum and its DCMU^r-mutant strain under diazotrophic growth conditions with varying concentrations of the inhibitor DCMU.

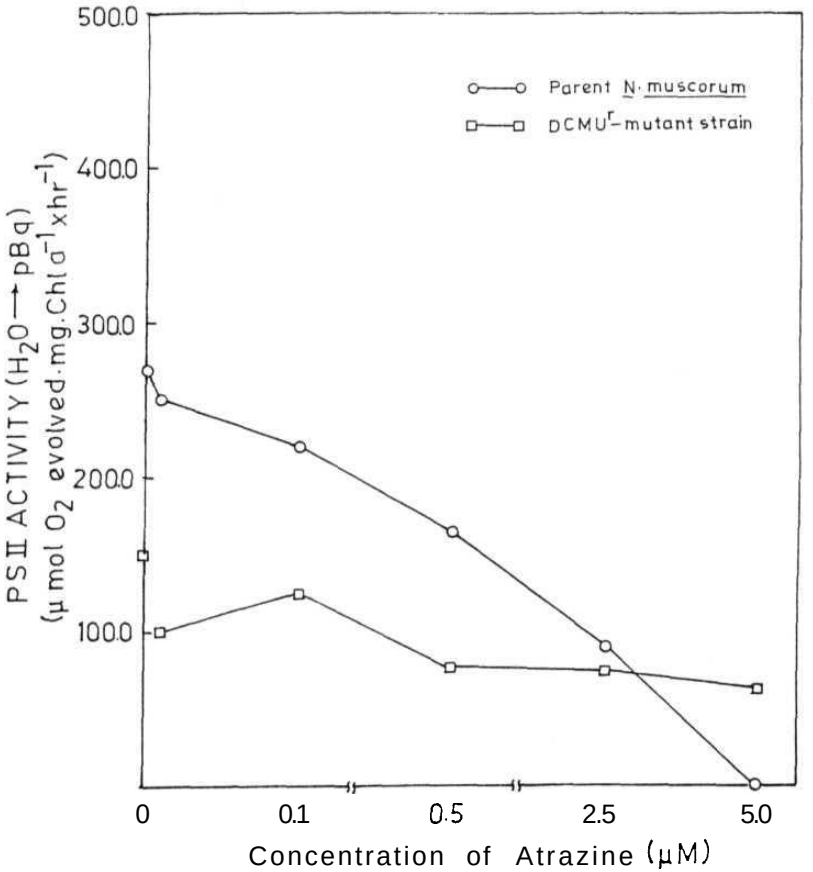


Fig. XIII. PSII electron transport activity in *Nostoc muscorum* and its DCMU^r-mutant strain under diazotrophic growth conditions with varying concentrations of the inhibitor Atrazine.

Effect of varying concentrations of Atrazine and DCMU on PSII activity in parent Nostoc muscorum and its Atrazine^r-mutant strain under diazotrophic growth conditions:

We also studied the response of photochemical activity of PSII of Atrazine -mutant and its parent to increasing concentrations of DCMU. As shown in Fig. XIV, PSII activity in the parent strain declined to a zero value with DCMU concentration of 1 μM. In comparison, the mutant strain PSII activity increased slightly in 0.1 μM DCMU and then started declining very slowly with the DCMU concentration upto 10 μ M. This observation also suggests, that the mutation to Atrazine-resistance renders photosystem II activity resistant to DCMU inhibition and that Atrazine-resistance and DCMU-resistance both result from an alteration in a genetic determinant coding for a common target of PSII. We also studied the kinetics of PSII activity in the parent and its Atrazine^r-mutant strains with increasing concentrations of Atrazine. The results are shown in Fig. XV. The PSII activity of the parent declined linearly and reached a zero value in 5 u M Atrazine. The PSII activity of the mutant strain remained almost unaffected with the rise in Atrazine concentration. This observation finds an explanation to the complete resistance of diazotrophic growth of Atrazine -mutant to Atrazine in the growth medium.

PSI activity in parent Nostoc muscorum and its DCMU^r-mutant and Atrazine -mutant strains:

We examined the possibility of an alteration in the photochemical activity of PSI in parent, DCMU -mutant and Atrazine -mutant of N. muscorum. The results obtained by such studies are shown in Table XI. It is evident that mutation to DCMU-resistance or Atrazine-resistance has not influenced the PSI activity. PSII activity was also determined in spheroplasts prepared from N. muscorum, DCMU^r-mutant and Atrazine^r-mutant strain under diazotrophic growth conditions. The results obtained by such studies are shown in Table XI B.

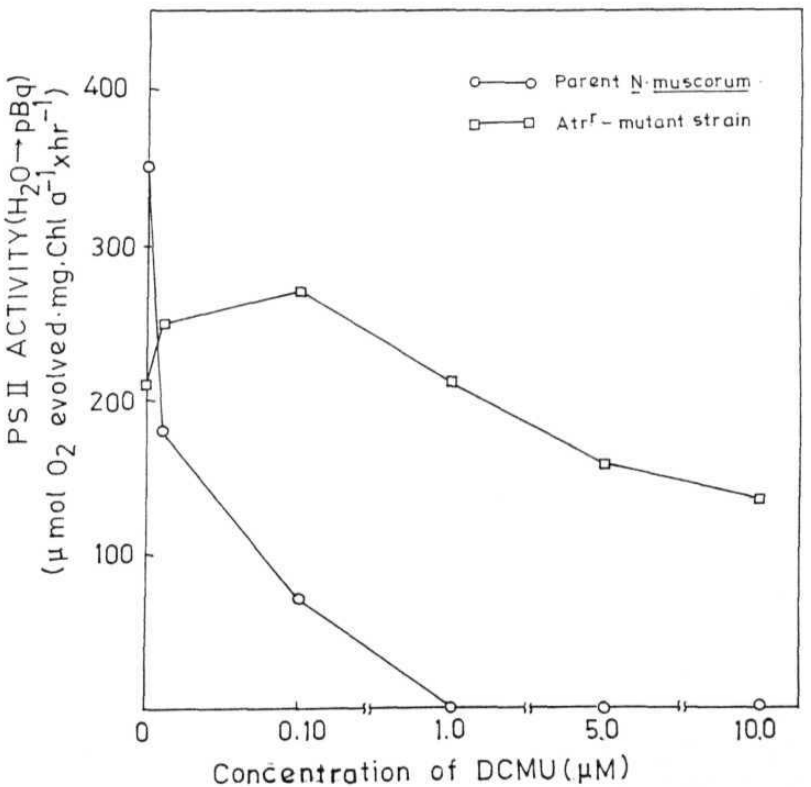


Fig. XIV. PSI1 electron transport activity in Nostoc muscorum and its Atrazine^r-mutant strain under diazotrophic growth conditions with varying concentrations of the inhibitor DCMU.

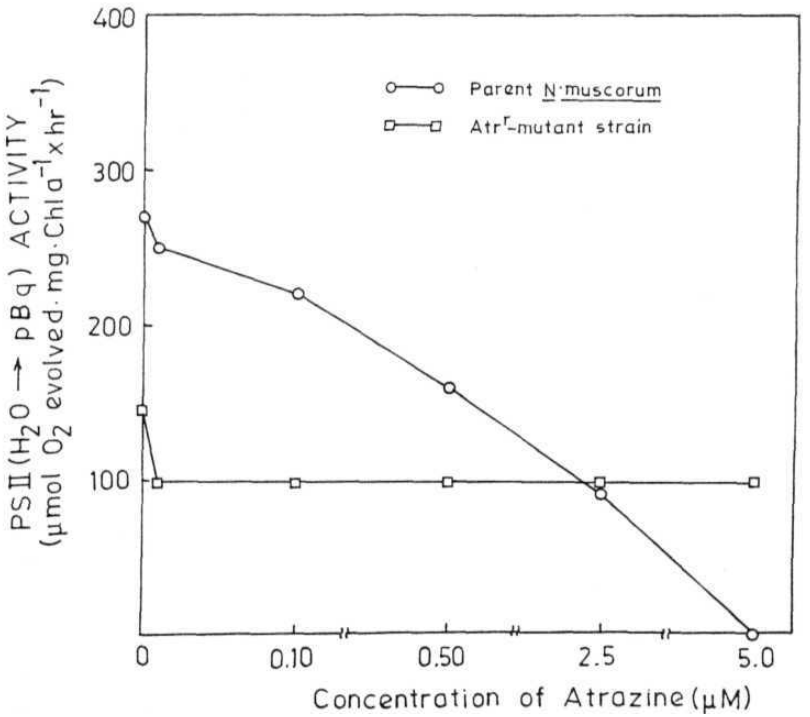


Fig. XV. PSII electron transport activity in Nostoc muscorum and its Atrazine^r-mutant strain under diazotrophic growth conditions with varying concentrations of the inhibitor Atrazine.

Table XI : Photochemical activities (PSII and PSI) in parent Nostoc muscorum, DCMU^r-mutant and Atrazine^r-mutant strains under diazotrophic growth conditions.
(A)

	Parent strain		DCMU^r-mutant strain		Atrazine^r-mutant strain	
	$H_2O \rightarrow$ pBQ	DCIP $\rightarrow$ MV	$H_2O \rightarrow$ pBQ	DCIP $\rightarrow$ MV	$H_2O \rightarrow$ pBQ	DCIP $\rightarrow$ MV
Control	350.0	356.24	175.0	357.03	210.0	383.87
+DCMU (5 μM)	0.0	356.24	125.0	357.03	165.0	380.80
+Atrazine(5 μ M)	0.0	358.40	62.5	357.03	100.0	382.50

PSII activity ($H_2O \rightarrow$ pBQ) expressed as μ mol. O_2 evolved. mg Chl $\underline{a}^{-1}$. hr^{-1}.

PSI activity (DCIP $\rightarrow$ MV) expressed as μ mol. O_2 consumed. mg Chl $\underline{a}^{-1}$. hr^{-1}.

All the results are an average of three independent experiments which did not vary by more than 10%.

For other details please see text.

Table XI : PSII activity in spheroplasts prepared from parent Nostoc muscorum, (B) DCMU^r-mutant and Atrazine^r-mutant strains under diazotrophic growth conditions

	Photosystem II activity		
	Parent strain	DCMU^r-mutant strain	Atrazine^r-mutant strain
Control	126.49	70.56	77.40
+DCMU (5 μM)	0.0	50.56	66.92

PSII activity expressed as μ mol O_2 evolved. mg Chl a $^{-1}$.hr $^{-1}$.

All the results are an average of three independent experiments.

For other details please see text.

Characteristics of Room temperature fluorescence emission spectra of whole cells in parent Nostoc muscorum and its DCMU -mutant and Atrazine -mutant strains in the presence and absence of either DCMU or Atrazine:

Changes in the fluorescence characteristics of PSI1 are a very reliable index of ascertaining the inhibitory effect of photosynthetic inhibitors on one or the other photochemical activities. We used this parameter in the intact cells of parent, DCMU -mutant and Atrazine^{r}-mutant strains to monitor the characteristics of in vivo fluorescence changes in the presence and absence of DCMU. DCMU induced nearly two-fold increase in fluorescence intensity of the parent strain. Such an increase in fluorescence intensity by DCMU was missing in DCMU -mutant cultures or Atrazine -mutant cultures after treatment with DCMU (Table XII). These results dearly show that mutation to DCMU-resistance or Atrazine-resistance has resulted in the loss of DCMU-induced rise in fluorescence intensity. Similar kinds of experiments were repeated with Atrazine. Results of such studies are shown in Table XIII. While Atrazine was found to cause more than 1.5 fold rise in fluorescence intensity in the parent strain, such an Atrazine-induced rise in fluorescence intensity was almost missing in DCMU^{r} or Atrazine^{r}-mutants. These results also suggest that mutation to Atrazine-resistance in the cyanobacteriurn has resulted in the loss of Atrazine-induced increase in fluorescence intensity.

Effect of DCMU and Atrazine on Nitrogenase and uptake hydrogenase activities of parent Nostoc muscorum, its DCMU^{r}-mutant and Atrazine^{r}-mutant strains:

DCMU and Atrazine effects were studied separately on nitrogenase activity and uptake-hydrogenase activity of parent N. muscorum, its DCMU and Atrazine^{r}-mutants. The results are shown in Table XIV. DCMU and Atrazine were found to be strongly inhibitory to cyanobacterial nitrogenase activity

Table XII : Room temperature fluorescence emission spectra of whole cells in parent Nostoc muscorum, DCMU -mutant and Artazine - mutant strains under diazotrophic growth conditions in the presence and absence of DCMU (5μM).

Strain	Peak position (nm)	Fluorescence intensity (a.u)		Ratio $r \frac{+DCMU}{-DCMU}$
		-DCMU	+DCMU	
Parent	682	30.75	65.5	2.13
$DCMU^r$-mutant	682	29.5	34.5	1.16
$Atrazine^r$-mutant	682	56.5	60.0	1.06

a.u. : arbitrary units.

For other details please see text.

Table XIII : Room temperature fluorescence emission spectra of whole cells in parent Nostoc muscorum, DCMU^r-mutant and Atrazine^r-mutant strains under diazotrophic growth conditions in the presence and absence of Atrazine (5 μM).

	Peak position (nm)	Fluorescence intensity (a.u.)		Ratio F +Atrazine / -Atrazine
		-Atrazine	+Atrazine	
Parent	682	30	51	1.70
DCMU^r-mutant	682	38	40	1.05
Atrazine^r-mutant	682	46	54	1.17

a.u. : arbitrary units.

For other details please see text.

Table XIV : Nitrogenase and uptake-hydrogenase activity in parent Nostoc muscorum, DCMU^r-mutant strain and Atrazine^r-mutant strains under diazotrophic growth conditions.

Strain	Nitrogenase activity			Uptake-hydrogenase activity		
	Control	+ DCMU	+ Atrazine	Control	+ DCMU	+ Atrazine
Parent	7.45	0.50	1.08	6.60	0.85	0.74
DCMU^r-mutant	5.80	4.75	ND	5.10	2.88	ND
Atrazine^r-mutant	6.81	ND	9.08	0.0	ND	0.0

Nitrogenase activity expressed as μ mol C_2H_4 formed. mg Chl $\underline{a}^{-1}$. hr^{-1}.

Uptake-hydrogenase activity expressed as μ mol H_2 consumed. mg Chl $\underline{a}^{-1}$. hr^{-1}.

All the above results are an average of three independent experiments which did not vary by more than 10%.

ND = Not determined.

For other details please see text.

and uptake-hydrogenase activity in the parent strain. Mutation to DCMU-resistance was found to have rendered nitrogenase activity and uptake-hydrogenase activity resistant to DCMU in DCMU^r-mutant strain. This strain was not studied with regard to Atrazine effect. Nitrogenase activity of Atrazine^r-mutant was found to rise significantly in the presence of Atrazine. The mutant strain was found to lack uptake-hydrogenase activity. Effect of DCMU on nitrogenase activity of Atrazine -mutant was also not studied. Mutation to Atrazine-resistance therefore seems to have been accompanied by loss of uptake-hydrogenase activity. This also indicates a close genetic linkage between Atrazine-resistant genetic determinant of PS11 activity and genetic determinant of uptake-hydrogenase activity. The availability of Hup characteristic in the Atrazine -mutant led us to examine the effect of H_2 on nitrogenase activity in the whole filaments of parent and Atrazine -mutant (Table XV).

Influence of H_2 on nitrogenase activity in parent Nostoc muscorum and its Atrazine -mutant strains:

Parent nitrogenase activity doubled in the presence of H_2. The activity of nitrogenase in the Atrazine^r-mutant on the other hand decreased by four-fold in the H_2 atmosphere. Atrazine addition to the mutant culture did not alter the H_2-mediated inhibition of nitrogenase activity. It is therefore, dear that uptake-hydrogenase enzyme has a role in preventing H_2-inhibition of nitrogenase activity in Nostoc muscorum.

Nitrogenase catalysed H_2-evolution in parent Nostoc muscorum, its DCMU -mutant and Atrazine -mutant strains:

We measured the nitrogenase catalysed H_2-production by the parent and mutant strains in the presence and absence of DCMU or Atrazine. Results of such studies are shown in Table XVI. Parent strain evolved significant amount

Table XV : Influence of H_2 on nitrogenase activity in parent Nostoc muscorum and its Atrazine^r-mutant (Hup) strains under diazotrophic growth conditions.

Growth condition	Nitrogenase activity		
	Parent strain	Atrazine^r-mutant strain	
		-Atrazine	+Atrazine
$-H_2$	7.50	6.80	9.08
$+H_2$	15.75	1.46	2.82

Nitrogenase activity expressed as μ mol C_2H_4 formed. mg Chl a .hr .

$+H_2$ indicates cultures incubated under H_2-atmosphere.

$-H_2$ indicates cultures incubated in the absence of H_2 atmosphere.

All the results are an average of three independent experiments which did not vary by more than 10%.

For other details please see text.

Table XVI : Nitrogenase catalyzed H_2-evolution under Argon atmosphere in parent Nostoc muscorum, DCMU^r-mutant and Atrazine^r-mutant strains under diazotrophic growth conditions.

Strain	H_2-evolution		
	Control	+DCMU	+Atrazine
Parent	10.74	0.0	0.0
DCMU^r-mutant	14.77	13.46	ND
Atrazine -mutant	10.43	ND	17.47

H_2-evolution expressed as μ mol H_2evolved. mg Chl a .hr .

All the results are an average of three independent experiments which did not vary by more than 10%.

For other details please see text.

ND : Not determined.

of H_2 under photoautotrophic growth conditions. This evolution of H_2 was stopped completely by DCMU or Atrazine. In comparison to parent, $DCMU^r$-mutant evolved higher level of H_2 and such photoproduction of H_2 was insensitive to DCMU. Effect of Atrazine on photo-evolution of H_2 was not seen in DCMU -strain. Atrazine -mutant in the absence of Atrazine showed as much production of H_2 as parental strain but, addition of Atrazine to it resulted in more than 1.5 fold rise in its H_2-evolving activity. Effect of DCMU on H_2 evolving activity of Atrazine -mutant was not examined.

Effect of tungsten on nitrogenase activity, uptake hydrogenase activity and nitrogenase catalysed H_2-evolution in parent Nostoc muscorum:

The nitrogenase mediated H_2-evolution and uptake-hydrogenase mediated H_2-consumption was studied in parent strain incubated under growth conditions containing Tungsten (W). Results are shown in Table XVII. W-free control cultures showed acetylene reducing (nitrogenase) activity which was decreased by more than 7-fold as a result of incubation with tungsten. H_2-evolution observed in control cultures also decreased by nearly 4-fold in W-incubated cultures. In comparison, uptake-hydrogenase activity of W-free and W-containing cultures did not differ significantly. W-induced loss in H_2-evolution was not reflected in great alteration of uptake-hydrogenase activity. This would mean that nitrogenase activity involved in H_2-production plays no significant role in the regulation of uptake-hydrogenase activity.

Growth kinetics of unicellular cyanabacterium Gloeocapsa:

We studied the growth kinetics of Gloeocapsa in different inorganic nitrogen-media. The results are shown in Fig. XVI. Growth of the cyanobacterium was best in NO_3-medium while growth in N_2 and NH_4-medium was almost similar. The more or less similar rate of growth in NH_4^+- and in

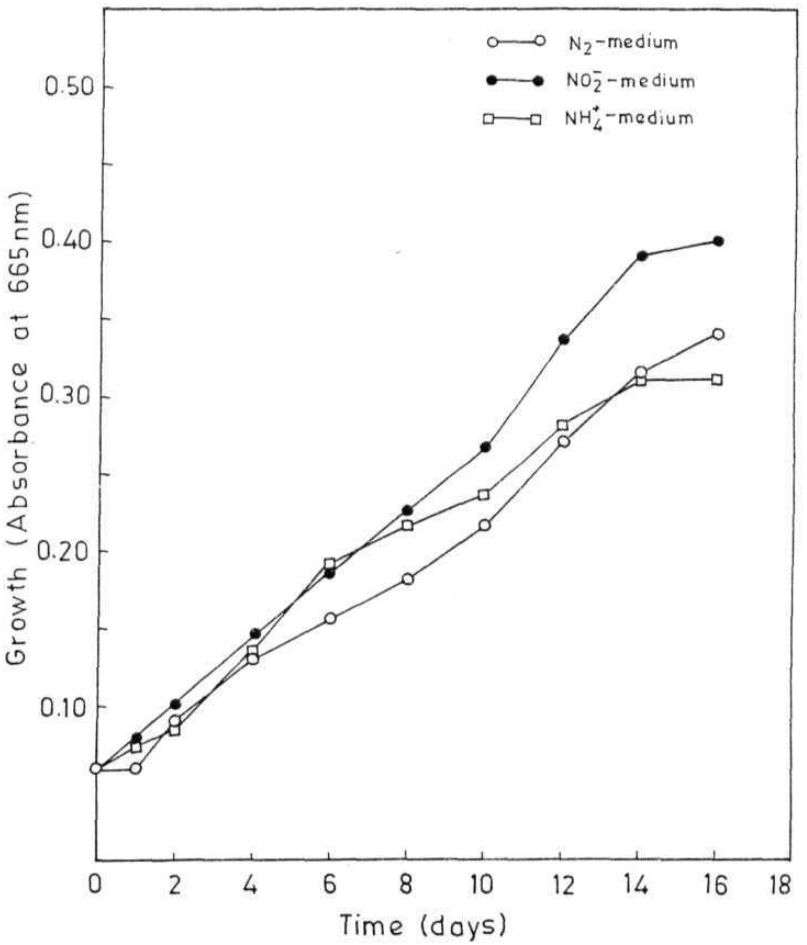


Fig. XVI. Growth kinetics of the unicellular diazotrophic cyanobacterium Gloeocapsa under N_2-assimilating, NO_2^--assimilating and NH_4^+-assimilating growth conditions.

Table XVII : Nitrogenase activity, H_2-evolution and uptake-hydrogenase activities in parent N. muscorum incubated with Tungsten.

Grwoth condition	Nitrogenase activity	H_2-evolution	Uptake-hydrogenase activity
Control	7.68	5.00	6.10
+ Tungsten	0.96	1.20	5.85

Nitrogenase activity expressed as u mol C_2H_4. formed. mg Chl a^{-1}.hr^{-1}

H_2-evolution expressed as μ mol H_2 evolved. mg Chl a . hr .

Uptake hydrogenase activity expressed as μ mol H_2 consumed. mg Chl a .hr

For other details please see text.

N_2-medium is surprising in the sense that the unicellular cyanobacterium under aerobic oxygenic photosynthetic conditions appears to assimilate N_2 as efficiently as NH_4^+. This could well mean that there is an efficient O_2-protection mechanism under aerobic N_2-fixing conditions in unicellular cyanobacterium Gloeocapsa.

Effect of fixed nitrogen sources on photosynthetic O_2-evolution, nitrogenase activity, uptake hydrogenase activity and H_2-evolution in unicellular diazotrophic cyanobacterium Gloeocapsa:

We compared the rate of photosynthetic O_2-evolution, nitrogenase activity and uptake-hydrogenase activity in Gloeocapsa growing with N_2, NO_3^- and NH_4^+ as nitrogen source (Table XVIII). We also measured H_2-evolution by the cyanobacterium under these conditions. Photosynthetic O_2-evolution was lowest in diazotrophic cultures, more or less same in NO_3^--assimilating cultures and significantly higher in NH_4^+-assimilating cultures. Nitrogenase activity was found in all the three nitrogen sources with maximum activity in N_2-medium and almost similar level in NO_3^--medium and nearly five fold lower in NH_4^+-medium. Uptake-hydrogenase activity was optimum in N_2-assimilating cultures, slightly lower in NH_4^+-assimilating cultures and still lower in NO_3^--assimilating cultures. However, the range of variation observed in uptake-hydrogenase activity with nitrogen source was not as high as that of nitrogenase activity. Diazotrophic cultures also evolved H_2 which was nearly more than 10-fold higher than that produced by NO_3^--assimilating or NH_4^+-assimilating cultures. The interesting thing about nitrogenase activity and uptake-hydrogenase activity is that they operate even in cultures growing with fixed nitrogen source like NO_3^- or NH_4^+. H_2-evolution although occurred significantly only in N_2-fixing cultures, its evolution continued occurring at a low or almost negligible level in NO_3^--assimilating or NH_4^+-assimilating cultures also.

Table XVIII : Photosynthetic 0_2-evolution, nitrogenase activity, uptake-hydrogenase activity and H_2-evolution in Gloeocapsa in various inorganic nitrogen media.

Nitrogen source	Photosynthesis	Nitrogenase activity	Uptake-hydrogenase activity	H_2-evolution
N_2-medium	111.08	1.70	5.36	11.28
NO_2^--medium	115.53	1.20	3.71	0.98
NH_4^+-medium	141.40	0.35	4.02	0.65

All the results are an average of three independent experiments which did not vary by more than 10%.
Photosynthetic 0_2 evolution expressed as μ mol 0_2 evolved. mg Ch l $\underline{a}^{-1}$. hr^{-1}.
Nitrogenase activity expressed as μ mol C_2H_4 formed. mg Chl $\underline{a}^{-1}$. hr^{-1}.
Uptake-hydrogenase activity expressed as μ mol H_2 consumed. mg Chl $\underline{a}^{-1}$. hr^{-1}.
H_2-evolution expressed as μ mol H_2 evolved. mg Chl $\underline{a}^{-1}$. hr^{-1}.
For other details please see text.

Effect of organic carbon source(Glucose) on nitrogenase activity and uptake hydrogenase activity in the cyanobacterium Gloeocapsa under diazotrophic growth conditions:

We examined the effect of darkness and glucose on nitrogenase and uptake-hydrogenase activity in Gloeocapsa growing under diazotrophic conditions. The experiments were done at the end of 24 hr incubation period and data are shown in Table XIX. Light-incubated cultures under autotrophic condition showed a **level** of nitrogenase activity which doubled **in** the presence of glucose. This finding indicates a role for glucose **metabolism** in optimizing nitrogenase activity under photoautotrophic growth conditions. Uptake-hydrogenase activity was present in autotrophic cultures incubated in light but not in glucose-containing light-incubated cultures. Glucose while enhancing nitrogenase activity inhibited completely uptake-hydrogenase activity under photoheterotrophic conditions in the cyanobacterium. Parallel experiments done in the dark showed a level of nitrogenase activity in its autotrophic cultures nearly as good as with light-incubated cultures. Glucose addition to dark autotrophic cultures also caused significant rise in cyanobacterial nitrogenase activity, thus glucose-mediated increase in nitrogenase activity occurs both under photoheterotrophic and chemoheterotrophic conditions. Uptake-hydrogenase activity of autotrophic dark cultures was nearly 2-3 fold lower than its corresponding cultures in light. This suggests a role for light in the activation of uptake-hydrogenase activity in the cyanobacterium. Glucose abolished the uptake-hydrogenase activity in the dark as well.

Table XIX : Nitrogenase activity and uptake-hydrogenase activity in Gloeocapsa growing diazotrophically in the presence and absence of glucose.

	LIGHT		DARK	
	- Glucose	+ Glucose	- Glucose	+ Glucose
Nitrogenase activity	1.70	3.43	1.88	2.38
Uptake-hydrogenase activity	5.36	*0.0*	*0.72*	*0.0*

Nitrogenase activity expressed as u mol C_2H_4 formed. mg chl a .hr

Uptake hydrogenase activity expressed as u mol H_2 consumed. mg Chl a .hr .

All the above results are an average of three independent experiments which did not vary by more than 10%.

For other details please see text.

DISCUSSION

'Q'$_B$ protein is the component of electron transport chain between PS11 and PSI in oxygenic photosynthetic systems like chloroplast and cyanobacteria. DCMU or Atrazine blocks PS1I activity by interaction with the 'Q'$_B$ protein, which on contact with the herbicide reduces its ability to participate in the electron transport chain thereby inactivating the PSI1 photochemical activity. In a recent detailed study aiming at finding out the structure-function relationship in the 'Q'$_B$ protein, Haselkorn and his group took a detailed study of the occurrence of psbA genes, their organization and expression under different stages of growth in Anacystis nidulans R_2. Their results showed that A.nidulans R_2 possesses three copies of psbA gene for the 'Q ' protein of PSII. This protein is essential for oxygenic photosynthesis and is the target for several herbicides which act by binding directly to the 'Q$_B$' protein of the photosynthetic apparatus. Mutants conferring resistance against DCMU or Atrazine conferred dominance phenotype even in the presence of other two copies which coded for the herbicide-sensitive phenotype. Their studies have shown for the first time the occurrence of a family of genes coding for the same phenotype in any prokaryote and that mutant phenotype is dominant over the normal phenotype (Golden et al, 1986).

Here we report the spontaneous occurrence of a mutation to DCMU-resistance or Atrazine-resistance in the diazotrophic heterocystous cyanobacterium N. muscorum. This cyanobacterium may as well have a family of genes coding for 'Q'$_B$ protein and mutation in one of these can result in expression of herbicide-resistance phenotype in view of its dominant nature as reported for A. nidulans R_2. Our results also suggest, that 'Q'$_B$ protein might be the mutated protein involved in conferring DCMU resistance as well as Atrazine resistant phenotype, because the two mutant phenotypes showed cross-resistant

relationship to each other. However, mutation to DCMU resistant phenotype resulted in reduced growth rate thereby suggesting slightly harmful nature of such mutant phenotype in N. muscorum. On the other hand, mutation to Atrazine-resistance did not seem to adversely effect the cyanobacterial diazotrophic growth. However, mutation to Atrazine-resistance was accompanied by the loss of uptake-hydrogenase activity. It does seem to indicate pleiotropic nature of Atrazine-resistant phenotype. Mutation to DCMU-resistance phenotype was not found to be associated with any specific loss in nitrogenase or uptake-hydrogenase activity. These results do indicate different loci of psbA gene involved in mutational control of DCMU-resistant and Atrazine-resistant phenotype. Inability of the parental strain to grow photoautrophically as well as diazotrophically with DCMU/Atrazine is as expected because the two herbicides are strong inhibitors of cyanobacterial photoautotrophic growth (Ho and Krogmann, 1982). One noteworthy feature of the Atrazine -mutant was its ability to show enhanced acetylene reducing activity or H_2-producing activity in the presence of the herbicide. We do not have any explanation at the moment to offer but, one can speculate that this herbicide may have a regulatory role in modulating nitrogenase activity under conditions of its non-inhibitory action on PSI1.

Since the discovery of uptake-hydrogenase activity in N_2-fixing cells and its suggested role in recycling of H_2, many questions have been raised about the uptake-hydrogenase in biology of N_2-fixing systems. What are the biochemical components of H_2-oxidation systems? How widely recycling mechanism of H_2 distributed in diazotrophs? What are the benefits and loss likely to result from H_2-recycling in the systems of its occurrence? and lastly what are the genetic determinants for H_2- recycling capacity? Detailed studies of these questions in Rhizobium has not settled the problem of beneficial

effect of uptake-hydrogenase activity in regulation of N_2-fixation in legumes (Evans et al, 1987).

H_2-evolution by nitrogenase is an energy wasting process. Therefore, an operation of active uptake-hydrogenase should be advantageous for N_2-fixation activity. Nevertheless, in a series of experiments with Nickel-depleted cultures of A. variabilis containing low levels of uptake-hydrogenase activity, no significant difference with respect to growth and nitrogenase activity in comparison to Nickel-grown cells containing high levels of uptake-hydrogenase was evident (Almon and Boger, 1984; Almon and Boger, 1987). These reports are similar to the reports published by Daday et al (1985) on the influence of uptake-hydrogenase activity and nitrogenase activity on diazotrophic growth. Thus it seems by the previous reports that, uptake-hydrogenase activity has no role in regulating nitrogenase activity and diazotrophic growth under normal growth conditions. Weisshaar and Boger (1985) have concluded that there are two pathways of H_2-utilization in heterocystous cyanobacteria - one mediated directly by nitrogenase for reduction of molecular N_2 to NH_3 or reduction of C_2H_2 to C_2H_4; and the other mediated by uptake-hydrogenase which can channel the H_2 into oxy-hydrogen reaction or into PSI-mediated production of reductant or ATP under anaerobic photosynthetic conditions. Chen et al (1986) has supported this concept by noting the operation of H_2-utilization in cyanobacterial strains deficient in uptake-hydrogenase activity. H_2 has been reported to enhance nitrogenase activity in heterocystous cyanobacteria (Benemann and Weare, 1974; Scherer et al, 1980). Our finding that external H_2 increased nitrogenase activity is in agreement with this. We have also isolated a Hup" mutant on the basis of the inability of the mutant strain to consume exogenous H_2. In such a mutant strain aerobic nitrogenase activity is as good as the aerobic nitrogenase activity of the parent strain. Examination

of the H_2 effect on the nitrogenase activity of the mutant revealed it to be toxic while under comparable conditions exogenous H_2, was found to augment significantly the aerobic nitrogenase activity of the parent strain. Since the parent strain showed consumption of exogenous H_2 and the mutant strain did not show consumption of exogenous H_2, we conclude that toxic effect of exogenous H_2 on the nitrogenase activity in the mutant strain is due to a mutational loss in the ability for H_2-consumption and that, this loss in the ability for H_2-consumption is because of mutational loss of uptake-hydrogenase activity. This mutational loss of uptake-hydrogenase activity has resulted in H_2-toxicity to nitrogenase activity. We therefore, conclude that uptake-hydrogenase in cyanobacterium like N. muscorum might have a role in preventing H_2-inhibition of nitrogenase activity. In addition, since exogenous H_2 increased nitrogenase activity in the parent strain, we further suggest that uptake-hydrogenase activity has a role in regulating nitrogenase activity through beneficial recycling of H_2 produced by nitrogenase during N_2-fixation.

A role of H_2 has been suggested in regulating uptake-hydrogenase activity in Rhizobium strains (Evans et al, 1987). H_2 is produced by nitrogenase as a side reaction of N_2-fixation and one could surmise that such a nitrogenase produced H_2 might act as an activator of uptake-hydrogenase activity in cyanobacteria. Tungsten(W), an analogue of Mo has been used by previous workers (Singh et al, 1978; Bagchi et al, 1985) to inactivate nitrogenase in N. muscorum and here we examined the consequence of such an inactivation on uptake-hydrogenase activity. Our results demonstrate that uptake-hydrogenase activity does not vary significantly with the inactivation of nitrogenase activity by W. Thus we conclude that, uptake-hydrogenase regulates nitrogenase activity by preventing H_2-inhibitory effect on the nitrogenase enzyme and by supplying reductant/ATP by recycling nitrogenase produced H_2 or exogenously supplied

H_2 and that nitrogenase activity plays no significant role in regulating uptake-hydrogenase activity. However, a detailed genetic analysis of the regulation of production and activity of the two enzymes should be undertaken to appreciate fully the implication and inter-dependence of photosynthesis and aerobic H_2 metabolism in diazotrophic cyanobacteria.

We studied the aerobic metabolism of H_2 in the aerobic diazotrophic unicellular cyanobacterium Gloeocapsa which is a naturally occurring multiple herbicide resistant strain containing genetic determinants for DCMU-resistance, Machete-resistance and Basalin-resistance (Singh, et al, 19S6; Singh, et al, 1987). This cyanobacterium shows nitrogenase activity in all inorganic nitrogen-media thereby suggesting it to be a partially derepressed diazotrophic strain occurring in nature. We also find evidence for the presence of uptake-**hydrogenase** activity in different nitrogen-media, thereby further suggesting nitrogen-derepression of uptake-hydrogenase activity as well in this cyanobacterium. This is the first report of a naturally occurring aerobic diazotrophic unicellular strain showing multiple herbicide resistant phenotype and derepression of nitrogenase and uptake-hydrogenase. The almost similar rate of growth of the cyanobacterium in N_2, NO_3 and NH_4-medium suggests that fixed nitrogen sources are no better source of nitrogen than atmospheric N_2 in this cyanobacterium. Such a situation might be the result of lack of nitrogen regulation of growth at genetic level, although there is no information at the moment available 10 say anything definitely and concretely about the occurrence of such a nitrogen-regulatory genetic system in control of nitrogen-nutrition and growth in cyanobacteria. In this strain as well, glucose is found inhibitory to uptake-hydrogenase activity and stimulatory to nitrogenase activity. This observation further indicate a role of uptake-hydrogenase in supplying reductant from H_2 to nitrogenase for N_2-fixation in the absence of organic carbon like

glucose which is a natural source of physiological substrate for generating reduced ferredoxin to serve directly as a reductant for nitrogenase activity. Gloeocapsa contains uptake-hydrogenase activity in N_2-assimilating, NO_3-assimilating and in NH_4^+-assimilating cultures under photoautotrophic growth conditions as well as under autotrophic condition in dark. Such a distribution of uptake-hydrogenase activity which is inhibited by glucose suggests, that organic carbon-limitation within the cell under all the given growth conditions is the reason for the constitutive presence of uptake-hydrogenase activity. Thus we conclude here, a role for organic carbon like glucose in regulating nitrogenase activity and uptake-hydrogenase activity in opposing pattern. We are inclined to believe that in cyanobacteria too uptake-hydrogenase activity is a mechanism to utilize H_2 as a reductant for reductive diazotrophic metabolism in the face of organic carbon-limitation.

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

Heterocystous and unicellular diazotrophic cyanobacteria are oxygenic photosynthetic organisms capable of efficient growth and multiplication at the expense of light, water and air. Much attention has been and is being focussed on these organisms in the hope that, photosynthetic reactions that generate reductant from water can be coupled to the reduction of nitrogen and protons, thus constituting a biophotolytic system in which solar energy can be used to generate the desired products NH_3 and H_2, the former as a source of nitrogen fertilizer and other industrial uses including as an easily exploitable source of H_2 and the latter mainly as a renewable source of future fuel in place of already progressively dwindling reserve of fossil **fuel.** Heterocysts of cyanobacteria are very efficient oxygen-protective systems for nitrogenase activity and N_2-fixation under aerobic photosynthetic conditions. The nitrogenase activity during N_2-fixation while converting N_2 to NH, also reduces H^+ to H_2 under aerobic conditions. If the aerobic atmosphere is replaced by Argon or a mixture of Argon and the gases excluding N_2, the nitrogenase enzyme would function wholly and exclusively as H_2 producing enzyme at the expense of light and water. It is this unique cyanobacterial feature which has rendered them very potential photobiological source of H_2.

Heterocystous and unicellular diazotrophic cyanobacteria also contain a membrane-bound H_2 oxidizing enzyme called uptake-hydrogenase capable of efficient functioning under oxygenic photosynthetic conditions. This enzyme is found to be localized in heterocysts in heterocystous cyanobacteria and in the same cell of unicellular diazotrophic cyanobacteria which contains nitrogenase enzyme. In respect of localization of nitrogenase

enzyme and uptake-hydrogenase enzyme, unicellular forms are comparable to heterocysts of heterocystous form.

Functional significance of such common localization of the two enzymes is considered to result in optimization of N_2-fixation. However, much basic studies are required to understand the physiological significance oi nitrogenase-uptake-hydrogenase interaction in cyanobacteria. In addition, the role of nutritional factors like carbon and nitrogen and energy factors like phototrophy and chemotrophy on relative regulation of the activities of nitrogenase and uptake-hydrogenase within, the unicells or heterocysts are almost unexplored. Furthermore, there are no genetic studies attempted to dissect out the functional interrelations of the two enzymes as well.

The present thesis has been an attempt to experimentally examine the functional interrelationship of the two enzymes in unicellular and heterocystous diazotrophic cyanobacteria in relations to genetic factors, carbon nutrition, nitrogen nutrition, phototrophy and chemotrophy.

The salient features of this study are:

1) Fixed nitrogen inhibits both nitrogenase activity and uptake-hydrogenase activity in heterocystous cyanobacteria mainly because of their inhibitory effect on heterocyst formation.

2) The activities of nitrogenase and uptake-hydrogenase within heterocyst is differentially regulated by fixed nitrogen and organic carbon.

3) Derepressed aerobic N_2-fixing cyanobacterial systems also become derepressed for uptake-hydrogenase system thereby indicating their common genetic and physiological regulation as well.

4) Regulatory unit responding to NH_4^+-repression signal seems to be distinctly different for heterocyst than that from nitrogenase system.

5) Isolated heterocysts contain nitrogenase, uptake-hydrogenase activity but lack active nitrate reductase enzyme.

6) Lack of nitrate reductase activity from heterocysts further supports the view that heterocyst is a physiologically specialized cell, mainly meant for carrying out metabolic processes capable of **optimizing** N_2-fixation.

7) The reason for localization of uptake-hydrogenase within heterocyst appears to be carbon-limited status of heterocysts.

8) Symbiotic association and chemotrophy, both conditions cause total lack of uptake-hydrogenase activity.

9) Physiological or genetical inactivation of nitrogenase activity does not seem to influence uptake-hydrogenase activity.

10) Inhibitors of photosynthesis blocks both the enzyme activities.

11) $DCMU^r$-mutant and $Atrazine^r$-mutant strains of Nostoc muscorum showed resistance to PSII activity in the presence of the herbicides.

12) Both the $DCMU^r$-mutant and Atrazine -mutant strains showed cross-resistant relationship.

13) The mutation to Atrazine-resistance was accompanied by the loss of uptake-hydrogenase activity.

14) The mutational loss of uptake-hydrogenase activity has resulted in H_2 toxicity to nitrogenase activity.

15) Uptake-hydrogenase activity has a role **in** regulating nitrogenase activity in Nostoc muscorum either by preventing H_2-inhibition of nitrogenase activity or through beneficial recycling of H_2 produced by nitrogenase during N_2-fixation.

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FEMS Microbiology Letters 49 (1988) 261-265
Published by Elsevier

FF.M 03073

Isolation and characterization of a metronidazole-resistant (*Mtn-R)* mutant strain of *Nostoc muscorum* affected in ammonium regulation of heterocyst formation and uptake hydrogenase activity

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Received 9 October 1987
Accepted 19 October 1987

Key words: Cyanobacterium; Heterocyst; Nitrogenase; Uptake hydrogenase; NADPH : ferredoxin **oxidoreductase; Glutamine synthetase;** Ammonium (methylammonium) **transport**

1 SUMMARY

The metronidazole-resistant (*Mtn-R*) mutant strain of *N. muscorum* produced drug-resistant **NADPH** : feiredoxin (Fd) oxidoreductase and showed derepression of heterocyst formation and uptake hydrogenase activity in NH_4^+-medium. The observation of NH_4^+-repression in regulation **of** nilrogenase **activity** alone in the mutant strain **suggests,** that **heterocyst** formation and nilrogenase activity are regulated by **two** separate NH_4^+-repression control systems, one specific for heterocyst and uptake hydrogenase and the other for nilrogenase. **The** partial drug-resistant NADPH : Fd oxidoreductase enzymatic activity seems to be the reason for drug-resistant growth of the **cyanobacterium in** N_2-medium and NH_4^+-medium.

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2. INTRODUCTION

Metronidazole (2-methyl-5-nitroimidazole, 1-ethanol), is **a** specific inhibitor **of** ferredoxin-linked reactions **in** phototrophs [1-4]. The drug has been successfully employed as screening agent for selecting out mutants defective in one or the other components **of** ferredoxin-dependent reductive reactions like N_2-fixation [5]. As expected, the drug has been shown to selectively inhibit photostimulated reduction of acetylene by intact filaments **of** *Anabaena* [6] and by isolated heterocysts [7]. Study of the mutants of heterocystous cyanobacteria resistant to growth inhibition by the drug with N, as nitrogen source are expected to provide interesting information about the functional linkage between ferredoxin-dependent activity and heterocyst differentiation.

We have isolated a metronidazole-resistant mutant (*Mtn-R*) strain of *Nostoc muscorum* and here we present evidences to show that mutation to drug resistance has been accompanied by production within heterocyst and vegetative cells of

partial drug-resistant NADPH : ferredoxin (Fd) oxidoreductase activity and by the loss of ammonium repression control of heterocyst formation and uptake hydrogenase activity.

3. MATERIALS AND METHODS

Nostoc muscorum was grown axenically in **nitrogen-free** (diazotrophically grown) and NH_4^+-supplemented modified Chu-10 medium [X] as in [9]. **Metronidazole** (100 $\mu g \cdot ml^{-1}$) was included **in** the growth medium whenever required.

Metronidazole-resistant mutant (*Mtn-R*) was **isolated on** combined nitrogen-free medium (N_2-medium) containing metronidazole by following the method of [10] and **the** stable, resistant mutants were maintained on NH_4^+-slants containing the inhibitor.

Heterocysts were isolated from the exponentially growing filaments of *N. **muscorum*** as in [11]. Such preparations had a vegetative cell contamination **of** less than *5%* **(as** observed in a light microscope) and showed a **biosynthetic** glutamine synthetase activity of 150 nmol product formed $min^{-1} \cdot mg^{-1}$ protein. Enzyme activities in vegetative cells was calculated by subtracting the activity **of** isolated heterocysts from that of whole filaments (containing both heterocysts and vegetative cells).

Growth was measured by increase in chlorophyll content as in [12]. Heterocyst frequency was calculated as percentage **of** total cells, by light-microscopic observations of the filaments of *N. muscorum*. Total protein was estimated by [13] as **in** [14].

Assays for NADPH : Ed oxidoreductase **(EC** 1.18.1.2) and glutamine synthetase (EC 6.3.1.2) activities in cell-free preparations of isolated heterocysts and whole filaments were performed as described by [15] and [16], respectively. Nitrogenase (EC 1.18.6.1) activity was measured as acetylene reduction as in [17] and uptake hydrogenase activity was measured by gas chromatography as in [18]. Ammonium (methylammonium) **transport** activity was estimated as in [19].

Total glucose (glycogen–glucose plus free glucose) and glycogen–glucose were estimated as in [20]. The value of free glucose was calculated by subtracting glyeogen-glucose from total glucose. The ratio of phycocyanin to chlorophyll-*a* was calculated from the absorption spectrum **of** methanol-soluble and water-soluble photosynthetic pigments as in [21].

4. RESULTS AND DISCUSSION

Both the parental strain and its drug-resistant (*Mtn-R*) mutant strain were compared for growth, nilrogenase activity, heterocyst frequency, uptake hydrogenase activity, glutamine synthetase activity and NADPH : Ed oxidoreductase activity in drug-free and drug-containing N_2-fixing and NH_4^+-assimilating cultures (Table 1). The drug-containing parental cultures did not grow in N_2-fixing or NH_4^+-assimilating conditions thus suggesting that growth inhibitory action **of** the drug is not nitrogen source-specific. A cellular factor common to N_2 assimilation and NH_4^+-assimilation, therefore, should most likely be the reason for the observed inhibitory effect of the drug on growth. It is known that reduced ferredoxin is the natural physiological reductant in N_2-reduction by nitrogenase in heterocystous cyanobacteria [22–24]. The same physiological reductant is also required in the assimilation of ammonia by the GS-GOGAT pathway in these cyanobactena [25]. Since metronidazole is a known specific inhibitor of ferredoxin-linked reactions, the drug-resistant diazotrophic growth or NH_4^+-supported growth is likely to result from mutational modification of the mechanism generating reduced ferredoxin. NADPH : Ed oxidoreductase is the suggested enzymatic pathway for the generation of reduced ferredoxin in cyanobacteria [24]. Since the drug was found almost completely inhibitory to this enzyme in the parental culture but not in **the** mutant culture, mutational production of partly drug-resistant NADPH : Fd oxidoreductase activity is suggested to be the biochemical reason for observed resistance of growth to the drug under diazotrophic or NH_4^+-assimilating conditions. It is important to note here that the mutant culture

Table I

Growth (μg Chl-a·ml^{-1}) **of culture, nitrogen ase activity** (nmol C_2H_4 formed·μg Chl-a^{-1}·h^{-1}), **heterocyst frequency** (number of **heterocysts/100 vegetative** cells), uptake hydrogenase activity (μmol H_2 consumed mg Chl-a^{-1}·h^{-1}), glutamine synthetase activity (μmol product formed mg^{-1} protein min^{-1}), and **NADPH : Fd** oxidoreductase (nmol DCPIP reduced·mg^{-1} protein·min^{-1}) activities in parent *N. muscorum* and its met ronidazole-resistant mutant (*Mtn-R*)**strains**

All the **above** values are an average **of** three **independent** experiments which did not vary by more than 10%. 6-day-old cultures were used for **estimation of** growth, heterocyst **frequency and** other **enzyme** activities. Heterocyst frequency and various **enzyme** activities were estimated with cultures treated or **untreated** with the drug for 12 h.

	Parent strain				*Mtn-R* strain			
	Diazotrophically grown		Grown with ammonia		Diazotrophically grown		Grown with ammonia	
	− Mtn	+ Mtn	− Mtn	+ Mtn	+ Mtn	− Mtn	+ Mtn	− Mtn
1. Growth	7.40	0.0	2.68	0.0	7.78	8.85	2.41	2.68
2. Nitrogenase activity	7.48	0.0	0.0	0.0	4.21	8.0	0.0	0.0
3. Heterocyst frequency (%)	5–6	5–6	0.0	0.0	8–10	8–10	8–9	8–9
4. Uptake hydrogenase activity	6.16	6.16	0.0	0.0	5.68	4.55	5.36	5.90
5. Glutamine synthetase (transferase) activity	1.60	1.60	0.46	0.46	1.00	1.00	0.58	0.76
6. NADPH : Fd oxidoreductase activity								
(a) Isolated heterocysts	622.78	38.12	–	–	199.16	180.16	76.25	70.0
(b) Vegetative cells	248.0	10.0	220.0	0.0	70.0	73.0	30.68	32.0

− Mtn: indicates cultures grown without metronidazole; + Mtn: indicates cultures grown with metronidazole; −: Indicates absence of heterocysts.

showed nearly **3-fold** less activity **of** NADPH : **Fd oxidoreductase as** compared **to** that **of** parental culture.

The **drug-free** cultures **of** parental and mutant strains showed almost similar level **of** growth, nitrogenase activity and **glutamine synthetase** activity under N_2-fixing and NH_4^+-assimilating **conditions,** thus suggesting no obvious direct influence **of** mutational change **on** these **phenotypes in** this cyanobacteria. However, the most remarkable impact of drug-resistant mutational change has been on the loss of NH_4^+-repression control of heterocyst formation and uptake hydrogenase activity (Table I). Heteiocyst frequency went up **significantly** and uptake hydrogenase activity declined slightly as a result of **mutational** loss of NH_4^+**-repression** control. However, the normal operation **of** NH_4^+-repression system in regulation **of** nitrogenase activity in the mutant strain clearly suggests the operation **of two** distinctly separate **ammonium-sensitive** regulatory units, one specific for nitrogenase and the other specific for heterocyst and uptake hydrogenase. Operation of **an** almost similar level **of** NH_4^+-transport activity in both strains (Fig. 1) rules out a role of defective ammonium transport system as the reason for production of heterocyst and uptake hydrogenase activity in NH_4^+-grown cultures of mutant strain. GS activity in mutant strain was nearly as good as that **in** the parental strain, thereby also ruling out a role of the activity of this enzyme in derepression of heleroeyst and uptake hydrogenase activity in the mutant strain. It is therefore suggested that NH_4^+-derepression has resulted from mutation alteration in the regulatory site of the gene(s) **of** heterocyst formation which no longer remains sensitive **to** the NH_4^+-generated repressor signal. The absence **of** nitrogenase activity in the NH_4^+-growing cultures of the mutant strain also for the first time demonstrates directly that NH_4^+ prevents nitrogenase biosynthesis not by preventing heterocyst formation as suggested by [26,27], but by a

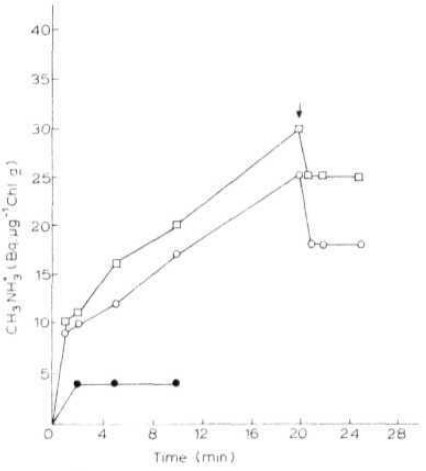

Fig. 1. [^{14}C]Methylammonium uptake by N_2-grown *N. muscorum* and its metronidazole-resistant mutant (*Mtn-R*) strain. Exponentially growing cells were harvested, washed and resuspended in 10 mM HEPES buffer, pH 7.0. Where required cells were pretreated with toluene (1%, v/v) for 15 min and then harvested, washed and resuspended in 10 mM HEPES buffer, pH 7.0. [^{14}C]Methylammonium was added at zero time and ^{14}C incorporation into cells measured over a period of 10 min. Arrow indicates the addition of ammonium chloride. ○, basic curve of parent *N. muscorum*; □, basic curve of metronidazole-resistant (*Mtn-R*) strain; ●, cells pretreated with 1% (v/v) toluene.

pathway separate from that involved in NH_4^+-repression of heterocyst formation. Such mutants would be extremely useful in analysing molecular details of the regulation and production of heterocyst-specific enzymes sensitive to NH_4^+-repression.

It is a well accepted view that uptake hydrogenase activity like nitrogenase activity is NH_4^+-repressible and is located within heterocyst [28,29]. The derepression of uptake hydrogenase activity along wilh the derepression of heterocyst formation in NH_4^+-grown cultures of mutant strain which do not show nilrogenasc activity suggest the operation of a common NH_4^+-repression regulatory unit for both systems, **heterocyst** formation and uptake hydrogenase aclivity (Table 1). However, it could as well be that heterocyst differentiation alone is under NH_4^+-repression control and that uptake hydrogenase **formation** and aclivity is a regulatory function of the heterocyst. Although diazolrophic growth of the mutant was resistant to the drug, the in vivo nitrogenase activity was still 50% drug-sensitive (Table 1). This could apparently mean that 50% reduction in acetylene reducing activity of the in vivo nitrogenase is not reflective of its N_2-fixing ability related to **diazolrophic** growth which was as good as that in drug-free parental culture.

The **mutant** and its parent were also **investigated** in respect of their protein content, glycogen content, glucose content and ratio of phycocyanin to chlorophyll (Table 2). Mutant strain produced more glycogen and glucose and less protein and **phycocyanin** to chlorophyll ratio than the parental

Table 2

Protein content, glycogen accumulation and phycocyanin/chlorophyll ratio in parent and *Mtn-R* strain of *N. must*

All the above values are an average of three independent experiments which did not vary by more than 10%.

	Parent strain		*Mtn-R* strain	
	Diazotrophically grown	Grown with ammonia	Diazotrophically grown	Grown with ammonia
Protein content (µg·ml^{-1} culture)	225.75	110.60	194.15	143.85
Glycogen-glucose (µg glucose·mg^{-1} protein)	179.70	17.0	407.20	6.95
Soluble glucose (µg glucose·mg^{-1} protein)	84.09	21.25	189.75	6.95
Phycocyanin/chlorophyll-*a* ratio	0.924	1.09	0.620	1.00

strain. Since it is now known that cyanobacterial uptake hydrogenase activity is inhibited by glucose in free-living *Anabaena cycadeae* **[14J,** the slightly lower activity **of** uptake hydrogenase enzyme in the **mutant** culture, despite its having **significantly** higher **heterocyst** frequency, may be because **of** its higher content **of** glucose and giyvogen.

The **mutation to** drug **resistance** appears **to** have **altered** two major cellular functions **in** *N. muscorum.* One. **the** production **of drug-resistant NADPH : Fd oxidoreductase** and **the** oilier, **derepression of** heterocyst formation and uptake hydrogena.se activity in NH_4^+-medium. These results merely suggest a close genetic linkage between these two **functions** in this cyanobacterium.

ACKNOWLEDGEMENTS

We thank the Dean, School of Life Sciences, **University** of Hyderabad, Hyderabad for **providing** necessary **facilities** and CSIR for financial **assistance.**

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CARBON NUTRITION AND THE REGULATION OF UPTAKE HYDROGENASE ACTIVITY IN FREE-LIVING AND SYMBIOTIC *ANABAENA CYCADEAE*

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(Accepted 25 April 1986)

SUMMARY

Anabaena cycadeae was grown with N_2 as nitrogen sourer, either photoautotrophically in light or with glucose as carbon source in darkness. The rave of growth was much slower in darkness but the heterocyst frequency was much the same; nitrogenase activity (on a chlorophyll basis) was ahout hull that of light-grown cells. Light-grown organisms contained uptake hydrogenase activity but dark-grown organisms did not. The addition of glucose to light-grown organisms was followed by the disappearance of uptake hydrogenase activity over the following 48 hours and the disappearance was independent of light. Heterocyst frequency' and nitrogenase activity were much less affected by glucose addition.

A. cycadeae growing symbiotically in cycad roots had much higher heterocyst frequency and nitrogenase activity than the free-living form but no detectable uptake hydrogenase activity.

It is suggested that the rate of supply of carbohydrate to the heterocyst controls the development of uptake hydrogenase activity and that the absence of this activity in the symbiotic cyanobacteria indicates that the organisms in the cycad roots have an ample supply of carbohydrate.

Key words: *Anabaena cycadeae*, coralloid roots, hydrogenase, nitrogenase, nitrogen fixation.

INTRODUCTION

Photoautotrophic aerobic nitrogen-fixing filamentous cyanobacteria produce nitrogenase and uptake hydrogenase predominantly within heterocysts (Peterson & Wolk, 1978; Eisbrenner *et al.*, 1978; Lambert & Smith, 1981; Houchins, 1984). Such cyanobacteria when grown in NH_4^+-medium show complete repression ot heterocyst formation and nitrogenase and uptake hydrogenase activities (Houchins, 1984), thereby suggesting some kind ol physiological interaction between the two enzymes within the hererocyst. Nitrogenase while fixing N_2 is also known to carry out a metabolically wasteful reaction in the form of H_2 production. The presence of the uptake hydrogenase system in N_2-fixing cells may aid N_2-fixation by recycling nitrogenase-generated H_2 thereby forming ATP and reductant, by protecting nitrogenase from O_2 toxicity through oxyhydrogen reaction and by removal of H_2 from the site of nitrogenase activity since it is a competitive inhibitor of N_2-fixation (Dixon, 1972; Bothe, Distler & Eisbrenner, 1978). Such beneficial effects of uptake hydrogenase have been reported only for the *Rhizobium-legume* association (Eisbrenner & Evans, 1983), There is a recent report that the nickel-mediated increase in uptake hydrogenase activity of the cyanobacterium *Anabaena cylindrica*

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is accompanied by no significant increase in cyanobacterial **nitrogenase** activity, nitrogen content or growth (Daday, Mackerras & Smith, 1985).

Heterocysts are differentiated cells physiologically unable **to** photoassimilate CO., and dependent upon vegetative cells for import of photosynthetically produced carbohydrate which provides the reductant and other necessary requirements **for** effective fixation of nitrogen (Wolk, 1968; Winkenbach & Wolk, 1973). Such compartmentation may imply that, under photoautotrophic N_2-fixing conditions, heterocysts are carbon-limited. In heterotrophic bacteria like *Rhizobium* **(Maier,** Hanus & Evans, 1979) and *Azotobacter* (Patridge *et al.,* 1980), organic carbon substrates strongly repress uptake hydrogenase activity and growth under carbon limitation results in derepression of the enzyme. Whether a similar type of control operates in heterocystous cyanobacteria can **be** analyzed in an organism capable **of** chemoheterotrophic nitrogen fixation in either the free-living or symbiotic state. We have used *A. cycadeae,* a symbiont from coralloid roots of *Cycas* capable of chemoheterotrophic **diazotrophic growth** with glucose or photo**diazotrophic** growth with or without glucose, **to** examine the effect of glucose on the uptake hydrogenase activity of heterocysts. If a carbon control operates, heterocystous cyanobacteria **in** a free-living or symbiotic state under heterotrophic conditions should not show uptake hydrogenase activity.

MATERIALS AND **METHODS**

Organisms

Anabaena cycadeae leinke, us described by Singh *et al.* (1983), was grown and maintained axenically by the method of Singh & Singh (1964). Cultures were grown in modified N_2-free Chu-10 medium (Gerloff, Fitzgerald & **Skoog,** 1950) **at** 28 °C at a photon flux ot 50 μE m $^{-2}$ 9 '. Coralloid roots from *Cycascircinalis* glowing under the soil surface and containing the cyanobacterium were collected and used as the source of the symbiotic system. The roots were washed repeatedly in distilled water; sections approximately 2 mm thickness from the tip regions were then **cut,** washed in distilled water and used (Lindblad, Hallbom & Bergaman, 1985).

Growth

Cultures were grown in **N_2-medium** in 250 ml conical Masks, each containing 100 ml medium under photoautotrophic growth conditions. Photoheterotrophic cultures were grown in N_2-medium containing 2% glucose (w/v) and incubated **in** light. **Chemoheterotrophic** conditions **for** cyanobacterial growth were obtained by wrapping flasks containing glucose-supplemented N_2-medium with aluminium foil. Growth rate was determined by following increase **of** protein content. Heterocyst frequency, nitrogenase activity and uptake hydrogenase activity were also measured.

Measurement of heterocyst frequency

Sections of coralloid roots of *Cycas,* were examined after crushing by light microscopy. Such sections invariably contained filaments with intact heterocysts. Heterocyst frequency was calculated as number of heterocysts per 100 vegetative cells.

activity measurement

Nitrogenase activity was measured using whole filaments by estimating the acetylene reduction activity (Stewart, Fitzgerald & Burris, 1967).

Uptakehydrogenase activity

Uptake hydrogenase activity was measured by the method of Tel-or, Luijk & Packer (1977). Cyanobacterial cells were harvested, washed and resuspended in 2 **ml** fresh N_2-medium in 15·0 ml capacity tubes sealed with rubber stoppers. H_2 was injected (2% v/v) with a gas-tight syringe. The tubes were incubated in an incubator under a photon flux of about 40 μE m $^{-2}$ s $^{-1}$ and temperature of 27+3 °C. 0·5 ml of the gas samples were withdrawn from the tubes and injected into a Perkin-Elmer **Sigma** 3B Dual FID chromatograph **provided** with **a molecular** sieve 5A column and **TCD** with argon as the carrier gas. Tubes containing buffer and H_2 served as a control. An increase in peak height after incubation, was considered to show H_2 evolution and a decrease, H_2-consumption.

Cellular protein was estimated by the method of Lowry *et al.* (1951) as modified by Bailey (1967) and described by Ramos, Guerrero & Losada (1984) using bovine serum albumin as a standard. Chlorophyll was estimated as described by MacKinney (1941).

RESULTS

Table 1 compares growth, heterocyst **frequency** and nitrogenase and uptake **hydrogenase** activities of **organisms** grown photoautotrophically or in darkness with glucose as carbon source. The two types of culture appeared identical in pigmentation and there was little difference in heterocyst frequency. The rate of growth was much slower (x $\frac{1}{8}$) under chemoheterotrophic conditions. The nitrogenase activity, on a chlorophyll basis, was **lower** (by **50%)** in the dark **cultures.** The most striking difference, however, was that uptake hydrogenase activity was completely undetectable in the chemoheterotrophically grown cultures.

Table 2 shows the effect of addition of glucose to cultures grown phototrophically. After the addition of glucose, cultures were incubated either in light or in darkness. Heterocyst frequency was little altered in either condition but nitrogenase activity (chlorophyll basis) decreased, the decrease being almost twice as great during 72 h incubation in darkness as compared to incubation in light. But again the most striking effect was on uptake hydrogenase activity. This decreased markedly in the 24 h following glucose addition and after 48 h was undetectable; this effect **of** glucose addition was independent **of** light.

Table .3 gives information about symbiotic *A. cycadeae*. Comparison with Table 2 shows that the heterocyst frequency and nitrogenase activity (chlorophyll basis) were much higher in the symbiotic organisms than in the free-living ones; but no **uptake** hydrogenase activity was detectable in the symbiotic organisms, irrespective **of** whether or not they were preincubated in light or darkness for 24 h.

DISCUSSION

A. cycadeae grows photoautotrophically with molecular nitrogen as nitrogen source; mutants are known which can grow with N_2 in light only when glucose is added (Singh & Singh, 1964). This observation was initially difficult to

Table 1. *Generation time, heterocyst frequency, nitrogenase activity and uptake hydrogenase activity of* Anabaena cycadeae *grown photoautotrophically and chemoheterotrophically*

Characters	Photoautotrophic growth condition	Chemoheterotrophic growth condition
Generation time (h)	28	224
Heterocyst frequency (%)	5–6	4–5
Nitrogenase activity (nmol C_2H_4 formed μg Chl^{-1} h^{-1})	5·5	2·9
Uptake hydrogenase activity (μmol H_2 consumed mg Chl^{-1} h^{-1})	28·3	0

The photoautotrophic growth condition refers to growth in light in modified Chu-10 medium. Chemoheterotrophic growth condition refers to growth in the dark in modified Chu-10 medium containing 2 g glucose per 100 ml of N_2-medium. Heterocyst frequency, nitrogenase activity and uptake hydrogenase activities were estimated in cultures after three generations of growth under photoautotrophic and chemoheterotrophic conditions. The results are an average of three independent experiments.

Table 2. *The effect of adding glucose (2%) on heterocyst frequency, nitrogenase activity and uptake hydrogenase activity in photoautotrophically grown N_2-fixing cultures of* Anabaena cycadeae *when incubated in light or darkness for various time intervals*

Characters	Light incubation (h)				Dark incubation (h)			
	0	24	48	72	0	24	48	72
Heterocyst frequency (%)	5–6	5–6	5–6	5–6	5–6	5–6	4–5·5	4–5
Nitrogenase activity (nmol C_2H_4 formed μg Chl^{-1} h^{-1})	5·5	4·5	4·8	4·4	5·5	2·9	2·9	2·9
Uptake hydrogenase activity (μmol H_2 consumed mg Chl^{-1} h^{-1})	28·3	3·2	0	0	28·3	1·9	0	0

The values are an average of three independent experiments. Glucose was added at zero time.

Table 3. *Effect of incubation for 24 h in light or darkness on heterocyst frequency, nitrogenase activity and uptake hydrogenase activity of symbiotic* Anabaena cycadeae *in coralloid roots of* Cycas circinalis

Characters	Light	Dark
Heterocyst frequency (%)	24–27	24–27
Nitrogenase activity (nmol C_2H_4 formed μg Chl^{-1} h^{-1})	40·0	38·7
Uptake hydrogenase activity (μmol H_2 consumed mg Chl^{-1} h^{-1})	0	0

The values are an average of three independent experiments.

understand. But with the discovery that hcterocysts lack the ability to photoassimilate CO., to organic carbon and that nitrogen fixation in them depends on an import of carbohydrate from vegetative cells an interpretation became possible. The mutants may be blocked in the transport of carbohydrate from the **vegetative** cells to the heterocysts. This interpretation, in turn, suggests that the rate of transport of carbon to the heterocyst may be a limiting factor in nitrogen fixation and have other metabolic consequences.

This conclusion is supported by the findings reported here. With a free-living strain of *A. cycadeae* that can grow with N_2 either in light (+ CO_2) or in darkness with glucose, the presence of glucose allowed N_2-fixation to occur; nitrogenase was active in both cultures albeit that its activity, on a chlorophyll basis, in the **dark-grown** cultures was only about half of that in the light-grown ones. The rate of growth in darkness on glucose was much slower than in light, possibly because the rate of ATP formation was slower (Smith, 1982). But the most striking effect of the presence of glucose was on uptake hydrogenase activity. Such activity was readily detectable in cells grown in light in the absence of glucose but the activity fell by 80 to **90%** in 24 h **following** glucose addition and the fall occurred whether cultures were illuminated or not (Table 2). This finding is consistent with those from studies on *Rhizobium* (Maier *et at.,* 1979) and *Azotobacter,* (Patridge *et al.*, 1980) which show that uptake hydrogenase activity in these organisms is repressed in the presence ot glucose. Thus, it appears that the carbon limitation control of uptake hydrogenase which operates in *Rhizobium* and *Azotobacter* also operates in the hcterocysts ot cyanobacteria.

We can also conclude that, when in symbiotic association with cycad roots, *A, cycadeae* is not subject to carbon limitation. Table 3 shows that the symbiotic organism possesses many heterocysts and has high nitrogenase activity as Lindblad *et al.* (1985) have also shown with the cyanobiont in coralloid roots of *Zamia.*

In contrast, uptake hydrogenase activity is completely absent from the symbiotic *A, cycadeae.* Although other interpretations are possible, the simplest one is that the absence of uptake hydrogenase activity is the consequence of an ample supply of carbohydrate from the surrounding cycad root tissue.

We conclude that the rate of supply of available carbon to the heterocysts exerts an important control on the development of uptake hydrogenase activity.

Acknowledgements

Financial support from the Council of Scientific and Industrial Research and the University Grants Commission is gratefully acknowledged. We thank Professor Tauro and l)r Dadarwal of the Department of Microbiology, **HAU,** Hissar for providing facilities for gas chromatography.

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Symbiosis, 1 (198C)
Balaban Publishers, Philadelphia/Rehovot

Cycas circinalis-Anabaena cycadeae Symbiosis: Photosynthesis and the Enzymes of Nitrogen and Hydrogen Metabolism in symbiotic and cultured *Anabaena cycadeae*

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Received February 4, 1986; Accepted March 26, 1986

Abstract

A comparative study of photosynthesis, nitrogen and hydrogen metabolism was carried out on the cyanobiont of *Cycas circinalis* coralloid roots and the cultured isolate, *Anabaena cycadeae*. The cyanobiont showed lower rates of photosynthesis, had a higher heterocyst frequency and nitrogenase activity, and lacked uptake hydrogenase. Increase in light intensity caused stimulation of photosynthesis and nitrogenase activity of the cultured *Anabaena cycadeae* but the response in the cyanobiont was far less pronounced.

Activities of glutamine synthctase (both biosynthetic and transferase), aspartate dehydrogenase, alanine dehydrogenase and glutamate pyruvate transaminase, in the cyanobiont, were 30–60% lower than those in its free-living form *Anabaena cycadeae*. Glutamate oxaloacetate transaminase, on the other hand, was significantly higher in the cyanobiont. Glutamate dehydrogenase and nitrate reductase were undetectable.

The cyanobiont evolved hydrogen during nitrogen fixation, while the free-living isolate, *Anabaena cycadeae*, showed no hydrogen evolution, due to the presence of an uptake hydrogenase.

Keywords: *Anabaena cycadeae*, Cyanobacteria, *Cycas circinalis*, Hydrogen-metabolism, Nitrogen-metabolism, Photosynthesis, Symbiosis

Abbreviations: ADH, alanine dehydrogenase; AsDH, aspartate dehydrogenase; Chl, chlorophyll; DCMU,[3(3,4-dichlorophenyl)1,1-dimethylurea]; GDH, glutamate dehydrogenase; GOT, glutamate oxaloacetate transaminase; GPT, glutamate pyruvate transaminase; GS, glutamine synthctase; H_2ase, hydrogenase; N_2ase, nitrogenase; NR, nitrate reductase.

1. Introduction

N_2-fixing hetcrocystous cyanobacteria develop into association with algae, fungi, bryophytes, the water fern *Azolla*, gymnosperms and the angiosperm *Gunnera* (Stewart rt al., 1983). In symbiosis the **cyanobactcrium** becomes modified. Such modifications include cell size, ultrastructure, heterocyst frequency and enzymes of nitrogen metabolism (Stewart et al., 1980, 1983).

Cycads are the only gymnosperms involved in symbiosis with cyanobacteria. Most cycads examined have root **nodules** where a **hctcrocystous** cyanobacterium occurs as an endosymbiont in mucilage filled spaces of the cortex (Allen and Allen, **1965).** Using $^{15}N_2$ and C_2H_2, nitrogenase activity has been demonstrated in such root nodules (Bergersen **et** al., 1965; Bond, 1967; Grobbelaar et al., 1971; Renaut et al., 1975; Halliday and Pate, 1976; Lindblad et al., 1985). Nitrogen, fixed by the cyanobiont, has been shown to be rapidly transferred to the remainder of the plant (Bergersen et al., 1965; Renaut et al., 1975; Halliday and Pate, 1976).

Presently, there is no information regarding the levels of nitrogen metabolizing enzymes, except nitrogenase, in the cycad cyanobionts, although, in a number of other cyanobacterial associations it has been demonstrated that some of these enzymes are modified in the cyanobiont. **Similarly,** no work has been done on the hydrogen metabolism in this symbiosis. In this paper we have studied photosynthetic characteristics and activities of nitrogen and hydrogen metabolism enzymes in the cyanobiont of *Cyras circinalis;* for comparison, data have also been obtained on the free-living isolate, *Anabaena cycadeae.*

1. Materials and Methods

Organisms

The cyanobacterium from *Cycas circinalis* coralloid roots (root nodules) was isolated, purified and raised in pure culture as described earlier (Singh and Singh, 1964; Singh et al., 1983). This free-living strain is referred to as *Anabaena cycadeae.* Symbiotic cyanobacterium (referred to as cyanobiont) was freshly isolated from root nodules of *C. circinalis* grown in a nursery.

Isolation of the cyanobiont

Root nodules were washed in distilled water, cut into small pieces and then gently crushed, using a mortar and pestle, in Chu-10 medium (Gerloff et al., 1959) containing 1% PVP, to release the cyanobiont. Bulk of the host tissue was removed by passing the suspension through 2, 4, and 8 layers

of muslin cloth. The filtrate was then repeatedly centrifuged at 500xg to remove remaining host tissue debris.

Heterocyst frequency

Heterocyst frequency was calculated as percentage of total cells, by light microscope observations of the filaments of *A. cycadeae* and the cyanobiont.

N_2ase activity

N_2ase activity of intact coralloid roots, freshly isolated cyanobiont, and cultured *A. cycadeae* was measured using acetylene reduction assay. One ml cyanobacterial culture, or 1 g coralloid roots were placed in 7 ml serum vials. Coralloid roots were kept moist by placing a distilled water soaked filter paper in the vial. Acetylene was injected to a final concentration of 10% (v/v) of the air phase in the vial and the production of ethylene estimated, after incubating the vial for 30 min at 25°C and desired light intensity (sec text), as described before (Stewart et al., 1967) except that the column packing material was Porapak T.

Chl estimations

Cells were filtered on a Whatman GF/C filter paper and chlorophyll extracted in methanol at 4°C for 12 h in darkness. Chl content was calculated from absorption readings at 663 nm according to Mackinney (1041).

Protein estimations

The method of Lowry et al., (1951) was followed using bovine serum albumin as standard.

Oxygen exchange

O_2-evolution, by the cultured isolate and by the cyanobiont in intact root nodules, was measured polarographically at 25°C and desired light intensity (see text) using a Clark-type oxygen electrode as before (Rao et al., 1984).

Enzyme assays

NR was assayed in whole cells according to Manzano et al., (1976). Other enzymes were assayed in cell-free preparations. Cells were washed in 50 mM Tris-HCl buffer, pH 7.5, centrifuged and then ruptured by passage through a French Pressure Cell at 110 MPa. The extracts were then centrifuged at 30,000xg for 20 min and the supernatant liquids dialyzed overnight at 4°C

against the same buffer. Enzyme activities in such extracts were then assayed by coupling the reactions to NADH oxidation (NADPH in the case of GDH) followed at 340 nm, except in the case of GS transferase assays which were done by colourimetric measurements of γ-glutamylhydroxamate formation. GS (biosynthetic and transferase) activities were measured according to Sampaio et al., (1979), AsDH according to Haystead et al., (1973), and GPT according to Jäger and Weigel (1978). GDH (NADPH-dependent), ADH and GOT were measured according to Stewart and Rowell, (1977).

Hydrogen exchange measurements

This was done according to Tel-Or et al., (1977). Samples were placed in 15 ml capacity sample tubes sealed with rubber stoppers. H_2 gas was injected into these tubes to a final concentration of 2% (v/v) followed by incubation in a BOD incubator, at 3 klux light intensity and 27±1°C temperature. At 30 min time intervals, 0.5 ml gas samples were withdrawn and analysed on a Perkin-Elmer Sigma 3D Gas Chromatograph fitted with a MS 5A column and a thermal conductivity detector. Argon served as a carrier gas and tubes containing N2-medium and H_2 served as control. The rates of decrease and/or increase in the H_2 gas phase, relative to the control, were calculated and are expressed as H_2 uptake and/or H_2 evolution rates, respectively.

Analysis of photosynthetic pigments

The absorption spectrum of methanol-soluble and water-soluble photosynthetic pigments was obtained using a Gilford spectrophotometer scanning from 400 nm to 700 nm. Methanol-soluble pigments were extracted an above and water-soluble pigments were extracted by freezing-thawing the cyanobacterial cells suspended 0.05 M potassium phosphate buffer (pH 0.7). The *in situ* pigment composition of *A. cycadeae.* and the cynnobiont, in thin slices (0.1 mm thick) of root nodules, was analysed using a photoacoustic spectrometer (E.G. and G. Princeton Applied Research Corporation, USA; model 6001), scanning from 500 nm to 700 nm wavelengths, at room temperature, with 40 Hz modulation frequency as detailed by Balasubramanian and Rao, (1982).

3. Results and Discussion

Photosynthctic characteristics

We measured the absorption spectra of mcthanol-soluble and water-soluble pigments of the cyanobiont and the cultured isolate and found the phycoerytrin peak to be absent in the ryanobiont (data not shown). To ensure

that the absence of the phycoerythrin peak was not due to the limitation of the extraction procedure we also measured *in situ* composition of the photosynthetic pigments using photoacoustic spectroscopy. Again, phycoerythrin was found to be absent in the cyanobiont, otherwise, *A. cycadeae* and the cyanobiont were similar in respect of Chl a and phycocyanin spectral characteristics (Fig. 1). The significance of the absence of phycoerythrin, in the cyanobiont, is not clear at present, however, it may indicate the nitrogen-limiting status of the cyanobiont. Similar results have been found in the case of *Peltigera aphthosa* cyanobiont (Rai, 1980; Stewart et al., 1981).

The free-living *A. cycadeae* showed a progressive increase in the O_2-evolution rate with increase in light intensity from 0.5 klux to 15 klux (Fig. 2). No further increase was observed beyond 15 klux light intensity (data not shown). In contrast, the intact root nodules, containing cyanobiont, did not show any O_2-evolution upto 2 klux light intensity but showed a relatively low level of O_2-evolution thereafter. O_2-evolution in response to increasing light intensity was also far less pronounced as compared to that in the free-living *A. cycadcae.* DCMU was found to inhibit O_2-evolution in both cases, suggesting the occurrence and operation of PS II in the cyanobiont when provided with light. That is, the cyanobiont retains the photosynthetic capacity although it occurs in root nodules under the soil where the availability of light would be negligible. The fact that the rate of O_2-evolution in intact root nodules was low and showed a slow response to increase in light intensity may have two explanations. First, previous studies on cyanobacteria indicate that under low light conditions of growth there are more PS I reaction centres than PS II, and that in high light both PS I and PS II reaction centres are similar in number (Kawamura et al., 1979; Vierling and Alberte, 1980). Since the cyanobiont functions under heterotrophic growth conditions in root nodules, with negligible light availability, the number of PS II reaction centres may be limiting. Second, there may be a limitation of light energy reaching the cyanobiont within the root nodules because of the intervening cycad root tissues. However, when the cyanobiont was separated from the root nodules and 02-evolution measured under saturating light conditions (15 klux) in such freshly isolated cyanobiont cells, a rate of 45 μmol oxygen evolved mg^{-1} Chl a h^{-1} was found. This was less than half of the O_2 evolution rate in the free-living *A. cycadcac* under similar conditions (111 μmol O_2 evolved mg^{-1} chl *a* h^{-1}). This clearly indicated that even with full light availability the rate of photosynthetic evolution in the cyanobiont was much lower. Thus the low number of O_2-evolving PS II reaction centers may be the main reason for low rates of photosynthetic O_2 evolution in the cyanobiont, as discussed above.

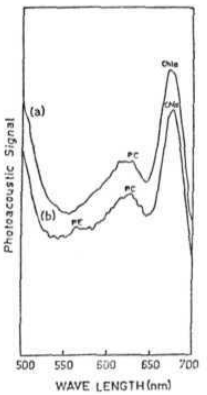

Figure 1. Photoacoustic spectrum of cyanobiont in intact root nodules (a) and the free-living *Anabaena cycadeae* (b). PE, phycoerythrin; PC, phycocyanin; Chl, chlorophyll a

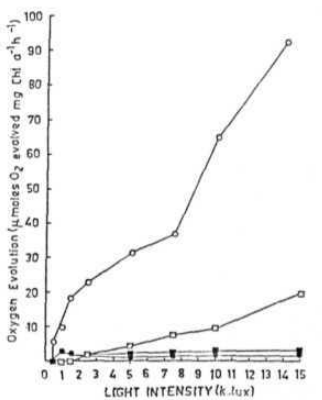

Figure 2. Effect of light intensity on photosynthetic O_2 evolution. ○, free-living *Anabaena cycadeae*; □, cyanobiont in intact root nodules; •, free-living *Anabaena cycadeae*, 5μM DCMU added; ▪, cyanobiont in intact root nodules, 5μM DCMU added.

Hydrogen metabolism

H_2 evolution was undetectable in the free-living N_2-fixing cultures of *A. cycadeae* but an H_2 evolution rate of 4μmol mg^{-1} Chl *a* h^{-1} was found in intact root nodules. In contrast, H_2 uptake was undetectable in the intact root nodules while free-living *A. cycadeae* showed an H_2 uptake rate of 28μmol rag"[1] Chl *a* h^{-1}. These findings clearly indicate that the development of symbiotic association between *A. cycadeae* and *C. circinalis* results in the loss of the cyanobacterial uptake H_2ase activity.

The reasons for the absence of uptake H_2ase activity in the cyanobiont are not yet clear. However, catabolizable organic carbon substrates are known repressors of uptake H_2ase activity in *Azotobacter* and *Rhizobium* (Partridge et al., 1980; Maier et al., 1979). Keeping in view the hctcrotrophic mode of cyanobiont's nutrition in the root nodules, it is tempting to suggest that the loss of uptake H_2ase activity in symbiosis may have been caused by the inhibitory effect of catabolizable organic carbon moving from the cycad to the cyanobiont as in the case of *Azotobacter* and *Rhizobium* mentioned above.

Nitrogen metabolism

The N_2ase activity of the cyanobiont, freshly removed from the cycad root nodules, was much higher than that of the free-living *A. cycadeae*. The cyanobiont showed a N_2ase activity of 40 nmol C_2H_2 reduced μg^{-1} Chl a h^{-1} as compared to 4 nmol C_2H_2 reduced μg^{-1} Chl *a* h^{-1} in the case of free-living *A. cycadeae*. This compared poorly with the optimum N_2ase activity of the cyanobiont in intact root nodules (see Fig. 3), prompting us to use intact root nodules in further experiments. High levels of N_2ase activity observed with the cyanobiont can be explained by the fact that the cyanobiont showed an average heterocyst frequency of 25%, as against a value of 5% detectable in the free-living *A. cycadeae*. Most free-living cyanobacteria show a heterocyst frequency of the 5-6% (Stewart, 1980). Our values for the heterocyst frequency of the cyanobiont are comparable to those reported by Grilli-Caiola (1980). However, it should be emphasized that our values are for a mixed population of the cyanobiont, isolated from whole root nodules. These values are likely to vary along the root as reported for *Zamia* (Lindblad et al., 1985). A similar argument applies to our N_2ase values.

Further studies with intact root nodules, in order to avoid disturbing the cyanobiont, showed that upto 24 h the N_2-fixation activity of the cyanobiont was rather similar both in dark and in upto 2.5 klux light intensity (40 nmol C_2H_2 reduced μg^{-1} Chl a h^{-1}). In contrast N_2ase activity of the free-

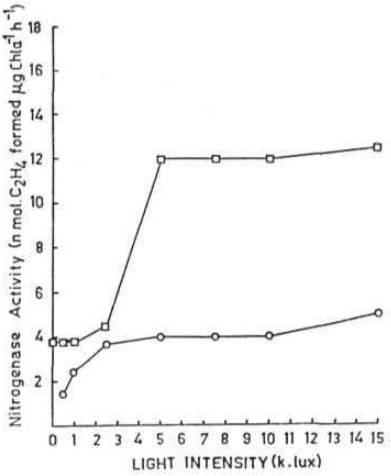

Figure 3. Effect of light intensity on nitrogenase activity in free-living *Anabaena cycadeae* and the cyanobiont in intact root nodules. ○, free-living *Anabaena cycadeae*; □, cyanobiont in intact root nodules ($\times 10^{-1}$). Please note that the N_2ase values of the cyanobiont are reduced by factor of 10 before plotting.

living *A. cycadeae* declined sharply and became undetectablc within 3 h of darkness (data not shown). It is evident that N_2ase activity in the cyanobiont was sustained through chemoheterotrophic metabolism, a mode of nutrition prevailing under natural conditions where the cyanobacterium occurs in root nodules below the soil surface in darkness.

When root nodules were subjected to light intensity above 2.5 klux, N_2ase activity increased rapidly reaching a maximum at 5 klux and remaining constant thereafter (Fig. 3). The lack of a significant effect of light intensity below 2.5 klux on the N_2ase activity of the cyanobiont may be due to the cyanobiont's inability to receive enough light because of the intervening cycad root tissues. Free-living *A. cycadeae*, in contrast, showed a light intensity optimum of 2.5 klux for maximum N_2ase activity.

The fact that O_2-evolution and N_2ase activity do not seem to show an identical response to increase in light intensity (see Fig. 2 and 3), may suggest that N_2ase is more directly dependent on the non-O_2-evolving PS I, rather

Table 1. Activities of various nitrogen-metabolising enzymes in *Cycas circinalis* cyanobiont and in free-living *Anabaena cycadeae*. (values are mean ±SEM; n=5).

Enzymes	Enzyme Activity (nmol product formed min^{-1} mg^{-1} protein) Cyanobiont	*Anabaena cycadeae*
GS transferase	966±20	1474±30
GS biosynthetic	43±2	60±2
ADH (aminating)	10±1	18±1
GDH (NADPH-dependent)	ND	30±2
AsDH (aminating)	7±1	19±1
GPT	27±2	54±3
GOT	22±1	12±1
NR (Fd-dependent)	ND	ND (0.2)[a]

[a] Represents activity in cells grown on medium supplemented with 20 mM KNO_3.
ND: not detectable.

than the O_2-evolving PS II. PS I has been shown to be able to supply with ATP and reductant from a pool of organic carbon intermediates (Tel-Or and Stewart, 1976).

Other nitrogen-metabolising enzymes, involved in primary ammonia assimilation and transamination reactions, also showed differences between the free-living *A. cycadeae* and the cyanobiont in the root nodules (Table 1). NR was found to be absent in the cyanobiont as well as in the free-living *A. cycadeae*. However, NR activity was detectable when the free-living *A. cycadeae* was grown on nitrate-supplemented medium, supporting the earlier contention that NR of *A. cycadeae* is nitrate-inducible (Bagchi et al., 1985). NADPH-dependent GDH activity was present in the free-living *A. cycadeae* but undetectable in the cyanobiont. There was a reduction in the activities of AsDH, ADH, GPT and GS (both biosynthetic and transferase), and an increase in the activity of GOT, in the cyanobiont as compared to those in the free-living *A. cycadeae*. It is interesting to note here that while in lichens and *Azolla* the GS levels are reduced by over 90% and 70%, respectively, here in the case of the *C. circinalis* cyanobiont the reduction level of GS seems much smaller (Table 1). This points to a possibility that the cycad cyanobiont, unlike those in lichens and *Azolla* (Rai et al., 1981, 1983; Peters et al., 1980),

transfers fixed-N2 to its eukaryotic partner not entirely as ammonia but does so, at least partially, in the form of organic-N. This should merit further research studies.

Acknowledgements

Financial support from INSA and CSIR is thankfully acknowledged.

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Nitrate reductase activity in isolated heterocysts of the cyanobacterium *Nostoc muscorum*

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Received 19 September 1984

The **nitrate reductase,** nitrate reductase **apoprolein** and nitrate reductase Mo-cofactor activities were measured in **the cell-free extracts** of isolated heterocysts and whole filaments of the **cyanobacterium** *Nostoc muscorum*. **The nitrate** reductase activity of N_2- and NO_3-grown whole **filaments** was 3.25 and 7.14 **nmol** NO_2 formed $min^{-1} \cdot mg$ protein $^{-1}$, respectively. No nitrate reducatase activity was found **in** the extracts of **isolated** heterocysts. However, when the heterocyst extract was supplemented with nitrate reductase apoprolein from the whole **filaments, a reconstituted nitrate reductase activity** of 6.4 nmol NO_2 formed $min^{-1} \cdot mg$ protein $^{-1}$ was detectable. Therefore, it was concluded that nitrate reductase in *Nostoc* ***muscorum*** was localized in the **vegetative** cells, that the heterocysts lacked niirate reductase activity due to the lack of apoprotein, and that the Mo-cofactor was present in helerocysts.

Cyanobacterium *Heterocvst* *Nitrate reduclase* *Nitrate* ***reductase apoprolein Nitrate reductase Mo-cofactor***
Nostoc muscorum

1. INTRODUCTION

Heterocysts of cyanobacteria are the sites of **aerobic nitrogen-fixation** and show various structural, **physiological,** biochemical and genetic **modifications** essential for **their function** [1,2]. **In** heterocystous **cyanobacteria** nitrogenase is localized in heterocysts and CO_2-fixation in vegetative cells [1,2]. Various enzymes involved in the reductive **pentose phosphate** cycle have been found to be absent **in** heterocysts [3]. **The** primary assimilation of **ammonia,** generated during N_2-fixation, occurs in heterocysls and **fixed** nitrogen is transferred to vegetative cells in the form **of** glutamine (4]. The CO_2-fixation occurs in vegetative cells and fixed carbon **is** transferred to heterocysts where it is **utilized** for generating **reductant** and energy for **nitrogen-fixation** [1]. Thus, there is a close interrelation between heterocysls and vegetative cells with regard to their carbon and nitrogen metabolism.

The specific activities of many of the nitrogen metabolizing enzymes of heterocysts and vegetative cells have been measured for comparison [2]. The glutamine **synthetase** [4,5], alanine dehydrogenase [5,6], glutamate dehydrogenase [5], glutamate oxaloacetate transaminase [5,6] and glutamate pyruvate transaminase [5] activities have been found to be present both in the heterocysts and in **the** vegetative cells. However, the nitrogenase was found lo be localized in the heterocysts [7–12] and **glutamate** synthase in vegetative cells only [4,13]; see however [14,15]. Activities of enzymes responsible for both the synthesis and breakdown of cyanophycin, arginine-poly(aspartic acid) synthetase and cyanophycinase, respectively, have been reported to be much higher in the heterocysts than in the vegetative cells [16].

At present no information is available with regard to the enzymes of nitrate metabolism in the heterocysts of cyanobacteria. We have studied the nitrate reductase activity in isolated heterocysts of *Nostoc muscorum* and show here that the nitrate reductase activity is absent in heterocysts due to the lack of nitrate reductase apoprotein.

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2. MATERIALS AND METHODS

NostOC muscorum was grown axenically in **nitrogen-free** and NO_3^--supplemented Chu-10 medium [17] as in [18]. *Escherichia coli* strain W was grown aerobically in Luria broth [19] supplemented with 1 mmol-dm^{-3} sodium molybdate.

Heterocysts were isolated from the exponentially growing filaments of *N. muscorum* as in [20]. Such heterocyst preparations had a glutamine synthetase biosynthetic activity of 174 nmol product formed • min^{-1} • mg protein^{-1} and a glutamine synthetase transferase activity of 3840 nmol product formed • min^{-1} • mg protein^{-1} (both assayed as in [21]). Vegetative cells were less than 5% of the total.

The protein estimations were made as in [22]. In vitro nitrate reductase activity was measured as in [23].

Mo-cofactor preparation from *E. coli* was essentially the same as in [24]. To prepare Mo-cofactor from heterocysts, heterocysts were isolated from cultures grown in Mo-containing medium, washed and resuspended in Tris buffer (50 mmol-dm^{-3}, pH 7.5) containing 100 mmol-dm^{-3} NaCl, 300 mmol dm^{-3} sucrose, 1 mmol-dm^{-3} EDTA and 5 mmol • dm^{-3} $MgCl_2$. The cell-free extract was then prepared by passage through a French Pressure Cell at 110 MPa followed by centrifugation at 30 000 x *g* for 20 min at 4°C. The supernatant was used as a source of Mo-cofactor for complementation analysis.

For preparation of Mo-cofactor-free nitrate reductase (nitrate reductase apoprotein), 200 mm cell-free extract, prepared as above, incubated with 200 mm^{-1} molybdate-GSH solution (10 mmol-dm^{-3} GSH and 5 mmol-dm^{-3} sodium molybdate in 100 mmol dm^{-3} sodium acetate buffer, pH 4.5). This brought the pH of the reaction mixture to 4.8. After 30 s incubation at 30°C (acid incubation) the pH was adjusted to 7.2 by adding 200 mmol • dm^{-3} K_2HPO_4 and incubated for 15 min at 30°C (neutral incubation).

Reconstituted nitrate reductase activity was measured by mixing equal volumes of the nitrate reductase apoprotein (cofactor-free nitrate reductase) and the Mo-cofactor. This mixture was incubated for 10 min at 30°C to achieve complementation and then nitrate reductase activity was assayed as mentioned above.

3. RESULTS AND DISCUSSION

The aim of this study was to establish whether or not the heterocysts contain nitrate reductase activity. For this we isolated heterocysts from *N. muscorum* filaments and measured the nitrate reductase activity in the cell-free extracts. For comparison, data were also obtained on nitrate reductase activity in the cell-free extracts of *N. muscorum* filaments grown in nitrogen-free (filaments containing both heterocysts and vegetative cells) and in nitrate media (filaments containing vegetative cells only).

The nitrate reductase activity was undetectable in the extracts of heterocysts while a nitrate reductase activity of 3.25 and 7.14 nmol NO_2^- formed • min^{-1} mg protein^{-1} was observed in the extracts of filaments grown in nitrogen-free and nitrate media, respectively (table 1). The higher level of nitrate reductase activity in nitrate-grown filaments of *N. muscorum* was consistent with earlier reports [25,26]. However, the absence of nitrate reductase in heterocysts, but its presence in whole filaments containing vegetative cells and heterocysts, was noteworthy. It suggested that nitrate reductase activity was localized in the vegetative cells only.

We further investigated whether the lack of nitrate reductase activity in heterocysts was due to the absence of nitrate reductase Mo-cofactor, nitrate reductase apoprotein or both. First, nitrate reductase apoprotein (Mo-cofactor-free nitrate reductase), prepared from isolated heterocysts, was supplemented with increasing amounts of Mo-cofactor prepared from *E. coli*, but nitrate reductase activity could not be restored. This indicated the absence of nitrate reductase apoprotein in the heterocysts. It could be argued that the Mo-cofactor from *E. coli* may not be compatible with the nitrate reductase apoprotein from *N. muscorum*. However, this is not so. The nitrate reductase Mo-cofactor from *E. coli* reconstituted nitrate reductase activity when added to the apoprotein preparations from *N. muscorum* filaments, grown in nitrogen-free or nitrate medium, suggesting that the Mo-cofaclor of *E. coli* was compatible with the apoprotein of nitrate reductase from *N. muscorum*.

Secondly, the nitrate reductase apoprotein preparations from whole filaments of *N. muscorum*, grown in nitrogen-free medium, were sup-

Table 1

Nitrate reductase **activity** in **cell-free** extracts **of** isolated heterocysts and whole **filaments** of *Nostoc muscorum* grown in **nitrogen-free** and **nitrate** medium

Enzyme source	Activity (nmol NO_2^- formed · min^{-1} · mg protein^{-1})
N_2-grown *N.* ***muscorum*** extract	3.25
Nitrate-grown *S.* ***muscorum*** extract	7.14
Isolaied heterocyst extract	not detectable
Apoprotein from N_2-grown *N.* ***muscorum***	not detectable
Apoprotein from **nitrate-grown** *N. muscorum*	not detectable
Isolated heterocyst extract **(Mo-cofactor)** supplemented **with apoprotein** preparation from N_2-grown *N.* ***muscorum***	6.25[a]
Isolated heterocyst **extract (Mo-cofactor)** supplemented with **apoprotein preparation** from **nitrate-grown** *N.* ***muscorum***	

[a]**Specific** activity expressed per mg protein of the **heterocysl** extract. The amount of **apoprotein** preparations used were similar in both eases (protein **concentration 2** mg · cm^{-3})

plemented **with Mo-cofactor** prepared from isolated heterocysts. Under such conditions a reconstituted nitrate reduetase activity of 6.25 nmol NO_3^- formed • min^{-1} · mg $protein^{-1}$ was delected. A similar level of reconstituted nitrate **reduetase** activity was detected when Mo-cofactor preparations from heterocysts were complemented with nitrate **reduetase** apoprotein from whole filaments of *N. muscorum* grown in nitrate medium (table 1). The fact that the filaments grown in nitrogen-free medium had a lower nitrate reduetase activity than those grown in nitrate medium while their apoprotein preparations showed similar levels of reconstituted nitrate reduetase activity **when complemented** with similar amounts of Mo-cofactor from isolated heterocysts, suggested that the Mo-cofactor may have been limiting in the vegetative cells of *N,* ***muscorum*** grown in nitrogen-free medium (nitrogen-fixing condition) and that heterocysts had a higher level of Mo-cofactor than vegetative cells. The higher nitrate reduetase activity in filaments of *N. muscorum* grown in nitrate medium therefore may have been due to the increased level of Mo-cofactor resulting from the lack of competition, between nitrate **reduetase** and nitrogenase, for the Mo-cofactor.

It should be mentioned that these **apoprotein** preparations did not show any nitrate reducla.se activity unless supplemented with Mo-cofactor preparations (from *E. coll,* isolated **heterocysts** or whole filaments). Furthermore, addition of Mo or ferredoxin, alone or together, did not restore nitrate reductase activity **in** the apoprotein preparations. This ruled out the possibility that reconstituted nitrate reduetase activity in apoprotein preparations supplemented with Mo-cofactor from heterocysts may have been due to Mo or ferredoxin rather than to the Mo-cofactor.

It is clear from the above observations that the nitrate reduetase Mo-cofactor is present in heterocysts and that the absence of nitrate reduetase in heterocysts is due to the absence of nitrate reduetase apoprotein. The presence, in heterocysts, of Mo-cofactor which can complement the apoprotein from vegetative cells and show reconstituted nitrate reduetase activity is consistent with the view expressed earlier [25,27] that the Mo-cofactor of nitrate reduetase **in** *N. muscorum* may be a precursor for the Fe-Mo-cofactor of nitrogenase.

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