PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF NITROGEN METABOLISM IN *ANTHOCEROS — NOSTOC* SYMBIOSIS

By

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(Mayashree Borthakur)

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ABBREVIATIONS

ADP	= Adenosine 5'-diphosphate
ATP	= Adenosine 5'-triphosphate
BOD	= Biological oxygen demand
BSA	= Bovine serum albumin
C	= Carbon
°C	= Degree centrigrade
C_2H_2	= Acetylene
C_2H_4	= Ethylene
Chl	= Chlorophyll
cm	= Centimeter
cm^{-2}	= / Square centimeter
EDTA	= Ethylene diamine tetra acetic acid
E.M.	= Electron microscopy
Fd	= Ferredoxin
Fv	= Flavodoxin
g	= Gram
GOGAT	= Glutamate synthase
GS	= Glutamine synthetase
HEPES	= 4-(2-Hydroxyethyl)-1-piperazine ethane sulphonic acid
HRP	= Horse radish peroxidase
IgG	= Immuno globulin G
l	= Litre
m	= Mole
M	= Molar

mA	= milli ampere
MES	= 2-(N-morpholino) sulphonic acid
mg	= Milligram
Min	= Minute (s)
ml	= Milliliter
mM	= Milli molar
µmol	= Micro mole
µM	= Micro molar
μm^2	= micro meter square
MSX	= L-Methyonine-DL-sulphoximide
N	= Nitrogen
NAD	= Nicotinamide adenine dinucleotide
NADH	= Nicotinamide adenine dinucleotide reduced
NH_4^+	= Ammonium
Nif	= Nitrogen fixing
NO_3^-	= Nitrate
nm	= nano meter
NR	= Nitrate reductase
OD	= Optical density
PAGE	= Poly acrylamide gel electrophoresis
PBS	= Phosphate buffered saline
PE	= Phycoerythrin
pH	= Hydrogen ion concentration
PS	= Photosystem
psi	= Pounds per square inch
RuBisCo	= Ribulose 1,5-bisphosphate carboxylase/oxygenase
s	= Second (s)

SDS	= Sodium dodecyl sulphate
SE	= Standard error
TBS	= Tris buffered saline
TEM	= Transmission electron microscopy
W	= Watt
Wt.	= Weight

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CHAPTER 1

General Introduction

Nitrogen is one of the most abundant elemental constituents of all living matter. It is an integral component of enzymes, proteins, nucleic acids, chlorophylls and other related compounds which are essential for maintaining structural and functional integrity of living systems.

Molecular nitrogen, which constitutes about 78% of the earth's total atmosphere, can not be directly used as nitrogen source by vast majority of organisms. Instead, they require combined nitrogen. Therefore, in modern day agriculture, chemical fertilizers are being used increasingly as the source of combined nitrogen. However, certain prokaryotic organisms possess the ability to reduce atmospheric nitrogen to ammonia under normal physiological conditions and therefore, are self sufficient. There is a global attempt to understand the physiological, biochemical and the genetic aspects of these primitive organisms so as to help find an alternative to chemical fertilizers. Among such organisms, cyanobacteria which are diazotrophic in nature and constitute one of the largest sub-group of gram negative prokaryctes, are of special interest as they have simple growth requirements combining oxygenic photosynthesis and nitrogen fixation (Singh, 1961; Sprent, 1979; Stewart, 1980; Carr & Whitton, 1982; Gallon & Chaplin, 1987; Fay & Van Baalen, 1987; Rai, 1990).

1.1 Cyanobacteria:

Cyanobacteria are prokaryotes possessing chlorophyll *a* and phycobiliproteins. They are photoautotrophs performing oxygenic photosynthesis, using water as eletron donor, in light. Some are also capable of performing photosynthesis using H_2S as electron donor, many fix atmospheric N_2, and some are facultative photo- or chemoautotrphs. Among cyanobacteria, a wide range of morphological diversity exists. They include unicellular, multicellular filamentous, branched filamentous and non-filamentous colonial strains. The filamentous forms consist of a maximum of three types of cells: vegetative cells, heterocysts and akinets (*see* Carr & Whitton, 1982; Fay & Van Baalen, 1987).

Vegetative cells are the site of photosynthesis and CO_2-fixation. CO_2 fixation occurs through calvin cycle (Stanier, 1977; Stewart, 1977; Allen, 1984). Oxidative pentose phosphate pathway is the main catabolic route. Oxidative phosphorylation and cyclic and non-cyclic phosphorylation generate ATP. Vegetative cells contain a number of storage bodies such as cyanophycean starch (glycogen) (Simon, 1971), carboxysomes (polyhedral bodies) which contain ribulose 1,5-bisphosphate carboxylase/oxygenase (Stewart & Codd, 1975; Codd & Marsden, 1984), and polyphosphate bodies (Grillo & Gibson, 1979).

Morphologically, heterocysts are distinctive cells with thick cell envelopes. They are enclosed by a unique glycolipid layer surrounded by polysaccharides which together impede the entrance of oxygen (Wolk, 1982; Murry & Wolk, 1989). They

represent about 3-7% of the total cells present in N_2-grown cultures. This proportion increases to almost double in nitrogen starved cultures (Kulasooria *et al.*, 1972). In presence of combined nitrogen sources, the frequency of heterocysts in the filaments of filamentous cyanobacteria decreases (Fogg, 1949). Heterocysts are larger than the vegetative cells and show a number of structural, biochemical and genetic changes during their development from vegetative cells (Wolk, 1982; Golden *et al.*, 1985; Haselkorn *et al.*, 1987). Heterocysts are the site of dinitrogen fixation (Fay *et al.*, 1968; Stewart, 1980; Janaki & Wolk, 1982; Bergman *et al.*, 1986). The key enzyme of nitrogen fixation, nitrogenase, is present and functional in the vegetative cells of non-heterocystous cyanobacteria (Nagatani & Haselkorn, 1978; Gallon & Chaplin, 1987). Heterocysts lack photosystem II (PS II) activity, thereby avoiding evolution of O_2 from water (Reinmann & Thornber, 1979; Alberte *et al.*, 1980) and have a high respiratory O_2 consumption rate (Haury & Wolk, 1978; Walsby, 1982; Jenson & Cox, 1983; Sprent *et al.*, 1987). These two processes together contribute to the protection of nitrogenase from O_2 damage. Heterocysts lack glutamate synthase (GOGAT) (Thomas *et al.*, 1977; Rai *et al.*, 1982) and nitrate reductase (Kumar *et al.*, 1985; Rai & Bergman, 1986; Rai *et al.*, 1992) and hence avoid competition for molybdenum and reductant (reduced Fd) with nitrogenase. They also lack ribulose 1,5-bisphosphate carboxylase enzyme and therefore do not fix CO_2 (Codd & Stewart, 1977; Codd *et al.*, 1980; Cossar *et al.*, 1985). The demand for fixed carbon is met from the neighbouring vegetative cells (Wolk,

1968; Stewart, 1980; Bothe *et al.*, 1984; Stewart *et al.*, 1985; Bergman *et al.*, 1986). They possess very little or no phycobiliproteins (Stewart & Rodgers, 1977; Stewart *et al* 1983; Lindblad, 1987). Ammonia produced by the action of nitrogenase is assimilated by the action of glutamine synthetase (GS) which is present in the heterocysts at levels two-fold higher than that in vegetative cells (Stewart *et al.*, 1975; Dharmawardene *et al.*, 1973; Thomas *et al.*, 1977). Such high level of GS are probably required in heterocysts for the assimilation of N_2-derived ammonia (Renstrom-Kellner *et al.*, 1990). Glutamine thus produced in the heterocysts is transported to adjacent vegetative cells where it is converted to glutamate by glutamate synthase (GOGAT) (Stewart *et al.*, 1975; Stewart, 1977). Part of the glutamate produced in the vegetative cells through the activity of GOGAT is transported back to the heterocysts to provide substrate for glutamine synthesis by GS (Thomas *et al.*, 1977).

Akinets are the single cells serving as perenating bodies. They are usually larger, their cell wall thicker and protoplasm more granular than the vegetative cells. They are associated with reproduction and survival under adverse conditions (Fogg *et al.*, 1973; Nichols & Adams, 1982).

1.2 Cyanobacterial symbiosis:

In general, symbiosis means the permanent living together of two or more dissimilar organisms involving exchange of metabolites between the symbionts. In most circumtances, this

interaction is mutually beneficial for both (all) partners. N_2-fixing cyanobacteria form symbiotic associations with a variety of plants as well as animals. Among plants representative hosts are found among algae (diatoms), fungi (about 8% of all lichens), bryophytes (liverworts, hornworts and mosses), pteridophytes (*Azolla*), gymnosperms (Cycads), and angiosperms (*Gunnera*). Among animals marine sponges and echiruoid worms as well as non-photosynthetic protists belonging to group glaucophyta and bacteria form symbiosis with cyanobacteria (Rai, 1990a; Bergman *et al.*, 1992).

Table: 1.1 N_2-fixing symbioses involving cyanobacteria:

Host plant	Cyanobacteria	Comments
Angiosperms:		
Gunnera	*Nostoc* sp.	Cyanobacterium located inside host cell in stem nodules.
Gymnosperms:		
Cycads(e.g. *Cycas angulata*, *Macrozamia communis*)	*Nostoc* sp.	Nodules found on coralloid roots. Occurs naturally only in southern hemisphere.
Pteridophytes:		
Azolla	*Anabaena azollae*	Occupies mucilage-filled cavities on the ventral surface of the dorsal lobes of the leaves. Widely used in as green manure/ biofertilizer in rice cultivation.
Bryophytes:		
Anthoceros *Notothylas* *Blasia* *Cavicularia*	*Nostoc* sp.	Cyanobacteria occupy mucilage-filled cavities on undersurface of liverwort/hornwort gametophyte thallus.
Sphagnum	*Nostoc* sp.	Cyanobacteria occupy the hyaline cells of the moss.

Host	Cyanobacteria	Comment
Fungi:		
Cyanolichens:	*Nostoc* sp. *Scytonema* sp. *Fischerella* sp. *Calothrix* sp.	Nitrogen-fixing lichens consist of two-membered associations between fungi and cyanobacteria or three-membered associations which contain a green alga as well. About 8% of the 18,000 species of lichens contain cyanobacteria.
Algae:		
Rhizosolenia *Haniaulus membranaceus*	*Calothrix*	Marine diatoms.
Rhopalodia	Coccoid cyanobacteria	Thin-walled unicellular cyanobacteria.
Host animals:		
Echiuroid worms		
Marine sponges:	*Aphanocapsa phormidium*	Occurs inter or intracellularly throughout the sponge tissue or in superficial tissue.
Hair of polar bear	*Aphanocapsa* *Gloeocapsa*	
Prokaryotes:		
Bacteria:	*Pleurocapsa minor.*	
Nonphotosynthetic protists:		
Glaucophytes *Paulinella*	*Cyanelles*	

1.2.1 Association with Angiosperms:

Gunnera is the only angiosperm genus capable of entering into a symbiotic association with *Nostoc*. The symbiosis between the two partners is facultative; the two partners can be

isolated, cultivated and reconstituted easily (Bergman *et al.*, 1992; Johansson *et al.*, 1992; Bonnett, 1990). The cyanobiont provides fixed nitrogen to the host plant which fully meets the latter's requirements for combined nitrogen (Osborne, 1989; Bonnett, 1990). The cyanobacteria infect specialized mucilage-filled glands at the base of the leaves on the stem of the host. This symbioses is unique in the sense that here, in contrast to other symbiosis, the cyanobiont enters the *Gunnera* cells and resides intracellularly (Johansson *et al.*, 1992; Stewart *et al.*, 1983; Rai, 1990a). The cyanobiont undergoes pronounced morphological, physiological and biochemical alterations. A gradient in the heterocyst frequency occurs, being about 20% in the younger colonies and higher in the older parts (Silvester, 1976). However, the nitrogenase activity is highest in the younger colonies near the plant apex, where the heterocyst frequency has not yet reached the maximum (Soderback *et al.*, 1990). Photosystem II (PS II) and the accessory pigments of PS II are absent and *in vivo* CO_2-fixation is negligible in the cyanobiont and the fixed carbon requirements of the latter is met by the host (Silvester, 1976). The primary ammonia assimilating enzyme, glutamine synthetase (GS), is found to be evenly distributed in the vegetative cells and heterocysts of the cyanobiont. The GS activity is more in the apex where the nitrogenase activity is also high (Soderback, 1992).

1.2.2 Association with Gymnosperms:

Within gymnosperms, all members of the order cycadales,

develop symbiotic associations with cyanobacteria. The cyanobiont (*Nostoc sp.*) occupies the middle cortical zone of the coralloid roots (Lindblad & Bergman, 1990). The vegetative cells possess chlorophyll *a* and phycobiliproteins and have a fully developed photosynthetic apparatus (Lindblad *et al.*, 1985). The vegetative cells also contain carboxysomes with the CO_2 fixing enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCo) (Lindblad *et al.*, 1985). Thus, the cyanobiont is similar to the free-living *Nostoc*. However, the heterocyst frequency increases gradually from being low in the younger parts to being higher in the older parts of the coralloid roots (Lindblad & Bergman, 1990). Nitrogenase activity has been found to be about seven times higher in the cyanobiont than in the free-living isolate (Kumar *et al.*, 1986). The primary ammonia assimilating enzyme (GS) activity and cellular and subcellular level of GS is found to be comparable to those of free-living N_2-fixing cyanobacteria (Lindblad & Bergman, 1986). However, the activity of GS declines towards the basal parts of the roots. The CO_2-fixation is negligible in the cyanobiont and fixed carbon may be provided by the host plant (Lindblad & Bergman, 1990). Using $^{15}N_2$, it has been clearly demonstrated that the fixed nitrogen is rapidly transferred from the cyanobiont to the host *Cycas* and *Macrozamia* (Renaut *et al.*, 1975; Bergersen *et al.*, 1965). Analysis of freshly extracted xylem sap from coralloid roots in a number of cycads showed that glutamine and a smaller amount of glutamic acid are the principal translocated N-solutes from coralloid roots to the rest of the cycad (Pate *et al.*, 1988).

1.2.3 Association with Pteridophytes:

Among pteridophytes, water fern *Azolla* develops symbiosis with the nitrogen-fixing cyanobacterium, *Anabaena*. The cyanobiont resides in the cavities on the ventral surface of the dorsal lobe of each leaf (frond). Within the cavities simple and branched hair-cells developed, having the structural characteristics of transfer cells (Peters *et al.*, 1982; Calvert *et al.*, 1985). The branched hair-cells have been suggested to be involved in transfer of fixed-nitrogen to *Azolla* from *Anabaena*, while single hair-cells may participate in the transfer of fixed carbon in the opposite direction (Calvert *et al.*, 1985). Sucrose is the major product of photosynthesis in *Azolla* and seem to be transported to the *Anabaena*. Several ultrastructural changes occur in the process of heterocyst differentiation (Neumueller & Bergman, 1981). Heterocyst frequency increases with leaf age, reaching upto 30% in the mature cavities (Hill, 1975; 1977; Braun-Howland & Nierzwicki-Bauer, 1990). Nitrogen-fixation takes place in heterocysts and supplies the total nitrogen requirements of both *Azolla* and *Anabaena* (Peters & Mayne, 1974). Symbiotic *Anabaena azollae* has been found to fix nitrogen at a rate 4 to 18 times greater than that of free-living *Anabaena cylindrica* (Tang *et al.*, 1981). Both the activity and protein levels of the primary ammonia assimilating enzyme, GS, is drastically decreased in the cyanobiont (Orr & Haselkorn, 1982). Such low levels of GS are unable to assimilate all the ammonia produced during N_2-fixation by the cyanobiont resulting in its liberation and subsequent

uptake by the host. Levels of primary carboxylating enzyme RuBisCo are also significantly lower in the cyanobiont as compared to the free-living ***Anabaena***, thus making it dependent on fixed carbon availability from the host ***Azolla*** (Braun-Howland & Nierzwicki-Bauer, 1990).

1.2.4 Association with Bryophytes:

The division bryophyta includes three classes of terrestrial plants: musci (mosses), hepaticeae (liverworts) and anthocerotae (hornworts). The nitrogen-fixing cyanobacteria symbiotically associated with bryophytes, are species of ***Nostoc.*** In mosses, except for two species of ***Sphagnum***, the cyanobacteria form epiphytic associations. Stewart in 1966 first reported endophytic association of ***Sphagnum***. The cyanobacteria in ***Sphagnum*** are localized in hyaline cells. The cyanobacteria have been identified as ***Nostoc*** sp. Endophytic association of ***Nostoc*** with ***Sphagnum*** is neither frequent in occurrence nor as active in nitrogen fixation as epiphytic association (Basilier, 1980). Morphologically, both epiphytic and endophytic ***Nostoc*** are similar. Nitrogen fixed by the epiphytic ***Nostoc*** is transferred to the host showing a metabolic interaction between the two partners (Basilier, 1980). Association of liverworts with the nitrogen-fixing cyanobacteria is rare, only two instances each of both epiphytic and endophytic associations (***Blasia*** sp. and ***Cavicularia*** sp.) are reported till date (Meeks, 1990). The hornworts are represented by six genera which undergo symbiotic association with endophytic nitrogen-fixing cyanobacteria. These are

Anthoceros laevis, A. husnotti, A. punctatus, Phaeoceros laevis, Notothylas sp. and *Dendroceros* sp. In all cases the cyanobiont is *Nostoc.* The *Nostoc* communities are visible as dark, round spots (0.4 to 0.7 mm in diameter), with a distinct organized structure in the gametophytic tissue and are referred as the *Nostoc* colonies (Rodgers & Stewart, 1977; Enderlin & Meeks, 1983). The cellular morphology of the cyanobiont is markedly different from its free-living isolates (Rodgers & stewart, 1977; Duckett *et al.*, 1977; Enderlin & Meeks, 1983). The vegetative cells during symbiotic association enlarge and become spherical. Connection between adjacent vegetative cells and heterocysts and vegetative cells appears to be fragile. The heterocyst frequency increases relative to free-living cultures (30-45% as against 5-10%) and the cyanobiont heterocysts have less distinct polar nodules (Rodgers & Stewart, 1977; Enderlin & Meeks, 1983). The cyanobiont vegetative cells contain cyanophycin granules, phycobilisomes and carboxysomes and numerous glycogen granules. The heterocysts lack these inclusions, but may possess phycobiliproteins in soluble state (Meeks, 1990). The bryophyte responds to the association with increase in cavity surface area and formation of multicellular filaments that mediate the interchange of metabolites (Duckett *et al.*, 1977). The photosynthetic CO_2-fixation (Steinberg & Meeks, 1989) and exogenous NH_4^+ assimilation (Meeks *et al.*, 1985) rates in symbiotic *Nostoc* are five to eight times lower than that in the free-living isolate. Cyanobiont assimilates N_2-derived NH_4^+ via glutamine synthetase - glutamate synthase (GS -GOGAT) pathway.

The activity of GS is three times lower than free-living isolate but the GS protein concentration is found to be comparable, suggesting a post-translational modification of the enzyme in the cyanobiont (Meeks, 1990).

1.2.5 Association with Fungi:

Lichens are symbiotic associations between one or two photobionts and a fungus (mycobiont). The lichen thalli are quite distinct from either of the symbionts occurring in the free-living state. They represent an integration of a heterotrophic fungal partner (mycobiont) and an autotrophic photosynthetic partner (photobiont) in bipartite lichens. The photobiont in such lichens is either a cyanobiont or a phycobiont. In tripartite lichens, apart from the mycobiont there are two photobionts: a green alga (phycobiont) and a cyanobacterium (cyanobiont). About 8% of lichen species have cyanobacteria as photobiont. Such lichens are called **cyanolichens**. Within the lichen thallus, cyanobiont develops extracellularly except in the case of ***Geosiphon pyriforme***, where the ***Nostoc*** invaginates the plasmalemma of the fungus. The cyanobiont either occurs throughout the thallus or is restricted to a distinct symbiont layer in the upper cortex in bipartite lichens. In tripartite lichens, the cyanobiont occurs in special structures called cephalodia. Cephalodia may be external, occuring on the surface of the thallus (e.g. in ***Peltigera aphthosa, Placopsis, Pilophorus, Stereocaulon*** etc.) or internal, occuring inside the thallus. (e.g. in ***Lobaria*** and ***Sticia***).

Cyanobacteria occuring as lichen cyanobionts may be unicellular (*Gloeocapsa, Gloeothece, Synechocystis, Hyella*), filamentous heterocystous (*Calothrix, Nostoc, Scytonema*) or heterocystous branched filamentous (*Fischerella*). All are diazotrophic. Reproduction in lichens is mainly by vegetative means.

A comparative study between *Nostoc* isolate from *Peltigera canina* and the cyanobiont in the symbiotic thallus indicates that ultrastructure of both is similar except increase in cell size (Bergman & Hallbom, 1982). Heterocyst frequency in case of bipartite lichens is similar to that in the free-living isolate (maximum of 8%). However, in case of tripartite lichens heterocyst frequency is higher reaching upto 20% with corresponding high rates of nitrogen fixation (Bergman & Hallbom, 1982; Koriem & Ahmadjian, 1986). Cyanobiont is autotrophic in all lichens. It fixes CO_2 and transfers fixed carbon to the mycobiont. The mechanism of carbon fixation in cyanobiont is similar to that in free-living cyanobacteria. Primary carboxylation reaction mediated by RuBisCo fixes CO_2 via calvin cycle (C_3 cycle) (Stewart *et al.*, 1981; Drew, 1966). In several lichens dark CO_2 fixation also occurs via C_4 cycle. In *P. aphthosa* dark CO_2 fixation rates are about 17 to 20% of that in the light (Rai *et al.*, 1981b). Cyanobiont provides both fixed carbon and fixed nitrogen to mycobiont in bipartite lichens. The fixed carbon moves in the form of glucose. The transfer of fixed nitrogen is in the form of ammonia. In tripartite lichens, cyanobiont mainly provides fixed nitrogen. The fixed carbon comes

from phycobiont in the from of mannitol (Smith, 1974; 1980) which is converted to ribitol in mycobiont. Reasons for carbon transfer is not known, but nitrogen transfer is due to low levels of GS synthesis and activity in the cyanobiont (Rai *et al.*, 1980; Rai 1981a, 1983).

1.2.6 Association with Algae:

There are a few reports of occurrence of cyanobacteria in symbiosis with marine diatoms. The filamentous heterocystous cyanobacterium *Richelia intracellularis* occurs in the marine pennate diatom *Rhizosolenia*. The cyanobiont is reported to be responsible for most of the CO_2 fixation in the association (Mague *et al.*, 1974). However, the major function of the cyanobiont is to fix nitrogen and transfer the fixed nitrogen to the diatom (Rai, 1990a). Inclusion bodies resembling thin walled unicellular cyanobacteria characterised as coccoid cyanobacteria are found in the diatom *Rhopalodia* (Drum & Pankratz, 1965). The cyanobiont fixes nitrogen in the association (Floener & Bothe, 1980). Both partners fix CO_2.

1.3 Nitrogen Fixation and Nitrogenase:

The enzyme responsible for biological N_2-fixation (reduction of dinitrogen to ammonia) is nitrogenase (Gallon, 1980; Stewart, 1980; Hallenbeck, 1987; Smith *et al.*, 1987). It is a cold-labile and oxygen-sensitive enzyme present in some prokaryotes only. The enzyme complex consists of two component

proteins: Mo-Fe protein (dinitrogenase) and Fe-protein (dinitrogenase reductase). The dinitrogenase is a tetramer (mol. wt. 245 KD) of two pairs of different subunits. It also contains two molecules of Mo-Fe cofactor. The dinitrogenase reductase (mol. wt. 64 KD) is a dimer of two identical subunits. N_2 reduction occurs on dinitrogenase and the electrons for reduction are supplied by dinitrogenase reductase which in turn is reduced by ferredoxin (Fd) or flavodoxin (Fv). The latter receives electrons from reductants generated in intermediary metabolism. The reaction is highly endergonic requiring 4 to 5 ATP per pair of electrons transferred. In addition to N_2, nitrogenase can reduce a number of other substrates such as acetylene, azides, cyanides, nitrous oxides etc. During nitrogen reduction there is a concomitant reduction of protons resulting in H_2 formation. This represents a waste of energy and reductants. However, H_2 recycling by uptake hydrogenase in some diazotrophs reduces such losses.

Two alternative nitrogenases, in addition to the molybdenum nitrogenase, have been reported in some diazotrophs (Pau, 1991). There are preliminary indications that these may be present in some cyanobacteria also (Kentemisch *et al.*, 1988; Gallon & Chaplin, 1988; Bothe *et al.*, 1991).

Nitrogenase protein from various diazotrophs show remarkable similarity in molecular mass (Burris *et al.*, 1980; Braun-Hawland *et al.*, 1988; Bergman & Rai, 1989; Zehr *et al.*, 1990). Futhermore, antibodies raised against nitrogenase from one

diazotroph specifically cross react with nitrogenase from other diazotrophs (Smoker *et al.*, 1989; Stal & Bergman, 1990; Rai *et al.*, 1989, 1992). The structural genes for nitrogenase (*nif* H,D,K) are also highly conserved (Haselkorn, 1986). This enables use of immunological and genetic probes to study nitrogenase protein and the concerned genes in a variety of diazotrophs.

As N_2-fixation demands high input of energy, the process is subjected to rigorous control both at the nitrogenase activity and the synthesis levels. Oxygen rapidly and irreversibly inactivates nitrogenase and also represses its synthesis (St. John *et al.*, 1974; Eady *et al.*, 1978; Smith *et al.*, 1987a). Combined nitrogen sources such as nitrate, nitrite, ammonia and amino acids are preferred sources of nitrogen and nitrogenase is synthesized only when such sources are absent in the medium. Detailed studies on effect of ammonia suggest that it inhibits nitrogenase activity as well as synthesis. Ammonia inhibits nitrogenase activity by lowering membrane potential (Haaker *et al.*, 1980) or causes irreversible inactivation by modification of the Fe-protein (Gotto & Yoch, 1982; Reich & Böger, 1989). Primarily though, ammonia through its assimilation via GS, causes repression of nitrogenase synthesis in all diazotrophs (Magasanik, 1977; Shanmugam *et al.*, 1978; Stewart, 1980).

Diazotrophic cyanobacteria are broadly of two types: heterocystous and nonheterocystous. Among heterocystous forms nitrogenase is synthesized only in heterocysts (Bergman *et al.*, 1986). These are specialized cells having undergone a number of

biochemical and structural changes, including loss of O_2-evolving photosystem II, conducive to nitrogenase functioning (Wolk, 1982). Among nonheterocystous cyanobacteria nitrogenase is synthesized in all the cells although this poses a problem of protecting it from O_2 evolved during photosynthesis (Stal & Bergman, 1990; Rai *et al.*, 1992). Such organisms however employ a number of strategies including temporal separation of N_2-fixation and photosynthesis, to protect nitrogenase (see Fay, 1992).

1.4 Hydrogenase:

Hydrogenases catalyze H_2 oxidation and/or proton reduction. Considerable work has been done on hydrogen metabolism and hydrogenases of bacteria, cyanobacteria and diazotrophic symbioses (Adams *et al.*, 1981; Gogotov, 1986; Lambert & Smith, 1981; Houchins, 1984; Ewart & Smith, 1989a, b; Evans *et al.*, 1987; Huss-Danell, 1990). The aim has been to investigate hydrogenase-nitrogenase relationships with regard to the efficiency of N_2-fixation. At least two types of hydrogenases have been reported in diazotrophs. A reversible hydrogenase and an uptake hydrogenase. The latter plays an important role in recycling of hydrogen evolve during N_2-fixation by nitrogenase. It has been suggested that such recycling improves the N_2-fixation efficiency because it generates ATP and reductant, consumes O_2, thereby contributing to oxygen protection of nitrogenase, and prevents build up of hydrogen which is inhibitory to nitrogenase and photosynthesis (*see* Adams *et al.*, 1981; Lambert & Smith, 1981; Houchins, 1984; Antarikanonda *et*

al., 1980). In heterocystous cyanobacteria, reversible hydrogenase was suggested to be located in cytoplasm of both heterocysts and vegetative cells while uptake hydrogenase bound to the membranes in heterocysts (Houchins, 1984). However, later studies showed presence of both uptake and reversible hydrogenase activities in membrane bound as well as in soluble fractions (Rao & Hall, 1988; Ewart & Smith, 1989a, b; Kentemisch *et al.*, 1989; Papen *et al.*, 1986). In addition, H_2-evolution also occurs via nitrogenase in heterocysts (Chen *et al.*, 1986; Almon & Boger, 1988).

1.5 Ribulose 1,5-Bisphosphate Carboxylase / Oxygenase (RuBisCo):

In cyanobacteria, RuBisCo is present both in the cytoplasm (soluble form) and in specialized structures called carboxysomes (polyhedral bodies). The latter contain a very high concentration of RuBisCo. Each vegetative cell on an average contains 1-2 carboxysomes (Lang & Whitton, 1973; Wolk, 1973; Stewart & Codd, 1975; Stanier & Cohen-Bazire, 1977). In contrast to the vegetative cells, the heterocysts do not contain RuBisCo and they also lack carboxysomes (Stewart & Codd, 1975; Codd & Stewart, 1977; Cossar *et al.*, 1985; Rai *et al.*, 1989). Cyanobacteria fix CO_2 via calvin cycle. The key reaction of calvin cycle where CO_2 is introduced to react with ribulose 1,5-bisphosphate (RuBP) to form two molecules of 3-phosphoglyceric acid (3-PGA), is catalysed by RuBisCo (Shively *et al.*, 1973; Shively, 1974; Codd, 1988). RuBisCo is amongst the most abundant proteins in the autotrophic cells. It has a molecular weight of 550,000 and has

eight large and eight small subunits. This enzyme needs Mg^{+2} ions for its catalytic activity (Lorimer, 1981; Miziorko & Lorimer, 1983). In all autotrophs RuBisCo is a bifunctional enzyme capable of catalysing carboxylation or oxygenolytic cleavage of RuBP.

1.6 Glutamine synthetase:

In all N_2-fixing cyanobacteria, glutamine synthetase (GS), plays a central role in the assimilation of the ammonia produced by the action of nitrogenase (Haselkorn, 1978; Bothe, 1982). GS catalyses the synthesis of L-glutamine in the reaction:

$$\text{L-glutamate} + \text{ATP} + NH_4^+ \longrightarrow \text{L-glutamine} + \text{ADP} + \text{Pi}$$

(at pH 7 and 37° C with a free energy change -5.2 Kcal $mole^{-1}$).

GS is the key enzyme in the flow of ammonia nitrogen to organic compounds. The amide nitrogen of glutamine thus produced through the action of GS is utilised in the biosynthesis of AMP, CTP, tryptophan, histidine, glucosamine 6-phosphate and carbamoyl phosphate (Meister, 1974). In addition, GS may be coupled to GOGAT (Tempest *et al.*, 1970) and various transaminases to provide a pathway for ATP-dependent synthesis of most amino acids. GS-GOGAT pathway serves as the sole route of primary ammonia assimilation in diazotrophic cyanobacteria (Wolk *et al.*, 1976; Singh *et al.*, 1991). In heterocystous cyanobacteria, the ammonia produced by the action of nitrogenase in the heterocysts, is assimilated in the heterocysts by the action of GS. The GS level in nitrogen fixing heterocysts is generally two-fold higher than that of the vegetative cells. The increased level of GS in

heterocysts shows a correlation with expression of nitrogenase and seems essential for assimilation of N_2-derived ammonia (Renström-Kellner *et al.*, 1990). GS is also involved in mediating the effect of ammonia on nitrogenase repression in all diazotrophs (Magasanik, 1977; Shanmugam *et al.*, 1978; Stewart, 1980). Heterocysts lack GOGAT hence the glutamine is transported to the adjacent vegetative cells where it is further metabolised by the action of GOGAT (Wolk *et al.*, 1976). The native GS enzyme has twelve identical subunits of approximately 50,000 dalton molecular weight each arranged in two superimposed hexagonal rings. The biosynthetic activity of GS is Mg^{+2}-dependent and the transferase activity requires Mn^{+2} ions (Sampaio *et al.*, 1979). GS activity has been shown to be regulated as a function of nitrogen source available in the growth medium. In the unicellular cyanobacterium ***Synechocystis***, intracellular carbon-nitrogen balance plays an important role in the regulation of GS (Merida, *et al.*, 1991). In ***Anabaena*** sp. associated with ***Azolla*** and ***Nostoc*** cyanobiont of lichens, GS activity is regulated by repression of synthesis (Nierzwicki-Bauer & Haselkorn,1986; Orr & Haselkorn, 1982; Sampaio *et al.*, 1979; Stewart *et al.*, 1983). But GS is regulated by posttranslational modification in ***Nostoc*** sp. associated with ***Anthoceros punctatus*** (Joseph & Meeks, 1987). Genes encoding GS has been found to be induced directly by available ammonia in ***Rhizobium*** (Hirel *et al.*, 1987).

1.7 Phycobiliproteins:

Phycobiliproteins are accessory photosynthetic pigments in cyanobacteria. They constitute well over half of the total soluble proteins of the cyanobacterial cells (Bennet & Bogorad, 1973). Cyanobacteria possess phycobiliproteins in their vegetative cells along the thylakoid membranes (Stewart *et al.*, 1983; Lindblad, 1987) and heterocysts are found to be deficient in them (Stewart, 1980; Rai *et al.*, 1989). Phycobiliproteins are assembled into particles called phycobilisomes which are attached to the external surface of the thylakoid membranes. They occur as chromoproteins and are involved in the light phase of the photosynthesis along with chlorophylls and carotenoids. They are divided into two major groups on the basis of their colour. The red phycoerythrin and blue phycocyanin. Phycoerythrin and phycocyanin are composed of two different protein subunits designated as α (mol. wt. 19 KD) and β (mol. wt. 21 KD), which occur in 1:1 stoichiometric ratio. Each subunit carries covalently bound phycobilins. Depending upon its source, a subunit may carry 1-4 molecules of phycobilins. The phycobiliproteins also serve as a nitrogen reserve and undergo degradation during nitrogen limitation (Cohen-Bazire & Bryant, 1982; Ho & Krogman, 1982). Cyanobacteria under symbiotic condition possess phycobiliproteins in their vegetative cells along the thylakoid membranes (Stewart *et al.*, 1983; Lindblad, 1987) and heterocysts are found to be deficient in them (Stewart, 1980; Rai *et al.*, 1989).

1.8 *Anthoceros*:

Anthoceros belongs to the division bryophyta. It is cosmopolitan in distribution, mainly occurring in temperate and tropical regions and has about 200 species. About 25 species of *Anthoceros* have been reported in India.

The plant body is small, dorsiventral, prostate and dark green thallose gametophyte. The thallus is sub-orbiculous or variously lobed. The lobes may be divided, several layers thick in the middle, midrib either broad indistinct or absent. The dorsal surface of the thallus is smooth or velvety. On the undersurface of the *Anthoceros* thallus mucilage-filled cavities are present in which *Nostoc* colonies develop (Enderlin & Meeks, 1983). From the ventral surface rhizoids of smooth walled type develop. Scales and mucilage hair are absent. Vegetative reproduction takes place by the growth of apical region and progressive death of the older parts. *Anthoceros* may be homothallic or heterothallic. Sex organs are deeply embedded in the thallus. Antheridia develop in clusters from the hypodermal cells of the thallus and archegonia develop simply from the superficial cells of the thallus. Sporophytic generation begins as soon as fertilization takes place. A mature sporophyte consists of expanded or bulbous foot and smooth, slender, erect cylindrical capsule. A capsule presents all the stages of spores production at a time.

1.9 The cyanobiont:

The cyanobiont, ***Nostoc***, is a filamentous heterocystous cyanobacterium capable of photoautotrophic as well as heterotrophic growth (Rippka *et al.*, 1979). There is an apparent correlation between symbiotically competent cyanobacteria and the presence of hormogonia (Stewart *et al.*, 1980; Enderlin & Meeks, 1983). The hormogonia of symbiotic ***Nostoc*** strains are highly motile, small-celled filaments that lack gas vacuoles. The stimulation of hormogonia formation depends upon an extracellular low molecular weight, heat labile product of ***A. punctatus*** metabolism which is inhibited by excess NH_4^+ (*see* Meeks, 1990). The cyanobiont forms discrete colonies within preformed cavities in the ***Anthoceros*** gametophyte tissue (Enderlin & Meeks, 1983).

1.10 *Anthoceros-Nostoc* symbiosis:

Anthoceros-Nostoc symbiosis is an endophytic association where ***Nostoc*** colonies develop in the mucilage-filled cavities on the undersurface of the ***Anthoceros*** thallus. The ***Nostoc*** colonies are macroscopically visible as distinctly organised, dark, round spots (0.4 to 0.7 mm in diameter) in the gametophytic tissues of the ***Anthoceros*** (Rodgers & Stewart, 1977; Enderlin & Meeks, 1983). Oxygen microelectrode profiles of symbiotic ***Anthoceros-Nostoc*** tissue reveals an anaerobic environment in the symbiotic cavities containing ***Nostoc***. It appears that the symbiotic cavities of ***Anthoceros punctatus*** can physiologically replace the function of

heterocyst outer wall (Campbell & Meeks, 1992). ***Anthoceros*** responds to the endophytic presence of ***Nostoc*** by an increase in the cavity surface and prolification of multicellular papillae which acquire the characteristics of transfer cells and facilitate the exchange of metabolites between the two partners (Rodgers & Stewart, 1977; Duckett ***et al.***, 1977). The symbiotic ***Nostoc*** has a filament and cellular morphology markedly different from free-living cultures (Rodgers & Stewart, 1977; Enderlin & Meeks, 1983; Meeks, 1990). The symbiotic ***Nostoc*** vegetative cells enlarge and become spherical. The connection between adjacent vegetative cells and heterocysts and vegetative cells become fragile (Meeks, 1990). Symbiotic heterocysts tend to have less distinct polar nodules and the difference in the pigmentation is muted. The heterocyst frequency goes upto 43 - 45 % (Rodgers & Stewart, 1977; Enderlin & Meeks, 1983). The symbiotic vegetative cells contain nitrogen reserves in the form of cyanophycin granules, phycobilisomes and carboxysomes as well as numerous glycogen granules. The relative amount of these cellular inclusions are equal to or greater than those observed in the free-living vegetative cells of N_2-grown cultures. The heterocysts lack these inclusions both in free-living and symbiotic cyanobionts. The eukaryotic partner ***Anthoceros***, exerts some regulatory constraints on the growth, metabolism and cellular differentiation of the symbiotic ***Nostoc*** to bring about a balanced growth (Campbell & Meeks, 1992). The main function of symbiotic ***Nostoc*** is to provide fixed-nitrogen to its eukaryotic partner. ***In situ*** ^{13}N tracer experiments established that

symbiotic *Nostoc* releases 80 to 90% of its fixed nitrogen as ammonia to support growth of *Anthoceros* tissue (Meeks *et al.*, 1985). The photosynthetic capability of the symbiotic *Nostoc*, as measured by CO_2-fixation, O_2-evolution and the level of accessory pigments for photosystem II, is diminished by five to eight times as compared to free-living cultures (Meeks *et al.*, 1985). It is now established that *Nostoc* is capable of complete photosynthesis when in association with *Anthoceros*, though at a lower rate (Steinberg & Meeks, 1989) and can use its photosynthate to support nitrogenase activity (Steinberg & Meeks, 1991). Free-living *Nostoc* under diazotrophic conditions doubles every 45 h but in symbiosis, *Anthoceros-Nostoc* association doubles its biomass in 10 days as against the doubling time of 5 days for symbiont-free *Anthoceros* (Meeks, 1990). The size of the *Nostoc* colony in *Anthoceros* tissue, its frequency of heterocysts and rate of nitrogen fixation are influenced by the growth conditions of the *Anthoceros-Nostoc* association (Enderlin & Meeks, 1983; Steinberg & Meeks, 1991). Establishment of symbiosis also initiates several biochemical changes in the endophyte. Rate of nitrogen-fixation by symbiotic *Nostoc* is strongly dependent on association with the intact *Anthoceros punctatus* tissue. Rodgers & Stewart in 1977 demonstrated that the rate of acetylene reduction is 3.2 times higher than the free-living cultured isolates. Cyanobacteria assimilate NH_4^+ exclusively by the sequential activity of glutamine synthetase (GS) and glutamate synthase (GOGAT) via GS-GOGAT pathway (Meeks, 1981; 1990). The GS activity in the cyanobiont of *Anthoceros-Nostoc* is reduced by

three-to four-fold without similar decrease in the GS content (Joseph & Meeks, 1987). Thus, the capability to assimilate the fixed-N is reduced greatly and as much as 90% of the fixed-N is transported to the host as ammonia (Stewart & Rodgers, 1977; Meeks *et al.*, 1985).

1.11 Present study:

Diazotrophic cyanobacteria efficiently combine oxygenic photosynthesis and nitrogen fixation. Those diazotrophic cyanobacteria which form symbiotic associations with plants undergo major structural, physiological and biochemical changes relating to their carbon and nitrogen metabolism (see Rai, 1990). Some of these changes such as high heterocyst frequency, high nitrogenase activity and ammonia liberation have biotechnological implications such as photobiological production of ammonia and production of biofertilizers. By understanding the underlying mechanisms involved in the physiological/biochemical changes during the symbiotic state of the cyanobiont, it is hoped that free-living cyanobacterial strains could be modified on similar lines. Thus, there is a need to fully understand the symbiotic associations of cyanobacteria, particularly the physiological and biochemical changes in the cyanobiont's nitrogen metabolism and related processes.

Nostoc-Anthoceros symbiosis was chosen for this particular study since the symbionts can be axenically cultured and the symbiosis reconstituted in the laboratory. This thesis

incorporates details of the work done on general characterization of the cyanobiont, reconstitution of symbiosis, electronmicroscopic localization of key enzymes involved in nitrogen, carbon and hydrogen metabolism. For comparative purposes, some of these aspects have also been studied using the non-heterocystous cyanobacterium ***Plectonema boryanum.***

CHAPTER 2

MATERIALS AND METHODS

2.1 Culture methods:

2.1.1 Culture vessels:

All glasswares used for the experiments were of corning grade. They were thoroughly washed under running tap water after immersing them in chromic acid solution overnight and cleaning them in laboratory detergent. Finally, the glasswares were rinsed twice with double distilled water and then dried in hot air oven.

2.1.2 Sterilization:

All glasswares and chemicals used for experiments were autoclaved at 121° C (15 psi) for 15 mins. The heat labile chemicals were sterilized by ultrafiltration using Whatman membrane filters of pore size 0.45 μm. These were then added to previously sterilized nutrient medium inside a laminar hood. Water used for washing cultures and preparing media were also autoclaved.

2.1.3 Culture conditions:

All cultures were grown and maintained in sterilized media under the aseptic condition in a culture room at 25° C and photon fluence rate of 50 μmol m^{-2} s^{-1} on the surface of the

vessels. To obtain aerated batch cultures, fish tank aerators were used with a sterile membrane filter of pore size 0.45 μm.

2.2 Collection and maintenance of *Anthoceros punctatus*:

Anthoceros punctatus gametophytes bearing cyanobacterial colonies were collected during the month of September from North Eastern Hill University campus (Shillong, India) and brought to the laboratory. Thalli were washed thoroughly with double distilled water and placed on sand in plastic trays fitted with perspex lids. The thalli were kept moist with periodic spray of Praskaur's solution (Table 2.1) devoid of combined nitrogen. For ease, individual stock solutions were made for items 1-4. However, only one stock solution was made for items 5-16 (micronutrient elements).

Table: 2.1 Composition of Praskaur's solution (final concentration):

Chemicals	mg l^{-1}	Chemicals	mg l^{-1}
1. $MgSO_4.7H_2O$	100.00	9. H_3BO_3	0.61
2. $CaCl_2$	100.00	10. Al_2SO_4	0.56
3. KH_2PO_4	100.00	11. $SnCl_2.2H_2O$	0.28
4. $FeSO_4$	50.00	12. KBr	0.28
5. $LiCl_2$	0.28	13. $MnCl_2.4H_2O$	0.39
6. $CuSO_4$	0.56	14. $NiSO_4.6H_2O$	0.56
7. $ZnSO_4$	0.56	15. $Ca(SO_4)_2.6H_2O$	0.56
8. TiO_2	0.56	16. KI	0.28

The pH of the medium was adjusted to 5.6. Trays containing *Anthoceros* thalli were maintained in a BOD incubator at 20° C fitted with fluorescent light (photon fluence rate 35 μmol $m^{-2}s^{-1}$)

2.3 Axenic culture of *Anthoceros punctatus*:

In nature *Anthoceros* gametophytes are found to be always in symbiotic association with *Nostoc*. Hence, axenic *Anthoceros* gametophytes (without *Nostoc* colonies) were obtained by germinating surface sterilized spores. Using the method described by Meeks *et al* (1987), mature sporophytes were excised from the gametophytes and sterilized by 0.5% sodium hypochlorite solution for 0.5 to 2.0 min. These sporophytes were then washed extensively with sterile double distilled water. Sporophytes were split lengthwise and the spores were spread on agar plates containing basal medium (0.2 g l^{-1} NH_4NO_3, 0.1 g l^{-1} each of KH_2PO_4, $MgSO_4$, $CaCl_2$ and micronutrient solution (final concentration as given in Table 2.1). The pH of the medium was adjusted to 6.4 before autoclaving. The plates were kept in 14/10 h light (photon fluence rate: 35 μmol m^{-2} s^{-1})/dark cycle at 25° C. After 3 weeks of incubation, individual germlings were transferred to new plates and flasks containing basal medium supplemented with 5mM 2-(N-morpholino) sulphonic acid (MES, Sigma) as buffer and 1% glucose (w/v). Liquid cultures were raised in 50 ml conical flasks where germlings from agar plates were floated on liquid surface. Stock cultures of symbiont-free *Anthoceros* were fragmented with transfer loop and subcultured in fresh medium after every 7 days.

2.4 Isolation of *Nostoc* ANTH:

Anthoceros gametophytes were washed thoroughly with distilled water and then treated with 0.5% sodium hypochlorite solution for surface sterilization for 2 min. The macroscopically visible cyanobacterial colonies were carefully excised with sterile needles and washed with sterile distilled water. The colonies were then plated on nitrogen free $BG\text{-}11_o$ nutrient medium with 1% agar. Subsequently, plates were incubated in the culture room under fluorescent light (photon fluence rate of 50 μmol m^{-2} s^{-1}) at 25° C. After 3 weeks of incubation individual cyanobacterial colonies were picked up and transferred to liquid medium in test tubes. After growth, plating and selection processes were repeated until unialgal suspensions were achieved.

2.5 Purification of *Nostoc* ANTH:

Test tubes containing 2 cm deep nutrient agar ($BG\ 11_o$) were autoclaved and allowed to set. One cyanobacterial colony was placed on top of the agar surface in each test tube. The second layer of 2 cm deep nutrient agar was poured on top of the first layer containing the cyanobacterial colony. The second layer of agar also contained antibiotic polymixin-B-sulphate (0.01mg ml^{-1}) and cycloheximide (0.1 mg ml^{-1}). This was followed by addition of 5 ml of liquid medium in the tubes. The tubes were plugged with cotton wool and incubated in light at 25° C. Cyanobacterial filaments migrated through the antibiotic agar

layer and eventually appeared on top of the tubes in the liquid medium. These were then transferred to sterilized flasks containing fresh liquid $BG\text{-}11_o$ medium. The procedures were repeated till axenic cultures of *Nostoc* were obtained.

2.6 Maintenance of purified *Nostoc*:

The purified *Nostoc* was maintained on agar slants as well as in liquid $BG\text{-}11_o$ medium with periodic checks for any contamination by plating on nutrient agar containing 1% glucose. Aerated batch cultures were maintained for running experiments in the logarithmic growth phase by transferring them every fortnight into fresh sterilized nutrient medium.

Table: 2.2 Composition of $BG\text{-}11_o$ medium (stock solutions):

Macroelements	g^{-1}	Microelements	g^{-1}
1. K_2HPO_4	40.0	8(a) H_3BO_3	2.86
2. $MgSO_4.7H_2O$	75.0	(b) $MnCl_2.4H_2$	1.81
3. $CaCl_2.2H_2O$	36.0	(c) $ZnSO_4.7H_2O$	0.22
4. Citric acid	6.0	(d) $Na_2MoO_4.2H_2O$	0.39
5. Ferric ammonium citrate	6.0	(e) $CuSO_4.5H_2O$	0.079
6. Na_2CO_3	20.0	(f) $Co(NO_3)_2.6H_2O$	0.0494
7. EDTA (disodium salt)	1.0		

To prepare 1 l of $BG\text{-}11_0$ medium 1 ml of each macronutrient (1-7) and 1 ml of micronutrient (8) solution was measured out with the help of Gilson pipette and made upto 1000 ml with double distilled water. Before autoclaving, the pH of the medium was adjusted to 7.3. As and when required, KNO_3 or NH_4Cl was added as combined nitrogen source to a final concentration of 5 mM and 1 mM, respectively. For studies of photoheterotrophy and heterotrophy different carbohydrates were added to the nutrient growth medium in light or dark, respectively. These carbohydrate solutions were filtered through Whatman membrane filters for sterilization and added to previously autoclaved growth medium under aseptic conditions on a laminar flow table. For dark incubations, the culture flasks were wrapped with aluminium foil.

2.7 Reconstitution of axenic *Anthoceros punctatus-Nostoc* symbiosis:

Reconstitution of *Anthoceros punctatus-Nostoc* symbiosis was performed in liquid medium. Axenic culture of *Nostoc* isolate and axenic *Anthoceros* gametophytes were incubated together in 50 ml of *Anthoceros* medium devoid of combined nitrogen. After about two weeks of incubation under environmental conditions (described for the growth of *Anthoceros*), the gametophytes were rinsed with sterile medium and transferred to fresh nitrogen-free medium. Formation of symbiotic association was confirmed by the appearance of cyanobacterial colonies within the *Anthoceros* gametophyte and by the dinitrogen dependent growth of the

associated tissue. Surface growth of *Nostoc* on *Anthoceros* gametophytes was removed by 2-day incubation in liquid medium supplemented with 250 units/ml of penicillin G (Sigma). The penicillin treatment was terminated by removal of the tissue from the supplemented medium, washing it three or four times in sterile medium and placing it in fresh growth medium.

2.8 Growth parameters:

Cyanobacterial growth was measured using the following parameters:

2.8.1 Chlorophyll:

Chlorophyll *a* was measured using the method described by Mackinney (1941). A known amount of cyanobacterial suspension (5 ml) was centrifuged and the pellet was suspended in 5 ml of methanol. This was mixed thoroughly with the help of a vortex mixture and incubated overnight at 4° C in dark. The solution was then vortexed, centrifuged, and filtered. The absorbance of the supernatant was read at 663 nm using a Jasco spectrophotometer (UVIDEC 610). The concentration of chlorophyll *a* was calculated as followed:

Chlorophyll *a* (µg/ml) = O.D. at 663 nm x 12.63.

2.8.2 Protein:

Protein content of the cyanobacteria was measured according to Bradford (1976) as per details given below:

2.8.2.1 Extraction of protein:

Five ml of cyanobacterial culture was centrifuged and the pellet was resuspended in 1 ml of distilled water. The cells were disrupted by ultrasonication using a Soniprep 150 (MSE) fitted with a microprobe (16 micron amplitute for 5 min). The supernatant was collected after centrifugation at 3000 g for 5 min and used for protein determination.

2.8.2.2 Estimation of protein:

Reagents:

A. Coomassie Brilliant Blue G-250 (100mg) dissolved in 50 ml of 95% ethanol.

B. 100 ml of 85% (w/v) phosphoric acid.

Bradford reagent: Reagent B was added to reagent A and the resulting solution was diluted to a final volume of 1 liter. The final concentrations of the reagents were 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol, 8.5% (w/v) phosphoric acid.

Bovine serum albumin (BSA) was used as standard (range 10 to 100 µg).

Procedure:

To 0.1 ml of the cyanobacterial protein extract 5 ml of Bradford reagent was added and mixed gently. Absorbance was read at 595 nm. A calibration curve was prepared by using standard

bovine serum albumin solution for determination of cyanobacterial protein content.

2.9 Measurement of specific growth rate:

Specific growth rate was measured using the following formula of Guillard (1973).

$$K = \frac{\log_{10} (N_1/N_0)}{t_1 - t_0}$$

Where K = Specific growth rate (divisions per day).

N_0 and N_1 = Chl *a* concentration (O.D. at 663 nm) at time t_0 and t_1, respectively.

t_0 and t_1 = time, in days, at the beginning (t_0) and end of the growth (t_1).

2.10 Enzyme assays:

2.10.1 Nitrogenase:

Nitrogenase activity was measured using acetylene reduction method (Stewart *et al.*, 1967). Five ml of cyanobacterial culture was taken in 15 ml serum vials. Acetylene gas was injected to a final concentration of 10% (v/v) of the air phase in the vials. After incubating the vials in light at photon fluence rate of 50 μmol m^{-2} s^{-1} at 25° C for 1 h, 1 ml gas

sample was analyzed for ethylene produced, using a Tracor 540 gas chromatograph fitted with a porapak T column (stainless steel column 6' x 1/8'' packed with porapak T mesh size 80 / 100) and a flame ionization detector.

2.10.2 Glutamine synthetase:

Glutamine synthetase (GS) biosynthetic activity was measured as described by Sampaio *et al* (1979).

2.10.2.1 Extraction of enzyme:

Cyanobacterial culture was harvested by centrifugation and the pellet was washed twice in buffer A (50 mM Tris-HCl buffer pH 7.5) and resuspended in buffer B (buffer A supplemented with 5 mg ml^{-1} each of $MgCl_2$ and B-mercaptoethanol, 10 mM sodium glutamate and 1.0 mM EDTA). Cyanobacterial cells were disrupted by ultrasonication, subjected to centrifugation at 35,000 g for 30 min, and the supernatant dialyzed in buffer B (without sodium glutamate) at 4° C overnight.

2.10.2.2 GS biosynthetic assay:

Production of ADP was coupled to oxidation of NADH to study biosynthetic activity of GS. The reaction mixture contained (in a final volume of 3 ml), 1 ml enzyme extract, 150 µmol Tris-HCl buffer pH 7.5, 3 µmol ATP, 200 µmol NH_4Cl, 60 µmol sodium glutamate, 150 µmol each of $MgCl_2$ and KCl, 0.45 µmol NADH,

0.5 µmol phosphoenol pyruvate, 20 units of lactate dehydrogenase and 8 units of pyruvate kinase. The rate of oxidation of NADH was measured in a Jasco spectrophotometer (UVIDEC 610) (λ, 340 nm; Temp. 30*x* C).

2.10.3 Nitrate reductase:

Nitrate reductase (NR) activity was measured as described by Manzano *et al* (1976). Five ml cyanobacterial culture was taken and centrifuged. The pellet obtained was washed with NR buffer. The NR buffer contains 50 mM tris (Trisma), 0.1 M NaCl, 0.3 M sucrose, 1 mM KNO_3, 1 mM EDTA and 5mM $MgCl_2$. The solution was centrifuged again and 2 ml NR buffer and 2% toluene (final concentration) was added to the pellet. The mixture was shaken vigorously for 20 mins. Toulene was removed, and the cell suspension pelleted and resuspended in fresh buffer to a final volume of 5 ml. 2.5 ml of freshly prepared assay mixture was then added. The assay mixture contained 20 mM KNO_3, 100 mM glycine-KOH, 4 mM methyl viologen, 0.2 M $NaHCO_3$ and 0.2 mg of sodium dithionate in 0.1 ml of $NaHCO_3$. The mixture was incubated in dark for 5 to 7 min. 0.2 ml of 1 M zinc acetate was added to the mixture and shaken to stop the reaction. The above mixture was centrifuged and the supernatant was analyzed for nitrite (*see* below). NR specific activity was expressed as nmol nitrite produced min^{-1} mg $protein^{-1}$.

2.10.4 Nitrite estimation:

Nitrite was estimated colorimetrically as described by Snell & Snell (1949).

Reagents:

A. 1% (w/v) sulphanilamide in 3 M HCl.

B. 0.02% (w/v) N-(1-Naphthyl ethylene diamine dihydrochloride) in distilled water.

C. Potassium nitrite solution was prepared in the range of 10 to 100 nmol ml^{-1}. This was used as standard.

Procedure:

To 1 ml of sample, 1 ml of sulphanilamide and 1 ml of N-(1-Naphthyl ethylene diamine dihydrochloride) was added. The solution was mixed thoroughly and after 15 min, absorbance was read at 540 nm. A calibration curve was prepared by using standard potassium nitrite solution for estimation of nitrite.

2.10.5 Nitrate uptake:

Nitrate uptake was estimated by measuring the disappearance of nitrate from the medium. Exponentially growing cyanobacterial cells were harvested by centrifugation, washed thoroughly with sterile medium and resuspended in the same medium to a final density of 200 μg protein ml^{-1}. Uptake was initiated

by the addition of 100 μM KNO_3. From the samples withdrawn at time intervals, cells were separated by centrifugation and cell-free supernatants were analysed for the residual nitrate. For measurement of nitrate, absorption difference at 202 nm and 250 nm was used (Calero *et al.*, 1980). Uptake activities were expressed in nmol nitrate taken up min^{-1} mg^{-1} protein.

2.11 Oxygen exchange:

Oxygen evolution and consumption was measured polarographically by using a Clark-type oxygen electrode (Rank) installed in a 3 ml Plexiglass container with magnetic stirring. Measurement involved: adding 3 ml cyanobacterial culture to the sample chamber of the non-polarized electrode and allowing each sample to equilibriate for 5 min with stirring. The electrode was then polarised and the linear rate of oxygen evolution was obtained in light supplied by a 100 w tungsten filament bulb which was shielded from the sample by a water bath acting as heat filter at a photon fluence rate of 50 μmol m^{-2} s^{-1} at 25° C. Oxygen consumption was measured in dark with the chamber wrapped with aluminium foil.

2.12 Antibodies:

Rabbit anti-***Rhodospirillum rubrum*** nitrogenase Fe-protein, -***Anabaena*** PCC 7120 GS, -***Phormidium persicinum*** PE, -***Sinapis alba*** RuBisCo and -***Alcaligene latus*** hydrogenase were gifts from Dr. S. Nordlund (University of Stockholm, Sweden), Professor R.

Haselkorn (University of Chicago, USA), Dr. D. Guard-Friar (New York state department of Health, USA), Dr. R. Olemüller (University of Freiburg, Germany) and Dr. D. J. Arp (University of California, USA), respectively. Secondary antibodies (goat anti-rabbit IgG conjugated to colloidal gold size 5, 10 or 15 nm, and conjugated to horseradish peroxidase) were obtained from Amersham and Bio-rad, respectively.

2.13 Immunogold labelling:

Immunogold labelling was done as described by Bergman *et al* (1985). Cells were fixed in 2.5% (w/v) glutaraldehyde in 50 mM sodium cacodylate buffer (pH 7.2) for 1 h and then washed once in cacodylate buffer and twice in palade buffer. These were then subjected to gradual dehydration in ethyl alcohol (20-100%) and twice in propylene oxide. Samples were embedded in Epon (TAAB, Berkshire, U.K). Ultrathin sections of glutaraldehyde-fixed and Epon-embedded samples were cut with a LKB Ultramicrotome using a diamond knife and picked up on gold grids (300 µm mesh) covered with carbon coated formver film. The sections were etched in a 5% aqueous solution of H_2O_2 for 5 to 10 min; washed twice in PBS buffer (20 mM phosphate buffer, pH 7.4 and 0.9% NaCl) containing 0.1% Tween-20. Sections were incubated for 1 h in primary antibodies (rabbit anti-nitrogenase Fe-protein, GS, PE, RuBisCo or hydrogenase) diluted 1:10 (v/v) in PBS buffer containing tween-20 and 1% bovine serum albumin. Thereafter, the grids were washed twice in PBS buffer and incubated for 30 min in secondary

antibodies (goat anti-rabbit IgG conjugated to colloidal gold) diluted 1:20 (v/v) in PBS buffer containing 1% bovine serum albumin and 0.1% Tween-20 and then washed twice in PBS, once in distilled water and finally in double distilled water using a gentle jet wash. These were left to air dry and then stained with 4% aqueous uranyl acetate for 15 min. After washing these were then stained in lead citrate for 5 min. These were then washed again and dried. Sections were observed under a Transmission Electron Microscope and for quantitation, 10-15 photographs were taken.

2.14 Transmission electron microscopy and quantitation of immunolabel:

Transmission electron microscopy (TEM) was performed using a Zeiss EM 10 transmission electron microscope operated at 60 kV. From the TEM photographic prints, relative levels of various antigens were estimated by counting impressions of gold particles in various cell types. They were converted to number of gold particles per μm^2 cell area taking into account the magnification of the prints used and the area counted. In total, 100 μm^2 cell area was counted in each case and the values presented are means (±SE) from 10-15 counts. In control experiments primary antibody was omitted during immunolabelling. For background labelling calculations, a similar procedure was followed by counting gold particles per unit area outside the cells.

2.15 Immunospecific Western blotting:

Immunospecific western blotting was performed as detailed earlier (Braun-Howland *et al.*, 1988). Cyanobacterial cells were harvested by centrifugation (3000 *g*) for 5 min. The pellet was then resuspended in sodium dodecyl sulphate (SDS)-sample buffer (1:1, v/v). The sample buffer consists of 10 mM-Tris/HCl (pH 8.0), 1.0 mM Ethylene diamine tetra acetic acid (EDTA; sodium salt), 2.5% (w/v) SDS, 5.0% (v/v) β-mercaptoethanol, and 0.01% (w/v) bromophenol-blue. These samples were boiled for 5 min and then centrifuged at 15,000 *g* for 5 min. The supernatant was used as sample for SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) (10-15% gradient) (Laemmli, 1970). The separated proteins were electroblotted onto nitrocellulose membranes using a Sartoblot II semi-dry electroblotting unit (Sartorius, Gottingen, FRG). Electroblotting was completed at 4 mA cm^{-2} in 15 min. Six layers of filter paper soaked in transfer buffer containing 48 mM Tris-HCl (pH 9.2), 39 mM glycine, 1.3 mM SDS and 20% (v/v) methanol were used on both sides of the gel with membrane. The membrane was then incubated for 1 h with gentle agitation in a blocking solution containing 20 mM Tris-HCl (pH 7.5), 500 mM NaCl (Tris-buffered saline, TBS) and 3% (w/v gelatine). The membrane was then washed twice in TTBS [0.05% (v/v) Tween-20 in TBS] and then incubated with the primary antibody (1:500 dilution in TTBS supplemented with 1% (w/v) gelatine) for 2 h to overnight. After thorough washing of the membrane in TTBS the second antibody goat anti-rabbit IgG

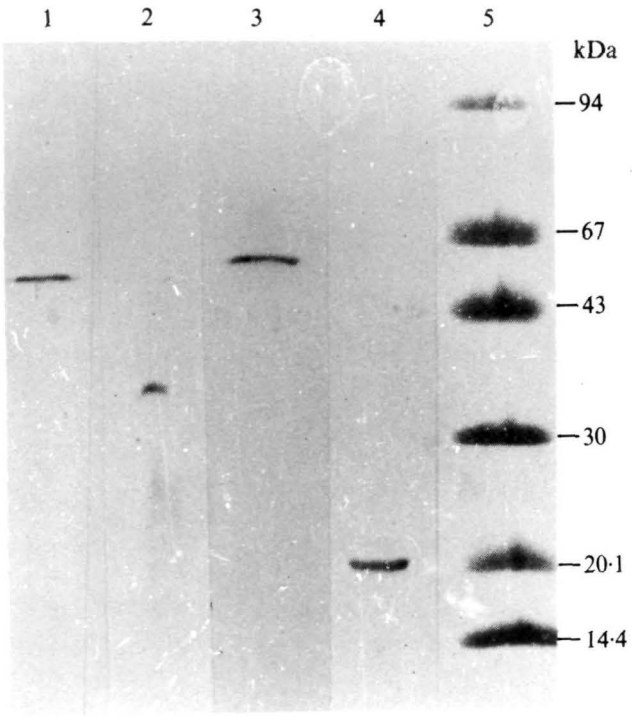
conjugated to horseradish peroxidase (Janssen Pharmaceutica, Beerse, Belgium), was applied in a 1:3000 dilution in TTBS and incubation was carried out for 1 h. The horseradish peroxidase (HRP) colour development was carried out as per instructions of the manufacturer.

The specificity of the antibodies against nitrogenase Fe-protein, RuBisCo, GS and PE were tested against crude extracts of N_2-fixing *P. boryanum* cells by western blotting (Fig. 2.1). All antibodies were found to be monospecific, recognizing a single polypeptide of known subunit molecular mass relating to their respective antigens (nitrogenase Fe-protein, 36 KDa; GS, 53 KDa; RuBisCo, 56 KDa; PE, 20 KDa). Specificity of these antibodies have also been tested against heterocystous cyanobacteria and found to be monospecific (Bergman & Rai, 1989). Hydrogenase antibodies have already been shown to be monospecific recognizing a single polypeptide of molecular mass 55 KDa, and have been previously used for specific localization of hydrogenase in *Nostoc* sp. (Lindblad & Sellstedt, 1990).

2.16 Immobilization:

Immobilization was done as described by Musgrave *et al*, (1982). Cells were harvested by centrifuging cyanobacterial culture at 2000 *g* for 15 min and resuspended in 50 ml of fresh $BG\text{-}11_o$ medium. 3 g alginic acid (sodium salt) (Sigma, USA) was dissolved in 50 ml of double distilled water at 45° C and allowed to cool to room temperature. The cyanobacterial suspension and

Fig. 2.1. SDS-PAGE and immunoblotting of cell extracts from N_2-fixing *P. boryanum*. Nitrate-grown cells were transferred to N_2-medium and nitrogenase induced as described in Chapter 5 (Fig. 5.1b). Immunoblotts of GS (lane 1), nitrogenase Fe-protein (lane 2), RuBisCo (lane 3) and PE (lane 4) are shown. Lane 5, molecular mass markers.


1
2
3
4
5
kDa
94
67
43
30
20·1
14·4

the alginic acid solution were mixed thoroughly and calcium alginate beads were prepared by dropping the mixture drop by drop into a solution of 0.1 M calcium chloride using a syringe canula. The beads (about the size of 2 mm diameter) remained in the calcium chloride solution for 30 min at 5° C for hardening. These beads were then harvested and rinsed with fresh $BG\text{-}11_o$ medium before proceeding with further experiments.

2.17 Chemicals and gases:

All the supplies for electron microscopy were obtained from Agar Aids, for electrophoresis from phermacia and for immunoblotting from Bio-Rad. All other chemicals were from Sigma. Gases were obtained from AGA Special Gases, Stockholm; Matheson (USA) and Indian Oxygen Ltd.

CHAPTER 3

GENERAL CHARACTERIZATION OF THE SYMBIOTIC, FREE-LIVING, IMMOBILIZED AND RECONSTITUTED *NOSTOC* ANTH CYANOBIONT

3.1 Introduction:

The nitrogen-fixing cyanobacteria when occurring in symbiosis undergo various morphological, structural, physiological and biochemical changes to facilitate the development of the symbiosis (*see* Rai, 1990; Smith & Douglas, 1987; Gallon & Chaplin, 1987). In the present study of *Anthoceros-Nostoc* symbiosis, the *Nostoc* colonies are found to occur within the mucillage-filled cavities on the undersurface of the *Anthoceros* gametophyte thalli as dark green compact, round spots of about 0.6-0.7 mm in diameter. In symbiosis, 40-45% of the cyanobacterial cells change to heterocysts to provide the fixed-nitrogen demand of the eukaryotic partner and the cyanobiont itself fixes very little or no carbon. The demand for the fixed carbon by the cyanobiont is met by the eukaryotic partner (Rodgers & Stewart, 1977). In case of bryophyte-cyanobacterial symbiosis, nitrogen fixation by the cyanobacterial partner allows the eukaryotic partner to grow well over a wide range of environmental conditions (pH, temperature, light and moisture content) in the absence of other sources of combined nitrogen (Rodgers & Stewart, 1977). It has already been pointed out that ammonia is the preferred source of inorganic nitrogen in

cyanobacteria and excess of ammonia leads to repression of nitrogenase (Stewart, 1980; Singh *et al.*, 1983; Macherras & Smith, 1986) and utilization of other inorganic/organic nitrogen compounds (Guerrero *et al.*, 1981; Bagchi *et al.*, 1985b). Moreover, ammonia is an obligate intermediate of N_2 and nitrate assimilation in these microbes. It is interesting to note the effects of different sugars on growth and nitrogenase activity of the cyanobiont and to determine the preferred fixed-carbon source of cyanobiont, which, in natural symbiosis is provided for by the eukaryotic partner for best symbiotic results.

A better understanding of free-living ***Nostoc* ANTH** in terms of growth, heterocyst differentiation, photosynthetic oxygen evolution, respiratory oxygen consumption and effects of various carbon and nitrogen sources on the growth and nitrogenase activity is essential to appreciate the morphological and physiological modifications that occur when free-living ***Nostoc* ANTH** enters into symbiotic association with its eukaryotic partner ***Anthoceros***.

3.2 Methods and materials:

Nostoc* ANTH** was isolated from ***Anthoceros gametophytic thalli, then purified and grown in axenic aerated batch cultures in $BG\text{-}11_o$ medium (Rippka *et al.*, 1979), at 25 ± 1° C at a photon fluence rate of 50 μmol m^{-2} s^{-1} (for details see **"MATERIALS AND METHODS"**). As and when required different sugars were added to the growth medium as fixed-carbon source, and NH_4Cl, KNO_3 and

glutamine as nitrogen source to a desired final concentration. The medium was buffered with 10 mM lit^{-1} HEPES-NaOH (pH 7.5).

Chl *a*, photosynthetic O_2 evolution, respiratory O_2 consumption, nitrogenase activity and specific growth rate were measured as detailed in "**MATERIALS AND METHODS**".

3.3 Characterization of symbiotic *Nostoc* ANTH (cyanobiont):

Freshly isolated *Nostoc* ANTH from the undersurface of the *Anthoceros* gametophytes was characterized in terms of morphological changes, heterocyst differentiation and nitrogenase activity before purification and culturing in the N_2-medium.

Microscopic studies of freshly isolated symbiotic *Nostoc* of *Anthoceros-Nostoc* association showed marked difference in the cellular morphology from the free-living cultures of the same strain. Under the pressure of a microscopic slide cover slip, there is predominance of single or double cells instead of filaments in case of symbiotically associated *Nostoc*. It seems that the connections between vegetative cells, and between heterocysts and adjacent vegetative cells are rather fragile. The vegetative cells were bigger and though somewhat distorted, were spherical. such vegetative cells contained numerous cyanophycin granules which serve as N-reserve thereby indicating that these are not N-starved (Fig. 3.1). The polar nodules of the heterocysts were less distinct. The most pronounced morphological change in the cyanobiont was its much higher heterocyst frequency compared to the free-living counterpart. Table 3.1 shows the

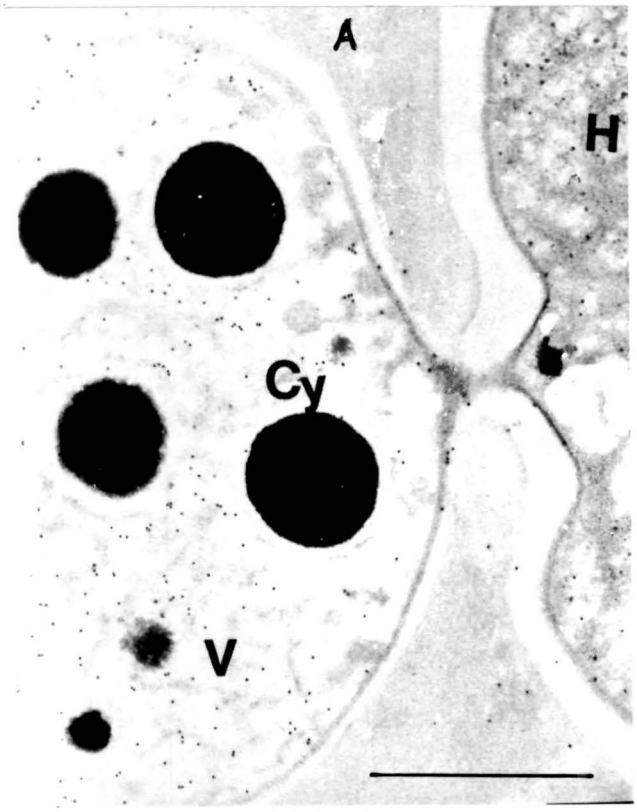

A
H
Cy
V

Table: 3. 1. Heterocyst frequency and nitrogenase activity of the *Nostoc* ANTH cyanobiont taken from different parts of the *Anthoceros* gametophyte:

Portions ofthe gametophyte	Heterocyst frequency (%)	Nitrogenase activity (nmol C_2H_4 produced mg^{-1} dry wt. h^{-1})
Tip	2.91	1.456
Middle	19.20	2.018
Base	45.80	1.135

heterocyst frequency of symbiotic ***Nostoc*** in the young, mature and old colonies from the tip, middle and basal portions of the ***Anthoceros*** gametophyte. Heterocyst frequency of 2.9%, 19.2% and 45.8% were noted in the young, mature and old colonies, respectively. This agrees with the findings of Rodgers & Stewart (1977) that the heterocyst frequency of the cyanobiont in association with bryophytes can vary with the age of colony, as was also the case with ***Nostoc*** cyanobiont in Lichens (Englund, 1977) and cycads (Lindblad *et al.*, 1985). The nitrogenase activity was 1.456, 2.018 and 1.135 nmol C_2H_4 produced mg^{-1} dry wt. h^{-1} in the tip, middle and basal portions of the gametophyte, respectively. Nitrogenase activity was maximum in the mature colonies rather than the old colonies where heterocyst frequency was found to be maximum. This may be due to the occurrence of many double or multiple heterocysts noted in the cyanobiont residing in the old colonies occurring towards the basal portion of the ***Anthoceros*** gametophyte. Such heterocysts may be mixture of healthy and decaying heterocysts lacking nitrogenase and/or nonfunctional heterocysts lacking nitrogenase activity due to lack of C-supply/restricted C-supply since those heterocysts in the middle do not have connecting vegetative cells. Multiple heterocysts in ***Anthoceros*** cyanobiont are known to contain nitrogenase (*see* chapter 4). therefore, restrictive fixed carbon supply to the multiple heterocysts is certainly one of the reasons for lower level of nitrogenase activity in the older colonies, a case similar to the cycad cyanobiont (Bergman *et al.*, 1986). Although the fixed carbon is supplied by the eukaryotic

partner (Rodgers & Stewart, 1977), it probably enters heterocysts via vegetative cells and heterocysts occurring in a chain with no direct contact with vegetative cells may receive little or no fixed carbon at all leading to insufficient amount of carbohydrate content for the efficient functioning of nitrogenase enzyme.

3.4 Characterization of free-living cultured *Nostoc* ANTH cyanobiont:

The free-living *Nostoc* ANTH was characterized in terms of growth (chlorophyll *a*), heterocyst differentiation (heterocyst frequency), nitrogen-fixation (nitrogenase activity), photosynthesis (O_2-evolution) and respiration (O_2-consumption).

3.4.1 Growth:

The growth of *Nostoc* ANTH in terms of chlorophyll *a* content at the expense of N_2, NH_4^+ and NO_3^- is shown in the Fig. 3.2. The cyanobiont was capable of utilizing N_2 as nitrogen source for its growth. The addition of NH_4^+ or NO_3^- in the medium increased the growth. These results suggest that *Nostoc* ANTH in free-living state can utilize N_2, NH_4^+, and NO_3^- as nitrogen source like other free-living cyanobacteria, and that NH_4^+ and NO_3^- serve as better sources of nitrogen than N_2.

3.4.2. Effects of sugars on the growth:

In symbiosis, host *Anthoceros* meets the fixed-carbon

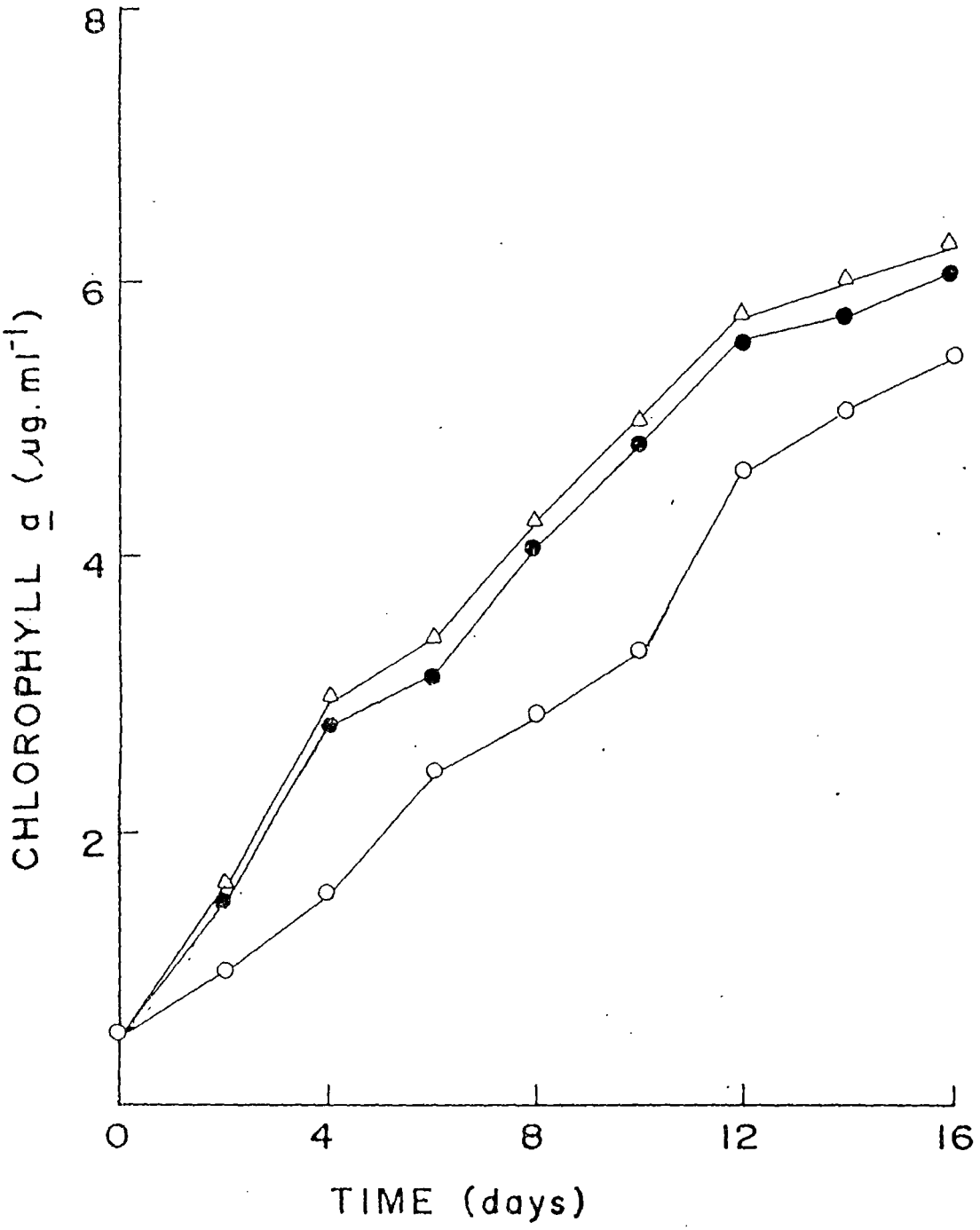


Fig. 3.2. Growth of *Nostoc* ANTH in BG-11_0 medium (○), BG-11_0 + 5 mM KNO_3 (●) and BG11_0 + 2 mM NH_4Cl (△).

demand of the cyanobiont *Nostoc* (Stewart & Rodgers, 1977). To determine whether the isolated cyanobiont in its free-living state can utilize various sugars as carbon source for its growth, the growth of *Nostoc* ANTH was measured in the absence and presence of glucose, fructose, sucrose, galactose, maltose, lactose, mannose, xylose, arabinose and rhamnose.

Figs. 3.3 to 3.7 show the growth of *Nostoc* ANTH in terms of chlorophyll *a* in the presence and absence of different sugars. It is evident from the data of figs. 3.3 to 3.7 that all the sugars employed, except mannose, arabinose and rhamnose, enhanced the growth of *Nostoc* ANTH in presence of light. The optimum concentration of the sugars that supported the best growth were: glucose (50 mM), fructose (50 mM), maltose (50 mM), sucrose (20 mM), galactose (20 mM), lactose (20 mM). It is clear from the above growth experiment that the requisite concentration of sugars for best growth of free-living *Nostoc* ANTH varied significantly. These results suggest that *Nostoc* ANTH may vary in its ability to utilize different carbon sources. Glucose was found to enhance the growth of *Nostoc* ANTH considerably much more than any other sugar employed for the growth experiment, suggesting that glucose is best favoured by the cyanobiont as fixed carbon source in its free-living state and the cyanobiont possess an efficient uptake and metabolism mechanism for this sugar. It is probable that in natural symbiosis, glucose is the form of fixed-carbon that is transported from host *Anthoceros* to the cyanobiont. The slower growth stimulation by fructose,

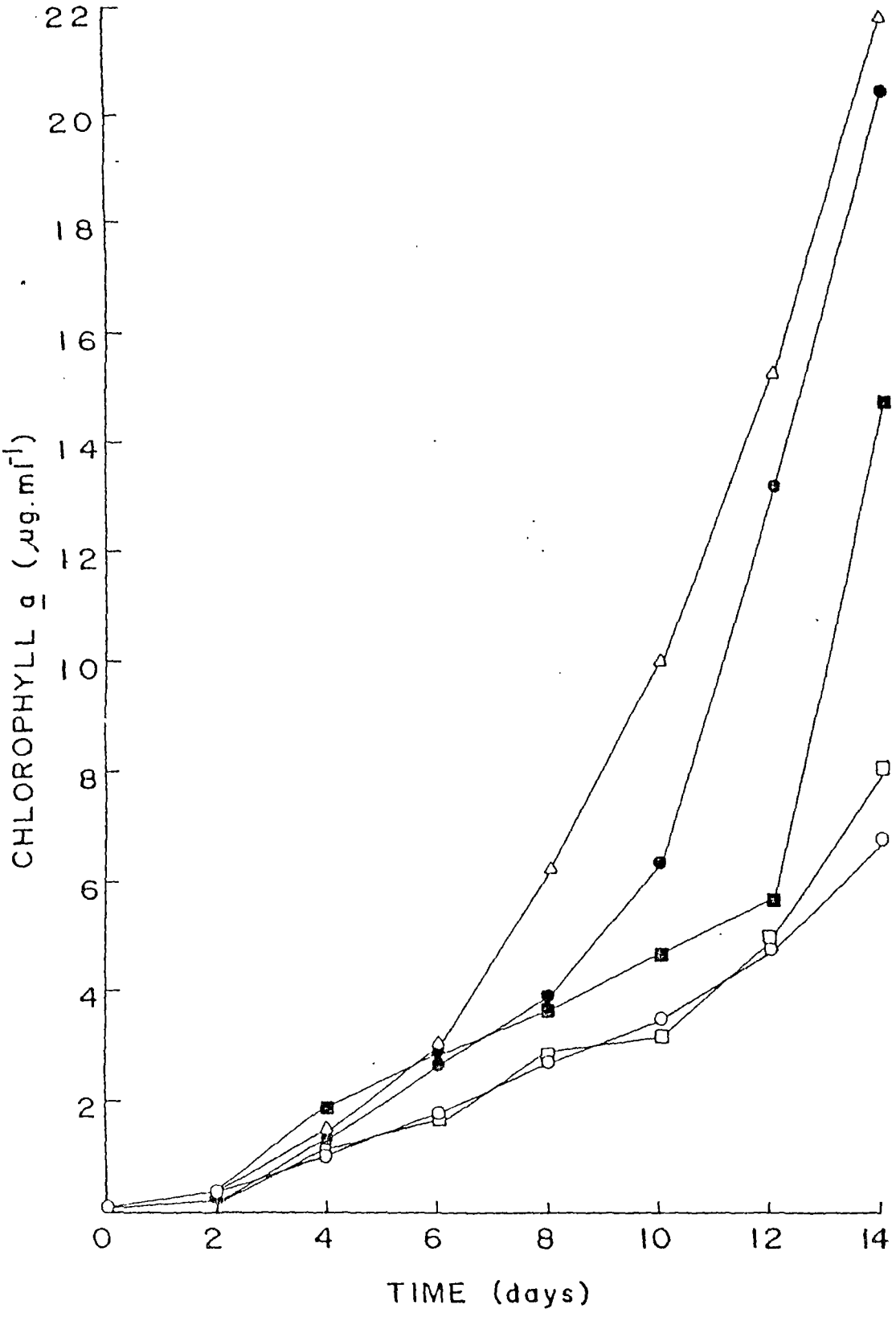


Fig. 3.3. Photoheterotrophic growth of *Nostoc* ANTH on BG-11_0 medium (○), BG-11_0 medium + glucose (●, 20 mM; △, 50 mM) and BG-11_0 + Fructose (□, 20 mM; ■, 50 mM).

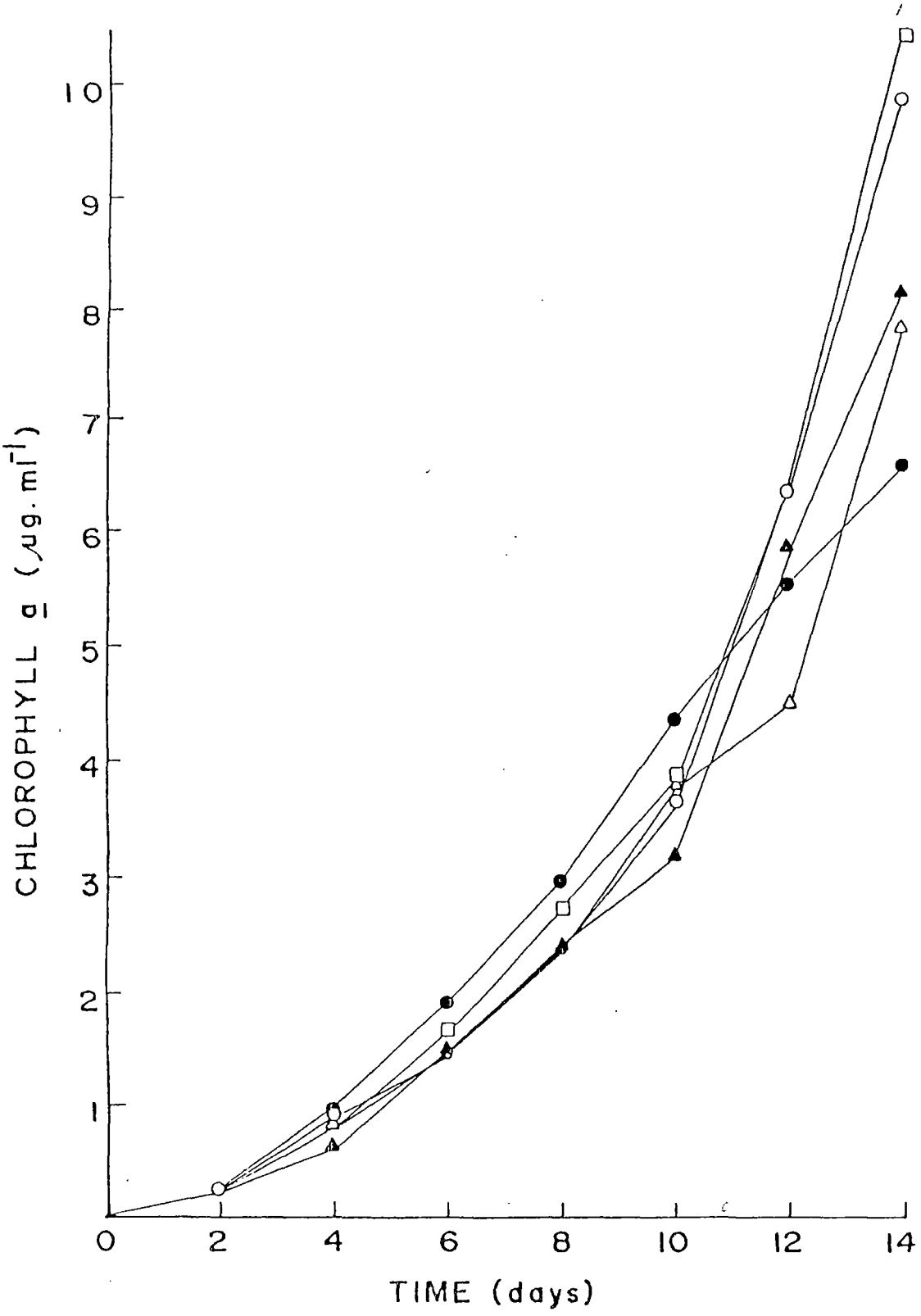


Fig. 3.4. Photoheterotrophic growth of *Nostoc* ANTH on BG-11_0 medium + sucrose (○, 20 mM; ●, 50 mM) and BG-11_0 + maltose (△, 20 mM;▲, 40 mM;□, 50 mM).

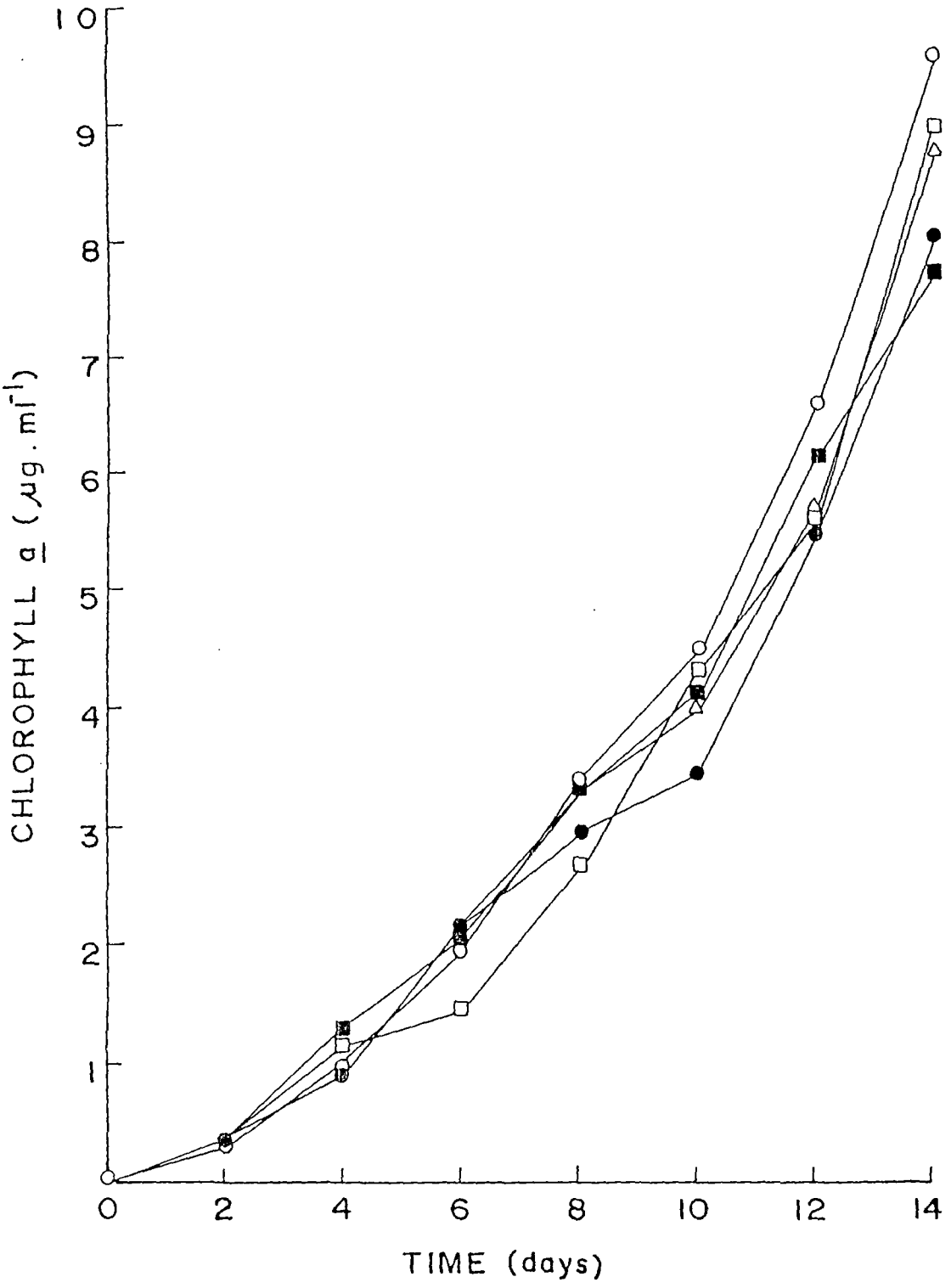


Fig. 3.5. Photoheterotrophic growth of *Nostoc* ANTH on BG-11_0 medium + lactose (○, 20 mM; ●, 30 mM; △, 50 mM) and BG-11_0 + galactose (□, 20 mM; ■, 40 mM).

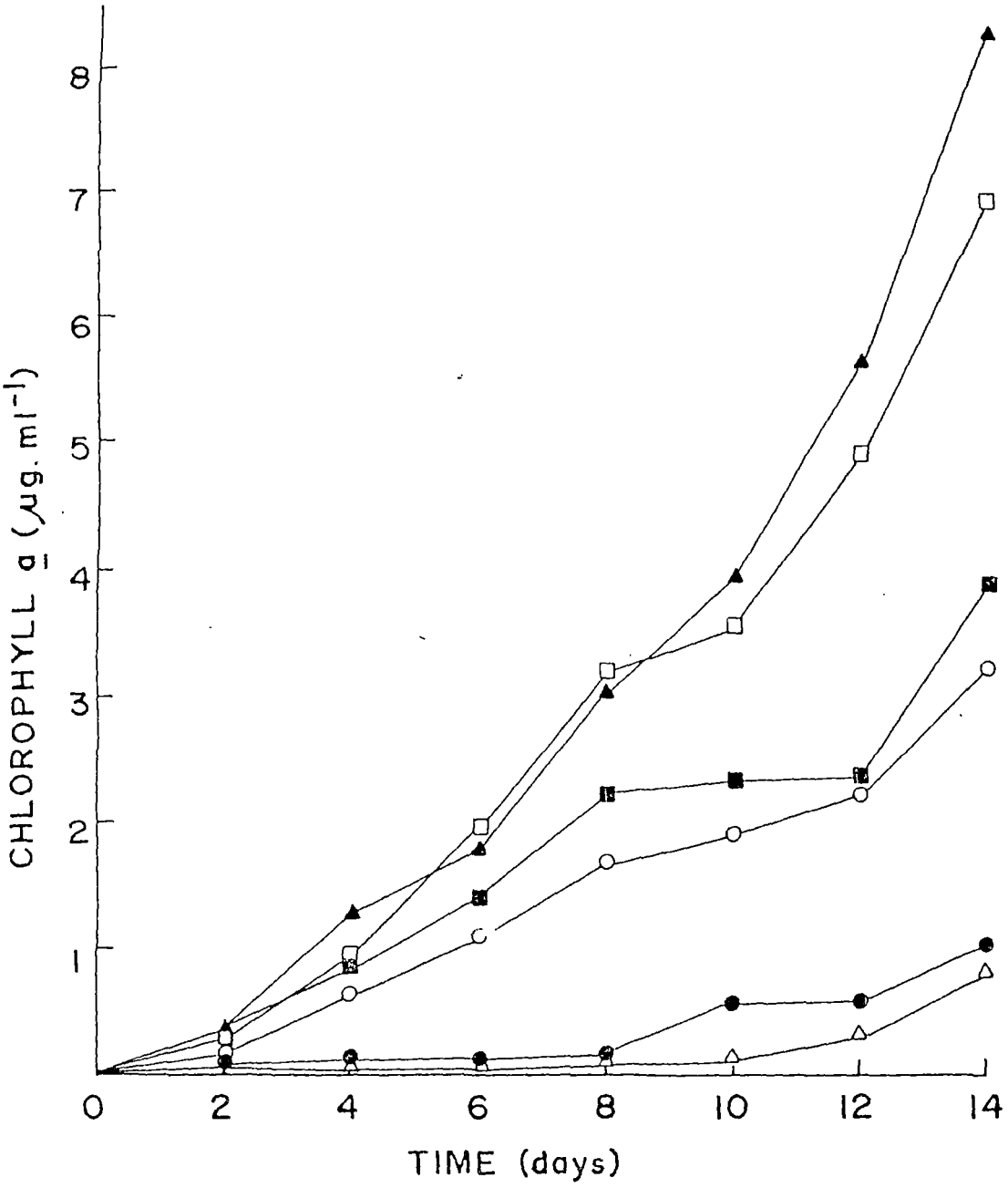


Fig. 3.6. Photoheterotrophic growth of *Nostoc* ANTH on BG-11_0 medium + mannose (○, 5 mM; ●, 10 mM; △, 20 mM) and BG-11_0 medium + Xylose (▲, 5 mM; □, 10.mM; ■, 50 mM).

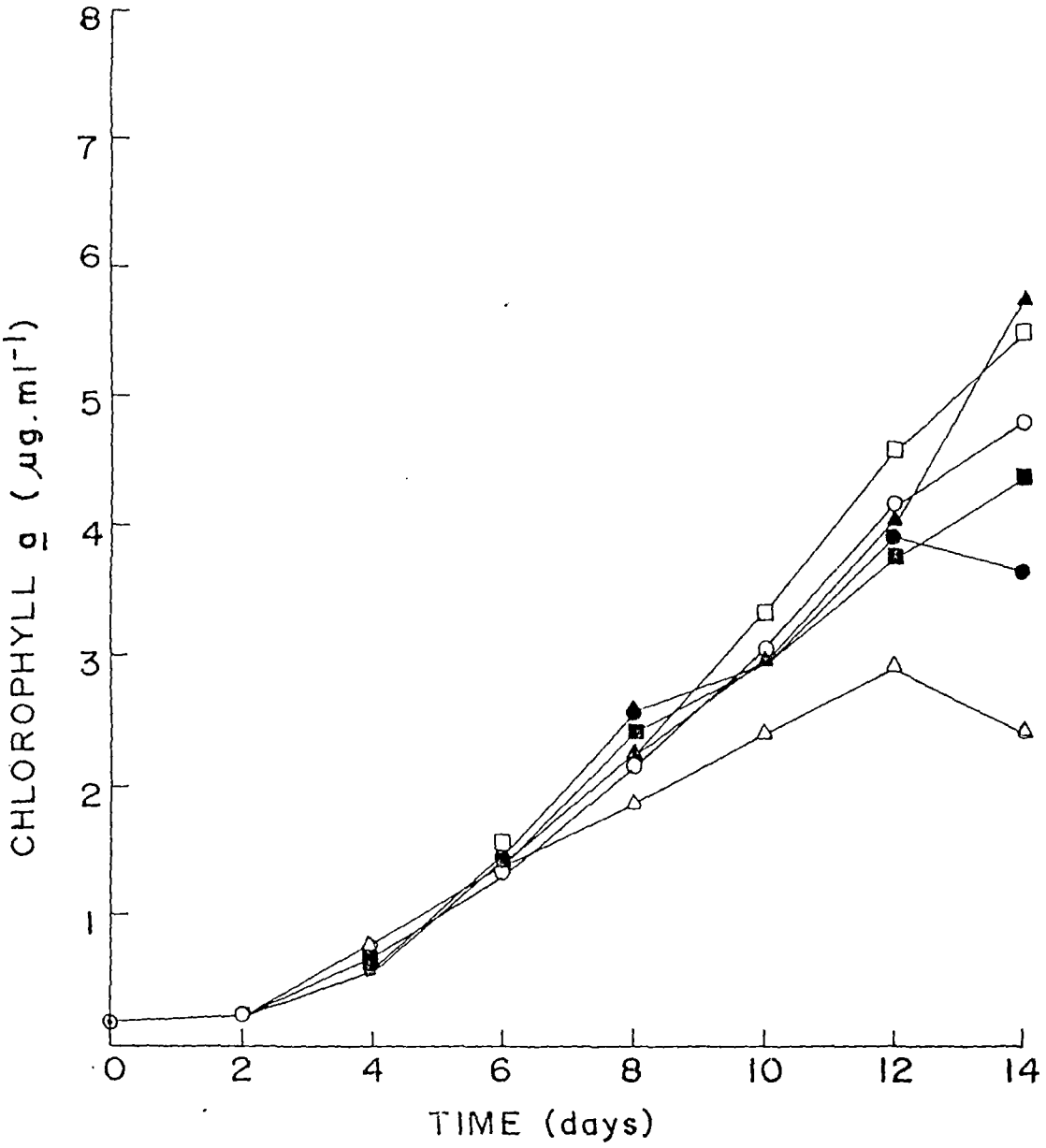


Fig. 3.7. Photoheterotrophic growth of *Nostoc* ANTH on BG-11_0 medium + arabinose (○, 5 mM; ●, 10 mM; △, 50 mM) and BG-11_0 medium + rhamnose (▲, 5 mM; □, 10 mM; ■, 20 mM).

maltose, sucrose, lactose, galactose can be explained by slower uptake and/or a less efficient metabolism of these sugars by ***Nostoc*** **ANTH**. The fact that growth was poorly supported by mannose, arabinose, xylose and rhamnose indicates that there may be a less efficient uptake system in the cyanobiont for these sugars.

3.4.3 Effects of nitrogenous compounds on nitrogenase activity:

For the determination of nitrogenase activity exponentially growing cultures were used. Whenever needed, NH_4Cl, KNO_3 and glutamine were added to the medium (final concentration of 1 mM, 5 mM and 5 mM, respectively).

Fig. 3.8 presents data on the nitrogenase activity of the whole filaments of the ***Nostoc*** **ANTH** in the presence and absence of various nitrogen sources and MSX (a glutamate analogue and an irreversible inhibitor of glutamine synthetase). It is evident from data of fig. 3.8 that ***Nostoc*** **ANTH** was able to fix N_2 and utilized it as nitrogen source. Addition of NH_4^+ to the N_2-grown cells resulted in significant inhibition (64%) of nitrogenase activity within 2 h. The inhibition of nitrogenase activity by NH_4^+ could have been exerted by NH_4^+ itself of by a product of NH_4^+ assimilation. MSX was used to distinguish between these two alternatives. MSX causes drastic irreversible inhibition of GS, thus preventing the assimilation of NH_4^+ by this route. Addition of MSX to the medium overcame the inhibitory effect of ammonia

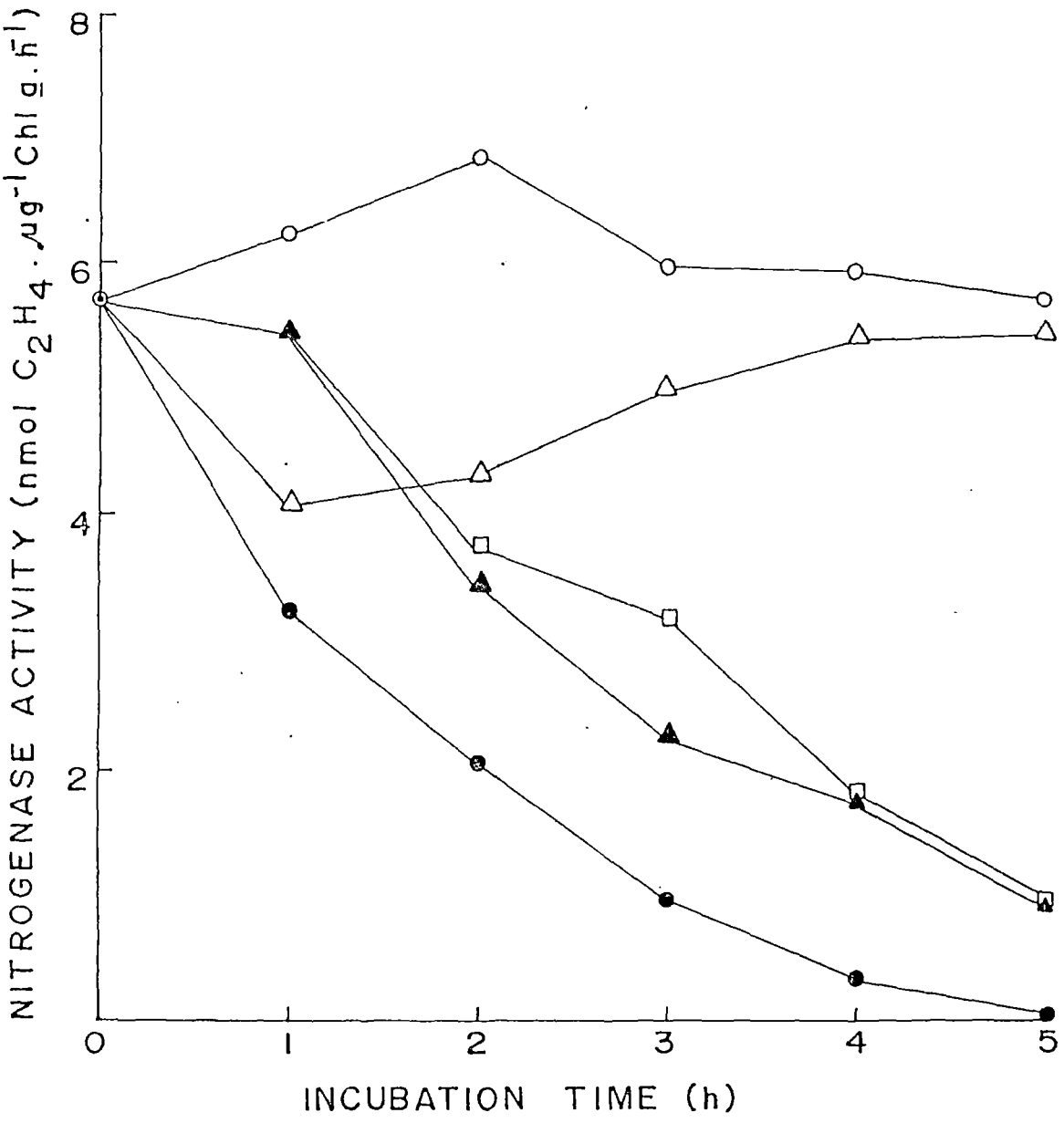


Fig. 3.8. Effect of ammonium and glutamine on the nitrogenase activity on *Nostoc* ANTH. ○, N_2-medium; ●, NH_4^+-medium (N_2-medium supplemented with 2 mM NH_4Cl); △, NH_4^+-medium + 10 μm MSX; ▲, glutamine medium (N_2-medium supplemented with 5 mM glutamine); □, glutamine medium + MSX.

(Fig. 3.8). The transient decline in nitrogenase activity was probably due to time taken for inhibition of GS activity by MSX. These results indicate that NH_4^+ assimilation through GS is necessary for NH_4^+ to repress nitrogenase. Repression of nitrogenase does not therefore, appear to be exerted by NH_4^+ itself or atleast not by NH_4^+ alone, but it requires the activity of GS and probably involves the participation of an organic nitrogen.

Glutamine is the primary product of ammonia assimilation in cyanobacteria (Meeks *et al.*, 1977; Wolk *et al.*, 1976). To determine whether inhibition of nitrogenase activity is related to production of glutamine via GS, or not, glutamine was added to N_2-grown cultures. The addition of glutamine to the cultures caused about 60% inhibition in nitrogenase activity within 3 h. The effect of glutamine on nitrogenase activity was also observed in the presence of MSX. These results indicate that unlike ammonium, glutamine inhibits nitrogenase activity both in MSX-treated and untreated cells and therfore, it is concluded that an organic nitrogenous compound which may be glutamine itself (or a glutamine derivative) acts as an inhibitor of nitrogenase activity in the cyanobiont *Nostoc* ANTH.

The Fig. 3.9 shows the changes in the nitrogenase activity level in *Nostoc* ANTH cells grown in N_2-medium and then transferred to nitrate containing medium. N_2-grown cells showed a higher level of nitrogenase activity. Addition of nitrate to the N_2-grown cells, resulted in a decrease in the nitrogenase

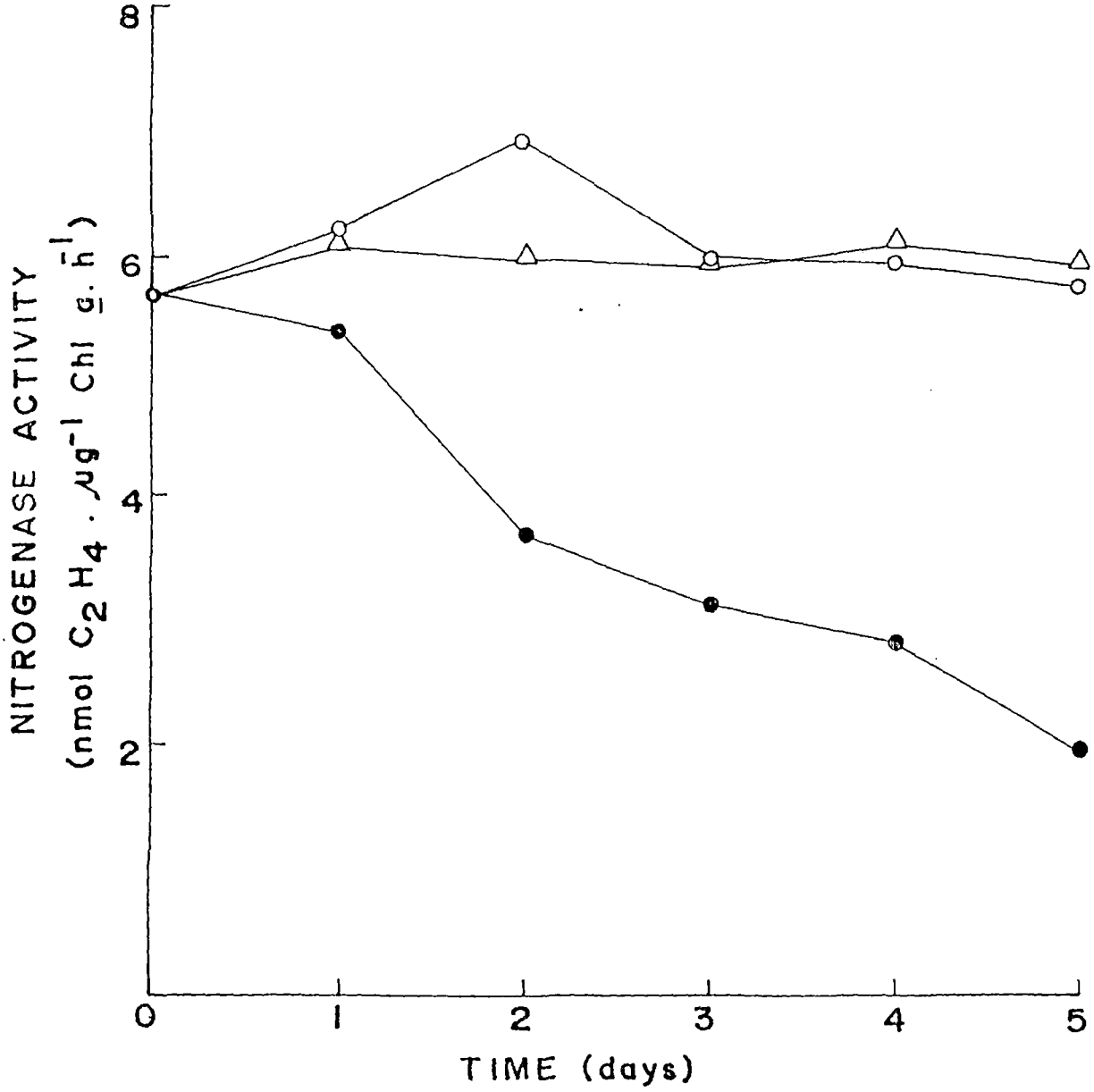


Fig. 3.9. Effect of nitrate on the nitrogenase activity of *Nostoc* ANTH. ○, N_2-medium; ●, nitrate-medium (N_2-medium supplemented with 2 mM calcium nitrate); △, nitrate-medium + 10 μm MSX.

activity after a lag period of 1 h. This time lag was consistent with the necessity for the nitrate to be first reduced to nitrite and then to ammonia. After 3 to 4 h, nitrogenase activity dropped by 50% of its original value. This result suggest that nitrate induced inhibition of nitrogenase activity is not exerted by nitrate itself but it requires the reduction of nitrate.

Further to determine whether the nitrate inhibition of nitrogenase activity was caused by nitrate itself or by a product of ammonia assimilation, experiments were conducted in which GS, the primary enzyme of ammonia assimilation, had been inactivated by MSX. The addition of MSX to the medium prevented the nitrate inhibition of nitrogenase activity. This suggests that nitrate inhibition of nitrogenase activity is neither exerted by nitrate itself nor by product of its mere reduction to ammonia but the assimilation of the latter is necessarily required for the inhibition of nitrogenase in ***Nostoc*** **ANTH.**

It is interesting to note here that while addition of ammonia, eventually led to total inhibition of nitrogenase activity, NO_3^- did not do so. This is consistent with the observations of Renström-kellner *et al* (1990) on ***Anabaena cylindrica.*** Since heterocysts lack NO_3^--uptake and reductase (Kumar *et al.*, 1985; Rai & Bergman, 1986), those heterocysts which are present in the culture continue fixing nitrogen while new heterocysts are not formed. That is, while ammonia prevents heterocyst development as well as nitrogenase in preformed heterocysts, NO_3^- affects nitrogenase only by preventing

differentiation of heterocysts (Renström-Kellner *et al.*, 1990; Rai, 1992).

3.4.4 Effects of sugars on nitrogenase activity:

The cyanobiont of *Anthoceros punctatus* has been shown to be deficient in carbon fixation by Rodgers & Stewart (1977) and the required fixed carbon moves from the host to the cyanobiont (Stewart & Rodgers, 1977). The heterocysts are deficient in primary carboxylating enzyme, RuBisCo (Codd *et al.*, 1980; Cossar *et al.*, 1985) and in free-living state fixed-carbon moves from vegetative cells to the heterocysts. To determine whether the externally supplied sugars can enhance the nitrogenase activity, the effect of sugars on nitrogenase activity was studied.

The data in the Table 3.2 show that all the sugars used enhanced the nitrogenase activity in ***Nostoc* ANTH** as compared to control cultures (without sugar). Maximum enhancement in the nitrogenase activity was observed in light with glucose as carbon source, indicating that this sugar is favoured by the cyanobiont. This is consistent with the fact that glucose also supported best growth (Fig. 3.3). The fact that nitrogenase activity is poorly supported by fructose, sucrose, maltose, lactose and galactose indicated the presence of a less efficient mechanism of uptake/metabolism of these sugars. These results are in contrast with the results obtained in case of ***Anabaena azollae*** in which fructose supports the best growth (Rosen & Tel-Or, 1986). These results also suggest that although glucose is

Table: 3.2. Effect of sugars on nitrogenase activity in *Nostoc* ANTH. *Nostoc* ANTH cultures grown in glucose, fructose, sucrose, maltose, lactose and galactose (50 mM each) were used for the estimation of nitrogenase activity. Dark conditions were achieved by wrapping the flasks with aluminium foil:

Sugars	Nitrogenase activity (nmol C_2H_4 produced μg^{-1} Chl*a* h^{-1})	
	Light	Dark
Control (without sugars)	3.09	0.08
Glucose	12.85	2.92
Fructose	9.05	1.26
Sucrose	8.65	1.18
Maltose	4.79	1.07
Lactose	4.19	0.12
Galactose	3.55	0.66

the most favoured carbon source for nitrogenase activity yet the cyanobiont cells are not necessarily limited in the use of single photosynthate but possess alternative mechanism to utilize fructose, sucrose, maltose, lactose and galactose as well, although to a lesser extent.

When the *Nostoc* **ANTH** cells were incubated in dark in the presence of glucose, the specific activity of nitrogenase was several fold lower than the cultures incubated with glucose in the presence of light, although it was definitely higher as compared to the control cells incubated in darkness in the absence of sugar. This finding is consistent with all other sugars employed (Table 3.2). The low activity in dark as compared to light was possibly because of limited ATP formation in dark (Bottomley & Stewart, 1977). These results suggest that illumination also plays an important role in the regulation of sugar uptake and nitrogenase activity in the cyanobiont (***Nostoc* ANTH**) and the rate of sugar uptake/transport to heterocysts may be a limiting factor for nitrogen-fixation.

3.4.5 Effects of combined nitrogen sources and sugars on heterocyst differentiation:

Combined nitrogen sources when available during growth, inhibit the heterocyst differentiation as well as nitrogenase activity in filamentous cyanobacteria (Fogg *et al.*, 1973). To see the effect of combined nitrogen sources and sugars on heterocyst differentiation, heterocyst frequency was calculated in the

cultures grown in the presence of these nitrogen sources and sugars.

To obtain undifferentiated filaments of ***Nostoc* ANTH**, N_2-grown cells were repeatedly transferred and grown in growth medium supplemented with 5 mM KNO_3. They were washed thoroughly with distilled water and inoculated in medium containing different concentrations of KNO_3, NH_4Cl, glucose, fructose, sucrose, maltose, lactose, galactose, mannose and xylose and the heterocyst frequency was recorded and are presented in the Table 3.3. It is evident from the data in Table 3.3 that higher concentration of nitrogen sources significantly inhibited heterocyst differentiation. At a concentration of 1 mM KNO_3 and 0.5 mM NH_4Cl inhibition of heterocyst differentiation were 41% and 43%, respectively. Total inhibition was observed in ***Nostoc* ANTH** cultures grown in 5 mM KNO_3 and 1 mM NH_4Cl, suggesting that ammonium-nitrogen appears to be a stronger inhibitor of heteroyst differentiation as compared to NO_3^-.

The data in Table 3.4 show that all sugars enhanced the heterocyst differentiation in ***Nostoc* ANTH**, except mannose and xylose which also could not enhance growth and nitrogenase activity. These results indicate to the fact that a direct correlation exists between heterocyst differentiation and nitrogenase activity. ***Nostoc* ANTH** cells grown in presence of glucose showed the maximum increase in heterocyst frequency, further indicating that glucose probably is the most favoured

Table: 3.3. Effect of KNO_3 and NH_4Cl on the heterocyst frequency of the free-living *Nostoc* ANTH.

Nitrogen source	Concentration (mM)	Heterocyst frequency (%)
Control	0.0	14.72
KNO_3	1.0	8.69
	5.0	0.00
	10.0	0.00
NH_4Cl	0.1	13.02
	0.5	8.44
	1.0	0.00

Table: 3.4. Effect of sugars on heterocyst frequency of free-living *Nostoc* ANTH:

Sugars	Concentration (mM)	Heterocyst frequency (%)
Control	0.00	13.95
Glucose	10.00	16.91
	15.00	17.78
	20.00	18.19
	25.00	19.94
	50.00	21.64
Fructose	10.00	16.72
	15.00	17.64
	20.00	18.80
	25.00	18.80
	50.00	19.02
Maltose	10.00	16.75
	15.00	18.39
	20.00	19.39
	25.00	20.40
	50.00	21.02
Sucrose	10.00	16.60
	15.00	17.50
	20.00	12.90
	25.00	14.32
	50.00	15.35
Lactose	10.00	14.21
	15.00	15.31
	20.00	16.12
	25.00	18.13
	50.00	18.77
Galactose	10.00	17.00
	15.00	13.03
	20.00	13.98
	25.00	11.80
Xylose	5.00	12.09
	10.00	13.78
	20.00	12.52
Mannose	5.00	6.52
	10.00	9.03

carbon source and there exists an efficient uptake and metabolism mechanism for this sugar in *Nostoc* **ANTH**. Comparatively less increase in heterocyst frequency by other sugars may be due to less efficient uptake and /or metabolism of these sugars. Overall, glucose seems to be the best form of fixed-carbon in *Nostoc* **ANTH** as it supported best growth, nitrogenase activity and also enhanced heterocyst differentiation. These characteristics are similar to the symbiotic *Nostoc* **ANTH**, thus suggesting that glucose seems to be the form of fixed-carbon transported from host *Anthoceros* to symbiont *Nostoc*.

3.4.6 Photosynthesis and respiration:

Photosynthesis and respiration of the cyanobiont was estimated by measuring O_2-evolution and O_2-consumption, respectively. Fig. 3.10 provides data on the trend in photosynthetic O_2-evolution (photosynthesis) and O_2-consumption (respiration) which were measured over a period of 336 h. The free-living *Nostoc* **ANTH** showed a progressive increase in the O_2-evolution and O_2-consumption with increase in time upto 216 h. No further increase in the rate of photosynthesis or respiration was observed beyond this time and it declined after this point to a rate which was equal to the initial value at zero time.

3.5 Characterization of immobilized *Nostoc* ANTH cyanobiont:

In symbiotic condition, growth of *Nostoc* **ANTH** is restricted as the cyanobiont occupies the confined mucilage-

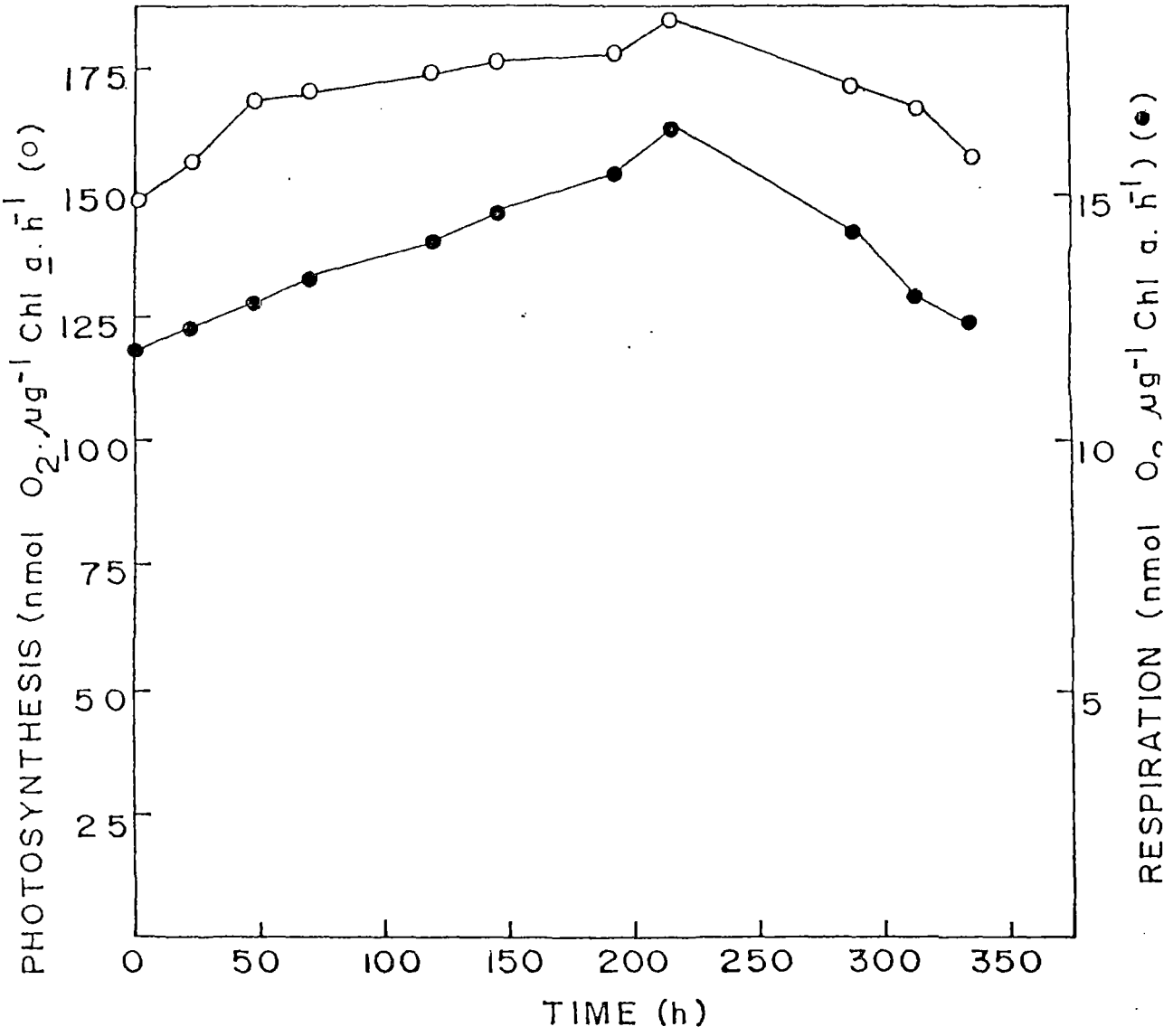


Fig. 3.10. Rates of photosynthetic O_2-evolution (○) and respiratory O_2-consumption (●) by free-living *Nostoc* ANTH.

filled cavities of the *Anthoceros* gametophytic thallus. The process of immobilization also provides similar restricted growth conditions for the cyanobiont. Immobilizing microbial cells leads to increased biocatalytic capacity due to increased cell densities, stabilization of enzyme activities and possibilities of continuous operations (Brouers & Hall, 1986). Rao and Hall (1984) have also shown that immobilization of cyanobacteria could enhance the efficiency of nitrogen-fixation. It has also been shown that metabolic activities of the cells and their efficiency may undergo changes due to immobilization (Webb & Mavituna, 1987; Shi *et al.*, 1987). Further, to study the immobilization caused changes in free-living ***Nostoc* ANTH**, the cells were immobilized in calcium alginate gel. The cyanobiont in the immobilized condition was characterized in terms of growth (Chl *a*), nitrogen-fixation, heterocyst frequency, photosynthetic O_2-evolution and respiratory O_2-consumption.

3.5.1 Growth:

Growth was measured by estimating increase in chlorophyll *a* content of calcium alginate beads. The generation time of immobilized ***Nostoc* ANTH** was found to be 144 h in contrast to its free-living counterpart which had a generation time of 24 h in N_2-medium. Thus, immobilization of the cells reduces the growth of ***Nostoc*** by almost 80% in case of ***Nostoc* ANTH**.

3.5.2 Nitrogen-Fixation:

Fig. 3.11 show the initial effect of entrapment of the cells in calcium alginate gel beads on the ability of ***Nostoc* ANTH** to reduce acetylene. Freshly prepared beads showed only about 18% acetylene reduction activity to that of free-living cells before immobilization (0.9 as against 5.1 nmol C_2H_4 produced μg^{-1} Chl *a* h^{-1}). The nitrogenase activity recovered to 50% of its original value within 48 h (Fig. 3.11) and 100% recovery was obtained between 144 h to 192 h. Nitrogenase activity actually exceeded its original value of 5.1 nmol C_2H_4 produced, chl a^{-1} h^{-1} between the period of 192 h to 240 h, and thereafter it declined and remained constant. This agrees with the findings of Brouers & Hall (1986) that during immobilization there is certain amount of increase in the enzyme activity.

3.5.3 Effects of ammonium and nitrate on the nitrogenase activity of immobilized *Nostoc* ANTH:

Free-living *Nostoc* ANTH showed inhibition of nitrogenase activity when combined nitrogen sources such as ammonium and nitrate were added to the growth medium (Fig. 3.12). To determine the effect of combined nitrogen sources on immobilized cells, the free-living and immobilized cells were grown in the presence of 5 mM KNO_3 and NH_4Cl each. Fig. 3.12 a shows that ammonium and nitrate severely inhibited nitrogenase activity in free-living *Nostoc* ANTH whereas the nitrogenase inhibition by these nitrogen sources was only about 30-40% in immobilized cells (Fig. 3.12 b).

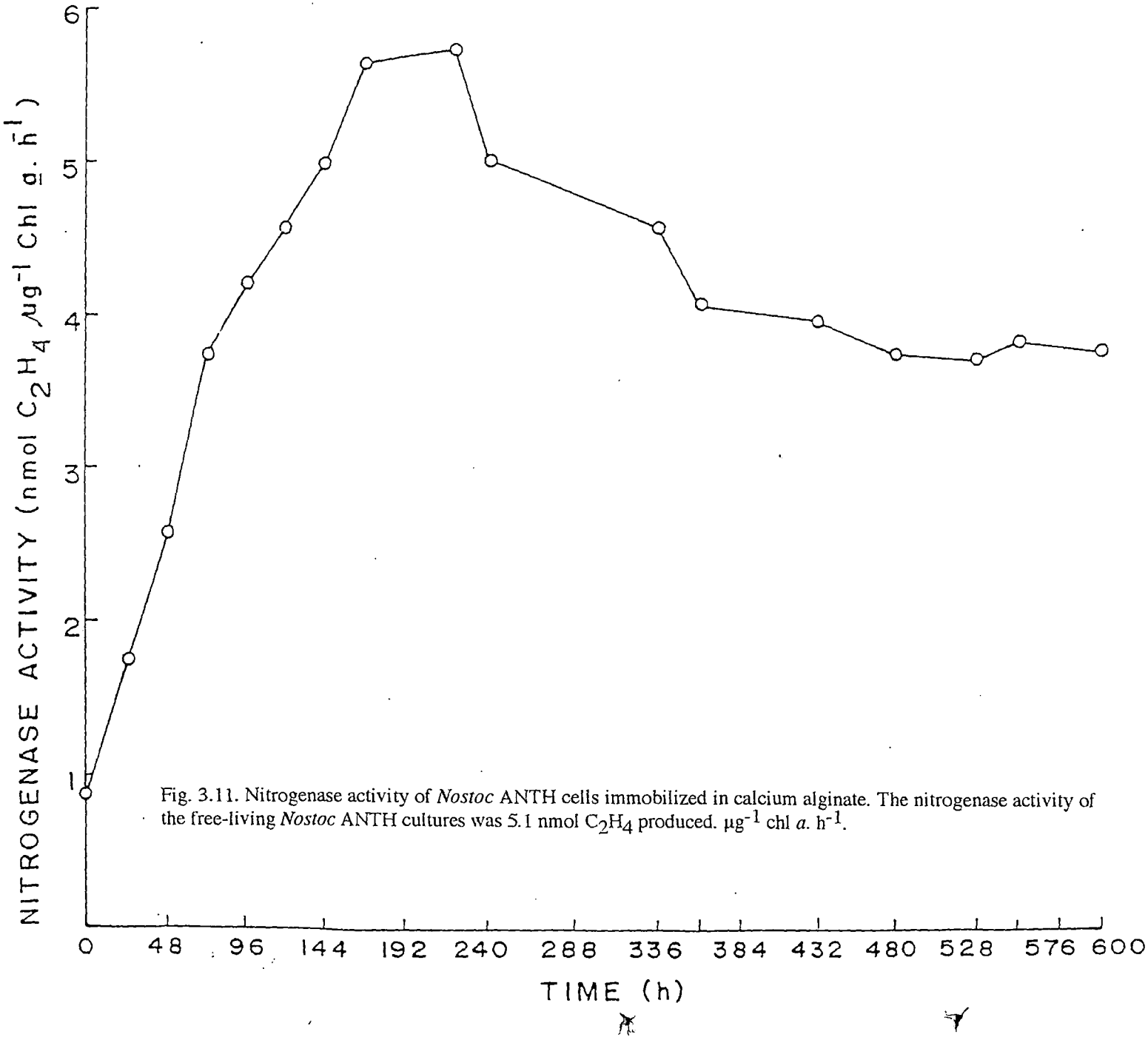
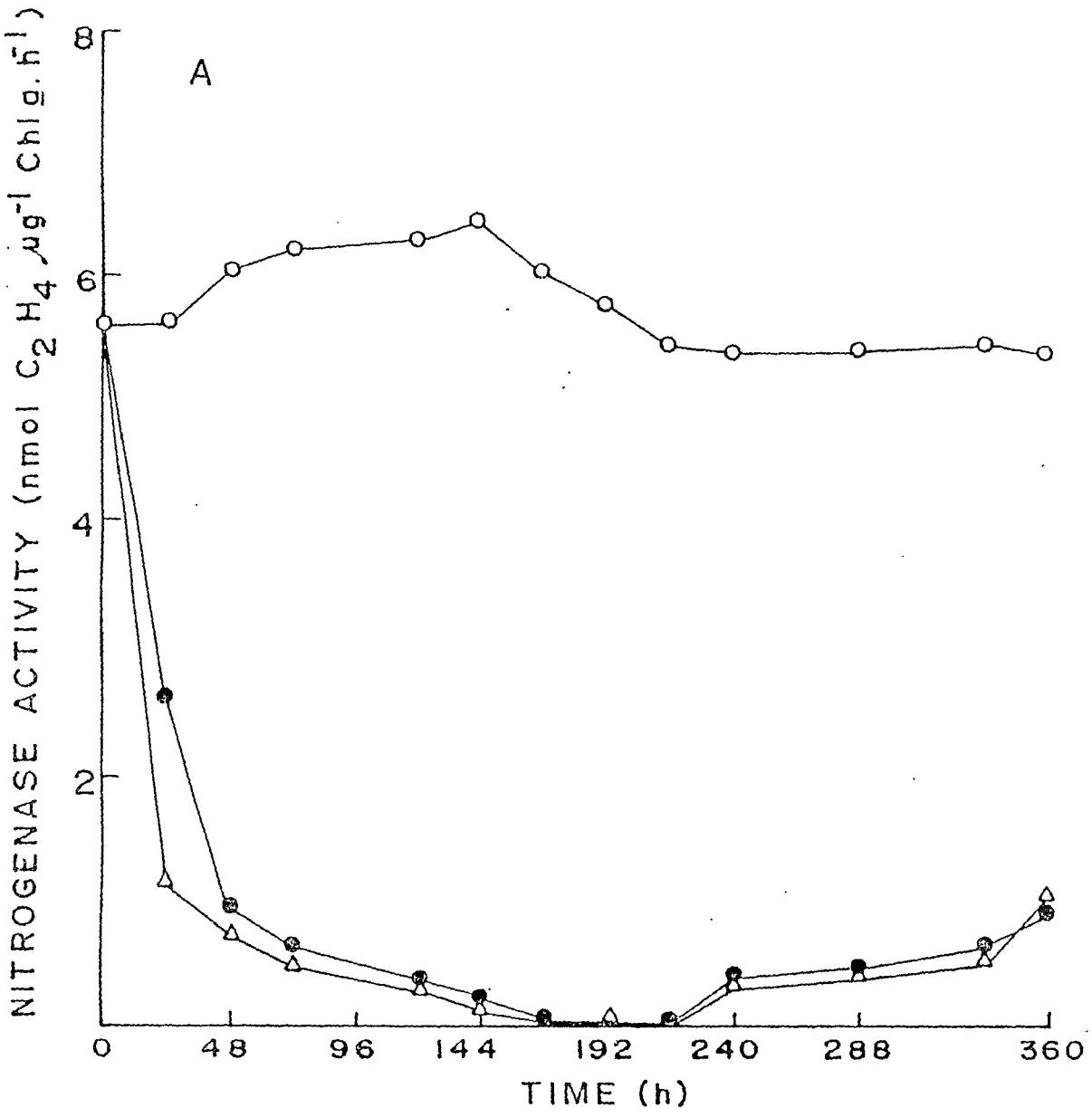
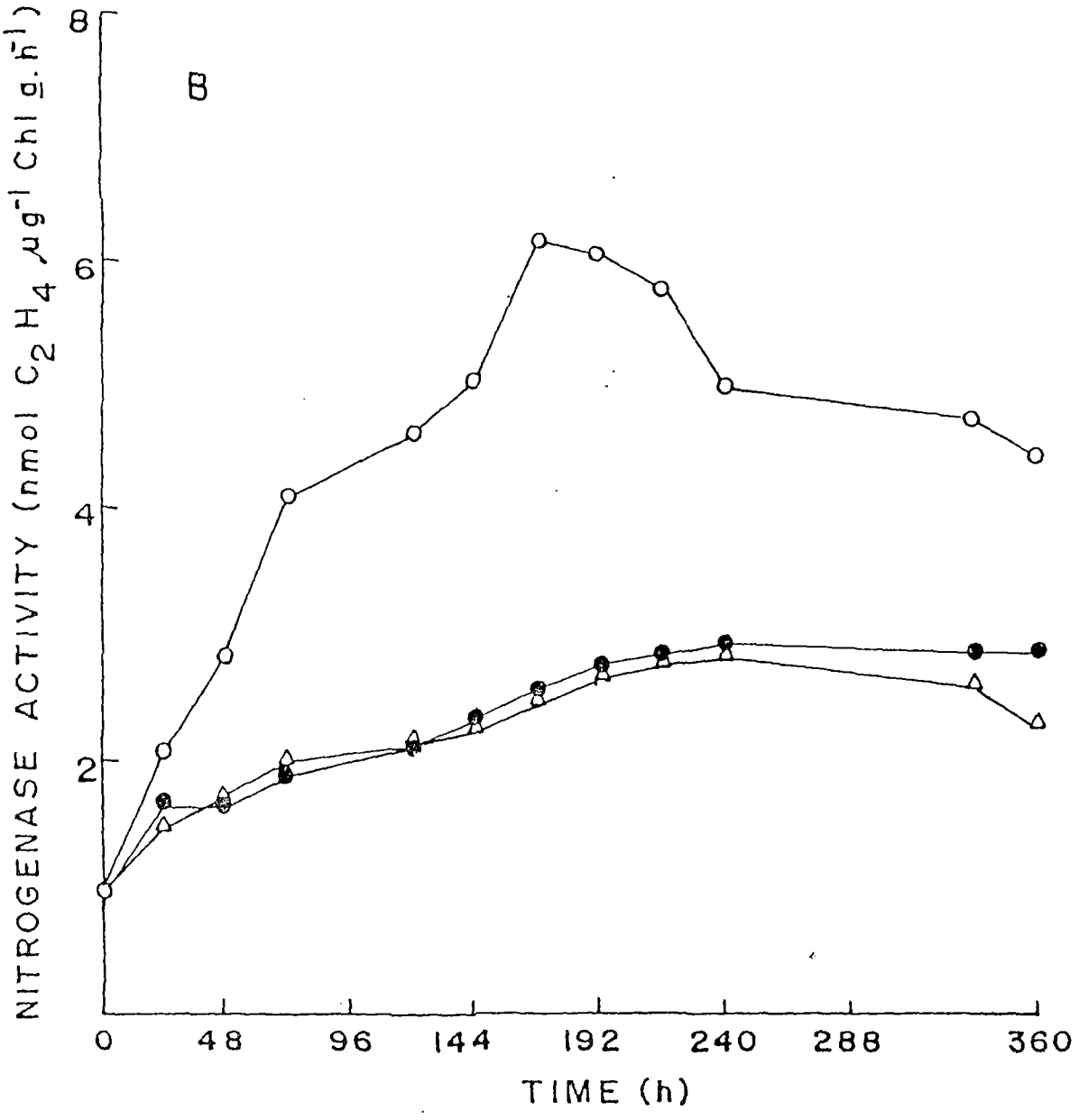


Fig. 3.11. Nitrogenase activity of *Nostoc* ANTH cells immobilized in calcium alginate. The nitrogenase activity of the free-living *Nostoc* ANTH cultures was 5.1 nmol C_2H_4 produced. μg^{-1} chl *a*. h^{-1}.

Fig. 3.12. Effect of nitrate and ammonia on nitrogenase activity of free-living (a) and immobilized (b) *Nostoc* ANTH ○, N_2-medium; ●, nitrate-medium; △, ammonium-medium.


A
NITROGENASE ACTIVITY (nmol C_2H_4 μg^{-1} Chl g. h^{-1})
8
6
4
2
0
48
96
144
192
240
288
360
TIME (h)


B
NITROGENASE ACTIVITY (nmol C_2H_4 μg^{-1} Chl a.h^{-1})
8
6
4
2
0
48
96
144
192
240
288
360
TIME (h)

This indicates that the cyanobiont in the immobilized state can go on fixing atmospheric nitrogen even in the presence of ammonium and nitrate. This may be due to the lower rate of uptake/metabolism of these nitrogen sources by the immobilized cells inside the beads, since the cells grow much slowly and/or the calcium alginate hinders their uptake.

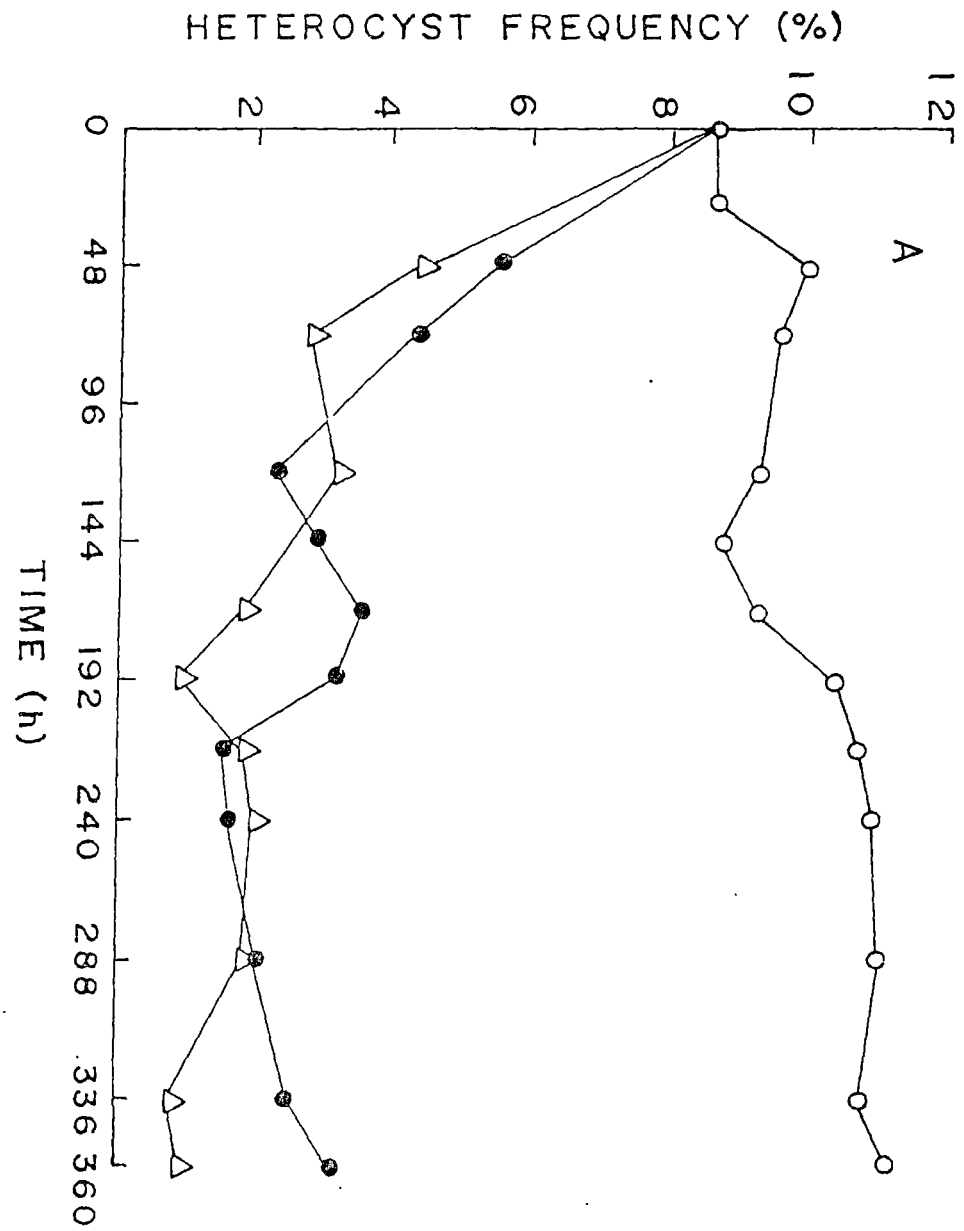
3.5.4 Effects of ammonium and nitrate on heterocyst frequency:

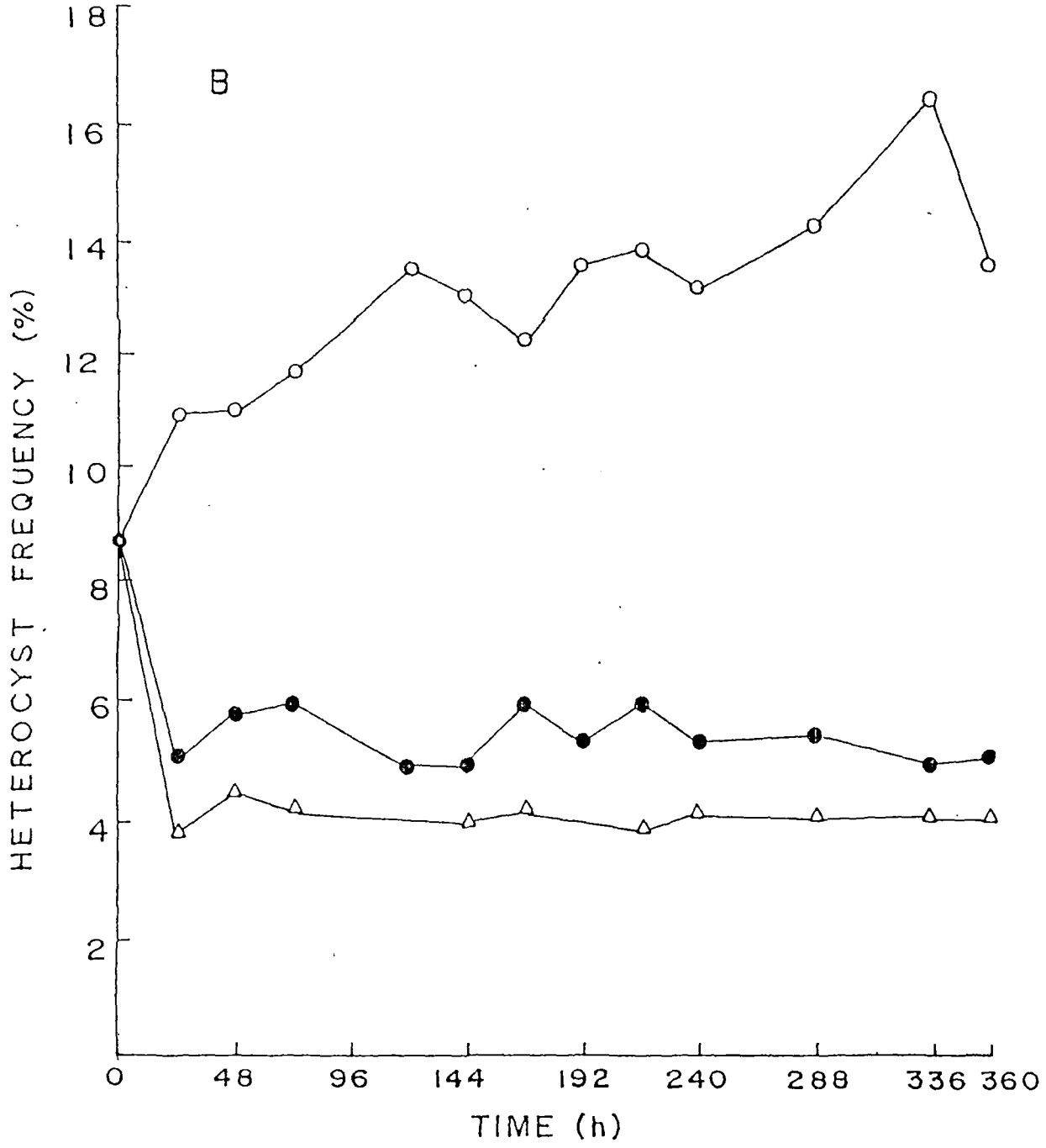
To determine the effect of combined nitrogen sources on the heterocyst differentiation of the entrapped cyanobacterial cells, the heterocyst frequency was estimated in immobilized cells of the cyanobiont grown in the presence of N_2, NO_3^-, and NH_4^+ as sole nitrogen source. Figs. 3.13 a and b show that nitrate and ammonium caused inhibition of heterocyst differentiation in free-living *Nostoc* ANTH. In contrast, immobilization increased the heterocyst frequency in N_2-grown cultures as compared to free-living cells. Nitrate and ammonia only partially inhibited the heterocyst differentiation in the immobilized cells, further indicating, immobilization restricts access/metabolism of these nitrogen sources by immobilized cyanobacterial cells.

3.5.5 Photosynthesis and respiration:

Fig. 3.14 presents data on the photosynthetic O_2-evolution and respiratory O_2-consumption trends of immobilized cells. The rates of photosynthesis and respiration showed severe

Fig. 3.13. Effect of combined nitrogen sources on heterocyst frequency of free-living (a) and immobilized (b) *Nostoc* ANTH.○, N_2-medium; ●, nitrate-medium; △, ammonium-medium.


HETEROCYST FREQUENCY (%)
2
4
6
8
10
12
A
0
48
96
144
192
240
288
336
360
TIME (h)


B
HETEROCYST FREQUENCY (%)
18
16
14
12
10
8
6
4
2
0
48
96
144
192
240
288
336
360
TIME (h)

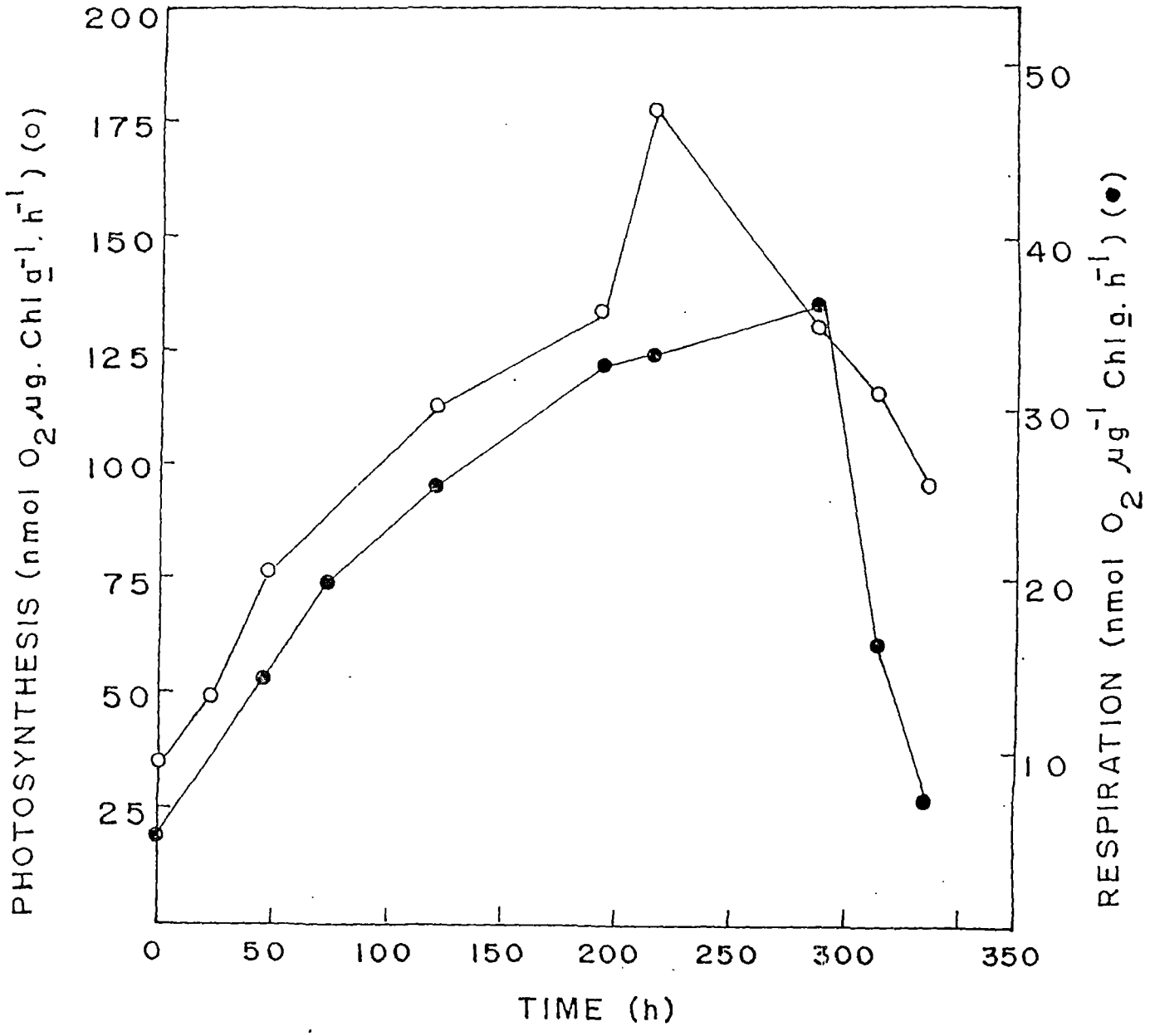


Fig. 3.14. Rates of photosynthetic O_2-evolution (○) and respiratory O_2-consumption (●) in the immobilized cells of *Nostoc* ANTH. The corresponding rates in free-living cultures of *Nostoc* ANTH were 135 nmol O_2 evolved μg^{-1} chl *a* h^{-1} and 100 nmol O_2 consumed μg^{-1} chl *a* h^{-1}.

inhibition upon immobilization (respiration dropped by 80% and photosynthesis by about 75%). However, immobilized cells did recover from the initial shock and recovered its full activity between 200 to 300 h. This initial drop in photosynthesis and respiration may explain the reason that low nitrogenase activity in the initial stages of immobilization was because of less ATP generation due to less photosynthetic activity.

3.6 Regeneration of *Nostoc* ANTH from desiccated calcium alginate beads:

Calcium alginate beads with *Nostoc* ANTH filaments were left to dry inside the culture room at room temperature. Periodically these were washed thoroughly with distilled water and were put in nutrient medium without combined nitrogen (BG-11_o) to check the viability of these desiccated, immobilized cyanobacterial cells. The calcium alginate beads swellup in the medium and after a gap of 7 to 8 days filaments of *Nostoc* ANTH become visible in the liquid medium. These were then inoculated in fresh BG-11_o medium and their heterocyst frequency, nitrogenase activity and growth in terms of chlorophyll *a* content were measured. These parameters were found to be comparable to the free-living *Nostoc* ANTH. This process was repeated every 4-5 months. Desiccated immobilized *Nostoc* ANTH cyanobiont remained viable upto 5 years.

3.7 Reconstitution experiments:

It has been pointed out by Rodgers & Stewart (1977) that the morphological and physiological changes that occur in the cyanobacteria when it undergoes symbiosis with an eukaryotic partner are mediated by the host. To identify the interactions between the host ***Anthoceros*** and the symbiont ***Nostoc*** that regulate cellular differentiation and metabolic activities in symbiosis, reconstitution seems to be the logical way to start from pure cultures of both the partners and study the progressive changes in the reconstituted thallus. It has already been established that ***Anthoceros-Nostoc*** symbiosis can be reconstituted from the axenic cultures of the partners under laboratory conditions (Rodgers & Stewart, 1977; Meeks *et al.*, 1983). Reconstitution experiments were conducted in the laboratory using pure axenic cultures of both host and the cyanobiont.

The data in Table 3.5 show the heterocyst frequency and nitrogenase activity of reconstituted ***Anthoceros-Nostoc*** gametophytic thallus. For nitrogenase activity, 30-40 mg fresh weight of reconstituted thallus (2, 3, 4 weeks old) and axenic ***Anthoceros*** were incubated in 2 ml of growth medium in 6.4 ml vials under air plus 10% (v/v) acetylene with 50 µmol m^{-2} s^{-1} light at 25° C for 60 min. The rate of acetylene reduction was measured and it was found to be 1.98, 4.91 and 4.63 nmol C_2H_4 mg^{-1} thallus dry weight h^{-1}, respectively. Axenic ***Anthoceros*** did not show any nitrogenase activity. The heterocyst frequency of the (2, 3 and 4 weeks) old thalli were 19.87, 31.3 and

Table: 3.5. Heterocyst frequency and nitrogenase activity of reconstituted *Anthoceros-Nostoc* thallus:

Sample	Heterocyst frequency (%)	Nitrogenase activity (nmol C_2H_4 produced mg^{-1} thallus dry wt. h^{-1})
Anthoceros-Nostoc		
2 weeks	19.87	1.98
3 weeks	31.30	4.91
4 weeks	29.73	4.63
Axenic *Anthoceros* (No *Nostoc* cyanobiont)	0.00	0.00

29.73%, respectively. These results suggest that ***Anthoceros-Nostoc*** symbiosis can be reconstituted successfully from their axenic cultures and development of symbiosis from the stage of initiation to a fully developed functional symbiotic state can be characterized.

CHAPTER 4

Anthoceros-Nostoc Symbiosis: Immuno-electronmicroscopic localization of nitrogenase, glutamine synthetase, phycoerythrin, ribulose - 1 , 5-bisphosphate carboxylase/ oxygenase and hydrogenase in the cyanobiont and the cultured (free-living) isolate *Nostoc* ANTH.

4.1 Introduction:

N_2-fixing cyanobacteria form associations with plants that ranges from algae to angiosperms (Rai, 1990; Bergman *et al.*, 1992). The cyanobacterial partner (cyanobiont) in all these symbioses is known to fix N_2 and make fixed-nitrogen available to the eukaryotic partner (*see* Stewart *et al.*, 1983). In some symbioses (e.g. in lichens and *Azolla*) the cyanobiont is photosynthetically active. However, in bryophytes, cycads and *Gunnera*, the cyanobiont is photosynthetically inactive and dependent on the eukaryotic partner for its fixed carbon (Stewart *et al.*, 1983; Meeks 1990; Lindblad & Bergman 1990; Bergman *et al.*, 1992). The transfer of metabolites between the cyanobiont and the eukaryotic partner is linked to physiological and biochemical changes in the cyanobiont (Rai, 1990; Meeks 1990; Braun-Hawland & Nierzwicki-Bauer, 1990; Lindblad & Bergman 1990). Among the bryophyte-cyanobacterial symbiosis, the hornwort *Anthoceros punctatus* symbiosis is better characterized. N_2-fixing *Nostoc* spp. develop in cavities on the undersurface of the

Anthoceros gametophyte thallus. The cyanobiont in this association reportedly has an increased heterocyst frequency, is photosynthetically inactive and lacks phycobiliproteins (Rodgers & Stewart, 1977; Stewart & Rodgers, 1977; Meeks, 1990). The increased heterocyst frequency does not correlate with an increase in N_2-fixation (Rodgers & Stewart, 1977; Stewart & Rodgers, 1977). Whether this is due to limited availability of fixed carbon or to the presence of nonfunctional heterocysts lacking nitrogenase is not known. The activity of glutamine synthetase (GS) in the cyanobiont is three- to four-fold lower than that in the free-living isolate; this reduction is apparently due to regulation at the level of enzyme inactivation rather than synthesis (Joseph & Meeks, 1987). This is in contrast to the situation in lichens and *Azolla* cyanobionts, where the reduction in the GS activity has been attributed to low rates of enzyme synthesis (Stewart *et al.*, 1985; Nierzwicki-Bauer & Haselkorn, 1986). The relative distribution of GS between vegetative cells and heterocysts of the cyanobiont of *Anthoceros* is not known.

Using immunocytochemistry, the aims of this study were (1) to examine the content of GS protein and its relative distribution between heterocysts and vegetative cells, and (2) to check for presence of nitrogenase, phycoerythrin (PE), ribulose-1,5-biphosphate carboxylase/oxygenase (RuBisCo) and hydrogenase in vegetative cells and in single and multiple heterocysts in the cyanobiont from mature cyanobacterial colonies of the *Anthoceros*

punctatus gametophyte. For comparison, the free-living cultured isolate *Nostoc* **ANTH** was also studied.

4.2 Methods:

Anthoceros punctatus was collected and the cyanobiont (*Nostoc* **ANTH**) was isolated, purified and raised using N_2-medium (for full details *see* **"MATERIALS AND METHODS"**). These were then used for immunolabelling studies using nitrogenase, GS, RuBisCo, PE and hydrogenase antibodies. Purity of antibodies, immunogold labelling protocols, transmission electron microscopy and quantitation of immunolabel were as detailed earlier (*see* **"MATERIALS AND METHODS"**).

4.3 Results and Discussion:

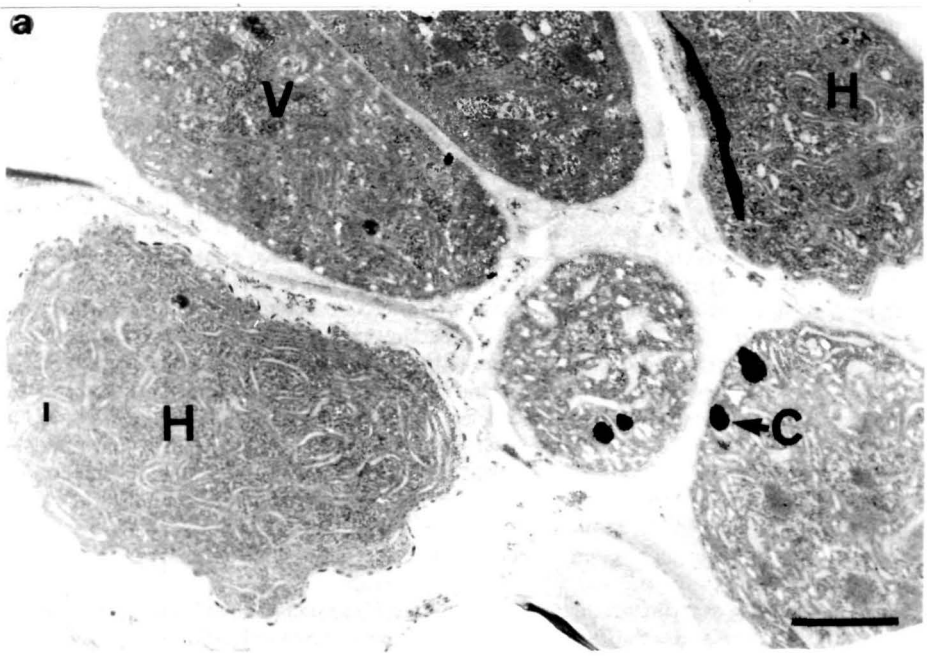
Biochemical characterization of the cyanobiont has been rather difficult because of the problem of obtaining enough clean material from the symbiotic association. Furthermore, in isolating the cyanobiont, whole thalli are generally used. This results in metabolically heterogeneous cyanobiont populations since cyanobionts are in different parts of the thallus, as indicated by varying heterocyst frequency and enzymatic activities (*see* Englund, 1977; Lindblad *et al.*, 1985; Rowell *et al.*, 1985). With the introduction of immunogold labelling these problems can be avoided and an antigen can be located precisely with little sample material. Here, this method has been used to study the mature cyanobacterial colonies from the mid-part of the

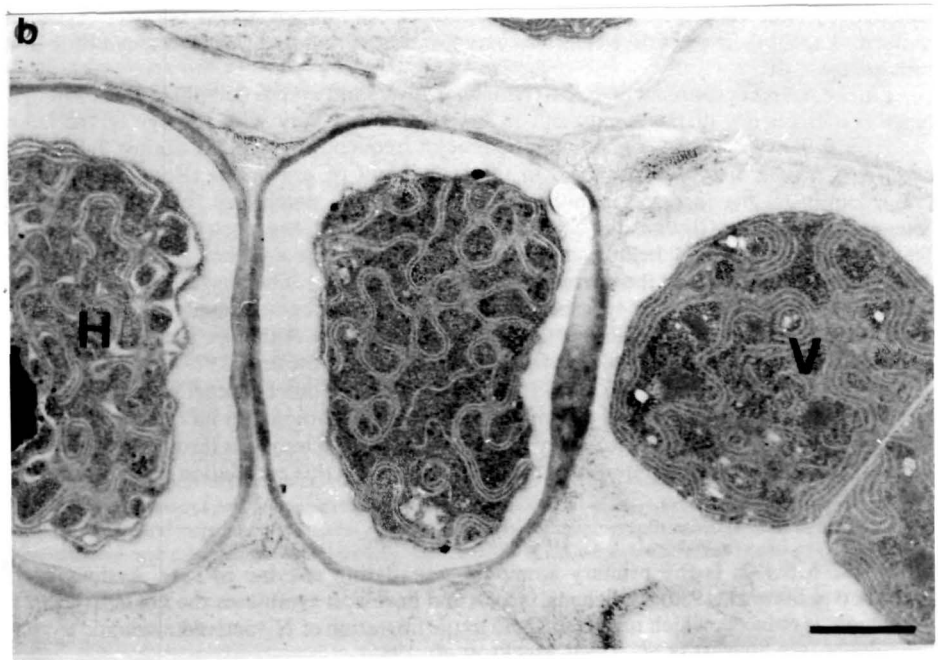
Anthoceros punctatus.

Secondary antibodies (goat anti-rabbit IgG) were first tested for nonspecific binding to the fixed cells. Virtually no label was detected in the absence of the primary antibody either in the cyanobiont or in *Nostoc* ANTH (Fig. 4.1 a,b). This showed that the antibodies were specific to rabbit IgG. The location of the specific antigens was then determined using primary antibodies to nitrogenase, GS, PE, RuBisCo and hydrogenase.

4.3.1 Nitrogenase:

In heterocystous cyanobacteria, nitrogenase is restricted to heterocysts under aerobic growth conditions (stewart, 1980; Janaki & Wolk, 1982). However, nitrogenase may be expressed in vegetative cells of non-heterocystous cyanobacteria both under microaerobic (Stewart & Lex, 1970; Rippka & Waterbury, 1977) and aerobic (Stal & Krumbein, 1985) conditions. Whether nitrogenase can be induced in the vegetative cells of heterocystous cyanobacteria is less certain (Flemming & Haselkorn, 1974; Murry *et al.*, 1984). Rippka & Stanier (1978) and Spence & Stewart (1987) isolated mutants of *Anabaena* lacking heterocysts in which N_2-fixation still occurs. Bergman *et al.* (1986) have shown that in lichens and in root nodules of cycads, nitrogenase is restricted to heterocysts only. However, the possibility of N_2-fixation in vegetative cells of the cyanobiont in *Anthoceros-Nostoc* symbiosis has been suggested (Rodgers & Stewart, 1977).

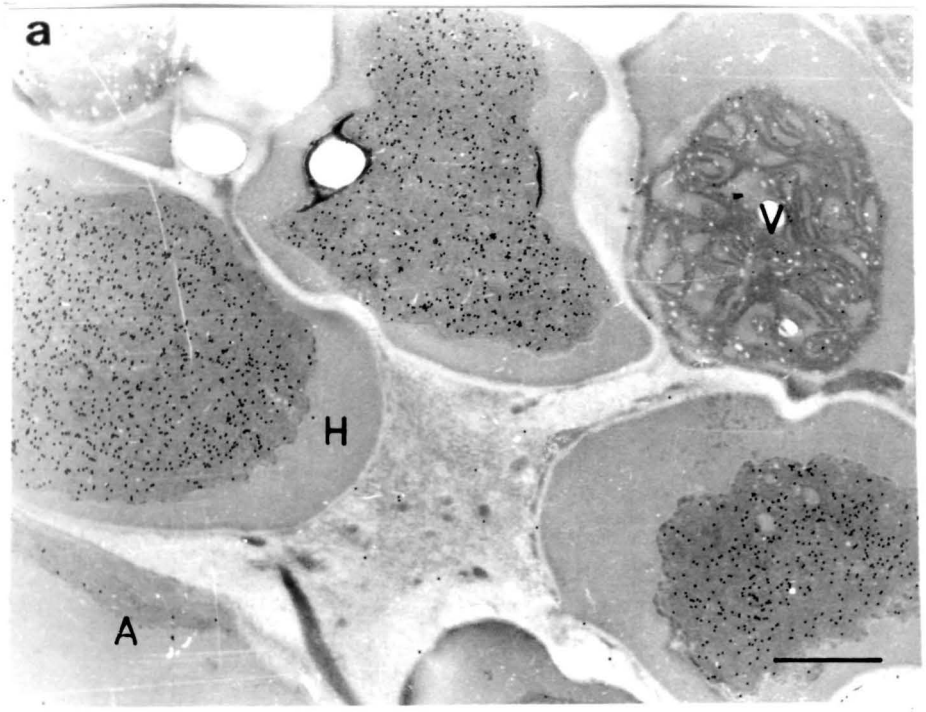

a
V
H
H
C

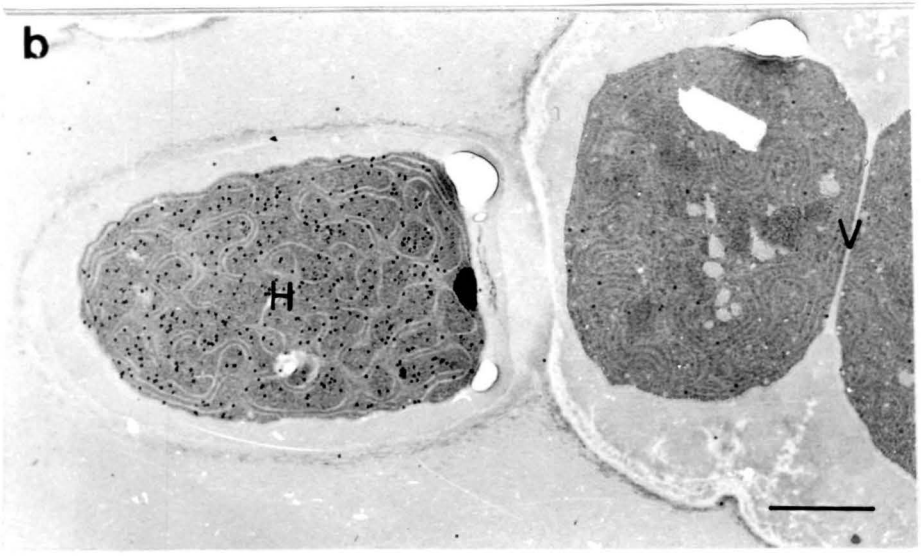

b
H
V

Immunolabelling experiments on the cyanobiont of *A. punctatus* showed that the nitrogenase was located in the heterocysts only (Fig. 4.2a). The pattern and intensity of labelling was similar in free-living *Nostoc* ANTH (Fig. 4.2b). The distribution of the antigen within the heterocysts was uniform. Labelling in vegetative cells was very low, suggesting that they contain little or no nitrogenase.

As in the *Nostoc* cyanobiont of lichens (Englund, 1977) and cycads (Lindblad *et al.*, 1985), the heterocyst frequency of the cyanobiont in bryophytes can vary with the age of the colony (Rodgers & Stewart, 1977). A maximum heterocyst frequency of 43%, as against 3-6% in the free-living *Nostoc*, has been observed in the cyanobiont of *Anthoceros* (Rodgers & Stewart, 1977). However, the increase in nitrogenase activity was much less than the increase in heterocyst frequency (Rodgers & Stewart, 1977), indicating the presence of nonfunctional heterocysts. Immunogold labelling showed that all the heterocysts of the cyanobiont, including the multiple ones, contained the nitrogenase protein (Fig. 4.2 a,b). This suggests that the supply of fixed carbon to multiple heterocysts occurring in chain may be restricted. A similar case has been found in the cycad symbiont (Bergman *et al.*, 1986). Although the photosynthate is provided by the eukaryotic partner in *Anthoceros* (Rodgers & Stewart, 1977), it probably enters the heterocyst via the vegetative cells. Heterocysts with no direct contact with the vegetative cells may receive little or no fixed carbon. In the cycad cyanobiont, an

Fig. 4.2. Localization of nitrogenase in the cyanobiont (a) and in the free-living cultured isolate *Nostoc* ANTH (b). Rabbit anti-*Rhodospirrilum rubrum* nitrogenase (Fe-protein) was used as primary antibody. Other details as in Fig. 4.1; A, *Anthoceros* tissue.


a
V
H
A


b
H
V

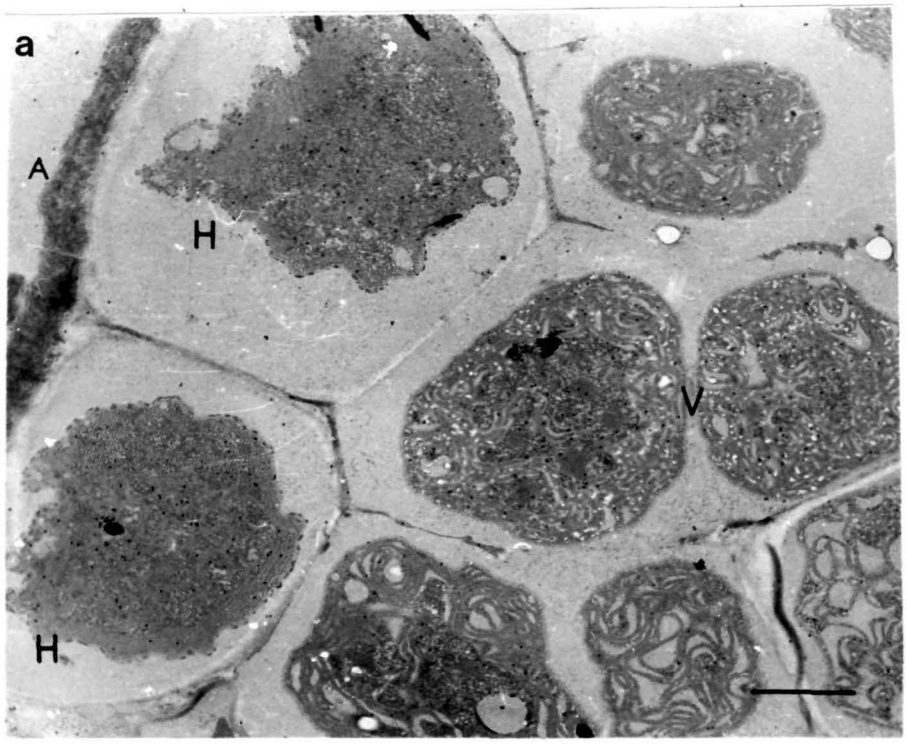
increase in nitrogenase activity correlates with an increase in the frequency of single heterocysts throughout the root (tip to base), but with increasing number of heterocysts this correlation is lost (Lindblad *et al.*, 1985).

4.3.2 Glutamine synthetase:

GS (EC 6. 3. 1. 2) is the primary ammonia-assimilating enzyme in the heterocystous cyanobacteria (Stewart, 1980). In lichens, *Azolla* and hornwort symbioses the GS activity in the cyanobiont is reduced, which may contribute to the liberation of N_2-derived ammonia from the cyanobiont (*see* Stewart *et al.*, 1983; Meeks *et al.*, 1985).

In the *Anthoceros-Nostoc* association, GS activity in the cyanobiont has been reported to be reduced three- to four-fold without a similar decrease in the GS content (Joseph & Meeks, 1987). This led to the conclusion that GS activity in the cyanobiont is regulated by a post-translational mechanism. However, the relative distribution of the inactive GS protein between heterocysts and vegetative cells is not known. The GS protein was found to be present in both heterocysts and vegetative cells (Fig. 4.3 a,b) and its distribution was uniform within the cells. As in other free-living cyanobacteria (Bergman *et al.*, 1985), labelling in *Nostoc* **ANTH** heterocysts was nearly two-fold higher than that in vegetative cells (Fig. 4.3b, Table 4.1). In contrast, the label in the cyanobiont was similar in

heterocysts and vegetative cells (Fig 4.3 a, Table 4.1). Multiple heterocysts also contained similar amounts of the label. The GS content was similar in the vegetative cells of the cyanobiont and ***Nostoc*** **ANTH**, but the cyanobiont heterocysts had 60% less GS protein than the heterocysts of ***Nostoc*** **ANTH**. Since the heterocysts are up to 50% of the total cell population, such reduction amounts to 40% of the total GS in the ***Nostoc*** **ANTH** filaments considering that heterocysts have two-fold higher GS than vegetative cells. Thus, reduction in GS activity of the cyanobiont is at least partly due to a reduction in GS content of the heterocysts. This contrasts with the situation in lichen and ***Azolla***, where reduction in GS activity is due only to decreased synthesis of GS (Stewart *et al.*, 1983; Nierzwicki-Bauer & Haselkorn, 1986). It also differs from cycad cyanobiont, which has full GS activity and normal GS content, with a distribution pattern in heterocysts and vegetative cells similar to that in heterocysts and vegetative cells of the free-living isolate (Lindblad & Bergman, 1986). However, the selective decrease in the GS content in heterocysts noted here resembles the situation in lichens (Hallbom *et al.*, 1986) and ***Azolla*** (Bergman, unpublished results), where the distribution of the residual GS in cyanbiont is similar in both heterocysts and vegetative cells. It is interesting to note here that when N_2-grown cultures of ***Anabaena cylindrica*** are transferred to ammonium medium, the few heterocysts which remain show a GS content similar to that of vegetative cells. (Renström-Kellner *et al.*, 1990), indicating a correlation between nitrogenase expression and increase in GS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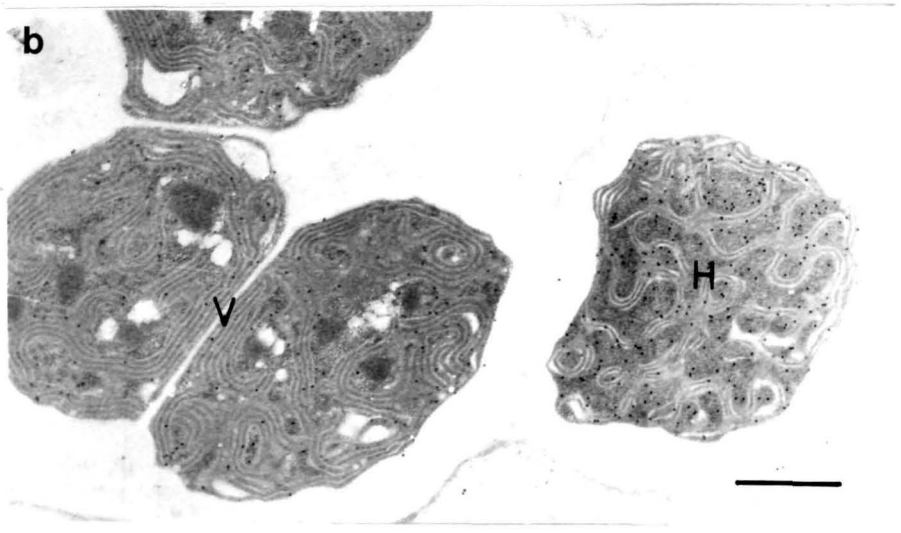
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Table: 4.1. GS content of heterocysts and vegetative cells of the cyanobiont and the free-living cultured isolate ***Nostoc* ANTH** from ***Anthoceros punctatus*:**

The GS contents given below are comparative and were calculated by counting the number of gold particles per unit cell area using TEM photomicrograph prints (at x 15000 magnification). The data Bare means ±SE of 70 counts from 15 photomicrographs taken from 5 ultrathin sections of three symbiotic colonies.

Sample	No. of gold particles cm^{-2}
Nostoc ANTH	
Vegetative cells	8.21 ± 1.35
Heterocysts	17.30 ± 3.65
Cyanobiont	
Vegetative cells	6.96 ± 1.30
Heterocysts	7.15 ± 1.71

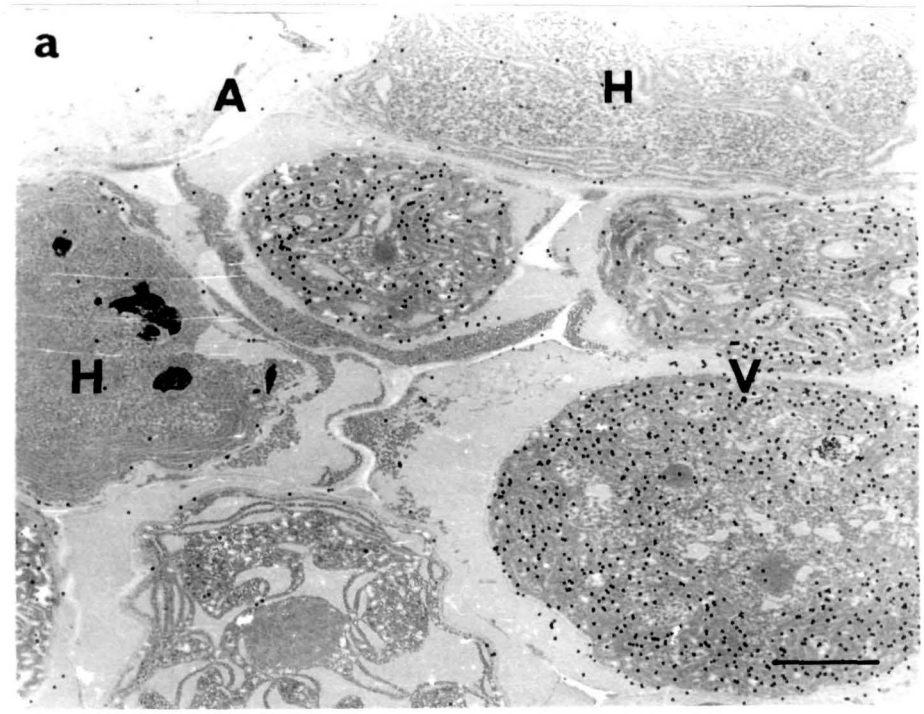
level in heterocysts, and a role for the latter in assimilation of N_2-derived ammonia.

4.3.3 Phycoerythrin:

Phycobiliproteins are accessory pigments of photosynthesis in cyanobacteria. They also serve as a nitrogen reserve and undergo degradation during nitrogen limitation (Cohen-Bazire & Bryant, 1982; Ho & Krogman, 1982). The cyanobionts of lichens, *Azolla* and cycads possess phycobiliproteins (Stewart *et al.*, 1982; Lindblad, 1987). However, Stewart & Rodgers (1977) were unable to detect phycobiliproteins in the cyanobiont of *A. punctatus*, although structures similar to phycobilisomes were noted in their TEM photomicrographs.

Immunolabelling experiments showed that phycobiliproteins were present in cyanobiont (Fig. 4.4). The pattern of labelling was similar to that in free-living *Nostoc* ANTH. The labelling of PE was associated with thylakoid membranes in vegetative cells. There was comparatively little labelling in heterocysts; this is consistent with the fact that heterocysts are generally deficient in phycobiliproteins (Stewart, 1980). The intensity of labelling was similar in vegetative cells of the cyanobiont and *Nostoc* ANTH. Cyanophycin granules (nitrogen reserves) were also noted in the cyanobiont (Fig 4.1), indicating that the cyanobiont was not nitrogen starved. Considering that the frequency of heterocysts (which have a very low phycobiliprotein content) is eight or nine times higher in the cyanobiont, the overall level of

Fig. 4.4. Localization of PE in the cyanobiont (a) and in free-living cultured isolate *Nostoc* ANTH (b) Rabbit anti-*Phorsidium persicinum* PE was used as primary antibody. Other details as in Figs. 4.1 and 4.2.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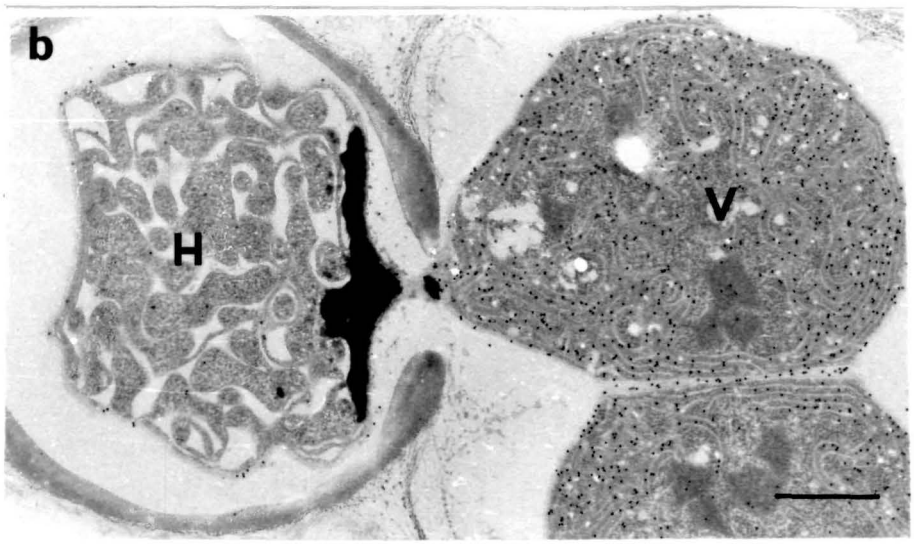
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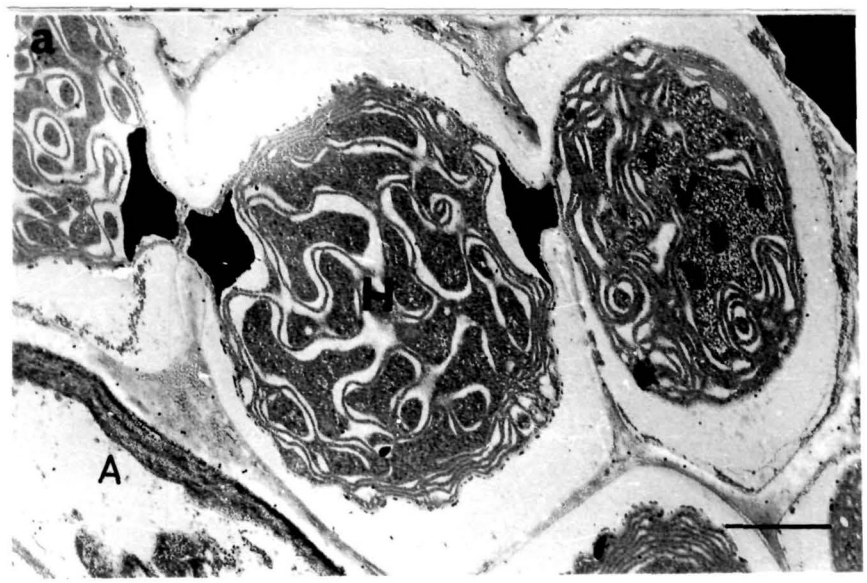
phycobiliproteins would be considerably lower than that of ***Nostoc* ANTH.**

4.3.4 Ribulose-1,5-biphosphate carboxylase/oxygenase:

RuBisCo (EC 4. 1. 1. 39) is the primary carboxylating enzyme in cyanobacteria, and cyanobacterial carboxysomes (polyhydral bodies) contain RuBisCo (*see* Codd & Marsden, 1984). The cyanobiont of the ***A. punctatus*** has been shown to be deficient not only in photosynthetic O_2-evolution but also in CO_2-fixation (Rodgers & Stewart, 1977).

In both the cyanobiont and free-living ***Nostoc* ANTH**, RuBisCo was present in vegetative cells, with little or no labelling in heterocysts (Fig. 4.5 a,b). This is consistent with the reported absence of RuBisCo in heterocysts (Codd *et al.*, 1980; Cossar *et al.*, 1985). The labelling within the vegetative cells of ***Nostoc* ANTH** was most prominent in carboxysomes, although label was also present in the cytoplasm. The pattern and intensity of labelling was similar in the cyanobiont. Thus the inability of the cyanobiont, in ***A. punctatus***, to fix CO_2 is not due to lack of RuBisCo. This has been confirmed by the observations of Steinberg & Meeks (1989) who found very low RuBisCo activity in the cyanobiont due to post translational modification of the enzyme. In case of cycad cyanobiont the *in vitro* RuBisCo activity is similar to that of free-living isolate; however, there is no *in vivo* CO_2-fixation by the cyanobiont (Lindblad *et al.*, 1987).

Fig. 4.5. Localization of RuBisCo in the cyanobiont (a) and in the free-living cultured isolate *Nostoc* ANTH (b). Rabbit anti-*Sinapis alba* RuBisCo was used as primary antibody. Other details as in Figs. 4.1 and 4.2. Note the heavy labelling in carboxysomes (arrow).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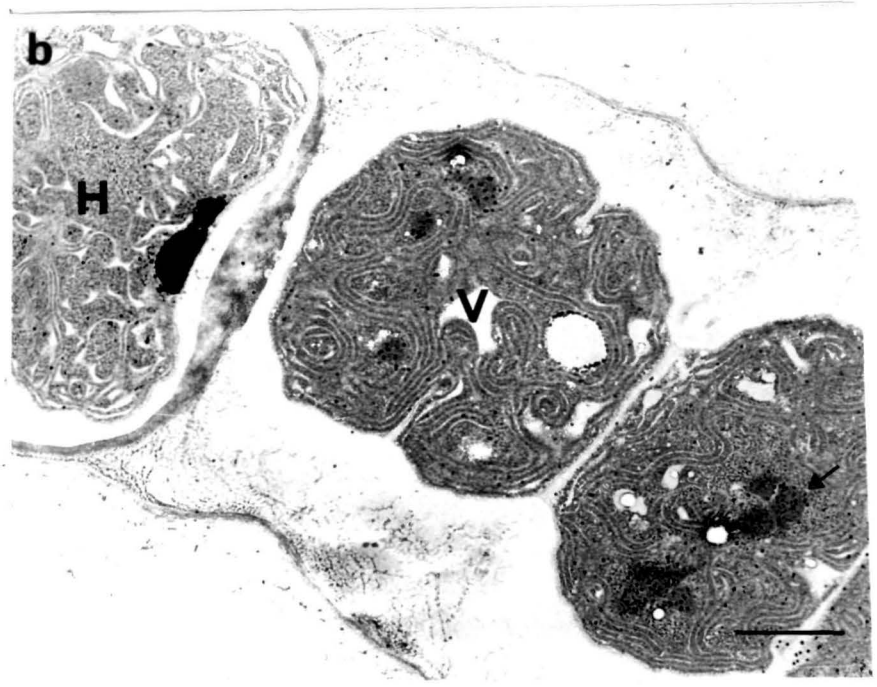
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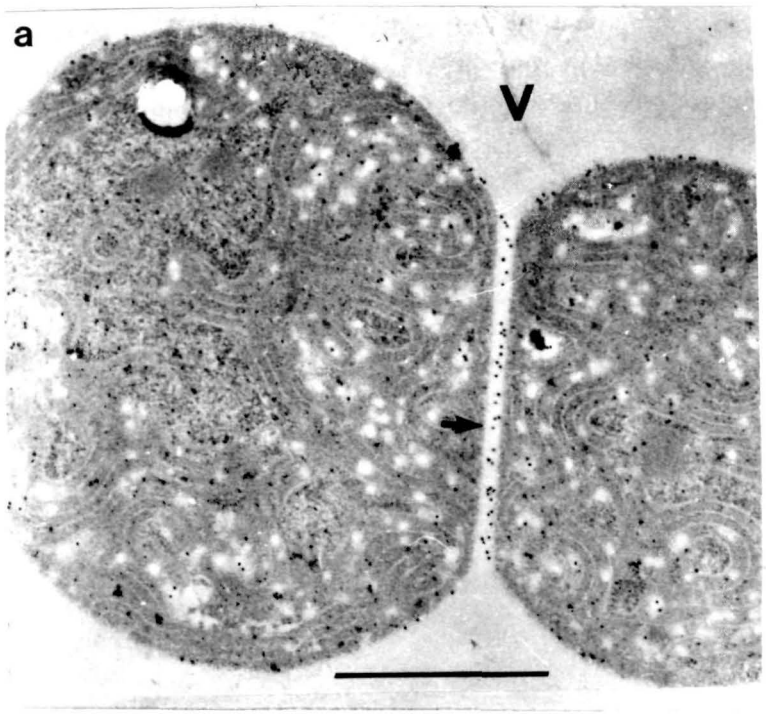
4.3.5 Hydrogenase:

Hydrogenases are important in diazotrophs for the recycling of hydrogen evolved during nitrogen-fixation (*see* Adams *et al.*, 1981; Lambert & Smith, 1981; Houchins, 1984; Antarikanonda *et al.*, 1980). Information about occurrence and localization of hydrogenase in cyanobacterial symbioses is lacking except for preliminary studies done about hydrogen uptake and evolution in cycad root nodule (Perraju *et al.*, 1986) and *Azolla* (Peters *et al.*, 1977).

Immunogold studies of hydrogenase in free-living (cultured) cyanobacterial isolate of *A. punctatus* showed that the hydrogenase antigen was present both in heterocysts and vegetative cells (Fig. 4.6). Most of the heterocysts observed had lower density of hydrogenase label as compared to the vegetative cells, though few heterocysts did have similar labelling density to that of vegetative cells. Within the cells hydrogenase antigen was evenly distributed with no preferential association to any particular structure. However, a high density of hydrogenase label was found along the plasma membrane particularly between the vegetative cells and along the plasma membranes being formed during cell division.

A quantitative analysis of the hydrogenase label distribution in heterocysts and vegetative cells showed that in the cyanobacterial isolate, heterocysts had significantly lower level of hydrogenase antigen (per unit cell area) as compared to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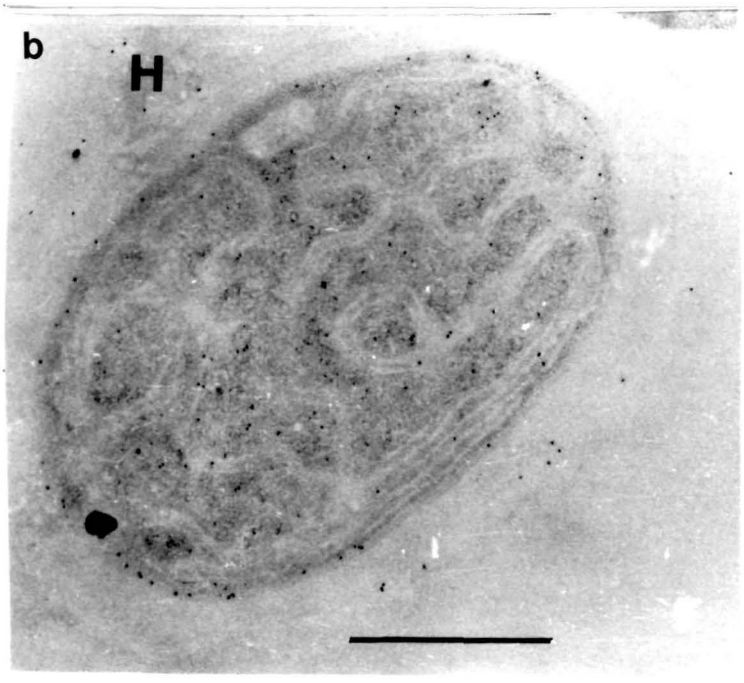
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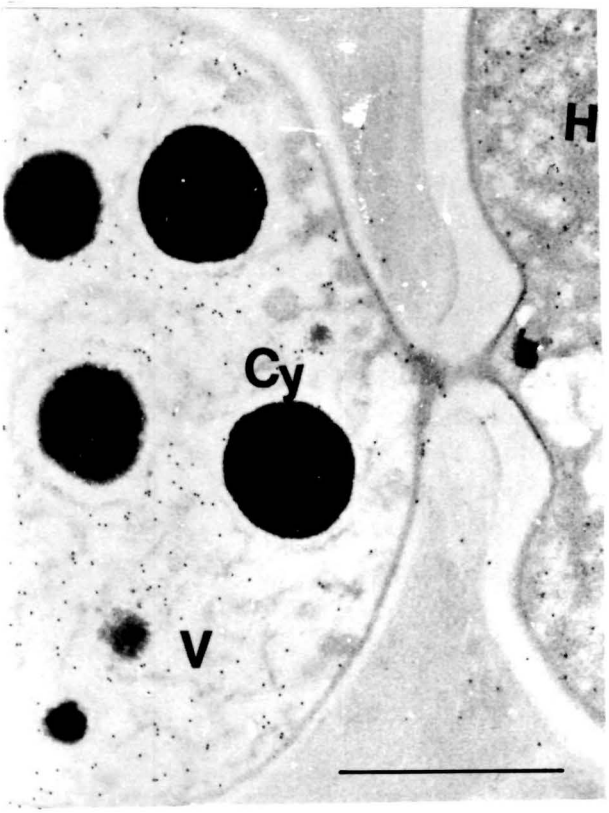
the vegetative cells (Fig. 4.8). The decrease in hydrogenase antigen level in heterocysts, as compared to vegetative cells, was 70% in *Nostoc* ANTH (Fig. 4.8).

In the case of *Anthoceros punctatus*, hydrogenase antigen was found to be present in the cyanobiont cells but none was detected in the *Anthoceros* tissue (Fig. 4.7). Within the cyanobiont cells,the pattern of hydrogenase labelling was similar to that in the cultured cyanobacterial isolate mentioned above. As in *Nostoc* ANTH, vegetative cells had a higher intensity of hydrogenase label than the heterocysts. The hydrogenase antigen levels in the heterocysts of the cyanobiont in *A. punctatus* and *Nostoc* ANTH was comparable. However, the hydrogenase antigen levels in the vegetative cells were 20% lower than in vegetative cells of *Nostoc* ANTH (Fig. 4.8). This contrasts with the observations in *Peltigera canina* and *Gunnera magellanica* symbioses where the cyanobionts had 60-70% lower hydrogenase levels than their respective free-living (cultured) *Nostoc* isolates (Fig. 4.8).

Presence of hydrogenase both in heterocysts and vegetative cells, and the higher intensities of hydrogenase label along the plasma membranes (Figs. 4.6-4.8) are similar to the observations of Lindblad and Sellstedt (1990) in *Nostoc* 73102. However, in contrast to *Nostoc* 73102, where heterocyst and vegetative cells had similar levels of hydrogenase (Lindblad & Sellstedt, 1990), a higher levels of hydrogenase was found in vegetative cells than in heterocysts of free-living (cultured) *Nostoc* cyanobionts (Fig.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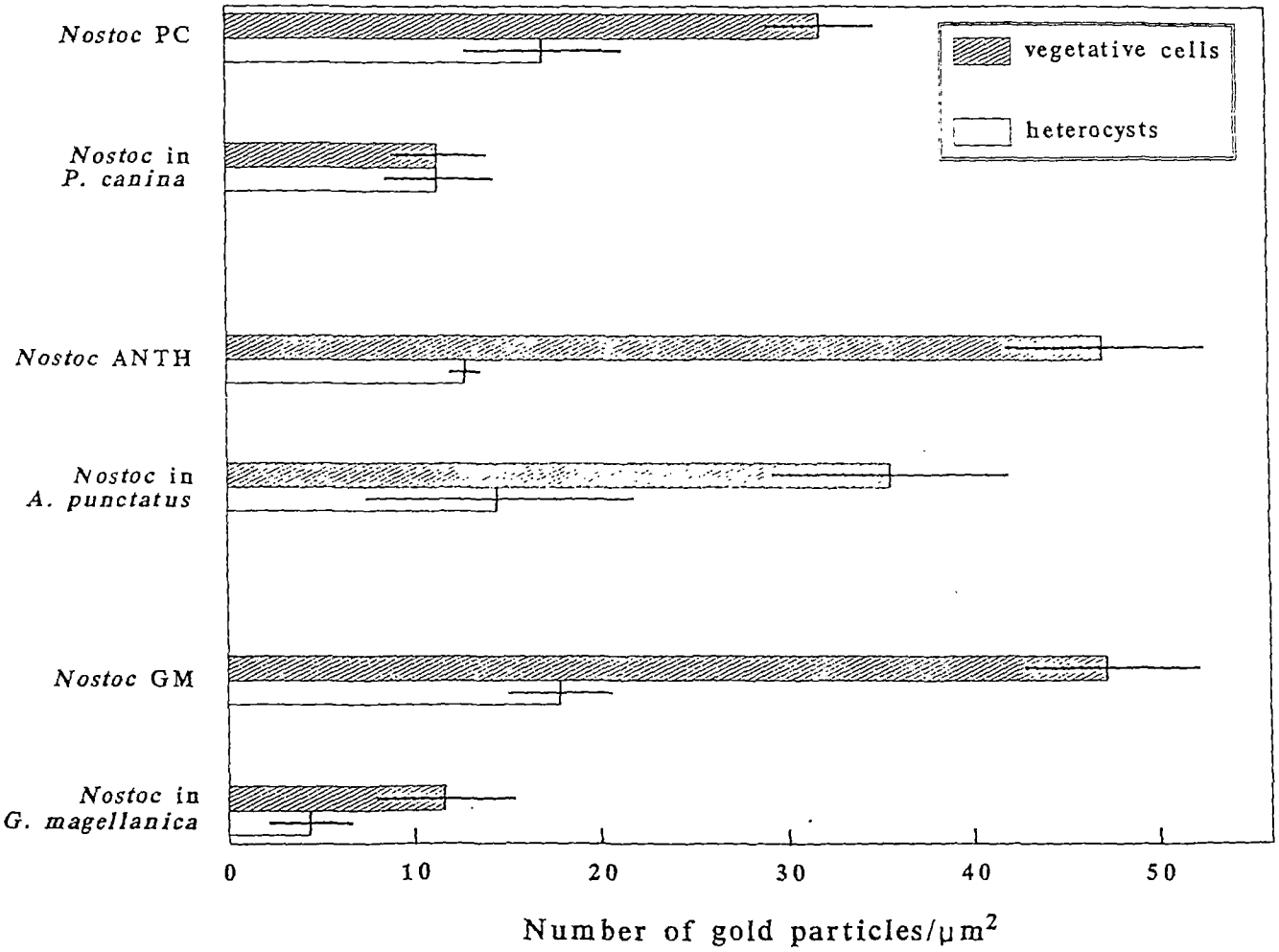
4.8). The difference may be due to the different strains of *Nostoc* used and/or due to difference in the immunolabelling protocols. While most heterocysts were found to possess lower levels of hydrogenase label than vegetative cells, a few heterocysts did have similar levels of hydrogenase to that in vegetative cells. It is possible that hydrogenase levels are reduced during heterocyst differentiation and that young heterocysts still retain hydrogenase levels similar to the vegetative cells. Comparative studies on *Peltigera canina* and *Gunnera magellanica* showed a marked decrease in the cytoplasmic level of hydrogenase which seems to relate to microaerobiosis.

Absence of hydrogenases in the eukaryotic partners is consistent with the fact that among eukaryotic organisms only algae are known to have hydrogenases (*see* Adams *et al.*, 1981). A comparison between the free-living and the symbiotic *Nostoc* in *A. punctatus* showed almost similar levels of hydrogenase as well as labelling pattern (Fig. 4.7, 4.8). However, while the intensity of hydrogenase label along the plasma membrane remained unchanged, there was significant decrease in the cytoplasmic levels of hydrogenase in the cyanobionts of *P. canina* and *G. magellanica*, particularly in the vegetative cells (Fig. 4.8 and Rai *et al.*, 1992). These results suggest that the synthesis of cytoplasmic hydrogenase, but not the plasma membrane associated hydrogenase, is significantly decreased in the cyanobionts of *P. canina* and *G. magellinica*. Such a decrease seems to correlate with a lowered oxygen tension. In *P. canina* and *G. magellianica* the cyanobionts reside in respiring nonphotosynthetic tissues but

Fig. 4.8. Cellular distribution and quantitative estimates of hydrogenase label in free-living (cultured) and symbiotic cyanobionts of *Peltigera canina*, *Anthoceros punctatus* and *Gunnera magellanica*. Bars indicate standard deviation.


Nostoc PC
Nostoc in *P. canina*
Nostoc ANTH
Nostoc in *A. punctatus*
Nostoc GM
Nostoc in *G. magellanica*
vegetative cells
heterocysts
0
10
20
30
40
50
Number of gold particles/μm²

in *A. punctatus* the cyanobiont is surrounded by photosynthetically active tissue (*see* Stewart *et al.*, 1983; Rai, 1988). This may also explain the lower levels of hydrogenase in heterocysts which are known to have lower oxygen tension than that in vegetative cells (*see* Stewart, 1990). Perraju *et al* (1986) suggested that heterotrophic mode of carbon nutrition may repress the uptake hydrogenase in symbiotic cyanobacteria but this does not seem to be the case here as mode of carbon nutrition in cyanobionts of both *A. punctatus* and *G. magellanoca* is heterotrophic but only in *G. magellanica* the cyanobiont showed decreased levels of hydrogenase. Furthermore, a decrease was also seen in the case of *P. canina* which is autotrophic. Another possibility that Ni availability may explain this decrease was ruled out because while Ni is necessary for hydrogenase activity, it is not necessary for hydrogenase synthesis (Ewart & Smith, 1989b).

CHAPTER 5

Nitrogen derepression, its regulation and metabolic changes associated with diazotrophy in the non-heterocystous cyanobacterium *Plectonema boryanum* PCC 73110

5.1 Introduction:

Cyanobacteria are O_2-evolving photosynthetic prokaryotes, many of which are also capable of autotrophic growth using N_2 as the sole nitrogen source (Stewart, 1980; Gallon, 1989). Cellular integration of N_2-fixation in cyanobacteria requires strategies for protection of nitrogenase from atmospheric and photosynthetically produced O_2, provision of ATP and reductant and efficient assimilation of N_2-derived ammonia. In some cyanobacteria this is achieved by development of specialized cells called heterocysts, resulting in spatial separation of photosynthesis (located in vegetative cells) and N_2-fixation (located in heterocysts); fixed carbon moved from the vegetative cells to heterocysts and fixed nitrogen from heterocysts to vegetative cells (Stewart, 1980; Wolk, 1982; Bergman *et al.*, 1986). Several other metabolic changes conducive to nitrogenase functioning occur during heterocyst development, including: (1) loss of photosynthetic O_2 evolution, phycobiliproteins and ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo); (2) increase in respiratory activity and in the activity of enzymes of oxidative pentose phosphate pathways; (3) loss of nitrate

reductase systems; (4) increased levels of glutamine synthetase (GS) necessary for assimilation of N_2-derived ammonia (Wolk, 1982; Kumar *et al.*, 1985, Rai & Bergman, 1986; Renström-Kellner *et al.*, 1990).

The strategies for O_2 protection during aerobic N_2-fixation by non-heterocystous cyanobacteria have been studied in detail, with conclusion of a temporal separation of N_2-fixation and photosynthesis (Gallon, 1992). However, very few studies have been conducted on non-heterocystous cyanobacteria which fix N_2 under microaerobic or anaerobic conditions. The reasons for lack of aerobic N_2-fixation in such cyanobacteria are not fully understood. Futhermore, virtually no information is available regarding levels of phycoerythrin (PE), GS, nitrate uptake, NR and RuBisCo under diazotrophic growth conditions in these cyanobacteria.

Plectonema boryanum PCC 73110 is a filamentous non-heterocystous cyanobacterium which fixes N_2 under microaerobic to anaerobic conditions (Stewart & Lex, 1970). In the present investigation, this strain was used to study the derepression, subcellular localization and regulation of nitrogenase, and changes in GS, RuBisCo, PE, nitrate uptake and NR when nitrate-grown cultures adapted to diazotrophic growth. The data are discussed in relation to the known facts about the above aspects in heterocystous cyanobacteria.

5.2 Methods:

5.2.1 Organisms and growth conditions:

Plectonema boryanum PCC 73110 (ATCC 29407 and UTEX 594) and *Gloeothece* PCC 6909 (ATCC 27152 and UTEX 795) were grown in batch cultures in BG-11 medium (Rippka *et al.*, 1979) at 25° C and a photon fluence rate of 20 μmol m^{-2} s^{-1}. *Oscillatoria limosa* (Stal & Bergman, 1990) was grown on artificial sea water medium ASN_3 (Rippka *et al.*, 1979) at 20° C and a photon fluence rate of 20 μmol m^{-2} s^{-1}. N_2-fixing cultures of *Gloeothece* and *O. limosa* were obtained by transferring the cultures to nitrogen-free media ('N_2-medium'; $BG\text{-}11_o$ and NO_3^--free ASN_3, respectively). Derepression of nitrogenase in *P. boryanum* was achieved as described below.

5.2.2 Nitrogenase derepression:

Aerated batch cultures of *P. boryanum* grown on $BG\text{-}11_o$ medium were harvested by centrifugation during the exponential phase. The cells were washed and resuspended in $BG\text{-}11_o$ medium to a cell density of 200 μg ml^{-1} (3 μg chlorophyll *a* ml^{-1}). These cultures were subdivided into 20 ml batches and transferred to 100 ml capacity serum stoppered Erlenmeyer flasks. These were sparged with desired gas mixture at specific times and maintained at 20° C and a photon fluence rate of 10 μmol m^{-2} s^{-1}. These flasks were directly used for acetylene reduction assay, each assay lasting 30 min; the cultures were not transferred to another vessel, in order to avoid air contamination.

5.2.3 Enzyme assays:

Nitrogenase, glutamine synthetase, nitrate reductase were done as detailed in the "MATERIALS AND METHODS".

5.2.4 Estimations:

Estimation of chlorophyll, proteins, O_2-exchange, NO_3 uptake, antibodies were done according to earlier description given in the "MATERIALS AND METHODS".

5.2.5 Procedures:

Transmission electron microscopy, immunolabelling and immunoblotting were performed as given in the "MATERIALS AND METHODS".

5.3 Results:

5.3.1 Derepression of nitrogenase:

No nitrogenase activity or protein could be detected in NO_3^- or NH_4^+-grown *P. boryanum* cultures either under air or with N_2/CO_2 (95:5, v/v) sparging (data not shown). Upon transfer to N_2-medium and periodic sparging with N_2/CO_2, development of acetylene reducing activity started after 30 h (Fig. 5.1a). The activity continues to increase during next 40 h, after which it steadily declined. The pattern and specific activity were similar to those noted by Stewart & Lex (1970). However, when the cells

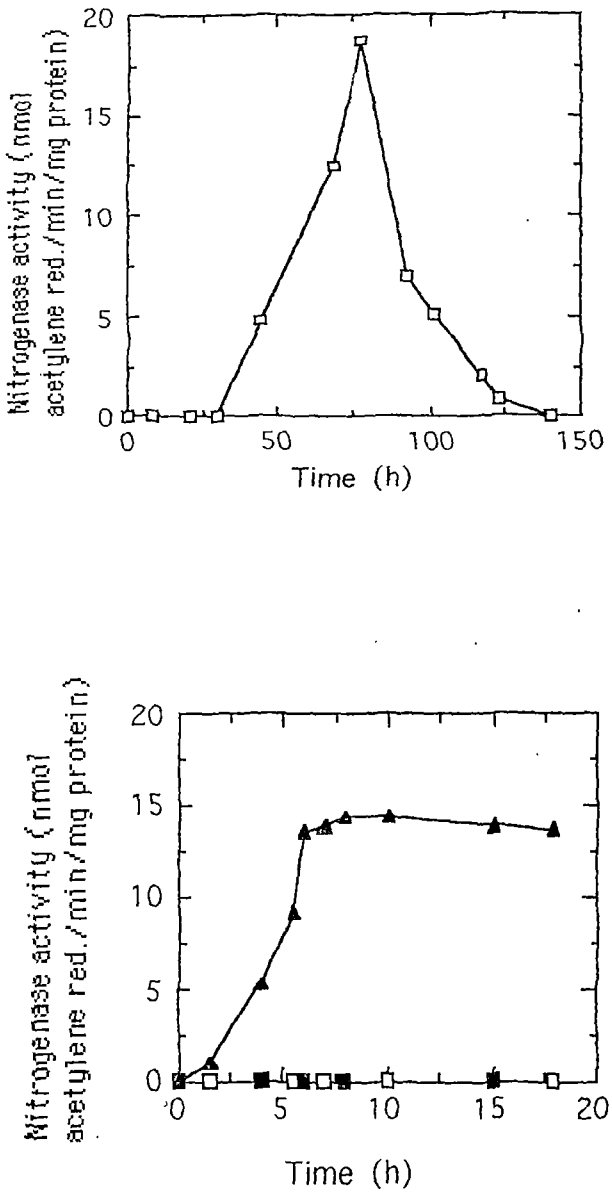


Fig. 5.1. Nitrogenase derepression in *P. boryanum*. (a) NO_3^--grown cells were transferred to N_2-medium as detailed in Methods and maintained at 25°C with a photon fluence rate of 10 μmol $m^{-2}s^{-1}$. Starting at zero time and at 6 h intervals they were sparged for 15 min with N_2/CO_2 (95:5, v/v) at a rate of 1000 ml h^{-1}. Nitrogenase activity was measured in 30 min assays under a N_2/CO_2 gas phase and under the light and temperature conditions mentioned above. In this and other figures nitrogenase activity quoted is the mean of that during the 30 min before the points shown. (b) Same as (a) except that prior to the start of N_2/CO_2 sparging (zero time) the cultures were nitrogen-starved for 24 h by incubating them in N_2-medium with continuous air sparging (2000 ml h^{-1}). Symbols in (b):▲, control;□, plus chloramphenicol (100 μg ml^{-1});■, plus rifampicin (100 μg ml^{-1}). Chloramphenicol and rifampicin were added at the start of N_2/CO_2 sparging (i.e. after the aerobic nitrogen-starvation period). This and other experiments (Figs. 5.2a, 5.3, 5.4, 5.5) were all done in duplicate. The values presented are means of two measurements from each replicate.

were nitrogen starved for 24 h under aerobic conditions prior to the periodic N_2/CO_2 sparging, acetylene-reducing activity developed much faster(within 2 h) and reached a peak by 6-7 h (Fig. 5.1b), with maximal specific activity similar to that in Fig. 5.1(*a*). To see if the faster development of nitrogenase activity was due to the activation of pre-existing nitrogenase protein developed during the 24 h nitrogen starvation, development of acetylene-reducing activity was followed in cells where chloramphenicol or rifampicin was added at the end of nitrogen starvation and just before the start of N_2/CO_2 sparging. As seen in Fig. 5.1(b), acetylene-reducing activity did not appear under such conditions. these results indicate that nitrogenase protein was absent during the aerobic nitrogen starvation period and that the derepression on N_2/CO_2 sparging was due to fresh synthesis of nitrogenase. An absence of nitrogenase protein in aerobic cultures was also observed by immunoblotting cell extracts of *P. boryanum* which has been nitrogen starved for 30 h under aerobic conditions (Fig. 5.2b, lane 1). These results show that nitrogenase derepression required both low cellular nitrogen and microaerobic to anaerobic conditions, and that the longer time required for nitrogenase derepression in Fig. 5.1(a) was due to the time required for the depletion of intracellular nitrogen reserves under non-optimal growth conditions.

Reexposure of N_2-fixing *P. boryanum* cells to air caused a rapid decline in acetylene-reducing activity, which became

undetectable after 90 min of exposure (Fig. 5.2a). To see whether the decline in nitrogenase activity was due to inactivation of the enzyme or to protein degradation/modification, immunoblots of cell extracts were done using *P. boryanum* cells exposed to air for increasing period of time after the appearance of peak nitrogenase activity (Fig. 4.3b). After 2 h of exposure to air, when nitrogenase activity has become undetectable, nitrogenase Fe-protein was still detectable (lane 3), although the cross-reaction was less intense than that in *P. boryanum* cells under N_2/CO_2 (lane 2). In both cases, only a single polypeptide of 36 kDa was detected corresponding to nitrogenase Fe-protein. These data indicate that loss of nitrogenase activity on exposure to air was due to nitrogenase inactivation followed by degradation. Such inactivation did not involve modification of Fe-protein to a higher molecular mass form as noted in other cyanobacteria (Ernst *et al.*, 1990; Reich & Böger, 1989; Smith *et al.*, 1987; Stal & Bergman, 1990). To see if the inactivation of nitrogenase was reversible, *P. boryanum* cells were transferred back to N_2/CO_2 atmosphere after 90 min exposure to air (Fig. 5.2a). Acetylene-reducing activity reappeared after 90 min and reached a peak in 3 h. Such reappearance of nitrogenase activity was sensitive to chloramphenicol and rifampicin indicating fresh nitrogenase synthesis to be necessary. Thus, inactivation of nitrogenase under air concluded to be irreversible.

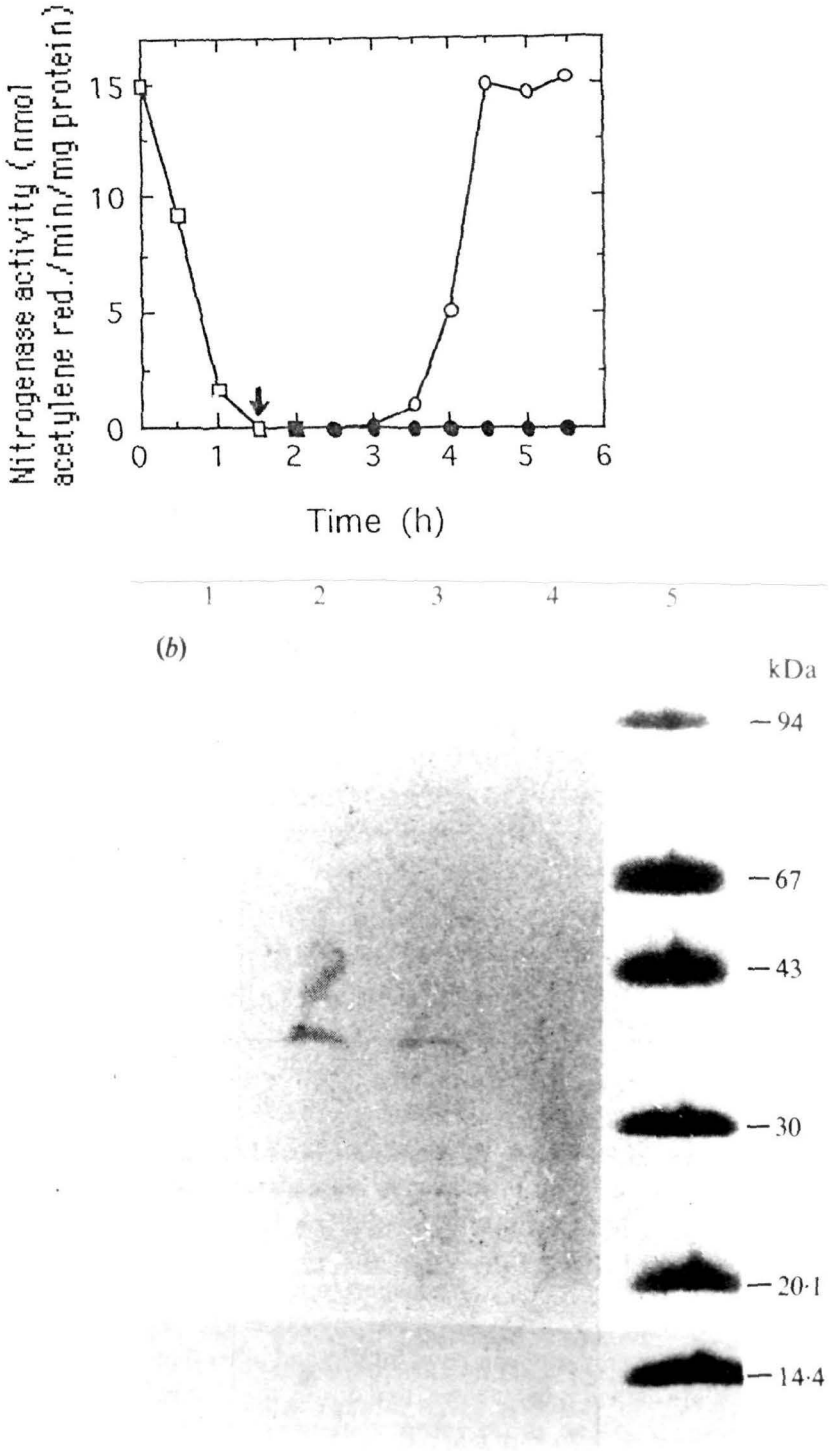


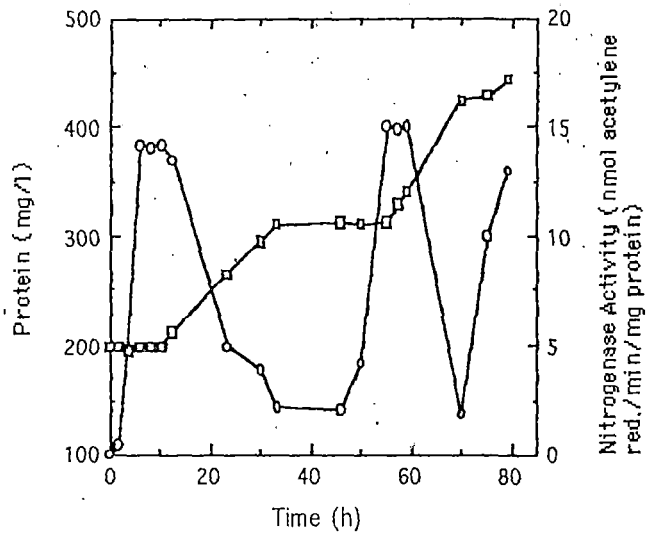
Fig. 5.2. (a) Loss of nitrogenase activity on exposure to air (□) and its regain upon transfer to a N_2/CO_2 atmosphere (○●) in *P. boryanum*. Nitrogenase derepression was achieved as in Fig 5.1(b) and after appearance of the nitrogenase peak, the cells were sparged with air for 5 min at the rate of 2000 ml h^{-1}. Nitrogenase activity was measured at 30 min intervals under aerobic conditions. At the time indicated by the arrow, the gas phase was changed to N_2/CO_2 in the absence (○) and presence (●) of chloramphenicol or rifampicin (both 100 μg ml^{-1}). (b) Detection of nitrogenase Fe-protein by immunoblotting in cell extracts of *P. boryanum*. Lane 1, extract from cells maintained in aerobic N_2-medium for 30 min; lane 2, extract from cells with peak nitrogenase activity; lane 3, extract from cells which were exposed to air for 2 h after appearance of the nitrogenase peak; lane 4, extract of cells which had been exposed to air for 4 h after appearance of nitrogenase activity; lane 5, molecular mass markers.

5.3.2 Growth, N_2 fixation and O_2 evolution:

When nitrogenase was derepressed as in Fig. 5.1(b), and cultures maintained under similar conditions over a prolonged period, repeated peaks of acetylene-reducing activity was observed (Fig. 5.3a). An increase in protein content, which was taken as indicative of growth, followed. A detailed analysis of one such peak of nitrogenase activity and growth phase (Fig. 5.3b) showed that during appearance of nitrogenase the rate of net O_2 evolution declined rapidly, becoming undetectable by the time nitrogenase activity reached its peak. No growth occurred during this period. Net O_2 evolution was detected again after 7 h. This coincided with growth and with a decline in nitrogenase activity. These data indicate that under the conditions used here, *P. boryanum* can grow photoautotrophically using N_2 as sole nitrogen source, in repeated cycles of nitrogen fixation and growth, and under such conditions there is a temporal separation of net O_2 evolution and nitrogenase activity. During the maximal N_2-fixing period, O_2 evolution may be balanced by respiratory O_2 consumption, resulting in the absence of net O_2 exchange.

5.3.3 Effects of NH_4^+ and NO_3^- and darkness on nitrogenase activity:

After nitrogenase derepression as in Fig. 5.1(b), the effects of darkness, NH_4^+ and NO_3^- were studied during the 6 h stable period when maximal nitrogenase activity was expressed. Transfer of N_2-fixing cells into darkness resulted in a rapid



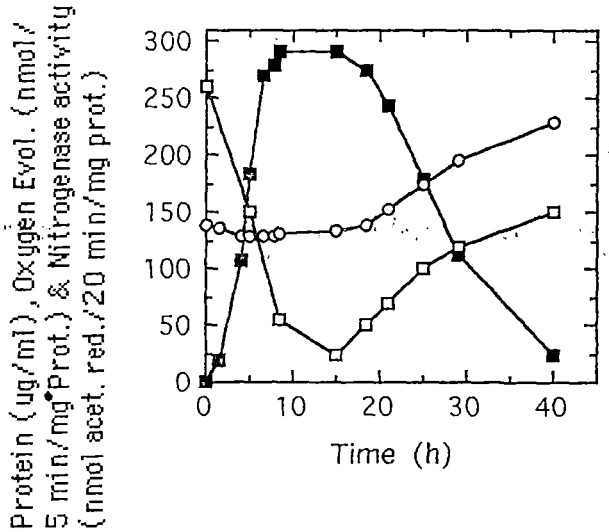


Fig. 5.3. (a) Nitrogenase activity (○) and protein content (□) of *P. boryanum*. (b) Nitrogenase activity (■), protein content (○) and net O_2 evolution rate (□) during the first phase of N_2-fixation in (a). Culture conditions and other details as in Fig. 5.1 (b) control.

decline in acetylene-reducing activity, which became undetectable within 2 h (Fig. 5.4). This decline was similar to that observed in air. ATP and/or reductant were probably the essential factors supplied by light reactions. Addition of 2 mM-NH_4Cl resulted in a slower decline of acetylene-reducing activity, perhaps because NH_4^+ may have acted by repressing nitrogenase synthesis rather than inhibiting the activity. NO_3^- did not affect nitrogenase activity during the initial 2 h of incubation, but a slow decline in activity was seen thereafter. The difference in the effects of NO_3^- and NH_4^+ may be due to slower rates of nitrate uptake and/or metabolism in N_2-fixing cultures (see Table 5.1).

5.3.4 NO_3^- uptake and NR activities:

NO_3^--grown cultures of *P. boryanum* showed NO_3^- uptake and NR activities (Table 5.1) similar to those reported earlier (Ida & Mikami, 1983). In contrast, NO_3^- uptake and NR activities of N_2-fixing cultures were only 10% of those in NO_3^--grown cells (activities measured after appearance of peak nitrogenase activity). These activities did not change during the period of nitrogenase decline that also corresponded to net O_2 evolution and growth (data not shown). NH_4^+-grown cells had no detectable levels of NO_3^- uptake and NR. Essentially similar results were found in the case of non-heterocystous cyanobacteria *Gloeothece* 6909 and *O. limosa*, which fix N_2 aerobically (Table 5.1). When N_2-fixing cells of *P. boryanum* were transferred to NO_3^- medium, NO_3^- uptake and NR activities increased reaching a maximum within

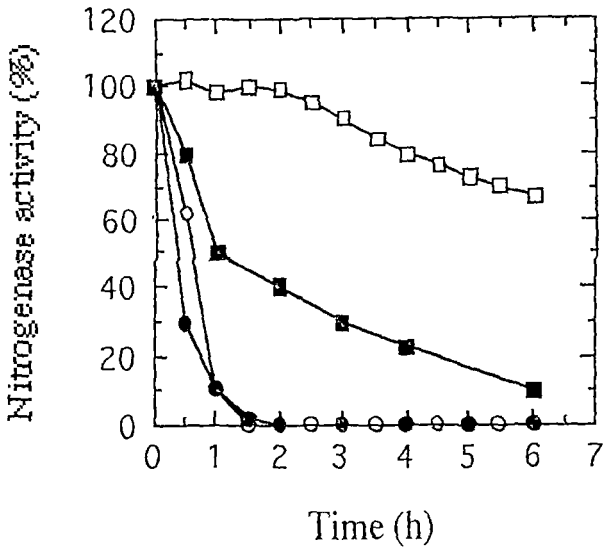


Fig. 5.4. Effects of air, darkness, NH_4^+ and NO_3^- on nitrogenase activity in *P. boryanum*. Nitrogenase activity was derepressed as in Fig. 5.1(b). After the appearance of the nitrogenase peak, the conditions were changed (zero time) and the response of nitrogenase activity was monitored during the next 6 h. ●, Cultures transferred to darkness; ■, NH_4Cl added to a final concentration of 2 mM; □, KNO_3 added to a final concentration of 10 mM; ○, cultures transferred to aerobic atmosphere. The NH_4Cl and KNO_3 solutions were sparged with N_2/CO_2 for 30 min before addition. The solutions were also buffered with 10 mM HEPES/NaOH (pH 7.5). One hundred percent activity represents 18 nmol C_2H_4 formed min^{-1} (mg protein)$^{-1}$.

Table: 5.1. NO_3^- uptake and NR activities in *P. boryanum*, *Gloeothece 6909* and *O. limosa* grown on N_2, NO_3^- or NH_4^+:
NO_3^- uptake activities are expressed as nmol NO_3^- taken up min^{-1} (mg protein)$^{-1}$ and NR activities as nmol NO_2^- formed min^{-1} (mg protein)$^{-1}$. NO_3^--medium represents BG-11 in the case of *Gloeothece 6909* and *P. boryanum*, and ASN_3 in the case of *O. limosa*. N_2-medium represents the respective nitrogen-free medium ($BG\text{-}11_0$ or NO_3^--free ASN_3). NH_4^+-medium represents N_2-media supplemented with NH_4Cl (final concentration 2 mM) and buffered with 10 mM-HEPES/NaOH (pH 7.5). The values presented are means ± SE of three measurements from a single experiment done in triplicate. ND, Not detectable.

Growth Medium	Nitrate uptake			Nitrate reductase		
	Plectonema	*Gloeothece*	*Oscillatoria*	*Plectonema*	*Gloeothece*	*Oscillatoria*
N_2-medium	0.6 ± 0.1	0.2 ± 0.05	0.2 ± 0.04	0.5 ± 0.1	0.2 ± 0.04	0.3 ± 0.1
NO_3^--medium	7.5 ± 0.3	4.9 ± 0.25	6.0 ± 0.30	7.8 ± 0.4	4.3 ± 0.10	4.7 ± 0.2
NH_4^+-medium	ND	ND	ND	ND	ND	ND

10-12 h (Fig. 5.5). The increase was sensitive to chloramphenicol. These result imply that the NO_3^- uptake and NR systems in *P. boryanum* are substrate-inducible and NH_4^+-repressible.

5.3.5 Nitrogenase localization:

Immunogold localization of nitrogenase Fe-protein in NO_3^--grown cells (Fig. 5.6a) and NH_4^+-grown cells (data not shown) showed label intensity similar to background (4-6 gold particles per μm^2 cell area). N_2-fixing *P. boryanum* cells showed nitrogenase antigen uniformly distributed throughout the cell without preferential association with any cell structure (Fig. 5.7a). All the cells in all the filaments examined had a similar pattern and intensity of labelling. Cells undergoing division also had nitrogenase label. The density of label was 95 ± 12 gold particles per μm^2 cell area which is comparable to to that in the heterocysts (unpublished data. See also Stal & Bergman, 1990). These results confirm the lack of nitrogenase in NO_3^-- and NH_4^+-grown cells and imply no spatial separation or subcellular compartmentalization of nitrogenase in *P. boryanum*. Similar results have been reported in *P. boryanum* 581 using antibodies against nitrogenase Mo-Fe protein (Smoker *et al.*, 1989).

5.3.6 GS activity and cellular localization of GS antigen:

Potential changes in GS activity and protein concentration during derepression of nitrogenase were examined in NO_3^--grown

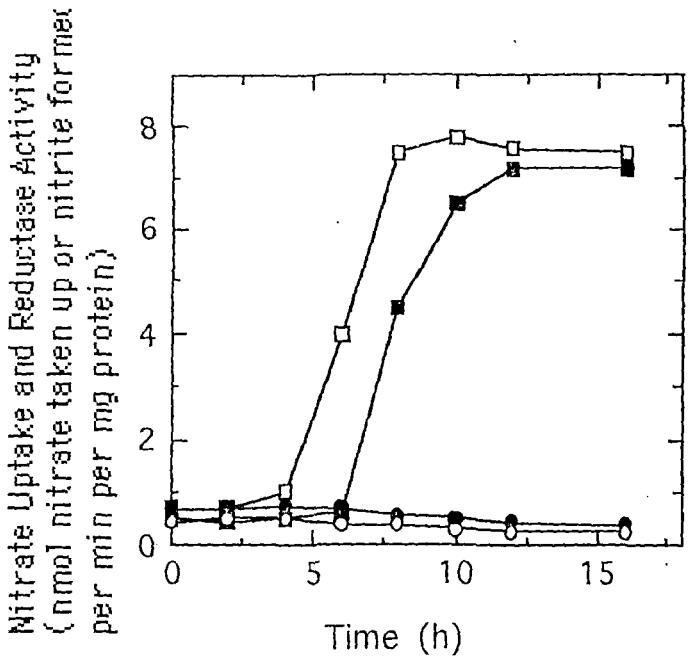
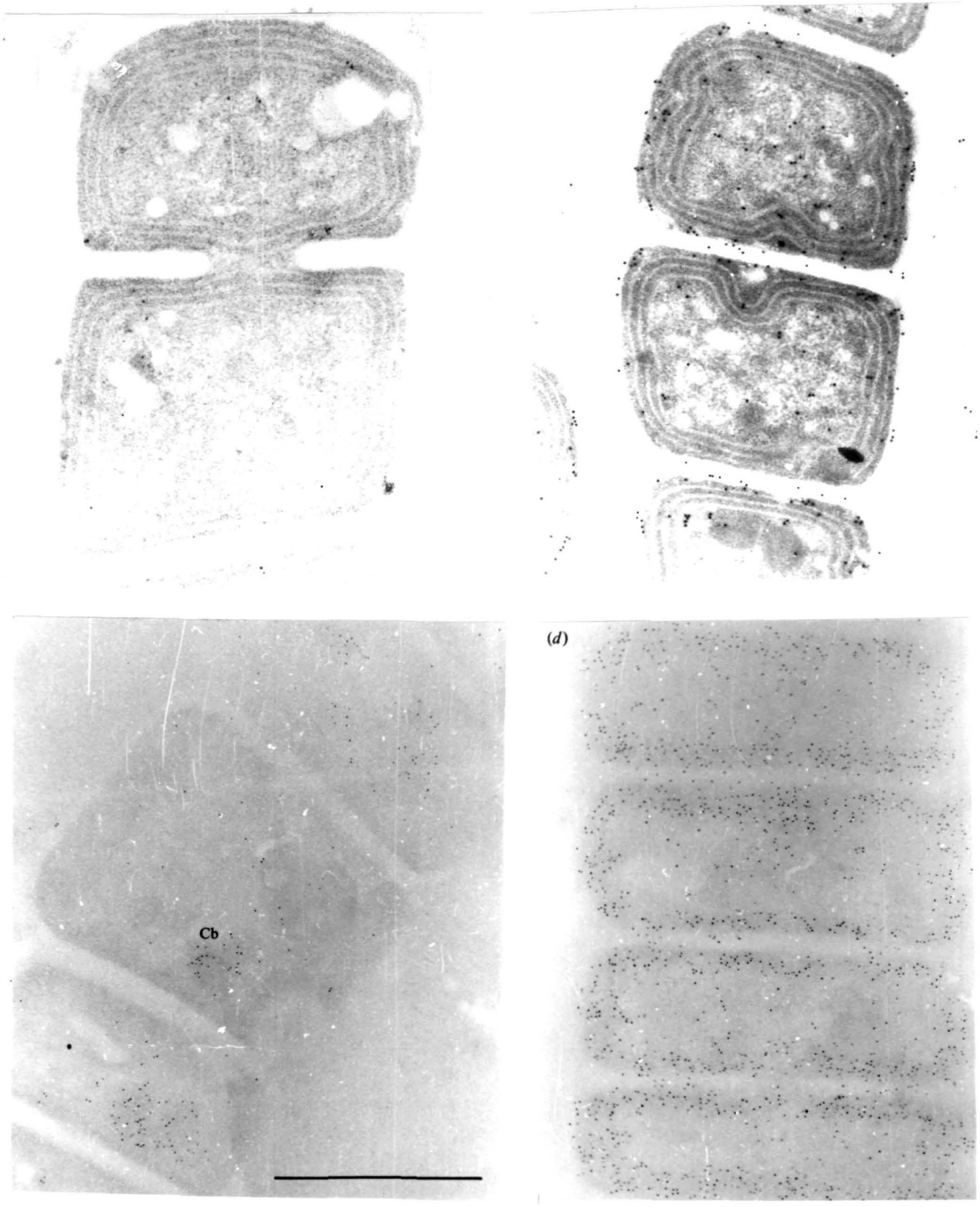


Fig. 5.5. Induction of NO_3^- uptake (□,●) and NR (○,■) activities on transfer of N_2-fixing *P. boryanum* cells to NO_3^- - medium (BG-11) without (□,■) or with (○,●) chloramphenicol (100 μg ml^{-1}). Nitrogenase was derepressed as in Fig. 5.1(b). After the appearance of the nitrogenase peak, the cells were harvested by centrifugation and resuspended in NO_3^--medium (zero time), then maintained under aerobic growth conditions. At time intervals samples were withdrawn and NO_3^- uptake rate and NR activities measured.

Fig. 5.6. Immunogold localization of nitrogenase (a), GS (b), RuBisCo (c) and PE (d) in nitrate grown cells of *P. boryanum*, Rabbit anti-*R. rubrum* nitrogenase Fe-protein (a), anti-*Anabaena* 7120 GS (b), anti-*S. alba* RuBisCo (c) and anti-*P. percicinum* PE (d) were used as primary antibodies at a dilution of 1:100. Goat anti-rabbit IgG conjugated to 5nm (a,c,d) or 10 nm (b) colloidal gold was used as secondary antibody at a dilution of 1:20. Cb, carboxysomes. Bar = 1 μm (all parts of the figure are at the same magnification).

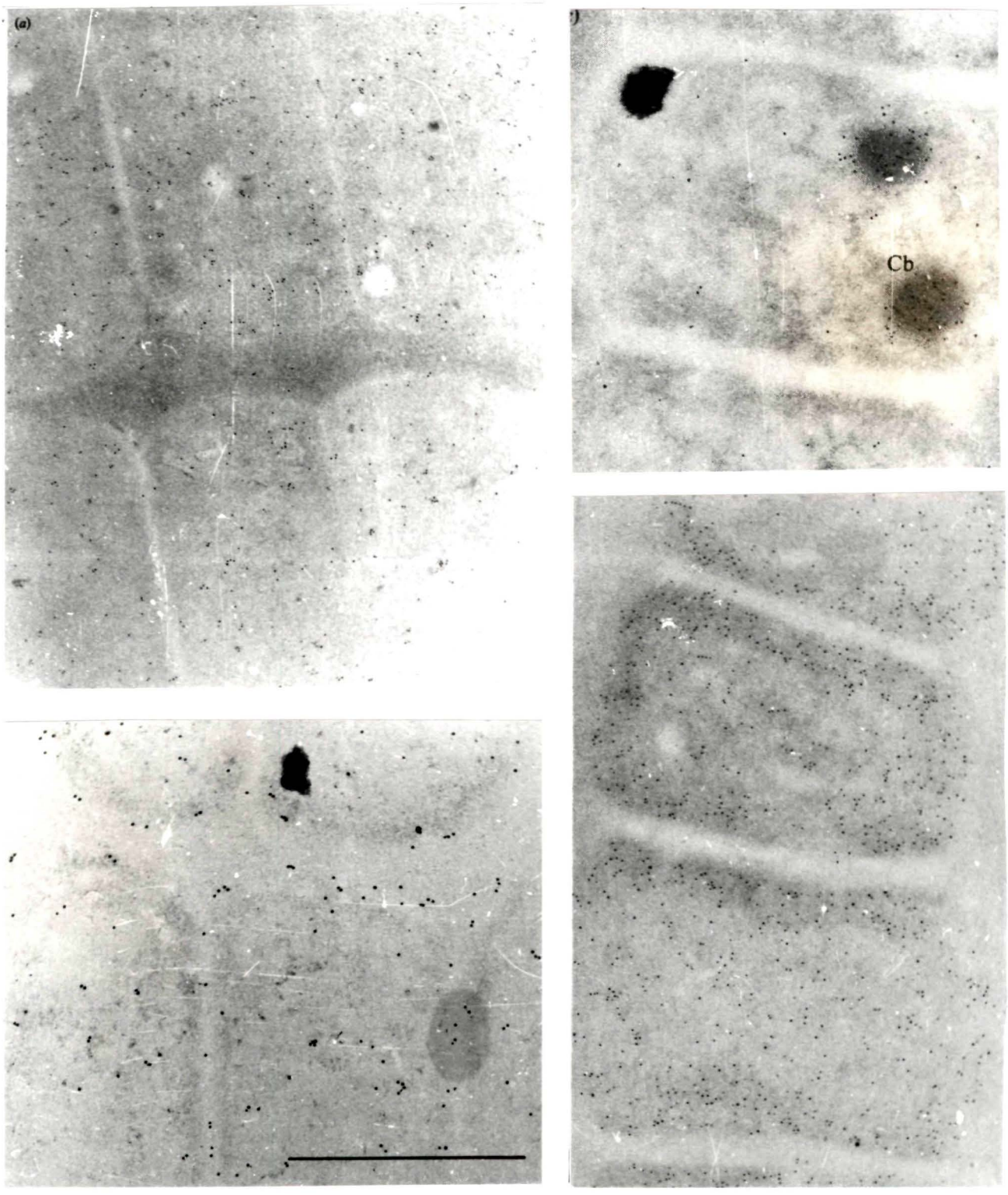

Cb
(d)

and N_2-fixing *P.boryanum* cells. GS biosynthetic activity in the two cultures was 45 ± 2.8 and 56 ± 3.1 nmol product formed min^{-1} (mg $protein^{-1}$), respectively. Immunolabelling experiments showed that the GS antigen was distributed throughout the cell both in NO_3^--grown and N_2-fixing cells (Figs. 5.6b, 5.7b). The relative density of the gold label were 22 ± 2.5 and 27 ± 2.6 gold particles per μm^2 cell area respectively. Thus, a 20% increase in GS activity and protein label occurred on nitrogenase derepression. This is in contrast to the report of Nagatani & Haselkorn (1978), who found no increase in GS activity during nitrogenase derepression under an argon atmosphere. The differences may have arisen due to different condition used for nitrogenase derepression, including the fact that we have used N_2/CO_2 (95:5, v/v) for nitrogenase derepression.

5.3.7 RuBisCo localization:

RuBisCo was localized in NO_3^--grown and N_2-fixing cells of *P. boryanum* (Figs. 5.6c, 5.7c). In the latter case, cells were processed for immunolabelling at the beginning of the appearance of nitrogenase, when the nitrogen stress, and therefore the difference in RuBisCo, is likely to be highest. RuBisCo was present in both NO_3^--grown and N_2-fixing cells. In both cases, an intense labelling was found in carboxysomes and a lower intensity in the cytoplasm. The overall level of RuBisCo in N_2-fixing cells was 20% lower than that in NO_3^--grown cells (43 ± 3.4 and 54 ± 4 gold particles per cell, respectively). Smoker *et al.* (1990)

Fig. 5.7. Immunogold localization of nitrogenase (a), GS (b), RuBisCo (c) and PE (d) in N_2-fixing *P. boryanum* cells. Nitrogenase derepression was achieved as in Fig. 5.1 (b). The cells used for immunolabelling were sampled either at the beginning (c,d) or at the peak (a,b) of nitrogenase activity. Other details as in Fig. 5.6.


(a)
Cb

noted a much higher (over 50%) reduction in RuBisCo levels on nitrogenase derepression in *P. boryanum* 581. However, the cells had been stressed for nitrogen for 40 h in an argon atmosphere lacking N_2. These results show that unlike the situation in heterocysts, derepression of nitrogenase in *P. boryanum* does not lead to total loss of RuBisCo.

5.3.8 Localization of PE:

During nitrogenase derepression in *P. boryanum*, a transient decrease in phycocyanin had been noted (Stewart & Lex, 1970; Weare & Benemann, 1974). To see if the PE concentration also change during nitrogenase derepression, immunogold labelling was examined in NO_3^--grown and N_2-fixing cells. In the latter case, cells for immunolabelling were taken at the beginning of the appearance of nitrogenase activity, when the differences are likely to be maximal. An intense PE labelling associated with thylakoid membranes was found both in NO_3^--grown and N_2-fixing cells (Figs. 5.6d, 5.7d). In both cases, the amount of PE was similar (447 ± 15 and 435 ± 18 gold particles per cells, respectively), indicating little or no degradation of PE in *P. boryanum* under the conditions of nitrogenase derepression used here.

5.4 Discussion:

Under a N_2/CO_2 atmosphere *P. boryanum* synthesized nitrogenase and showed nitrogenase activity in the absence of

combined nitrogen (Figs. 5.1, 5.3). These activities are among the highest reported by other workers using this strain (Stewart & Lex, 1970; Weare & Benemann, 1974; Nagatani & haselkorn, 1978; Rogerson, 1980; Pearson & Howsley, 1980; Giani & Kumbein, 1986). Since the cultures used were non-synchronous, development of nitrogenase in all the cells (including those undergoing division) and the uniform distribution throughout the cells (Fig. 5.7) argue against the possibility of nitrogenase being expressed during a particular phase of the life cycle or being compartmentalized within the cell (Mitsui *et al.*, 1986; Giani & kumbein, 1986). Similar patterns of labelling have been found in *O. limosa* (Stal & Bergman, 1990), *P. boryanum* UTEX 581 (Smoker *et al.*, 1989) and *Gloeothece* PCC 6909 (A. N. Rai & Bergman, unpublished results).

Futhermore, the fact that the nitrogenase occurred in all the cells in *P. boryanum* with the labelling intensity comparable to that of heterocysts (which form only 5-10% of the total cell population in heterocystous cyanobacteria), means that the total nitrogenase protein in *P. boryanum* filaments is likely to be 10-20 times higher than that in filaments of heterocystous cyanobacteria. However, nitrogenase activity in *P. boryanum* was only 2-3 fold higher than that reported in heterocystous cyanobacteria. It is likely that this is due to limited availability of ATP and reductant. Similar arguments may apply in cases of other nonheterocystous cyanobacteria e. g. *Gloeothece* and *Oscillatoria*.

O_2 removal or nitrogen limitation alone did not lead to derepression of nitrogenase. This was indicated by the lack of nitrogenase derepression under aerobic conditions irrespective of the nitrogen status of the cell, and by the fact that even on N_2/CO_2 sparging nitrogenase derepression occured only under nitrogen-limited conditions (Figs. 5.1, 5.2, 5.6a). These results are consistent with, and provide evidence for, the suggestion that nitrogenase derepression in *P. boryanum* requires both O_2 removal and nitrogen limitation (Nagatani & Haselkorn, 1978). Absence of nitrogenase derepression in the presence of combined nitrogen is consistent with earlier observations in cyanobacteria including *P. boryanum* (Stewart, 1980; Gallon, 1989; Stewart & Lex, 1970). However, the fact that O_2 removal or lowering of O_2 tension was necessary for nitrogenase derepression, despite a temporal separation of net O_2 evolution and nitrogenase activity, and that nitrogenase activity decline sharply on exposure to air or with the onset of net O_2 evolution endogenously (Fig. 5.3), suggests that the O_2-scavenging capacity of *P. boryanum* is much more limited than in other non-heterocystous cyanobacteria. Indeed, Weare & Benemann (1974) found respiration to be only limited significance in O_2-protection in *P. boryanum.*

Our studies showing irreversible inactivation of nitrogenase by O_2 in *P. boryanum* (Fig. 5.2) are consistent with the results of Weare & Benemann (1974). Nitrogenase Fe-protein has been shown to be modified to a higher molecular mass form by O_2 in some heterocystous cyanobacteria, which results in

reversible inactivation of the protein but renders it insensitive to O_2 damage (Smith *et al.*, 1987; Reich & Böger 1989; Ernst *et al.*, 1990). A similar modification has also been noted in nonheterocystous cyanobacteria *O. limosa* (Stal & Bergman, 1990; Villbrandt *et al.*, 1992), *Trichodesmium* (Ohki *et al.*, 1991) and *Synechocystis* BO-8402 (Brass *et al.*, 1992). The fact that such a modification was not found in *P. boryanum* (Fig. 5.2b) may explain why inactivation of nitrogenase in this strain was irreversible and resulted in degradation of the enzyme.

The temporal separation of N_2 fixation and net O_2 evolution noted here (Fig. 5.3) also confirms the findings of Weare & Benemann (1974). In addition, the results show that *P. boryanum* can grow photoautotrophically with repeated cycles of N_2 fixation and growth. Since nitrogenase was found to be irreversibly inactivated and degraded on exposure to air, and regain of nitrogenase activity required fresh nitrogenase synthesis (Figs. 5.2, 5.4), it is possible that repeated phases of N_2 fixation required fresh nitrogenase synthesis and that during the following microaerobic phase nitrogenase was degraded. Giani & Krumbein (1986) have demonstrated N_2 fixation and concomitant photoautotrophic growth in *P. boryanum* at lower light intensities at continous N_2/CO_2 flushing. Thus, depending on the culture conditions, *P. boryanum* seems capable of photoautotrophic growth either with repeated phases of N_2 fixation (when sparged with N_2/CO_2 periodically) or concomitantly with continous N_2 fixation when sparged with N_2/CO_2 continuously to remove any net

O_2 evolved, keeping the culture microaerobic). Although, the precise mechanism of temporal separation is not clear, it is possible that this is achieved by changes in rates of photosynthesis, with respiration balancing photosynthetic O_2 evolution during the growth that follows. This may occur due to transient changes in phycobiliprotein levels. Although we found no significant changes in PE levels (Figs. 5.6, 5.7), phycocyanin levels are known to change and repeated degradation and synthesis of phycocyanin has been noted earlier (Stewart & Lex, 1970; Weare & Benemann, 1974; Giani & Krumbein, 1986).

Mo may be a limiting factor in cyanobacterial cells (Bagchi *et al.*, 1985). Nitrogenase and NR are both molybdoenzymes and require reduced ferredoxin as electron donor (Guerrero & Lara, 1987). Indeed, Nagatani & Haselkorn (1978) have shown that in absence of Mo, nitrogenase proteins synthesized are inactive. Occurrence of NR and nitrogenase in the same cell is likely to lead to competition for Mo and reduced ferredoxin. Heterocystous cyanobacteria avoid such competition for Mo and reduced ferredoxin by spatial separation of nitrogenase and NR (Kumar *et al.*, 1985; Rai & Bergman, 1986). Such spatial separation is not possible in non-heterocystous cyanobacteria since nitrogenase is present in all the cells. A temporal separation of NR and nitrogenase was not found in non-heterocystous cyanobacteria tested (Table 5.1). However, that nitrate uptake and NR were found to be NO_3^--inducible, with very low activities in N_2-fixing cultures (Fig. 5.5; Table 5.1) means that these cyanobacteria are

able to efficiently minimize competition between NR and nitrogenase under diazotrophic growth conditions. It is noteworthy that N_2-fixing *P. boryanum* cells retain the capacity to develop nitrate uptake and NR (Fig. 5.5), unlike heterocysts, where, these systems are lost (Rai & Bergman, 1986).

As in heterocystous cyanobacteria, the GS-GOGAT pathway has been shown to be the route of primary NH_4^+ assimilation in *P. boryanum* (Meeks *et al.*, 1978). An increase in GS activity and protein level has been noted when vegetative cells differentiate into heterocysts (*see* Wolk, 1982; Bergman *et al.*, 1985). This has been shown to be linked to nitrogenase expression and to be necessary for assimilation of N_2-derived NH_4^+ (Renstrom-Kellner *et al.*, 1990). Our results showing an increase in GS with induction of nitrogenase (Figs. 5.6, 5.7) are consistent with the above view. The increase (20%) noted in *P. boryanum* is apparently less than that in heterocysts (100%). However, since heterocysts constitute only 5-10% of the total cell population, the overall increase in GS of a N_2-fixing culture of heterocystous cyanobacterium would be only 5-10%. Thus, the GS increase in *P. boryanum* is infact higher than that in heterocystous filaments. This may reflect the fact that nitrogenase activity and therefore, the likely rate of primary ammonia production, is 2-3 fold higher in *P. boryanum*. This findings also explain why the NO_3^- is less inhibitory and acts more slowly than NH_4^+. As mentioned above, No_3^- uptake and NR levels are very low in N_2-fixing cultures and availability of NO_3^- requires 3-4 h for

induction of NO_3^- uptake and NR activity (Table, 5.1; Fig. 5.5), while NH_4^+ assimilation remains active throughout. These effects resemble the effects of NO_3^- and NH_4^+ on nitrogenase activity and protein in heterocysts (Renstrom-kellner *et al.*, 1990). Thus, during N_2-fixation *P. boryanum* cells functionally resembles heterocysts. However, unlike heterocysts, N_2-fixing *P. boryanum* cells retains PE and RuBisCo (Figs. 5.6, 5.7), they retain the capacity to develop NO_3^- uptake and NR activity on nitrate availability (Fig. 5.5), and their PC levels change only transiently (Stewart & Lex, 1970; Weare & Benemann, 1974; Giani & Krumbein, 1986).

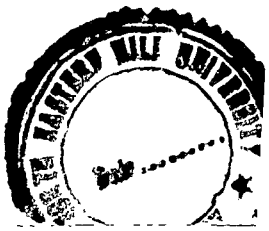
CHAPTER 6

SUMMARY

Nostoc ANTH cyanobiont was characterized in its symbiotic, free-living, immobilized and reconstituted states. Studies such as morphological and physiological changes occurring during above mentioned states as well as localization of various enzymes of nitrogen metabolism were performed. For comparative purposes non-heterocystous cyanobacteria *Plectonema boryanum* was studied with regard to nitrogenase derepression, its regulation and metabolic changes associated with diazotrophy. The findings are summarized below:

1. Characterization of symbiotic, free-living, immobilized and reconstituted *Nostoc* ANTH cyanobiont:

a) Axenic culture of *Anthoceros* was raised from spores. The cyanobiont *Nostoc* was isolated from *Anthoceros* gametophytic thalli and raised in axenic culture. Using these two axenic cultures of the symbionts, *Anthoceros-Nostoc* symbiosis was reconstituted in the laboratory under axenic conditions. Reconstitution was verified by microscopic observations of *Nostoc* colonies in the gametophytes of *Anthoceros*, growth of these *Nostoc* containing *Anthoceros* thalli in combined nitrogen free medium and the nitrogen fixation by these *Anthoceros* thalli. As the symbiosis progressed, heterocyst frequency increased



progressively to a maximum of 47% and nitrogen fixation also progressed similarly. Maximum nitrogenase activity was recorded in the middle portion of the thallus, although, the heterocyst frequency was highest in the basal portion of the thallus. This was because of occurrence of double or multiple heterocysts towards the base which although possess nitrogenase protein, get limited fixed carbon from the vegetative cells.

b) The cyanobiont isolated from the ***Anthoceros*** thalli is a ***Nostoc*** sp. (referred as ***Nostoc*** **ANTH**). It is capable of autotrophic growth using atmospheric N_2 and can also utilize other combined nitrogen sources such as ammonia and nitrate for its growth. The heterocyst frequency in the cultured free-living ***Nostoc*** **ANTH** was recorded to be 14% which is much lower than its heterocyst frequency in symbiotic state but definitely higher than other free-living cyanobacteria (5-7%).

c) Rate of nitrogen fixation and heterocyst frequency increased progressively when free-living ***Nostoc*** **ANTH** was supplied with sugars as fixed-carbon sources. Best response was obtained with the provision of glucose as fixed-carbon source. It is known that ***Anthoceros*** transfers fixed-carbon to the cyanobiont in symbiosis. Data obtained here using glucose indicated that the fixed-carbon transferred may be in the form of glucose and availability of fixed-carbon may be one of the reasons for high heterocyst frequency and higher nitrogenase activity in symbiotic ***Nostoc***.

d) Immobilization mimics one of the conditions that the cyanobiont faces while undergoing symbiotic association. Immobilization was done in calcium alginate beads which was found to be convenient and harmless. The cyanobiont initially undergoes shock upon immobilization but subsequently recovers fully. Nitrogenase activity showed a drop initially but recovered after a time gap of 144 h and subsequently exceeded its original value in free-living state. The effects of fixed nitrogen sources on heterocyst frequency and nitrogenase activity were milder than in free-living state. Similarly, rate of photosynthetic O_2-evolution and respiratory O_2-consumption showed severe inhibition upon immobilization. However, immobilized cells did recover fully between 200 h to 300 h. Drop in these two processes may explain the initial drop in nitrogenase activity as ATP and reductants necessary for nitrogenase activity are generated during photosynthesis.

e) ***Nostoc*** **ANTH** in immobilized state stayed viable for a long period (upto 5 years) in desiccated condition. These beads when put in nutrient medium swell up and ***Nostoc*** filaments grow out of the beads into the medium. It is interesting to note that the immobilized beads become the size of mustard seeds upon desiccation and are easily transportable and can be regenerated as and when required.

2. Immunoelectronmicroscopic localization of various enzymes: localization of nitrogenase, Glutamine synthetase (GS),

phycoerythrin (PE), ribulose 1,5-bisphosphate carboxylase oxygenase (RuBisCo) and hydrogenase was studied using immunochemical techniques in the cyanobiont and the free-living cultured isolate ***Nostoc* ANTH** of ***Anthoceros punctatus***. In both cases, nitrogenase was located in heterocysts only and was uniformly distributed within the cell. This put rest to the speculation that vegetative cells of cyanobionts in symbiosis may contain nitrogenase. GS was located both in heterocysts and vegetative cells, with a uniform cellular distribution in each cell type. Whereas heterocysts of ***Nostoc* ANTH** had about two-fold higher label than vegetative cells, labeling in heterocysts and vegetative cells of the cyanobiont was similar. While the GS content of the vegetative cells of the cyanobiont and ***Nostoc* ANTH** was comparable, the apparent GS content of the cyanobiont heterocysts was 60% less than that in ***Nostoc* ANTH** heterocysts. With this finding it becomes apparent that in all cyanobionts liberating ammonia, GS levels in heterocysts are repressed and become similar to that in vegetative cells. It seems higher level of GS in heterocysts is essential for assimilation of N_2-derived ammonia. PE and RuBisCo were located in vegetative cells only. PE was located on thylakoid membranes and RuBisCo in the carboxysomes and cytoplasm. Carboxysomes had much higher levels of RuBisCo than that in cytoplasm. In each case pattern and extent of labeling in the cyanobiont and ***Nostoc* ANTH** was similar. Hydrogenase was located both in vegetative cells and heterocysts of ***Nostoc* ANTH**; the former having consistently higher label than the latter. A similar pattern and level of hydrogenase labeling

was found in the cyanobiont cells residing in *Anthoceros punctatus* tissue. In *Nostoc* ANTH as well as in cyanobiont cells, a higher intensity of hydrogenase labeling was observed along the plasma membranes between vegetative cells. The eukaryotic partner did not show any hydrogenase antigen.

3. Nitrogenase derepression, its regulation and metabolic changes associated with diazotrophy in the non-heterocystous Cyanobacterium *Plectonema boryanum* PCC 73110: The regulation of nitrogenase derepression, plus the catalytic activity and protein concentration of glutamine synthetase (GS), nitrate reductase (NR), ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCo) and phycoerythrin (PE) were studied in filamentous non-heterocystous cyanobacterium *Plectonema boryanum* PCC 73110. Both nitrogen limitation and microaerobic incubation were essential for derepression of nitrogenase. Oxygen caused irreversible inactivation of nitrogenase, as well as repression of its synthesis. A temporal separation of nitrogen fixation and photosynthetic O_2-evolution was observed under N_2/CO_2 (95:5 v/v) atmosphere. Repeated peaks of nitrogenase and growth were observed. Immunogold localization showed that in N_2-fixing cultures, all cells, including those undergoing division, contained nitrogenase, and that the nitrogenase antigen was uniformly distributed throughout the cells without any preferential association with cellular structures. Thus, the earlier speculation about possible subcellular compartmentalization of nitrogenase in non-heterocystous

cyanobacteria seems unfounded. RuBisCo was mainly located in carboxysomes of both N_2-fixing and NO_3^--grown cells. Both N_2-fixing and NO_3^--grown cells showed similar levels of PE, which was associated with the thylakoid membranes. GS antigen was distributed throughout the cells and the relative amounts of this enzyme, as well as its activity, were 20% higher in N_2-fixing than NO_3^--grown cultures. NO_3^--uptake and NR systems were found to be NO_3^--inducible, with very low activities in N_2-fixing cultures. The latter may be important in avoiding competition for Mo between nitrogenase and NR. Thus, *P. boryanum* differs from heterocystous cyanobacteria in having nitrogenase in all the cells. However, as in heterocysts, GS levels go up on derepression of nitrogenase, although not to the same extent. Furthermore, NO_3^--uptake and reductase activities are lost under conditions of nitrogenase derepression, as has been noted on heterocyst development.

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PUBLICATIONS

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Anthoceros–Nostoc Symbiosis: Immunoelectronmicroscopic Localization of Nitrogenase, Glutamine Synthetase, Phycoerythrin and Ribulose-1,5-bisphosphate Carboxylase/Oxygenase in the Cyanobiont and the Cultured (Free-living) Isolate *Nostoc* 7801

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Localization of nitrogenase, glutamine synthetase (GS), phycoerythrin (PE) and ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) was studied with immunocytochemical techniques in the cyanobiont and the free-living cultured isolate *Nostoc* 7801 of *Anthoceros punctatus*. In both cases nitrogenase was located in heterocysts only and was uniformly distributed within the cell. GS was located both in heterocysts and vegetative cells, with a uniform cellular distribution in each cell type. Whereas heterocysts of *Nostoc* 7801 had about twofold higher label than vegetative cells, labelling in heterocysts and vegetative cells of the cyanobiont was similar. While the GS content of the vegetative cells of the cyanobiont and *Nostoc* 7801 was comparable, the apparent GS content of the cyanobiont heterocysts was 60% less than that in *Nostoc* 7801 heterocysts. PE and RuBisCO were located in vegetative cells only. PE was located on thylakoid membranes and RuBisCO in the cytoplasm and carboxysomes. In each case the pattern of labelling in the cyanobiont and *Nostoc* 7801 was similar.

INTRODUCTION

N_2-fixing cyanobacteria form symbiotic associations with plants that range from algae to angiosperms (Gallon & Chaplin, 1987; Smith & Douglas, 1987). The cyanobacterial partner (cyanobiont) in all these symbioses is known to fix N_2 and make fixed nitrogen available to the eukaryotic partner (see Stewart *et al.*, 1983). In some symbioses (e.g. in lichens and *Azolla*) the cyanobiont is photosynthetically active. However, in bryophytes, cycads and *Gunnera*, the cyanobiont is photosynthetically inactive and dependent on the eukaryotic partner for its fixed carbon (Smith & Douglas, 1987; Stewart *et al.*, 1983). The transfer of metabolites between the cyanobiont and eukaryotic partner is linked to physiological and biochemical changes in the cyanobiont (Stewart *et al.*, 1983; Meeks *et al.*, 1985; Nierzwicki-Bauer & Haselkorn, 1986; Lindblad *et al.*, 1987).

Among the bryophyte–cyanobacterial symbioses, the hornwort *Anthoceros–Nostoc* symbiosis is best characterized. N_2-fixing *Nostoc* spp. develop in cavities on the undersurface of the *Anthoceros* gametophyte thallus. The cyanobiont in this association reportedly has an increased heterocyst frequency, is photosynthetically inactive and lacks phycobiliproteins (Rodgers & Stewart, 1977; Stewart & Rodgers, 1977). The increased heterocyst frequency does not correlate with an increase in N_2-fixation rates (Rodgers & Stewart, 1977; Stewart & Rodgers, 1977). Whether this is due to limited availability of fixed carbon or to the presence of nonfunctional heterocysts lacking nitrogenase is not known. The activity of glutamine synthetase (GS) in the

Abbreviations: GS, glutamine synthetase; GDH, glutamic dehydrogenase; PE, phycoerythrin; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; TEM, transmission electronmicroscopy.

cyanobiont is three- to fourfold lower than that in the free-living isolate; this reduction is apparently due to regulation at the level of enzyme inactivation rather than synthesis (Joseph & Meeks, 1987). This is in contrast to the situation in lichen and *Azolla* cyanobionts, where the reduction in GS activity has been attributed to low rates of enzyme synthesis (Stewart *et al.*, 1983; Rowell *et al.*, 1985; Nierzwicki-Bauer & Haselkorn, 1986). The relative distribution of GS between vegetative cells and heterocysts of the cyanobiont in *Anthoceros* is not known.

Using immunocytochemistry, the aims of this study were (1) to examine the content of GS protein and its relative distribution between heterocysts and vegetative cells, and (2) to check for the presence of nitrogenase, phycoerythrin (PE) and ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) in vegetative cells and in single and multiple heterocysts in the cyanobiont from mature cyanobacterial colonies of the *Anthoceros punctatus* gametophyte. For comparison, the free-living cultured isolate *Nostoc* 7801 was also studied.

METHODS

Organisms. Nostoc sp. strain 7801 was obtained from Dr J. C. Meeks (Department of Bacteriology, University of California, Davis, USA). Axenic batch cultures were grown in BG-11_0 medium (Rippka *et al.*, 1979) at 25 °C and at a photon fluence rate of 70 μmol m^{-2} s^{-1}. *Anthoceros punctatus* gametophyte thalli bearing cyanobacterial colonies were collected from North-Eastern Hill University campus (Shillong, India) at the end of September, at around 11 a.m. The thalli were cleaned using distilled water and mature colonies from middle portions of the thalli were picked using a needle and fine forceps.

Antibodies. Rabbit serum containing antibodies raised against Fe-protein of nitrogenase (dinitrogenase reductase), purified from *Rhodospirillum rubrum*, was a gift from Dr S. Nordlund (University of Stockholm, Stockholm, Sweden) and has been previously used for specific localization of nitrogenase in free-living and symbiotic cyanobacteria (Bergman *et al.*, 1986). Antibody against GS protein, purified from *Anabaena* PCC 7120, was a gift from Professor R. Haselkorn (University of Chicago, Chicago, USA); it has been shown to be monospecific, and has been used for GS localization in cyanobacteria (Orr & Haselkorn, 1982; Bergman *et al.*, 1985). Antibodies against PE, purified from the cyanobacterium *Phormidium persicinum*, were a gift from Dr D. Guard-Friar and its specificity has been shown by Guard-Friar *et al.* (1986). These antibodies were also seen to be specific from the labelling pattern observed, i.e. high labelling in thylakoids of vegetative cells and comparatively little labelling in heterocysts, which are known to have little or no phycobiliproteins (see Stewart, 1980).

Rabbit serum containing antibodies raised against RuBisCO purified from *Sinapis alba* was a gift from Dr R. Oelmüller (University of Freiburg, Freiburg, FRG). The antibodies have been shown to be specific against chloroplast RuBisCO (Oelmüller & Mohr, 1986). Their specificity with cyanobacterial RuBisCO was assumed, indirectly, from the labelling pattern observed, i.e. high labelling in vegetative cells, particularly carboxysomes (which are known to be RuBisCO-containing bodies in cyanobacteria), and very low labelling in heterocysts. This pattern of labelling is identical to that observed using RuBisCO antibodies raised against the enzyme purified from cyanobacteria (see Cossar *et al.*, 1985).

Immunolabelling and transmission electron microscopy (TEM). Preparation of samples for TEM and the protocols for immunolabelling were essentially as described by Bergman *et al.* (1985) except that uncoated gold grids were used, the goat anti-rabbit IgG was conjugated to 15 nm size colloidal gold particles and different primary antibodies were used as required (see figure legends). Ultrathin sections of glutaraldehyde-fixed and Epon-embedded samples were cut with an LKB Ultramicrotome and picked up on gold grids (300 μm mesh). These were then labelled for 1 h with primary antibody (rabbit anti-nitrogenase, GS, PE or RuBisCO) followed by 30 min incubation with secondary antibody (goat anti-rabbit IgG conjugated to 15 nm size colloidal gold particles). After cross-reaction, the sections on grids were stained with uranyl acetate and lead citrate. TEM observations were made using a Jeol 100B electron microscope operated at 60 kV. Five ultrathin sections from three mature *Nostoc* colonies from *Anthoceros punctatus* gametophyte were used in each labelling experiment. However, except for GS, where 15 electronmicrographs were taken, only five or six electronmicrographs were taken. We present one set of representative micrographs, for each labelling experiment, which we assume reflect the functional symbiosis.

RESULTS AND DISCUSSION

Biochemical characterization of the cyanobiont has been rather difficult because of the problem of obtaining enough clean material from the symbiotic association. Furthermore, in isolating the cyanobiont, whole thalli are generally used. This results in metabolically heterogeneous cyanobiont populations since cyanobionts are in different stages of developing symbiosis in different parts of the thallus, as indicated by varying heterocyst frequency and

enzymic activities (see Englund, 1977; Lindblad *et al.*, 1985; Rowell *et al.*, 1985). With the introduction of immunogold labelling these problems can be avoided and an antigen can be located precisely with little sample material. Here, we have used this method to study the *Anthoceros–Nostoc* association in mature cyanobacterial colonies from the mid-part of the *A. punctatus* thallus.

The secondary antibody (goat anti-rabbit IgG) was first tested for any nonspecific binding to the fixed cells. Virtually no label was detected in the absence of the primary antibody either in the cyanobiont or in *Nostoc* 7801 (Fig. 1*a*, *b*). This showed that the antibody was specific to rabbit IgG. The location of the specific antigens was then determined using primary antibodies to nitrogenase, GS, PE and RuBisCO.

Nitrogenase

In heterocystous cyanobacteria, nitrogenase is restricted to heterocysts under aerobic growth conditions (Stewart, 1980; Janaki & Wolk, 1982). However, nitrogenase may be expressed in vegetative cells of non-heterocystous cyanobacteria both under microaerobic (Stewart & Lex, 1970; Rippka & Waterbury, 1977) and aerobic (Stal & Krumbein, 1985) conditions. Whether nitrogenase can be induced in vegetative cells of heterocystous cyanobacteria is less certain (Flemming & Haselkorn, 1974; Murry *et al.*, 1984). Rippka & Stanier (1978) and Spence & Stewart (1987) isolated mutants of *Anabaena* lacking heterocysts in which N_2-fixation still occurs. Bergman *et al.* (1986) have shown that in lichens and in root nodules of cycads, nitrogenase is restricted to heterocysts only. However, the possibility of N_2-fixation in vegetative cells of the cyanobiont in *Anthoceros–Nostoc* symbiosis has been suggested (Rodgers & Stewart, 1977).

Immunolabelling experiments on the cyanobiont of *A. punctatus* showed that the nitrogenase was located in heterocysts only (Fig. 2*a*). The pattern and intensity of labelling was similar in the free-living *Nostoc* 7801 (Fig. 2*b*). The distribution of the antigen within the heterocysts was uniform. Labelling in vegetative cells was very low, suggesting that they contained little or no nitrogenase.

As in the *Nostoc* cyanobiont of lichens (Englund, 1977) and cycads (Lindblad *et al.*, 1985), the heterocyst frequency of the cyanobiont in bryophytes can vary with the age of the colony (Rodgers & Stewart, 1977). A maximum heterocyst frequency of 43%, as against 3–6% in the free-living *Nostoc*, has been observed in the cyanobiont of *Anthoceros* (Rodgers & Stewart, 1977). However, the increase in nitrogenase activity was much less than the increase in heterocyst frequency (Rodgers & Stewart, 1977), indicating the presence of nonfunctional heterocysts. Immunogold labelling showed that all the heterocysts of the cyanobiont, including the multiple ones, contained the nitrogenase protein (Fig. 2*a*, *b*). This suggests that the supply of fixed carbon to multiple heterocysts occurring in chains may be restricted. A similar case has been found in the cycad cyanobiont (Bergman *et al.*, 1986). Although the photosynthate is provided by the eukaryotic partner in *Anthoceros* (Rodgers & Stewart, 1977), it probably enters the heterocysts via the vegetative cells. Heterocysts with no direct contact with the vegetative cells may receive little or no fixed carbon. In the cycad cyanobiont, an increase in nitrogenase activity correlates with an increase in the frequency of single heterocysts throughout the root (tip to base), but with increasing number of multiple heterocysts this correlation is lost (Lindblad *et al.*, 1985).

Glutamine synthetase

GS (EC 6.3.1.2) is the primary ammonia-assimilating enzyme in heterocystous cyanobacteria (see Stewart, 1980). In lichens, *Azolla* and hornwort symbioses the GS activity in the cyanobiont is reduced, which may contribute to the liberation of N_2-derived ammonia from the cyanobiont (see Stewart *et al.*, 1983; Meeks *et al.*, 1985).

In the *Anthoceros–Nostoc* association, GS activity in the cyanobiont has been reported to be reduced three- to fourfold without a similar decrease in the GS content (Joseph & Meeks, 1987). This led to the conclusion that GS activity in the cyanobiont is regulated by a post-translational mechanism. However, the relative distribution of the inactive GS protein between heterocysts and vegetative cells is not known.

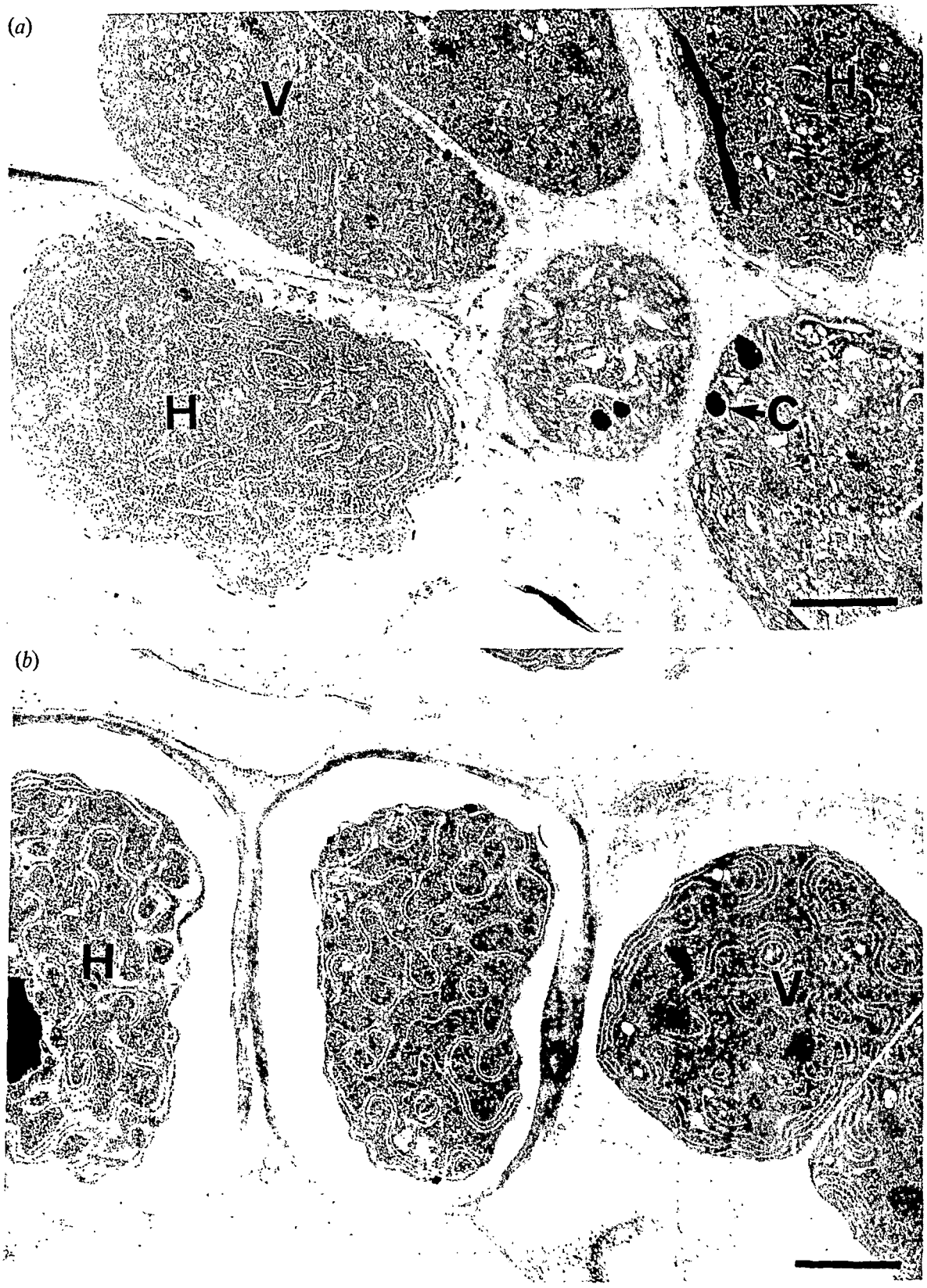

Fig. 1. Background (unspecific) labelling due to secondary antibody (goat anti-rabbit IgG, gold conjugated) in the cyanobiont (*a*) and in the free-living cultured isolate *Nostoc* 7801 (*b*). In these experiments primary antibody was omitted during immunolabelling. V, Vegetative cells; H, heterocysts; C, cyanophycin. Bar marker, 1 micrometer.

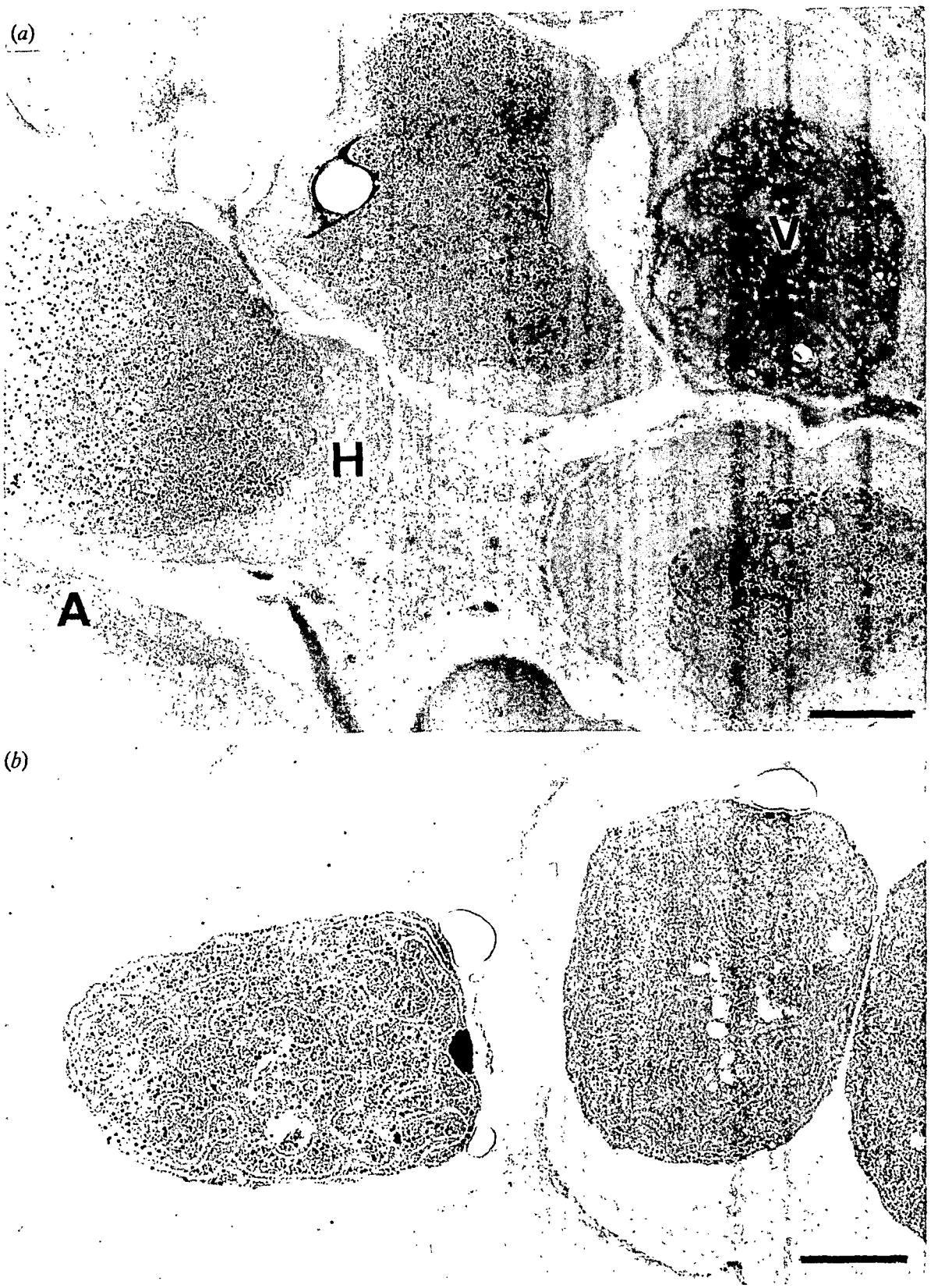

Fig. 2. Localization of nitrogenase in the cyanobiont (*a*) and in the free-living cultured isolate *Nostoc* 7801 (*b*). Rabbit anti-*Rhodospirillum rubrum* nitrogenase (Fe-protein) was used as the primary antibody. Other details as in Fig. 1; A, *Anthoceros* tissue.

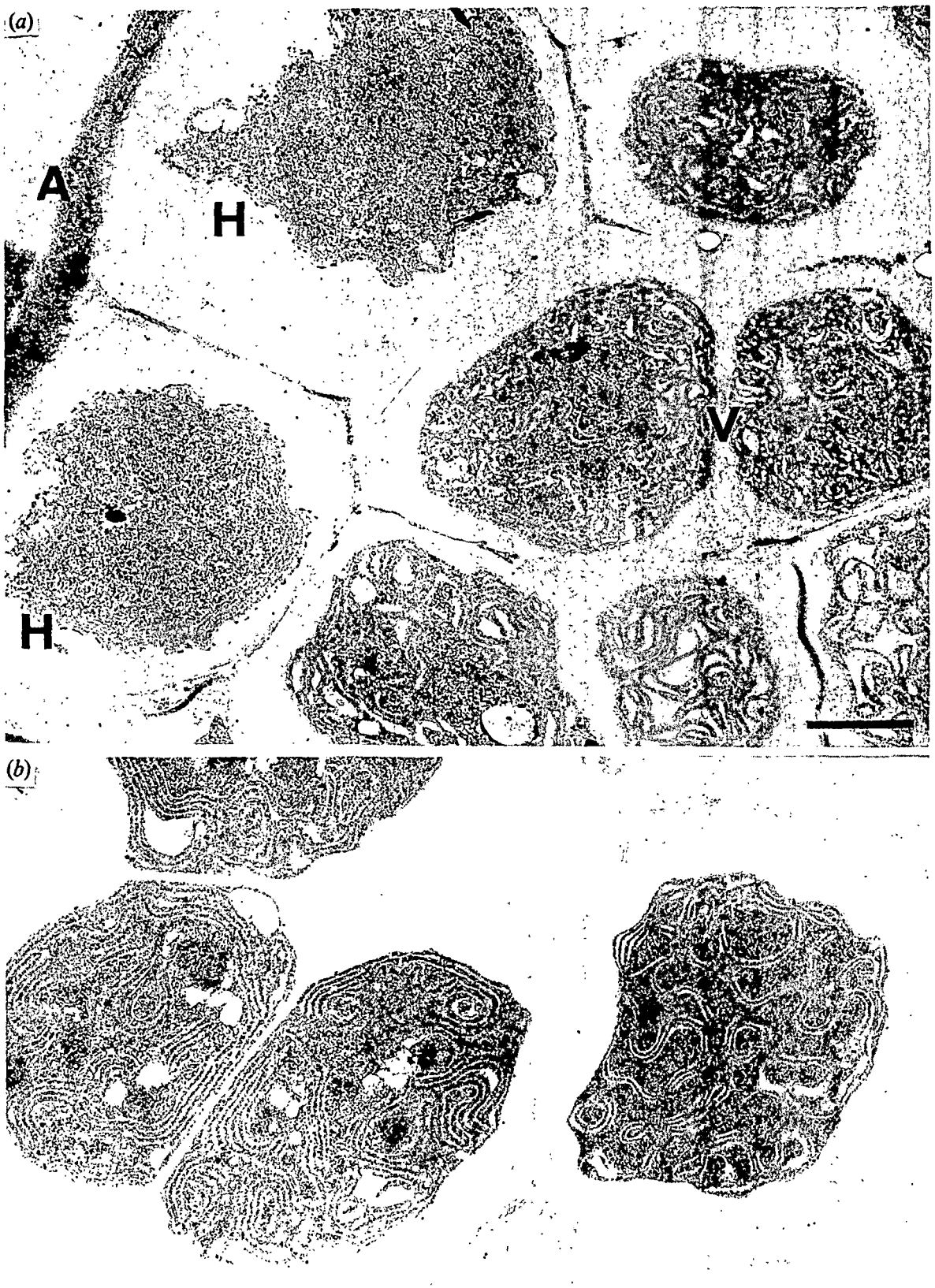

Fig. 3. Localization of GS in the cyanobiont (*a*) and in the free-living cultured isolate *Nostoc* 7801 (*b*). Rabbit anti-*Anabaena* 7120 GS was used as primary antibody. Other details as in Figs 1 and 2.

Table 1. *GS content of heterocysts and vegetative cells of the cyanobiont and the free-living cultured isolate Nostoc 7801 from Anthoceros punctatus*

The GS contents given below are comparative and were calculated by counting the number of gold particles per unit cell area using TEM photomicrograph prints (at × 15000 magnification). The data are means ± SE of 70 counts from 15 photomicrographs taken from five ultrathin sections of three symbiotic colonies.

Sample	No. of gold particles cm^{-2}
Nostoc 7801	
Vegetative cells	8·21 ± 1·35
Heterocysts	17·30 ± 3·65
Cyanobiont	
Vegetative cells	6·96 ± 1·30
Heterocysts	7·15 ± 1·71

The GS protein was present in both heterocysts and vegetative cells (Fig. 3*a*, *b*) and its distribution was uniform within the cells. As in other free-living cyanobacteria (Bergman *et al.*, 1985), labelling in *Nostoc* 7801 heterocysts was nearly twofold higher than that in vegetative cells (Fig. 3*b*, Table 1). In contrast, the label in the cyanobiont was similar in heterocysts and vegetative cells (Fig. 3*a*, Table 1). Multiple heterocysts also contained similar amounts of the label. The GS content was similar in vegetative cells of the cyanobiont and *Nostoc* 7801 but the cyanobiont heterocysts had 60% less GS protein than the heterocysts of *Nostoc* 7801. Thus, reduction in GS activity of the cyanobiont is at least partly due to a reduction in the GS content of heterocysts. This contrasts with the situation in lichens and *Azolla*, where reduction in GS activity is due only to decreased synthesis of GS (see Stewart *et al.*, 1983; Nierzwicki-Bauer & Haselkorn, 1986). It also differs from the cycad cyanobiont, which has full GS activity and normal GS content, with a distribution pattern in heterocysts and vegetative cells similar to that in heterocysts and vegetative cells of the free-living isolate (Lindblad & Bergman, 1986). However, the selective decrease in GS content in heterocysts noted here resembles the situation in lichens (Hällbom *et al.*, 1986) and *Azolla* (B. Bergman, unpublished results), where the distribution of residual GS in the cyanobiont is similar in both heterocysts and vegetative cells. It is interesting to note here that when N_2-grown cultures of *Anabaena cylindrica* are transferred to ammonium medium, the few heterocysts which remain show a GS content similar to that of vegetative cells (E. Renström, unpublished results).

Phycoerythrin

Phycobiliproteins are accessory photosynthetic pigments in cyanobacteria. They also serve as a nitrogen reserve and undergo degradation during nitrogen limitation (Cohen-Bazire & Bryant, 1982; Ho & Krogman, 1982). The cyanobionts in lichens, *Azolla* and cycads possess phycobiliproteins (see Stewart *et al.*, 1983; Lindblad, 1987). However, Stewart & Rodgers (1977) were unable to detect phycobiliproteins in the cyanobiont of *Anthoceros punctatus*, although structures similar to phycobilisomes were noted in their TEM photomicrographs.

Immunolabelling experiments showed that PE is present in the cyanobiont of *A. punctatus* (Fig. 4). The pattern of labelling was similar to that in free-living *Nostoc* 7801. The labelling of PE was associated with thylakoid membranes in vegetative cells. There was comparatively little labelling in heterocysts; this is consistent with the fact that heterocysts are generally deficient in phycobiliproteins (see Stewart, 1980). The intensity of labelling was similar in vegetative cells of the cyanobiont and *Nostoc* 7801. Cyanophycin granules (nitrogen reserves) were also noted in the cyanobiont (see Fig. 1), indicating that the cyanobiont is not nitrogen starved. Considering that the frequency of heterocysts (which have a very low phycobiliprotein content) is eight or nine times higher in the cyanobiont, the overall level of phycobiliproteins would be considerably lower than that of *Nostoc* 7801.

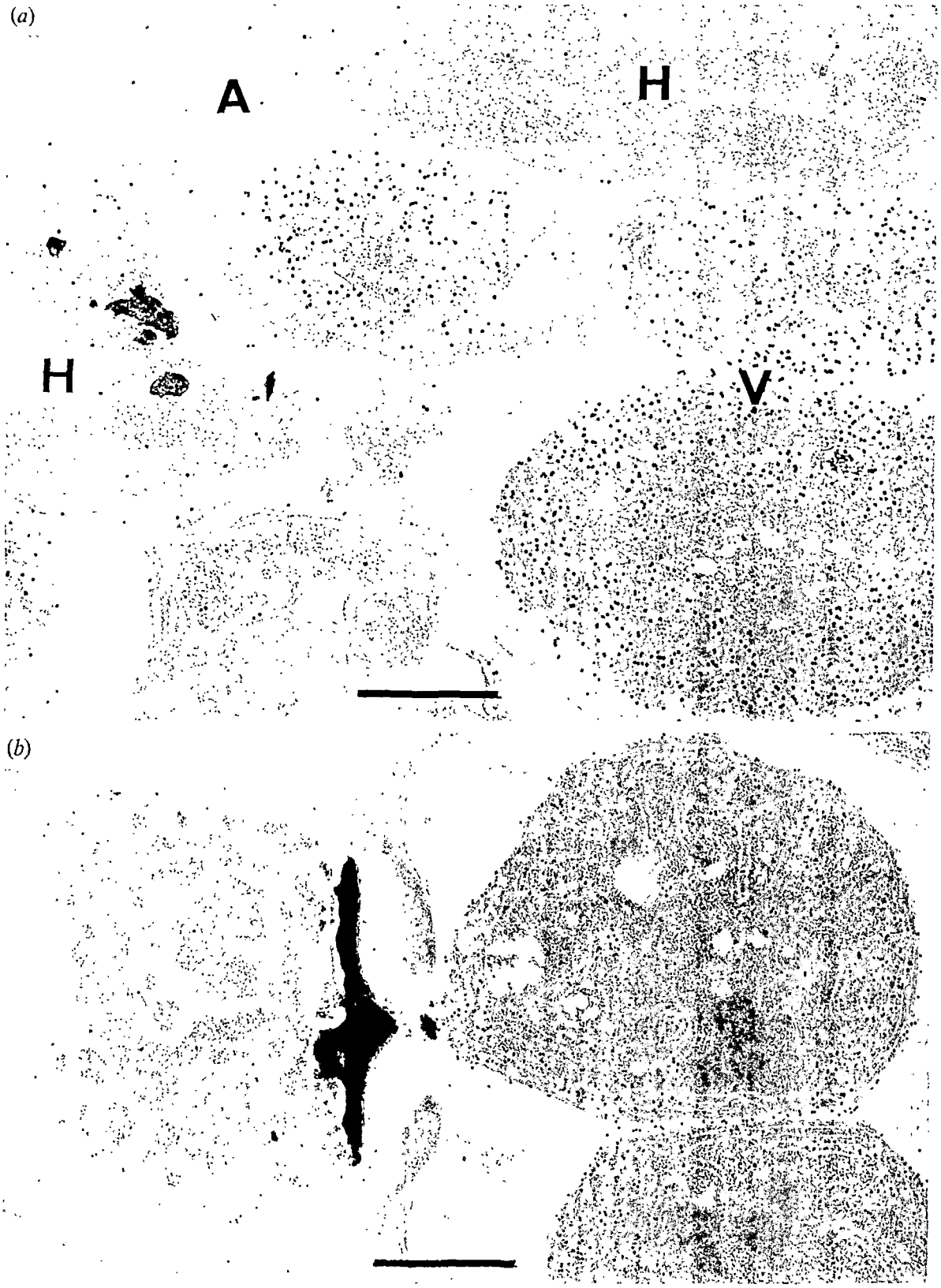

Fig. 4. Localization of PE in the cyanobiont (*a*) and in the free-living cultured isolate *Nostoc* 7801 (*b*). Rabbit anti-*Phormidium percicinum* PE was used as primary antibody. Other details as in Figs 1 and 2.

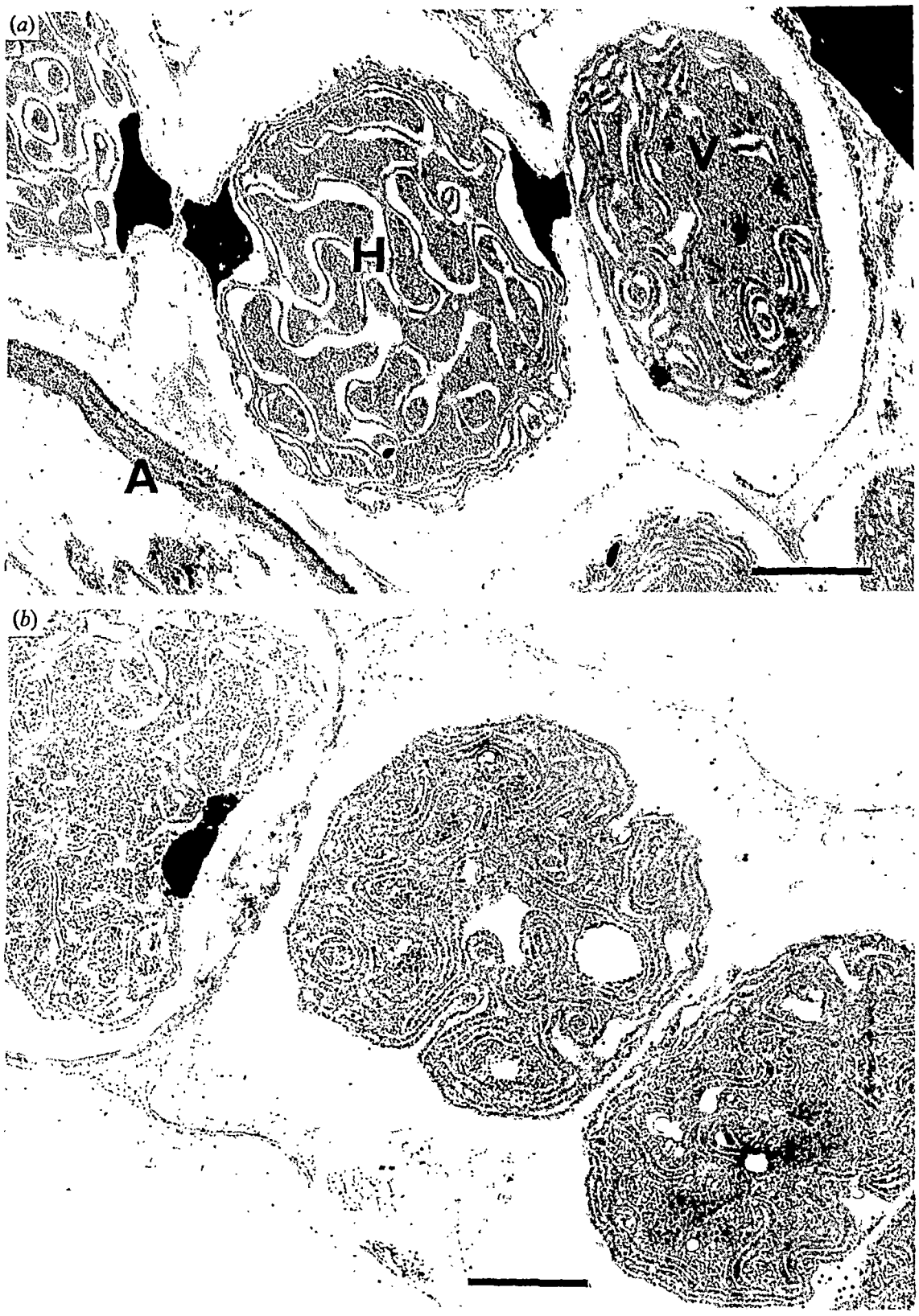

Fig. 5. Localization of RuBisCO in the cyanobiont (*a*) and in the free-living cultured isolate *Nostoc* 7801 (*b*). Rabbit anti-*Sinapis alba* RuBisCO was used as primary antibody. Other details as in Figs 1 and 2.

Ribulose-1,5-bisphosphate carboxylase/oxygenase

RuBisCO (EC 4.1.1.39) is the primary carboxylating enzyme in cyanobacteria, and cyanobacterial carboxysomes (polyhedral bodies) contain RuBisCO (see Codd & Marsden, 1984). The cyanobiont of *Anthoceros punctatus* has been shown to be deficient not only in photosynthetic O_2-evolution but also in CO_2-fixation (Rodgers & Stewart, 1977).

In both the cyanobiont and the free-living *Nostoc* 7801, RuBisCO was present in vegetative cells, with little or no labelling in heterocysts (Fig. 5*a*, *b*). This is consistent with the reported absence of RuBisCO in heterocysts (Codd *et al.*, 1980; Cossar *et al.*, 1985). The labelling within the vegetative cells was most prominent in carboxysomes, although label was also present in the cytoplasm. The pattern of labelling was similar in the cyanobiont and *Nostoc* 7801. Thus, the inability of the cyanobiont, in *A. punctatus*, to fix CO_2 is not due to lack of RuBisCO. In the case of the cycad cyanobiont, *in vitro* RuBisCO activity is similar to that of the free-living isolate; however, there is no *in vivo* CO_2-fixation by the cyanobiont (Lindblad *et al.*, 1987).

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Immunogold Localization of Hydrogenase in the Cyanobacterial-Plant Symbioses *Peltigera canina*, *Anthoceros punctatus* and *Gunnera magellanica*

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Abstract

Using immunoelectronmicroscopy, localization and levels of hydrogenase were studied in the cyanobionts of three cyanobacterial-plant symbioses: *Peltigera canina*, *Anthoceros punctatus* and *Gunnera magellanica*. Free-living (cultured) *Nostoc* isolates from all the three symbioses showed hydrogenase label in both vegetative cells and heterocysts; the former having consistently higher label than the latter. A similar pattern and level of hydrogenase labelling was found in the cyanobiont cells residing in *Anthoceros punctatus* tissue. In contrast, labelling in cyanobiont cells of *P. canina* and *G. magellanica* was much lower than that in the cells of their respective cultured cyanobacterial isolates. In the cultured cyanobacterial isolates as well as in the cyanobiont cells of all the symbioses studied, a higher intensity of hydrogenase labelling was observed along the plasma membranes between vegetative cells. The eukaryotic partners did not show any hydrogenase antigen in any of the symbioses studied. These data suggest lack of hydrogenase in the eukaryotic partners of all the three symbioses studied here and a reduction in the levels of hydrogenase protein in the cyanobionts of *P. canina* and *G. magellanica* but not of *A. punctatus*. The data are discussed in relation to the cyanobionts mode of carbon nutrition and oxygen levels in these cyanobacterial-plant symbioses.

Keywords: *Anthoceros punctatus*, cyanobacteria, *Gunnera magellanica*, hydrogenase, *Nostoc*, *Peltigera canina*, symbiosis

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1. Introduction

Hydrogenases are important in diazotrophs for the recycling of hydrogen evolved during nitrogen-fixation. It has been suggested that such a recycling improves the nitrogen-fixing efficiency because it regenerates ATP and reductant, consumes oxygen and thereby contributes to the oxygen protection of nitrogenase, and prevents buildup of hydrogen which is an inhibitor of nitrogenase and photosynthesis (see Adams et al., 1981; Lambert and Smith, 1981; Houchins, 1984; Antarikanonda et al., 1980). Hydrogen metabolism has been studied extensively in diazotrophic bacteria (Adams et al., 1981; Gogotov, 1986; Evans et al., 1987, 1988), cyanobacteria (Lambert and Smith, 1981; Houchins, 1984; Papen et al., 1986; Almon and Boger, 1988; Rao and Hall, 1988; Chen et al., 1989; Kentemich et al., 1989; Ewart and Smith, 1989a,b, 1990), and rhizobial and actinorhizal symbioses (Evans et al., 1987, 1988; Mellor and Werner, 1990; Huss-Danell, 1990). The aim has been to understand nitrogenase-hydrogenase relationships and to increase the efficiency of nitrogen-fixation in economically important plants like legumes and actinorhizal trees.

Cyanobacteria form diazotrophic symbioses with eukaryotic plants ranging from algae to angiosperms. In these symbioses, the cyanobacterial partner (cyanobiont) undergoes several structural and metabolic modifications resulting in increased rates of of nitrogen-fixation and transfer of fixed nitrogen from the cyanobiont to the eukaryotic partner (Stewart et al., 1983). Cyanobacterial symbioses have been studied extensively with regard to nitrogen and carbon metabolism (see Rai, 1988, 1990). A few studies have been done on hydrogen metabolism and localization of hydrogenase in the free-living (cultured) cyanobacterial isolates from cycads (Kumar et al., 1986; Daday and Smith, 1987; Lindblad and Sellstedt, 1990; Tredici et al., 1990) and *Azolla* (Chan-van-Ni and Gogotov, 1984). However, information about occurrence and localization of hydrogenase in cyanobacterial symbioses are virtually nonexistent, except for preliminary studies about hydrogen uptake and evolution in cycad root nodules (Perraju et al., 1986) and *Azolla* (Peters et al., 1977).

The aim of the present study was to investigate the occurrence and localization of hydrogenase in cyanobacterial symbioses, and to compare the levels of hydrogenase in free-living and symbiotic cyanobacteria. To cover the variety of conditions encountered by cyanobacteria in cyanobacterial-plant symbioses, three cyanobacterial symbioses and their free-living (cultured) cyanobionts were studied (*Peltigera canina* and *Nostoc* PC; *Anthoceros punctatus* and *Nostoc* ANTH; *Gunnera magellanica* and *Nostoc* GM). Our data indicate a significant reduction in the levels of hydrogenase protein in symbiosis which

correlates with reduced oxygen tention but not with the mode of carbon nutrition.

2. Materials and Methods

Organisms

Nostoc PC (the isolate from *P. canina*), *Nostoc* ANTH (the isolate from *A. punctatus*) and *Nostoc* GM (an isolate from *G. magellanica*) were grown axenically in BG-11_0 medium (Rippka et al., 1979) at 25°C and in continuous light (photon fluence rate: 50 μmol $m^{-2}s^{-1}$). Cells were harvested during mid log phase. *P. canina* and *A. punctatus* thalli were collected locally from the field. *G. magellanica* was grown outdoors, using non-fertilized sandy soil, in the Botanical Garden. In the cases of *A. punctatus* and *G. magellanica*, *Nostoc*-infected tissues were carefully separated using a scalpel and a stereomicroscope. Cells of the cultured isolates, small pieces of *P. canina* thallus, and the *Nostoc*-infected tissues of *A. punctatus* and *G. magellanica* were fixed, embedded, sectioned and immunolabelled as described below.

Hydrogenase antibodies

Hydrogenase antibodies, raised in rabbit against the hydrogenase holoenzyme purified from *Alcaligenes latus*, were a gift from Dr. D.J. Arp (University of California, USA; Doyle and Arp, 1987). These antibodies have been shown to be monospecific, recognizing a single polypeptide of Mr 55 kDa, and have been used for specific localization of hydrogenase in *Nostoc* PCC 73102 (Lindblad and Sellstedt, 1990).

Immunogold labelling

Fixation, embedding, sectioning and immunolabelling protocols were essentially the same as described previously (Bergman et al., 1985) with the following differences. Rabbit anti-*Alcaligenes latus* hydrogenase was used as primary antibody at a dilution of 1:1000 and the labelling with the primary antibody was done overnight at 4°C. The secondary antibody used was 1:20 dilution of goat anti-rabbit IgG conjugated to 10 nm size colloidal gold particles (obtained from Amersham International plc, Amersham, UK). In control experiments primary antibody was omitted.

Transmission electron microscopy and estimation of hydrogenase label

Transmission electron microscopy was performed using a Zeiss EM 10 Transmission Electron Microscope (TEM) operated at 60 kV. Relative levels of hydrogenase label was estimated by counting gold particles in various cell types using TEM photomicrograph prints. These values were converted to number of gold particles per μm^2 cell area taking into account the magnification of the prints and the cell area counted. A similar exercise was done to calculate background labelling by counting gold particles per unit area outside the cells/tissues.

3. Results

Occurrence and localization of hydrogenase in free-living (cultured) cyanobacterial isolates

Three *Nostoc* spp., isolated from *P. canina* (*Nostoc* PC), *A. punctatus* (*Nostoc* ANTH) and *G. magellanica* (*Nostoc*GM), and cultured in BG-11_0 medium, were used for immunolocalization of hydrogenase. As seen in Fig. 1, hydrogenase antigen was found to be present both in heterocysts and vegetative cells of *Nostoc* PC. Most of the heterocysts observed had a lower density of hydrogenase label as compared to vegetative cells. However, we did find some heterocysts with a labelling density similar to that in vegetative cells. Within the cell, the hydrogenase antigen was evenly distributed with no preferential association with any particular structure. However, a higher intensity of hydrogenase label was found along the plasma membranes, particularly between two vegetative cells and along the plasma membranes being formed during cell division. Similar patterns of hydrogenase labelling were found in *Nostoc* ANTH and *Nostoc*GM. Background (unspecific) labelling, as evidenced from the gold distribution outside the cells were less than 5% of the specific labelling observed inside the cells. In control experiments where the primary antibody was omitted, no gold particles were observed within or outside the cells (data not shown).

A quantitative analysis of the hydrogenase label distribution in heterocysts and vegetative cells showed that in all the cyanobacterial isolates, heterocysts had significantly lower levels of the hydrogenase antigen (per unit cell area) as compared to that in vegetative cells (Fig. 5). This decrease in the hydrogenase antigen level in the heterocysts, as compared to vegetative cells, was 47% in *Nostoc* PC, 70% in *Nostoc* ANTH, and 60% in *Nostoc* GM (Fig. 5).

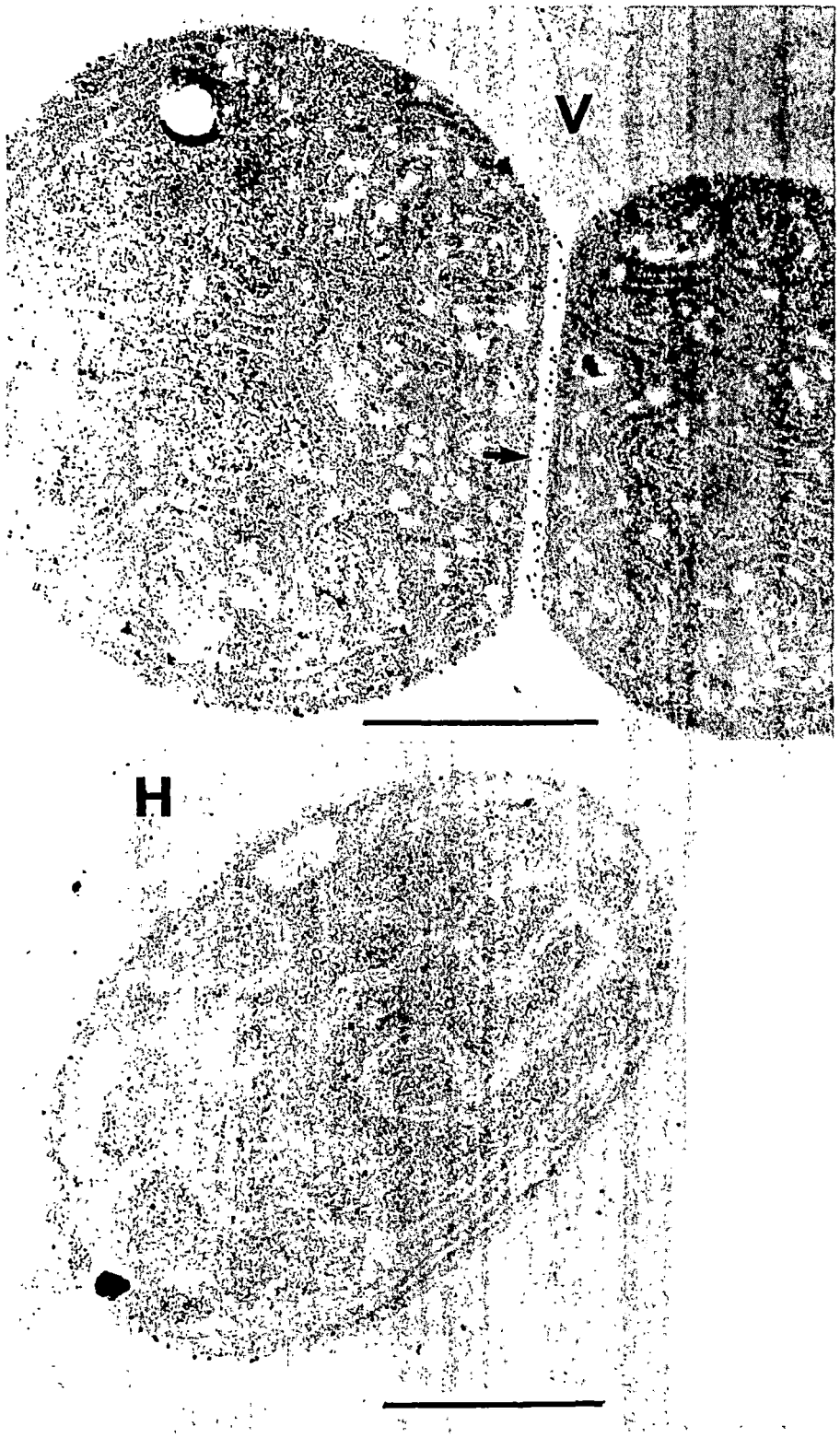

Figure 1. Immunogold localization of hydrogenase in *Nostoc* ANTH, the free-living cultured isolated from *A. punctatus*. Note the higher intensity of labelling along the cytoplasmic membranes at the cell junction (arrow). H, heterocyst; V, vegetative cell. Bar = 1 μm.

Occurrence and localization of hydrogenase in Peltigera canina

In the bipartite cyanolichen *P. canina*, hydrogenase antigen was detected both in heterocysts and vegetative cells of the cyanobiont however, labelling in the mycobiont was similar to the background (Fig. 2). The pattern of

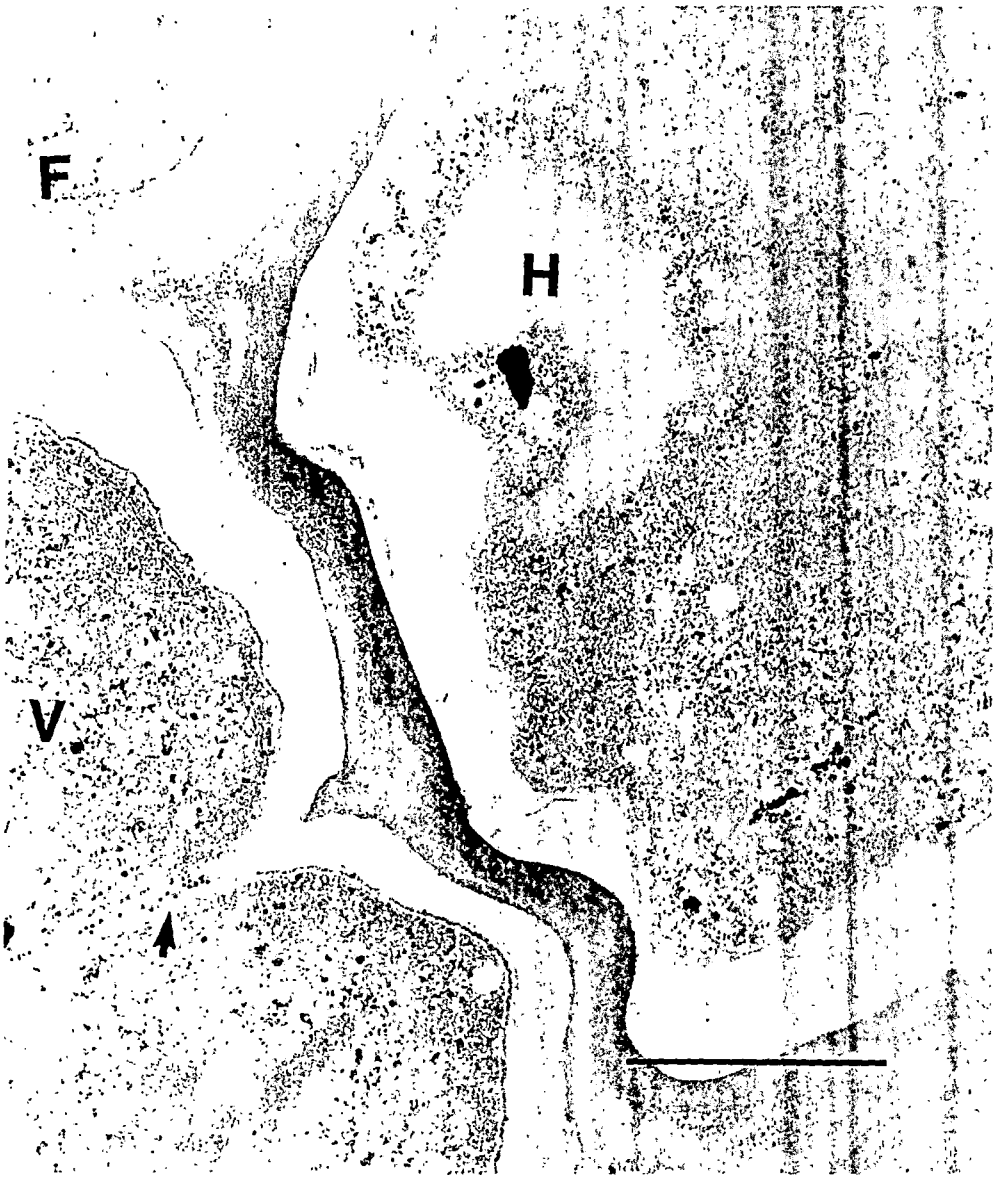

Figure 2. Immunogold localization of hydrogenase in *Nostoc* cells in *P. canina*. F, fungal hypha. Other symbols as in Fig. 1.

labelling in the cyanobiont cells was similar to that in the case of *Nostoc* PC including the high intensity of hydrogenase label along the plasma membranes between two vegetative cells. However, in contrast to the situation in *Nostoc* PC, heterocysts and vegetative cells of the cyanobiont had similar labelling intensities. Quantitative analysis of the hydrogenase antigen levels showed a

significant decrease in hydrogenase antigen level of the cyanobiont as compared to the free-living isolate *Nostoc* PC (Fig. 5). This decrease was much more pronounced in vegetative cells (65%) than in heterocysts (30%).

Occurrence and localization of hydrogenase in Anthoceros punctatus-Nostoc *symbiosis*

In the case of *A. punctatus*, hydrogenase antigen was found to be present in the cyanobiont cells but none was detected in the *Anthoceros* tissues (Fig. 3). Within the cyanobiont cells, the pattern of hydrogenase labelling was similar

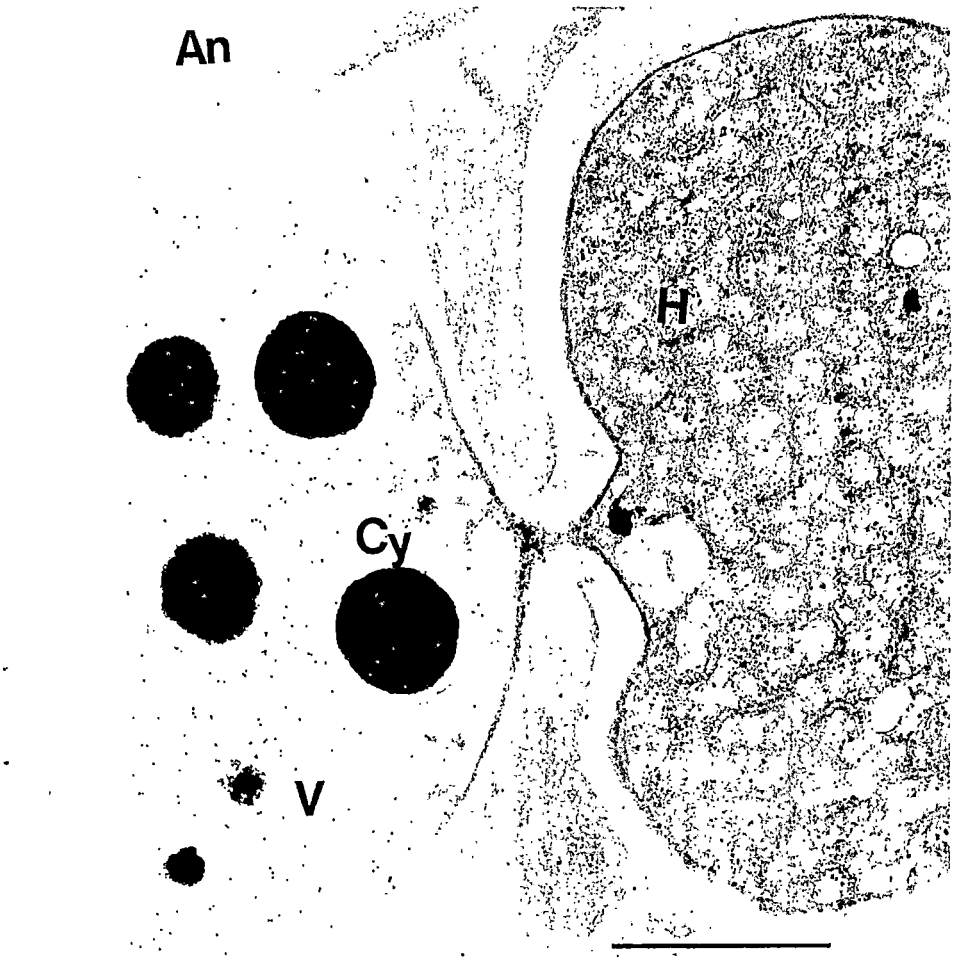

Figure 3. Immunogold localization of hydrogenase in *Nostoc* cells in *A. punctatus*tissue. An, *Anthoceros* tissue; Cy, cyanophycin granule. Other symbols as in Fig. 1.

to that in the cultured cyanobacterial isolates mentioned above. As in *Nostoc* ANTH, vegetative cells had a higher intensity of the hydrogenase label than the heterocysts. The hydrogenase antigen levels in the heterocysts of the cyanobiont in *A. punctatus* and *Nostoc* ANTH were comparable. However, the hydrogenase antigen levels in the vegetative cells were 20% lower than that in vegetative cells of *Nostoc* ANTH (Fig. 5). These results indicate that in *A. punctatus*, as in *P. canina*, hydrogenase was located in the cyanobiont cells and absent in the eukaryotic partner. However, in contrast to the situation in *P. canina*, there was little or no decrease in the hydrogenase levels of the cyanobiont in *A. punctatus*.

Occurrence and localization of hydrogenase in Gunnera magellanica-Nostoc *symbiosis*

In *Gunnera-Nostoc* symbiosis, the cyanobiont occurs intracellularly (Bonnett, 1990). Immunolabelling studies of *Nostoc* infected *G. magellanica* cells showed presence of hydrogenase antigen in the cyanobiont cells but no labelling was detectable in *Gunnera* cells (Fig. 4). The pattern of labelling was similar to that in the free-living (cultured) isolates, but the hydrogenase antigen levels were much lower in the cyanobiont cells (both in heterocysts and vegetative cells). A quantitative analysis of the hydrogenase label showed that hydrogenase level in heterocysts was 60% lower than that in vegetative cells. This is similar to the situation in the free-living (cultured) cyanobacterial isolate of *G. magellanica* (*Nostoc* GM; see Fig. 5). However, in symbiosis, there was a 75% decrease in the hydrogenase levels of both the heterocysts and the vegetative cells (Fig. 5). This decrease in the hydrogenase levels in the cyanobiont cells of *G. magellanica*bear resemblance to the situation in *P. canina* but differ from the situation in *A. punctatus*.

4. Discussion

In the present study we have discussed our data without making a distinction between uptake and reversible hydrogenases because of the following reasons. First, earlier studies on hydrogen uptake and evolution activities suggested occurrence of two hydrogenases in heterocystous cyanobacteria: a cytoplasmic reversible hydrogenase located both in heterocysts and vegetative cells and a membrane-bound uptake hydrogenase located in heterocysts (see Houchins, 1984). However, recent studies have shown hydrogen uptake by nitrogenase in heterocysts (Chen et al., 1986; Almon and Böger, 1988) and presence of both uptake and reversible hydrogenase activities in membrane bound as well

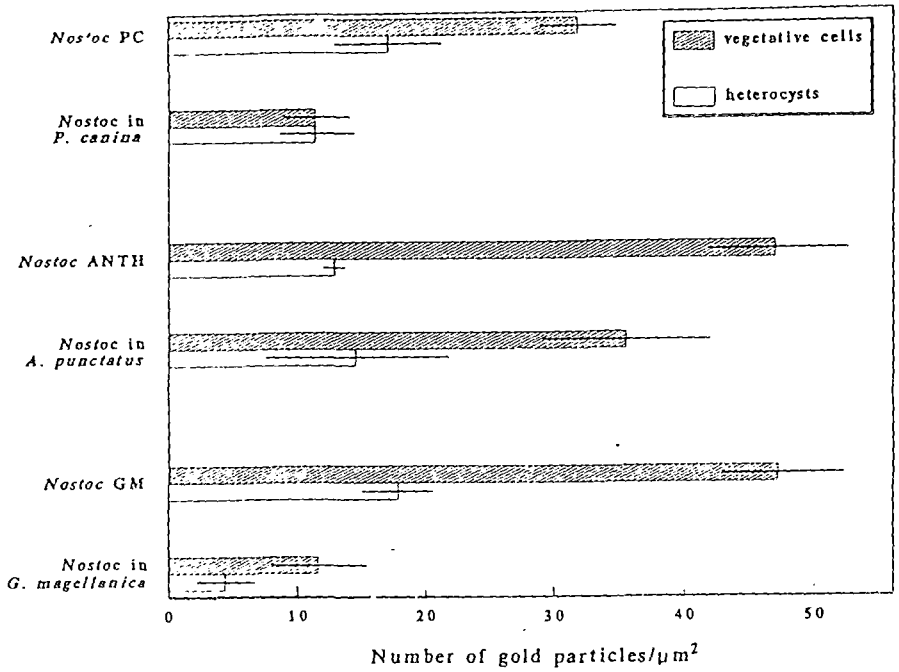

Figure 5. Cellular distribution and quantitative estimates of hydrogenase label in free-living (cultured) and symbiotic cyanobionts of *P. canina*, *Anthoceros punctatus*, and *G. magellanica*. In each case three grids containing several sections each were immunolabelled and 50–100 μm^2 cell area was used for counting gold particles (gold labelling associated with the plasma membranes was excluded in these counts). These values were plotted after subtracting the values for background labelling. The latter did not exceed 5% of the specific labelling. Bars indicate standard deviation.

as in soluble fractions (see Houchins, 1984; Rao and Hall, 1988; Ewart and Smith, 1989a,b; Kentemich et al., 1986; Papen et al., 1986). Indeed there have been suggestions of a single hydrogenase in cyanobacteria which may occur in different forms, working either in the direction of hydrogen oxidation or hydrogen evolution, depending on the physiological conditions (Adams et al., 1981; Lambert and Smith, 1981; Houchins, 1984). In view of this, the subcellular localization data (heterocysts vs vegetative cells or membrane-bound vs cytoplasmic) can not be interpreted in terms of uptake and reversible hydrogenases. Second, *A. latus* hydrogenase antibodies recognize a polypeptide of approximately 55 kDa in *Nostoc* 73102 cell extracts, which may correspond to the 50 kDa polypeptide reported to be present in both uptake and reversible hydrogenase proteins of *Anabaena cylindrica* (Lindblad and Sellstedt, 1990; Ewart and Smith, 1989a,b). Indeed the large subunits of uptake and reversible hydrogenases in cyanobacteria have molecular weights in the same range (see Houchins, 1984; Rao and Hall, 1988; Kentemich et al., 1989; Ewart and Smith,

1989a,b). Thus, in studies using antibodies raised against the large subunit, one can not exclude the possibility that the antibody recognizes both uptake and reversible hydrogenase antigens even if such antibodies recognize only one polypeptide.

Our results showing presence of hydrogenase both in heterocysts and vegetative cells, and the higher intensities of hydrogenase label along the plasma membranes (Figs. 1–4) are similar to the observations of Lindblad and Sellstedt (1990) in *Nostoc* 73102. However, in contrast to *Nostoc* 73102, where heterocysts and vegetative cells had similar levels of hydrogenase (Lindblad and Sellstedt, 1990), a higher levels of hydrogenase was found in vegetative cells than in heterocysts of all the three free-living (cultured) *Nostoc* cyanobionts studied here (Fig. 5). This difference in our findings may reflect the fact that different *Nostoc* strains were used in the two studies. Alternatively, it may be due to the difference in the immunolabelling protocols. For instance, the lower density of label in our studies may be better in highlighting the differences in subcellular levels of the hydrogenase antigen. We also found that while most heterocysts had lower levels of hydrogenase label than vegetative cells, a few heterocysts did have levels similar to that in vegetative cells. It is possible that hydrogenase levels are reduced during heterocyst differentiation and that young heterocysts still retain hydrogenase levels similar to that in vegetative cells. Such a decrease in hydrogenase levels of heterocysts may be the result of decreased synthesis of one or both hydrogenases if indeed heterocystous cyanobacteria have two hydrogenases.

Hydrogenase was absent in the eukaryotic partners of all the three cyanobacterial-plant symbioses (*P. canina*, *A. punctatus* and *G. magellanica*). This is consistent with the fact that among eukaryotic organisms only algae are known to have hydrogenase (see Adams et al. 1981). A comparison between the free-living and the symbiotic *Nostoc* in *A. punctatus* showed almost similar levels of hydrogenase as well as the labelling pattern (Figs. 3, 5). However, while the intensity of hydrogenase label along the plasma membrane remained unchanged, there was a significant decrease in the cytoplasmic levels of hydrogenase in the cyanobionts of *P. canina* and *G. magellanica*, particularly in the vegetative cells (Figs. 2, 4, 5). These results suggest that the synthesis of cytoplasmic hydrogenase, but not the plasma membrane associated hydrogenase, is significantly decreased in the cyanobionts of *P. canina* and *G. magellanica*. Such a decrease seems to correlate with a lowered oxygen tension. In *P. canina* and *G. magellanica* the cyanobionts reside in respiring nonphotosynthetic tissues but in *A. punctatus* the cyanobiont is surrounded by photosynthetically active tissue (see Stewart et al., 1983; Rai, 1988). This may also explain the

lower levels of hydrogenase in heterocysts which are known to have lower oxygen tension than that in vegetative cells (see Stewart, 1980). There has been an earlier suggestion that heterotrophic model of carbon nutrition may repress uptake hydrogenase in symbiotic cyanobacteria (Perraju et al., 1986) but this does not seem to be the case here. This is because the mode of carbon nutrition in cyanobionts of both *A. punctatus*and *G. magellanica* is heterotrophic but only in *G. magellanica* the cyanobiont showed decreased levels of hydrogenase. Furthermore, a decreease was also seen in the cyanobiont of *P. canina* which is autotrophic. Another possibility that Ni availability may explain this decrease was ruled out because while Ni is necessary for the hydrogenase activity, it is not necessary for hydrogenase synthesis (Ewart and Smith, 1989b).

Overall, our studies show that hydrogenase is present both in heterocysts and vegetative cells, the latter having higher levels of the enzyme; that in symbiosis the eukaryotic partners lack hydrogenase; and that while the levels of plasma membrane bound hydrogenase are similar in free-living and symbiotic cyanobionts, there is a decrease in the cytoplasmic levels of hydrogenase in cyanobionts occurring under microaerobic conditions. The fact that vegetative cells have nearly double the amount of hydrogenase than heterocysts, indicates an important role of the enzyme in cyanobacteria.which is not directly related to nitrogen-fixation as suggested earlier.

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Nitrogenase derepresssion, its regulation and metabolic changes associated with diazotrophy in the non-heterocystous cyanobacterium *Plectonema boryanum* PCC 73110

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The regulation of nitrogenase derepression, plus the catalytic activity and protein concentration of glutamine synthetase (GS), nitrate reductase (NR), ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and phycoerythrin (PE) were studied in the filamentous non-heterocystous cyanobacterium *Plectonema boryanum* PCC 73110. Both nitrogen limitation and microaerobic incubation were essential for the derepression of nitrogenase. Oxygen caused irreversible inactivation of nitrogenase, as well as repression of its synthesis. A temporal separation of N_2 fixation and net photosynthetic O_2 evolution was observed under a N_2/CO_2 (95:5, v/v) atmosphere. Repeated peaks of nitrogenase and growth were observed. Immunogold localization showed that in N_2-fixing cultures, all cells, including those undergoing division, contained nitrogenase, and that the nitrogenase antigen was uniformly distributed throughout the cells without any preferential association with cellular structures. Rubisco was mainly located in carboxysomes of both N_2-fixing and NO_3^--grown cells. Both N_2-fixing and NO_3^--grown cells showed similar levels of PE, which was associated with the thylakoid membranes. GS antigen was distributed throughout the cells and the relative amounts of this enzyme, as well as its activity, were 20% higher in N_2-fixing than in NO_3^--grown cultures. NO_3^- uptake and NR systems were found to be NO_3^--inducible, with very low activities in N_2-fixing cultures. The latter may be important in avoiding competition for Mo between nitrogenase and NR.

Introduction

Cyanobacteria are O_2-evolving photosynthetic prokaryotes, many of which are also capable of autotrophic growth using N_2 as the sole nitrogen source (Stewart, 1980; Gallon, 1989). Cellular integration of N_2 fixation in cyanobacteria requires strategies for protection of nitrogenase from atmospheric and photosynthetically produced O_2, provision of ATP and reductant, and efficient assimilation of N_2-derived ammonium. In some cyanobacteria this is achieved by development of specialized cells called heterocysts, resulting in spatial separation of photosynthesis (located in vegetative cells) and N_2 fixation (located in heterocysts); fixed carbon moves from vegetative cells to heterocysts and fixed nitrogen from heterocysts to vegetative cells (Stewart, 1980; Wolk, 1982; Bergman *et al.*, 1986). Several other metabolic changes conducive to nitrogenase functioning occur during heterocyst development, including: (1) loss of photosynthetic O_2 evolution, phycobiliproteins and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco); (2) increases in respiratory activity and in the activity of enzymes of the oxidative pentose phosphate pathway; (3) loss of nitrate uptake and nitrate reductase (NR) systems; (4) increased levels of glutamine synthetase (GS) necessary for assimilation of N_2-derived ammonia (Wolk, 1982; Kumar *et al.*, 1985; Rai & Bergman, 1986; Renström-Kellner *et al.*, 1990).

The strategies for O_2 protection during aerobic N_2-fixation by non-heterocystous cyanobacteria have been studied in detail, with the conclusion of a temporal separation of N_2-fixation and photosynthesis (see Gallon, 1989). However, very few studies have been conducted on non-heterocystous cyanobacteria which fix

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Abbreviations: ATCC, American Type Culture Collection; GOGAT, glutamate synthase; GS, glutamine synthetase; NR, nitrate reductase; PCC, Pasteur Culture Collection; PE, phycoerythrin; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; TEM, transmission electron microscopy; UTEX, culture collection of algae at University of Texas (formerly Indiana University Culture Collection).

N_2 only under microaerobic or anaerobic conditions. The reasons for the lack of aerobic N_2-fixation in such cyanobacteria are not fully understood. Under microaerobic to anaerobic conditions, a temporal separation of net O_2 evolution and nitrogenase activity has been observed in *Plectonema boryanum* (Weare & Benemann, 1974) and *Phormidium faveolarum* (Weisshaar & Böger, 1983). However, repeated peaks of alternating nitrogenase activity and oxygen evolution or growth have not been demonstrated. On the other hand, there are conflicting reports of N_2 fixation and concomitant growth in *Plectonema boryanum* (Rogerson, 1980; Pearson & Howsley, 1980; Giani & Krumbein, 1986). Furthermore, virtually no information is available regarding levels of phycoerythrin (PE), GS, nitrate uptake, NR and Rubisco under diazotrophic growth conditions in these cyanobacteria.

Plectonema boryanum PCC 73110 is a filamentous non-heterocystous cyanobacterium which fixes N_2 under microaerobic to anaerobic conditions (Stewart & Lex, 1970). In the present investigation, we used this strain to study the derepression, subcellular localization and regulation of nitrogenase, and changes in GS, Rubisco, PE, nitrate uptake and NR when nitrate-grown cultures adapted to diazotrophic growth.

Methods

Organism and growth conditions. Plectonema boryanum PCC 73110 (ATCC 29407 and UTEX 594) and *Gloeothece* PCC 6909 (ATCC 27152 and UTEX 795) were grown in batch cultures using BG-11 medium (Rippka *et al.*, 1979) at 25 °C and a photon fluence rate of 20 μmol m^{-2} s^{-1}. *Oscillatoria limosa* (Stal & Bergman, 1990) was grown on artificial sea water medium ASN_3 (Rippka *et al.*, 1979) at 20 °C and a photon fluence rate of 20 μmol m^{-2} s^{-1}. N_2-fixing cultures of *Gloeothece* and *O. limosa* were obtained by transferring the cultures to nitrogen-free media ('N_2-medium'; $BG\text{-}11_0$ and NO_3^--free ASN_3, respectively). Derepression of nitrogenase in *P. boryanum* was achieved as described below.

Nitrogenase derepression. Aerated batch cultures of *P. boryanum* grown on BG-11 medium were harvested by centrifugation during the exponential phase. The cells were washed and resuspended in $BG\text{-}11_0$ medium to a cell density of 200 μg ml^{-1} (3 μg chlorophyll *a* ml^{-1}). These cultures were subdivided into 20 ml batches and transferred to 100 ml capacity serum-stoppered Erlenmeyer flasks. These were sparged with the desired gas mixture at specified times and maintained at 25 °C and a photon fluence rate of 10 μmol m^{-2} s^{-1}. These flasks were directly used for acetylene reduction assay, each assay lasting 30 min; the cultures were not transferred to another vessel, in order to avoid air contamination.

Enzyme assays. Nitrogenase activity (EC 1.18.6.1) was estimated *in vivo* by gas chromatography using the acetylene reduction assay (Stewart *et al.*, 1967). GS biosynthetic activities (EC 6.3.1.2) were measured in cell extracts as described by Sampaio *et al.* (1979). Ferredoxin-dependent NR activity was measured in cells made permeable with toluene, using methyl viologen as electron donor, by following the formation of NO_2^- from NO_3^- as described by Manzano *et al.* (1976). NO_3^- was estimated colorimetrically as described by Snell & Snell (1949).

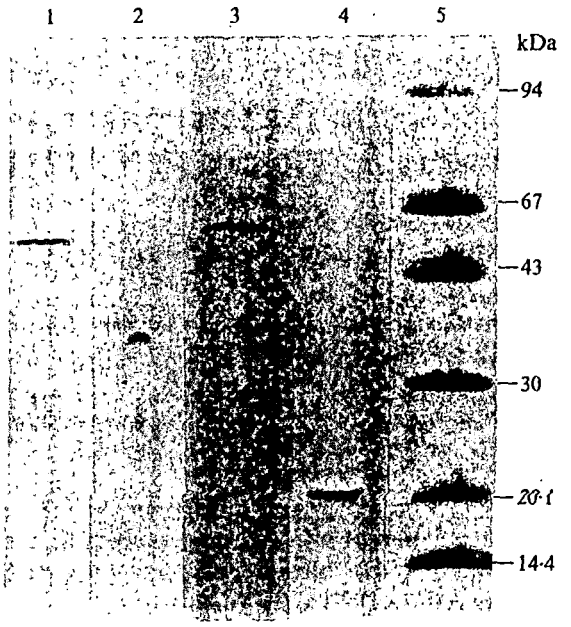


Fig. 1. SDS-PAGE and immunoblotting of cell extracts from N_2-fixing *P. boryanum*. NO_3^--grown cells were transferred to N_2-medium and nitrogenase induced as described in Fig. 2(*b*). Immunoblots of GS (lane 1), nitrogenase Fe-protein (lane 2), Rubisco (lane 3) and PE (lane 4) are shown. Lane 5, molecular mass markers.

NO_3^- uptake. This was measured by estimating the rate of disappearance of NO_3^- from the medium (final concentration 100 μM). NO_3^- was estimated by the difference in absorbance between 202 and 250 nm as described by Calero *et al.* (1980).

Protein and chlorophyll a. Protein concentration was measured according to Bradford (1976) and chlorophyll *a* according to MacKinney (1941).

O_2 exchange. O_2 evolution and consumption were measured using a polarographic Clark-type oxygen electrode installed in a 3 ml Plexiglass container with magnetic stirring. The measurements were done at 25 °C and at a photon fluence rate of 10 μmol m^{-2} s^{-1}.

Antibodies. Rabbit anti-*Rhodospirillum rubrum* nitrogenase Fe-protein, anti-*Anabaena* PCC 7120 GS, anti-*Phormidium persicinum* PE and anti-*Sinapis alba* Rubisco were gifts from Dr S. Nordlund (University of Stockholm, Sweden), Professor R. Haselkorn (University of Chicago, USA), Dr D. Guard-Friar (New York State Department of Health, USA) and Dr R. Oelmuller (University of Freiburg, Germany), respectively. The specificity of these antibodies was tested against crude extracts of N_2-fixing *P. boryanum* cells by Western blotting (Fig. 1). All antibodies were found to be monospecific, recognizing a single polypeptide of known subunit molecular mass relating to their respective antigens (nitrogenase 36 kDa; GS 53 kDa; Rubisco 56 kDa; PE 20 kDa) (see also Bergman & Rai, 1989). Secondary antibodies (goat anti-rabbit IgG conjugated to colloidal gold size 5 or 10 nm, and conjugated to horseradish peroxidase) were obtained from Amersham and Bio-Rad, respectively.

Western blotting. P. boryanum cells were harvested by centrifugation (3000 *g*, 5 min) and the pellet resuspended in sample buffer (1:1, v/v) consisting of 10 mM-Tris/HCl (pH 8), 1 mM-EDTA, 2·5% SDS, 5%

β-mercaptoethanol and 0·01 % bromophenol blue. These samples were boiled for 5 min and centrifuged at 15000 *g* for 5 min. Samples (1 μl) of the supernatant were used for SDS-PAGE and subsequent immunoblotting as described by Braun-Howland *et al.* (1988), using antibodies against nitrogenase Fe-protein, GS, PE and Rubisco at a dilution of 1:500 (v/v).

Immunogold labelling. Preparation of samples for transmission electron microscopy (TEM) and the protocols for immunolabelling were essentially the same as described before (Bergman *et al.*, 1985) except that the secondary antibody (goat anti-rabbit IgG) was conjugated to size 5 or 10 nm colloidal gold and that different primary antibodies were used as required (see figure legends). Five grids containing ultrathin sections of NO_3^--grown and N_2-fixing *P. boryanum* samples were immunolabelled. For quantification, 10–15 photographs were taken, of which one set of representative photographs is presented. In control experiments, the primary antibody was omitted.

TEM and quantification of the immunolabel. TEM was performed using a Zeiss EM 10 transmission electron microscope operated at 60 kV. Relative levels of various antigens were estimated by counting gold particles in various cell types using TEM photographic prints. These were then converted to number of gold particles per μm^2 cell area taking into account the magnification of the prints used and the area counted. In total, 100 μm^2 cell area was counted in each case and the values presented are means (±SE) from 10–15 counts. A similar exercise was done to calculate background labelling by counting gold particles per unit area outside the cells. Background labelling was also calculated in control experiments where primary antibody was omitted during immunolabelling.

Chemicals and gases. All the supplies for electron microscopy were obtained from Agar Aids, for electrophoresis from Pharmacia and for immunoblotting from Bio-Rad. All other chemicals were from Sigma. Gases were obtained from AGA Special Gases, Stockholm.

Results

Derepression of nitrogenase

No nitrogenase activity or protein could be detected in NO_3^-- or NH_4^+-grown *P. boryanum* cultured either under air or with N_2/CO_2 (95:5, v/v) sparging (data not shown). Upon transfer to N_2-medium and periodic sparging with N_2/CO_2, development of acetylene-reducing activity started after 30 h (Fig. 2*a*). The activity continued to increase during the next 40 h, after which it steadily declined. The pattern and specific activity were similar to those noted by Stewart & Lex (1970). However, when the cells were nitrogen starved for 24 h under aerobic conditions prior to the periodic N_2/CO_2 sparging, acetylene-reducing activity developed much faster (within 2 h) and reached a peak by 6–7 h (Fig. 2*b*), with maximal specific activity similar to that in Fig. 2(*a*). To see if the faster development of nitrogenase activity was due to the activation of pre-existing nitrogenase protein developed during the 24 h nitrogen starvation, development of acetylene-reducing activity was followed in cells where chloramphenicol or rifampicin was added at the end of nitrogen starvation and just before the start of

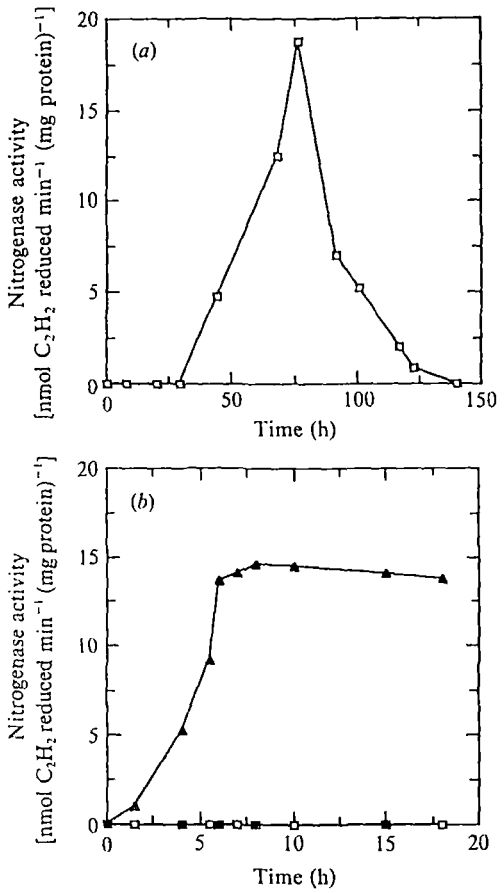


Fig. 2. Nitrogenase induction in *P. boryanum*. (*a*) NO_3^--grown cells were transferred to N_2-medium as detailed in Methods and maintained at 25 °C with a photon fluence rate of 10 μmol m^{-2} s^{-1}. Starting at zero time and at 6 h intervals they were sparged for 15 min with N_2/CO_2 (95:5, v/v) at a rate of 1000 ml h^{-1}. Nitrogenase activity was measured in 30 min assays under a N_2/CO_2 gas phase and under the light and temperature conditions mentioned above. In this and other figures the nitrogenase activity quoted is the mean of that during the 30 min before the points shown. (*b*) Same as (*a*) except that prior to the start of N_2/CO_2 sparging (zero time) the cultures were nitrogen starved for 24 h by incubating them in N_2-medium with continuous air sparging (2000 ml h^{-1}). Symbols in (*b*): ▲, control; □, plus chloramphenicol (100 μg ml^{-1}); ■, plus rifampicin (100 μg ml^{-1}). Chloramphenicol and rifampicin were added at the start of N_2/CO_2 sparging (i.e. after the aerobic nitrogen-starvation period). This and other experiments (Figs 3*a*, 4, 5, 6) were all done in duplicate. The values presented are means of two measurements from each replicate.

N_2/CO_2 sparging. As seen in Fig. 2(*b*), acetylene-reducing activity did not appear under such conditions. These results indicate that nitrogenase protein was absent during the aerobic nitrogen starvation period and that the derepression on N_2/CO_2 sparging was due to fresh synthesis of nitrogenase. An absence of nitrogenase protein in aerobic cultures was also observed by immunoblotting cell extracts of *P. boryanum* which had been nitrogen starved for 30 h under aerobic conditions (Fig. 3*b*, lane 1). These results show that nitrogenase

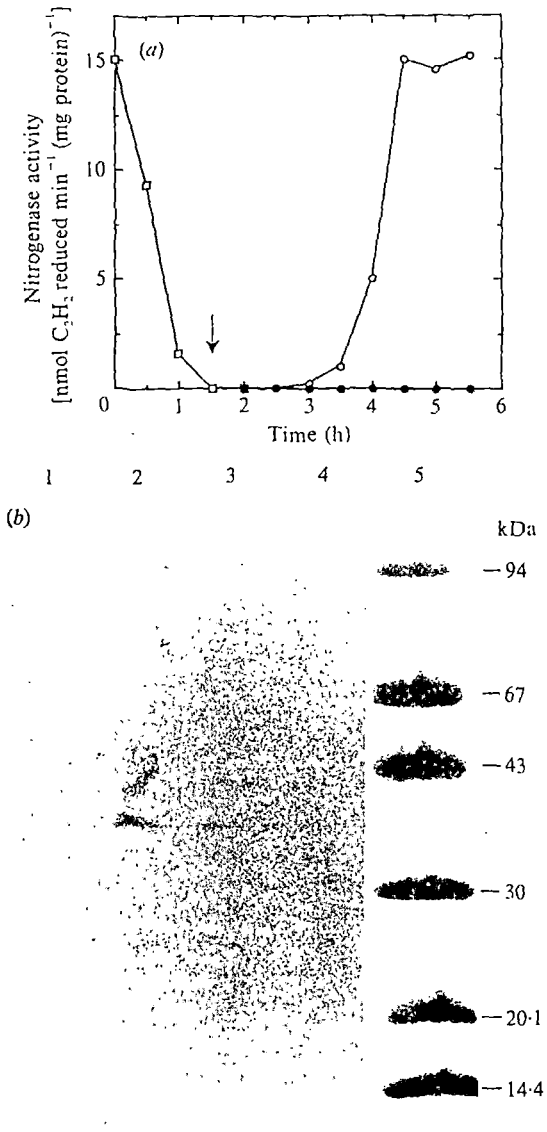


Fig. 3. (*a*) Loss of nitrogenase activity on exposure to air (□) and its regain upon transfer to a N_2/CO_2 atmosphere (○, ●) in *P. boryanum*. Nitrogenase derepression was achieved as in Fig. 2(*b*) and after appearance of the nitrogenase peak, the cells were sparged with air for 5 min at the rate of 2000 ml h^{-1}. Nitrogenase activity was measured at 30 min intervals under aerobic conditions. At the time indicated by the arrow, the gas phase was changed to N_2/CO_2 and nitrogenase activity measured at 30 min intervals under N_2/CO_2 in the absence (○) and presence (●) of chloramphenicol or rifampicin (both 100 μg ml^{-1}). (*b*) Detection of nitrogenase Fe-protein by immunoblotting in cell extracts of *P. boryanum*. Lane 1, extract from cells maintained in aerobic N_2-medium for 30 min; lane 2, extract from cells with peak nitrogenase activity; lane 3, extract from cells which were exposed to air for 2 h after appearance of the nitrogenase peak; lane 4, extract of cells which had been exposed to air for 4 h after appearance of nitrogenase activity; lane 5, molecular mass markers.

derepression required both low cellular nitrogen and microaerobic to anaerobic conditions, and that the longer time required for nitrogenase derepression in Fig. 2(*a*) was due to the time required for the depletion of

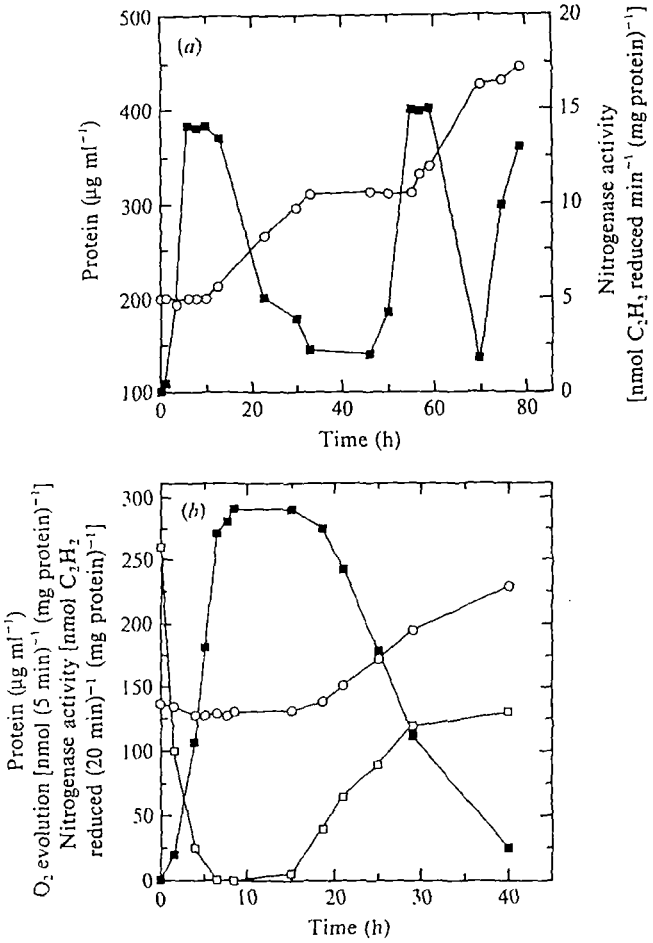


Fig. 4. (*a*) Nitrogenase activity (■) and protein content (○) of *P. boryanum*. (*b*) Nitrogenase activity (■), protein content (○) and net O_2 evolution rate (□) during the first phase of N_2 fixation in (*a*). Culture conditions and other details as in Fig. 2(*b*) control.

intracellular nitrogen reserves under non-optimal growth conditions.

Re-exposure of N_2-fixing *P. boryanum* cells to air caused a rapid decline in acetylene-reducing activity, which became undetectable after 90 min of exposure (Fig. 3*a*). To see whether the decline in nitrogenase activity was due to inactivation of the enzyme or to protein degradation/modification, immunoblots of cell extracts were done using *P. boryanum* cells exposed to air for increasing periods of time after the appearance of peak nitrogenase activity (Fig. 3*b*). After 2 h of exposure to air, when nitrogenase activity had become undetectable, nitrogenase Fe-protein was still detectable (lane 3), although the cross-reaction was less intense than that in *P. boryanum* cells under N_2/CO_2 (lane 2). In both cases, only a single polypeptide of 36 kDa was detected corresponding to nitrogenase Fe-protein. These data indicate that loss of nitrogenase activity on exposure to air was due to nitrogenase inactivation followed by degradation. Such inactivation did not involve modification of Fe-protein to a higher molecular mass form as

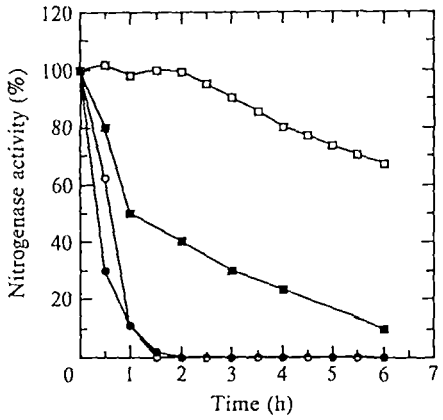


Fig. 5. Effects of air, darkness, NH_4^+ and NO_3^- on nitrogenase activity in *P. boryanum*. Nitrogenase activity was derepressed as in Fig. 2(*b*). After the appearance of the nitrogenase peak, the conditions were changed (zero time) and the response of nitrogenase activity was monitored during the next 6 h. ●, Cultures transferred to darkness; ■, NH_4Cl added to a final concentration of 2 mM; □, KNO_3 added to a final concentration of 10 mM; ○, cultures transferred to aerobic atmosphere. The NH_4Cl and KNO_3 solutions were sparged with N_2/CO_2 for 30 min before addition. The solutions were also buffered with 10 mM-HEPES/NaOH (pH 7·5). One hundred percent activity represents 18 nmol C_2H_4 formed min^{-1} (mg protein)$^{-1}$.

noted in other cyanobacteria (Ernst *et al.*, 1990; Reich & Böger, 1989; Smith *et al.*, 1987; Stal & Bergman, 1990). To see if the inactivation of the nitrogenase was reversible, *P. boryanum* cells were transferred back to a N_2/CO_2 atmosphere after 90 min of exposure to air (Fig. 3*a*). Acetylene-reducing activity reappeared after 90 min and reached a peak in 3 h. Such reappearance of nitrogenase activity was sensitive to chloramphenicol and rifampicin. Thus, inactivation of nitrogenase under air was concluded to be irreversible.

Growth, N_2 fixation and O_2 evolution

When nitrogenase was derepressed as in Fig. 2(*b*), and the cultures maintained under similar conditions over a prolonged period, repeated peaks of acetylene-reducing activity were observed (Fig. 4*a*). An increase in protein content, which was taken as indicative of growth, followed. A detailed analysis of one such peak of nitrogenase activity and growth phase (Fig. 4*b*) showed that during the appearance of nitrogenase the rate of net O_2 evolution declined rapidly, becoming undetectable by the time nitrogenase activity reached its peak. No growth occurred during this period. Net O_2 evolution was detectable again after 7 h. This coincided with growth and with a decline in nitrogenase activity. These data indicate that under the conditions used here, *P. boryanum* can grow photoautotrophically using N_2 as nitrogen source, in repeated cycles of N_2 fixation and growth, and under such conditions there is a temporal separation of net O_2 evolution and nitrogenase activity. During the maximal N_2-fixing period, O_2 evolution may be balanced by respiratory O_2 consumption, resulting in the absence of net O_2 exchange.

Effects of NH_4^+ and NO_3^- and darkness on nitrogenase activity

After nitrogenase derepression as in Fig. 2(*b*), the effects of darkness, NH_4^+ and NO_3^- were studied during the 6 h stable period when maximal nitrogenase activity was expressed. Transfer of N_2-fixing cells into darkness resulted in a rapid decline in acetylene-reducing activity, which became undetectable within 2 h (Fig. 5). This decline was similar to that observed in air. ATP and/or reductant were probably the essential factors supplied by light reactions. Addition of 2 mM-NH_4Cl resulted in a slower decline of acetylene-reducing activity, perhaps because NH_4^+ may have acted by repressing nitrogenase synthesis rather than inhibiting its activity. NO_3^- did not affect nitrogenase activity during the initial 2 h of incubation, but a slow decline in activity was seen thereafter. The difference in the effects of NO_3^- and NH_4^+ may be due to slower rates of nitrate uptake and/or metabolism in N_2-fixing cultures (see below).

Table 1. *NO_3^- uptake and NR activities in P. boryanum, Gloeothece 6909 and O. limosa grown on N_2, NO_3^- or NH_4^+*

NO_3^- uptake activities are expressed as nmol NO_3^- taken up min^{-1} (mg protein)$^{-1}$ and NR activities as nmol NO_2^- formed min^{-1} (mg protein)$^{-1}$. NO_3^--medium represents BG-11 in the case of *Gloeothece* 6909 and *P. boryanum*, and ASN_3 in the case of *O. limosa*. N_2-medium represents the respective nitrogen-free medium (BG-11_0 or NO_3^--free ASN_3). NH_4^+-medium represents N_2-media supplemented with NH_4Cl (final concentration 2 mM) and buffered with 10 mM-HEPES/NaOH (pH 7·5). The values presented are means ± SE of three measurements from a single experiment done in triplicate. ND, Not detectable.

Growth medium	Nitrate uptake			Nitrate reductase		
	Plectonema	*Gloeothece*	*Oscillatoria*	*Plectonema*	*Gloeothece*	*Oscillatoria*
N_2-medium	0·6 ± 0·1	0·2 ± 0·05	0·2 ± 0·04	0·5 ± 0·1	0·2 ± 0·04	0·3 ± 0·1
NO_3^--medium	7·5 ± 0·3	4·9 ± 0·25	6·0 ± 0·30	7·8 ± 0·4	4·3 ± 0·10	4·7 ± 0·2
NH_4^+-medium	ND	ND	ND	ND	ND	ND

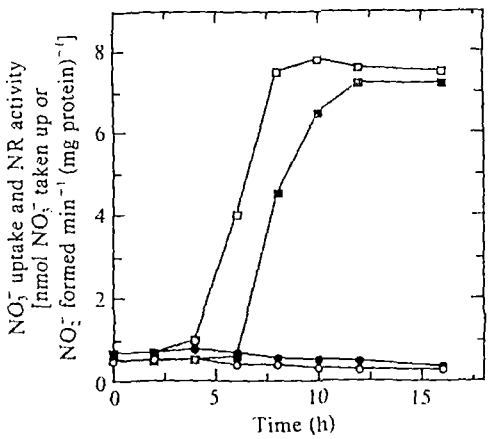


Fig. 6. Induction of NO_3^- uptake (□, ●) and NR (○, ■) activities on transfer of N_2-fixing *P. boryanum* cells to NO_3^--medium (BG-11_0) without (□, ■) or with (○, ●) chloramphenicol (100 μg ml^{-1}). Nitrogenase was derepressed as in Fig. 2(*b*). After the appearance of the nitrogenase peak, the cells were harvested by centrifugation and resuspended in NO_3^--medium (zero time), then maintained under aerobic growth conditions. At time intervals samples were withdrawn and NO_3^- uptake rate and NR activities measured.

NO_3^- uptake and NR activities

NO_3^--grown cultures of *P. boryanum* showed NO_3^- uptake and NR activities (Table 1) similar to those reported earlier (Ida & Mikami, 1983). In contrast, the NO_3^- uptake and NR activities of N_2-fixing cultures were only 10% of those in NO_3^--grown cells (activity measured after appearance of peak nitrogenase activity). These activities did not change during the period of nitrogenase decline that also corresponded to net O_2 evolution and growth (data not shown). NH_4^+-grown cells had no detectable levels of NO_3^- uptake or NR. Essentially similar results were found in the case of the non-heterocystous cyanobacteria *Gloeothece* 6909 and *O. limosa*, which fix N_2 aerobically (Table 1). When N_2-fixing cells of *P. boryanum* were transferred to NO_3^--medium, NO_3^- uptake and NR activities increased, reaching a maximum within 10–12 h (Fig. 6). The increase was sensitive to chloramphenicol. These results imply that the NO_3^- uptake and NR systems in *P. boryanum* are substrate-inducible and NH_4^+-repressible.

Nitrogenase localization

Immunogold labelling of nitrogenase Fe-protein in NO_3^--grown cells (Fig. 7*a*) and NH_4^+-grown cells (data not shown) showed label intensity similar to background (4–6 gold particles per μm^2 cell area). N_2-fixing *P. boryanum* cells showed nitrogenase antigen uniformly distributed throughout the cell without preferential association with any cellular structure (Fig. 8*a*). All the cells in all the filaments examined had a similar pattern and intensity of labelling. Cells undergoing division also had nitrogenase label. The density of label was 95 ± 12 gold particles per μm^2 cell area. These results confirm the lack of nitrogenase in NO_3^-- and NH_4^+-grown cells and imply no spatial separation or subcellular compartmentalization of nitrogenase in *P. boryanum*. Similar results have been reported in *P. boryanum* 581 using antibodies against nitrogenase Mo–Fe protein (Smoker *et al.*, 1989).

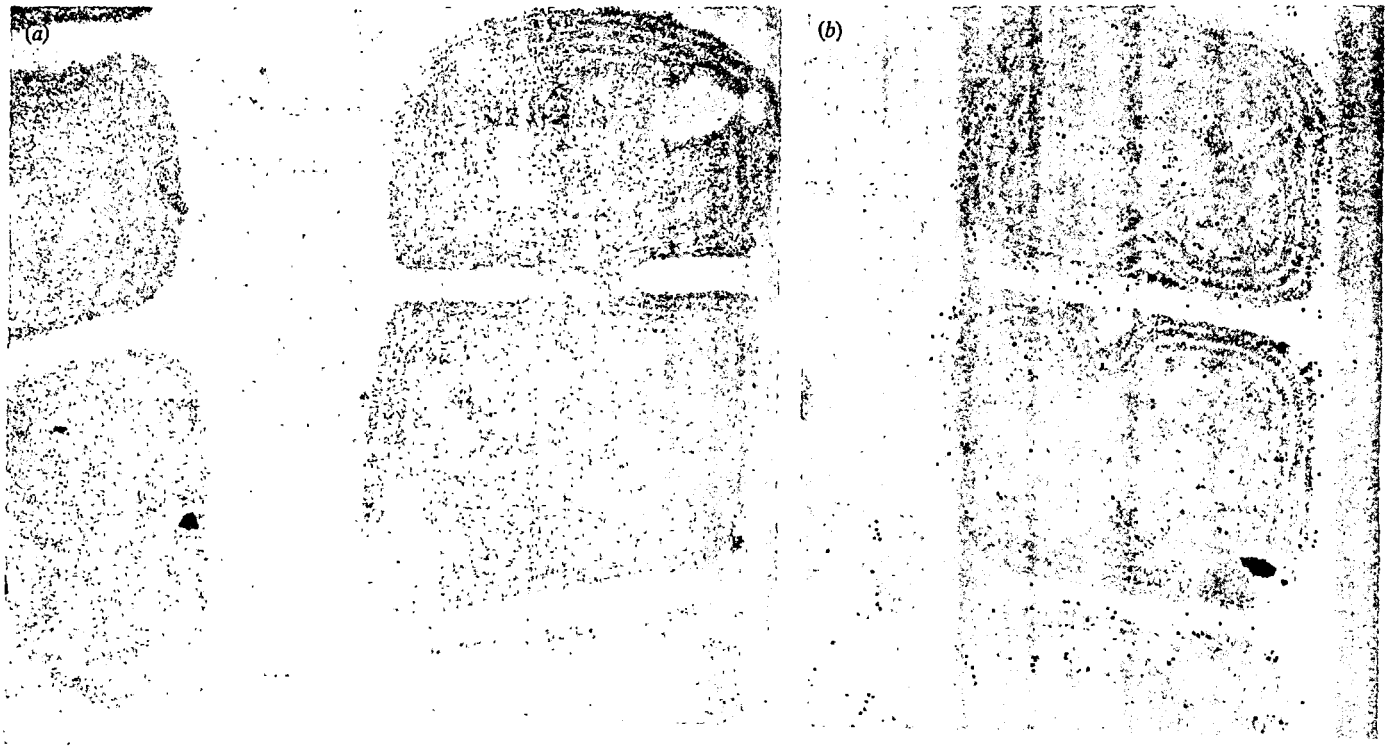
GS activity and cellular localization of GS antigen

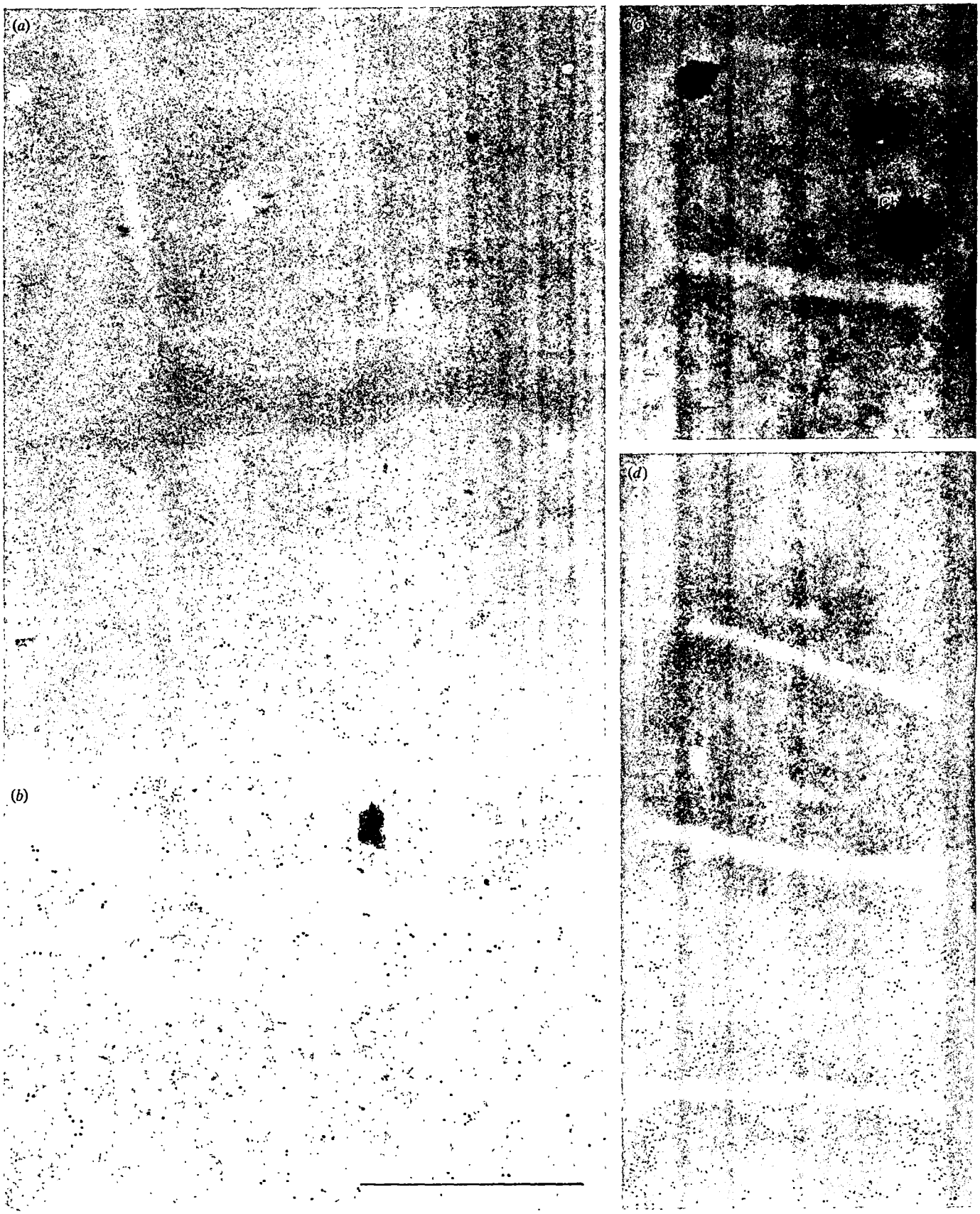
Potential changes in GS activity and protein concentration during derepression of nitrogenase were examined in NO_3^--grown and in N_2-fixing *P. boryanum* cells. GS biosynthetic activity in the two cultures was $45 \pm 2{\cdot}8$ and $56 \pm 3{\cdot}1$ nmol product formed min^{-1} (mg protein)$^{-1}$, respectively. Immunolabelling experiments showed that the GS antigen was distributed throughout the cell both in NO_3^--grown and in N_2-fixing cells (Figs 7*b*, 8*b*). The relative densities of the gold label were $22 \pm 2{\cdot}5$ and $27 \pm 2{\cdot}6$ gold particles per μm^2 cell area, respectively. Thus, a 20% increase in GS activity and protein label occurred on nitrogenase derepression. This is in contrast to the report of Nagatani & Haselkorn (1978), who found no increase in GS activity during nitrogenase derepression under an argon atmosphere. The differences may have arisen due to different conditions used for nitrogenase derepression, including the fact that we used N_2/CO_2 (95:5, v/v) for nitrogenase derepression.

Rubisco localization

Rubisco was localized in NO_3^--grown and in N_2-fixing cells of *P. boryanum* (Figs 7*c*, 8*c*). In the latter case, the cells were processed for immunolabelling at the beginning of the appearance of nitrogenase, when the nitrogen stress, and therefore the difference in Rubisco, is likely to be highest. Rubisco was present in both NO_3^--grown and N_2-fixing cells. In both cases, an intense labelling was found in carboxysomes and a lower intensity in the cytoplasm. The overall level of Rubisco in N_2-fixing cells was 20% lower than that in NO_3^--grown cells ($43 \pm 3{\cdot}4$ and 54 ± 4 gold particles per cell, respectively). Smoker *et al.* (1990) noted a much higher (over 50%) reduction

Fig. 7. Immunogold localization of nitrogenase (*a*), GS (*b*), Rubisco (*c*) and PE (*d*) in NO_3^--grown cells of *P. boryanum*. Rabbit anti-*R. rubrum* nitrogenase Fe-protein (*a*), anti-*Anabaena* 7120 GS (*b*), anti-*S. alba* Rubisco (*c*) and anti-*P. persicinum* PE (*d*) were used as primary antibodies at a dilution of 1:100. Goat anti-rabbit IgG conjugated to 5 nm (*a*, *c*, *d*) or 10 nm (*b*) colloidal gold was used as secondary antibody at a dilution of 1:20. Cb, carboxysome. Bar, 1 μm (all parts of the figure are at the same magnification).


(a)
(b)


(a)
(b)
(c)
(d)

in Rubisco levels on nitrogenase derepression in *P. boryanum* 581. However, the cells had been stressed for nitrogen for 40 h in an argon atmosphere lacking N_2. These results show that unlike the situation in heterocysts, derepression of nitrogenase in *P. boryanum* does not lead to a total loss of Rubisco.

Localization of PE

During nitrogenase derepression in *P. boryanum*, a transient decrease in phycocyanin has been noted (Stewart & Lex, 1970; Weare & Benemann, 1974). To see if PE concentrations also change during nitrogenase derepression, immunogold labelling of PE was examined in NO_3^--grown and in N_2-fixing cells. In the latter case, cells for immunolabelling were taken at the beginning of the appearance of nitrogenase activity, when the differences are likely to be maximal. An intense PE labelling associated with thylakoid membranes was found both in NO_3^--grown and in N_2-fixing cells (Figs 7*d*, 8*d*). In both cases, the amounts of PE were similar (447 ± 15 and 435 ± 18 gold particles per cell, respectively), indicating little or no degradation of PE in *P. boryanum* under the conditions of nitrogenase derepression used here.

Discussion

Under a N_2/CO_2 atmosphere *P. boryanum* PCC 73110 synthesized nitrogenase and showed nitrogenase activity in the absence of combined nitrogen (Figs 2, 4). These activities are among the highest reported by other workers using this strain (Stewart & Lex, 1970; Weare & Benemann, 1974; Nagatani & Haselkorn, 1978; Rogerson, 1980; Pearson & Howsley, 1980; Giani & Krumbein, 1986). Since the cultures used were non-synchronous, development of nitrogenase in all the cells (including those undergoing cell division) and the uniform distribution throughout the cells (Fig. 8) argue against the possibility of nitrogenase being expressed during a particular phase of the cell cycle or being compartmentalized within the cell (Mitsui *et al.*, 1986; Giani & Krumbein, 1986). Similar patterns of labelling have been found in *O. limosa* (Stal & Bergman, 1990), *P. boryanum* UTEX 581 (Smoker *et al.* 1989) and *Gloeothece* PCC 6909 (A. N. Rai & B. Bergman, unpublished results).

O_2 removal or nitrogen limitation alone did not lead to derepression of nitrogenase. This was indicated by the lack of nitrogenase derepression under aerobic conditions irrespective of the nitrogen status of the cell, and by the fact that even on N_2/CO_2 sparging nitrogenase derepression occurred only under nitrogen-limited conditions (Figs 2, 3, 7*a*). These results are consistent with, and provide evidence for, the suggestion that nitrogenase derepression in *P. boryanum* requires both O_2 removal and nitrogen limitation (Nagatani & Haselkorn, 1978). Absence of nitrogenase derepression in the presence of combined nitrogen is consistent with earlier observations in cyanobacteria including *P. boryanum* (Stewart, 1980; Gallon, 1989; Stewart & Lex, 1970). However, the fact that O_2 removal or lowering of O_2 tension was necessary for nitrogenase derepression, despite a temporal separation of net O_2 evolution and nitrogenase activity, and that nitrogenase activity declined sharply on exposure to air or with the onset of net O_2 evolution endogenously (Fig. 4), suggests that the O_2-scavenging capacity in *P. boryanum* is much more limited than in other non-heterocystous cyanobacteria. Indeed, Weare & Benemann (1974) found respiration to be of only limited significance in O_2-protection in *P. boryanum*.

Our studies showing irreversible inactivation of nitrogenase by O_2 in *P. boryanum* (Fig. 3) are consistent with the results of Weare & Benemann (1974). Nitrogenase Fe-protein has been shown to be modified to a higher molecular mass form by O_2 in some heterocystous cyanobacteria, which results in reversible inactivation of the protein but renders it insensitive to O_2 damage (Smith *et al.*, 1987; Reich & Böger, 1989; Ernst *et al.*, 1990). A similar form has also been noted in *O. limosa* (Stal & Bergman, 1990). The fact that such a modification was not found in *P. boryanum* (Fig. 3*b*) may explain why inactivation of nitrogenase in this strain was irreversible and resulted in degradation of the enzyme.

The temporal separation of N_2 fixation and net O_2 evolution noted here (Fig. 4) also confirms the findings of Weare & Benemann (1974). In addition, the results show that *P. boryanum* can grow photoautotrophically with repeated cycles of N_2 fixation and growth. Since nitrogenase was found to be irreversibly inactivated and degraded on exposure to air, and regain of nitrogenase activity required fresh synthesis of nitrogenase (Figs 3, 5), it is possible that repeated phases of N_2 fixation required fresh nitrogenase synthesis and that during the following microaerobic phase nitrogenase was degraded. Giani & Krumbein (1986) have demonstrated N_2 fixation and concomitant photoautotrophic growth in *P. boryanum* at lower light intensities with continuous N_2/CO_2 flushing. Thus, depending on the culture conditions, *P. boryanum* seems capable of photoauto-

Fig. 8. Immunogold localization of nitrogenase (*a*), GS (*b*), Rubisco (*c*) and PE (*d*) in N_2-fixing *P. boryanum* cells. Nitrogenase derepression was achieved as in Fig. 2(*b*). The cells used for immunolabelling were sampled either at the beginning (*c*, *d*) or at the peak (*a*, *b*) of nitrogenase activity. Other details as in Fig. 7.

trophic growth either with repeated phases of N_2 fixation (when sparged with N_2/CO_2 periodically) or concomitantly with continuous N_2 fixation (when sparged with N_2/CO_2 continuously to remove any net O_2 evolved, keeping the culture microaerobic). Although the precise mechanism of temporal separation is not clear, it is possible that this is achieved by changes in rates of photosynthesis, with respiration balancing photosynthetic O_2 evolution during N_2 fixation while increased rates of photosynthesis result in net O_2 evolution during the growth that follows. This may occur due to transient changes in phycobiliprotein levels. Although we found no significant changes in PE levels (Figs 7, 8), phycocyanin levels are known to change and repeated degradation and synthesis of phycocyanin has been noted earlier (Stewart & Lex, 1970; Weare & Benemann, 1974; Giani & Krumbein, 1986).

Mo may be a limiting factor in cyanobacterial cells (Bagchi *et al.*, 1985). Nitrogenase and NR are both molybdoenzymes and require reduced ferredoxin as electron donor (Guerrero & Lara, 1987). Indeed Nagatani & Haselkorn (1978) have shown that in absence of Mo, the nitrogenase proteins synthesized are inactive. Occurrence of NR and nitrogenase in the same cell is likely to lead to competition for Mo and reduced ferredoxin. Heterocystous cyanobacteria avoid such competition by spatial separation of nitrogenase and NR (Kumar *et al.*, 1985, Rai & Bergman, 1986). Such spatial separation is not possible in non-heterocystous cyanobacteria since nitrogenase is present in all the cells. A temporal separation of NR and nitrogenase activity was not found in the non-heterocystous cyanobacteria tested (Table 1). However, the fact that nitrate uptake and NR were found to be NO_3^--inducible, with very low activities in N_2-fixing cultures (Fig. 6; Table 1) means that these cyanobacteria are able to effectively minimize competition between NR and nitrogenase under diazotrophic growth conditions. It is noteworthy that N_2-fixing *P. boryanum* cells retain the capacity to develop nitrate uptake and NR (Fig. 6), unlike heterocysts, where these systems are lost (Rai & Bergman, 1986).

As in heterocystous cyanobacteria, the GS-GOGAT pathway has been shown to be the route of primary NH_4^+ assimilation in *P. boryanum* (Meeks *et al.* 1978). An increase in GS activity and protein level has been noted when vegetative cells differentiate into heterocysts (see Wolk, 1982; Bergman *et al.*, 1985). This has been shown to be linked to nitrogenase expression and to be necessary for assimilation of N_2-derived NH_4^+ (Renström-Kellner *et al.*, 1990). Our results showing an increase of GS with derepression of nitrogenase (Figs 7, 8) are consistent with the above view. These findings also explain why NO_3^- is less inhibitory and acts more slowly than NH_4^+. As mentioned above, NO_3^- uptake and NR levels are very low in N_2-fixing cultures and availability of NO_3^- requires 3–4 h for induction of NO_3^- uptake and NR activity (Table 1, Fig. 6), while NH_4^+ assimilation remains active throughout. These effects resemble the effects of NO_3^- and NH_4^+ on nitrogenase activity and protein in heterocysts (Renström-Kellner *et al.*, 1990). Thus, during N_2 fixation *P. boryanum* cells functionally resemble heterocysts. However, unlike heterocysts, N_2-fixing *P. boryanum* cells retain PE and Rubisco (Figs 7, 8), they retain the capacity to develop NO_3^- uptake and NR activity on nitrate availability (Fig. 6), and their PC levels change only transiently (Stewart & Lex, 1970; Weare & Benemann, 1974; Giani & Krumbein, 1986).

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