ROLE OF AMMONIUM AND GLUTAMINE TRANSPORT SYSTEMS IN NITROGEN CONTROL OF NITROGENASE IN NITROGEN-FIXING CYANOBACTERIA

BY

Reddy Shetty Prakasham

THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENT OF THE DEGREE OF
DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY

DEPARTMENT OF BIOCHEMISTRY
SCHOOL OF LIFE SCIENCES

NORTH-EASTERN HILL UNIVERSITY

SHILLONG-793014

INDIA

1990

Regn No. and Date.
313 of 29-2-1988

Thesis

This thesis is dedicated to my

Professor A. N. RAI

'BIOCHEMISTRY'

SHILLONG

I certify that the thesis entitled "**Role of Ammonium and Glutamine Transport Systems in Nitrogen Control of Nitrogenase in Nitrogen-fixing Cyanobacteria**" submitted by Mr. **Reddy Shetty Prakasham** for the degree of **Doctor of Philosophy** of the **North-Eastern Hill University, Shillong**, embodies the record of original investigation carried out by him under my supervision. He has been duly registered and the thesis presented is worthy of being considered for the award of the Ph.D. degree. This work has not been submitted for any other degree of this or any other University.

Signature of the Supervisor

Place: **Shillong**

Date: 9th April 1990

Forwarded by:

R. Kalyanasundaram 9/4/90

Head,

Department of Biochemistry,

North-Eastern Hill University,

Shillong - 793 014, INDIA.

Department of Biochemistry
North-Eastern Hill University
Shillong - 793 014, INDIA

Telephone : National (0364) 25136
International + 91 - 364 - 25136
Telegrams : NEHU

STATEMENT

I submit this thesis for the degree of **Doctor of Philosophy (in Biochemistry)** of **North-Eastern Hill University**. I declare that this thesis records details of experiments carried out (in the Department of Biochemistry) by myself, is of my own composition and has not been previously accepted in part or whole for a higher degree of this or any other university.

R. P

(R. PRAKASHAM)

CONTENTS

ACKNOWLEDGMENTS

I owe my sincere thanks to Professor **Amar Nath Rai** for introducing me into this field, his supervision, constant encouragement and interest during this study. I am grateful to Prof. **H.N. Singh** (University of Hyderabad, Hyderabad) and Prof. B. **Bergman** (University of Uppasala, Sweden) for supply of the cyanobacterial strains. Thanks are also due to Mr. **P.K. Prabhakaran** for his help in amino acid analysis, to **INSA, NEHU** and **UGC** for financial assistance and **Head, Department of Biochemistry (NEHU)** for providing research facilities. I express my sincere gratitude to Prof. R. Lalthantluanga, Dr. M.Y. Khan, Dr. R. Sharma, Dr. R.N. Sharan, Dr. A. Alam, Prof. E.R.S. Talpasayi, Dr. Surendra Singh, Dr. Sudhir Kr. Agarwal, Miss. M. Borthakur, Mr. Bijoy Das and all my colleagues in the School of Life Sciences, who helped me during this study.

R. P

(R. PRAKASHAM)

List of contents

List of Tables

List of figures

ABBREVIATIONS

ADP = Adenosine 5'-diphosphate

ATP = Adenosine 5'-triphosphate

ATS = Ammonium transport system

BSA = Bovine serum albumin

C = Carbon

Chl = Chlorophyll

cm = Centimeter

cm^3 = Milliliter

DCMU = Dichlorophenyl dimethylurea

dm^3 = Liter

DNA = Deoxyribonucleic acid

e^- = Electron

FMN = Flavin mononuclotide

GOGAT = Glutamate synthase

GS = Glutamine synthetase

h = Hour(s)

Het^+ = Heterocystous

Het^- = Non heterocystous

HEPES = 4-(2-Hydroxyethyl)-1-piperazine ethane sulphonic acid

LDH = Lactate dehydrogenase

MA = Methylamine

min = Minute(s)

mm^3 = Microliter

MSX = L-Methionine-DL-sulphoximine

N = Nitrogen

NAD = Nicotinamide adenine dinucleotide

NADH = Nicotinamide adenine dinucleotide reduced

NADP = Nicotinamide adenine dinucleotide phosphate

NADPH = Nicotinamide adenine dinucleotide phosphate reduced

Nif^+ = Nitrogen fixing

Nif^- = Non nitrogen fixing

NIR = Nitrite reductase

NR = Nitrate reductase

OD = Optical density

PK = Pyruvate kinase

POPOP = 1,4-Bis[2-(5-phenyloxazolyl)]benzene

PPO = 2,5-Diphenyloxazole

PS = Photosystem

RNA = Ribonucleic acid

TCA = Trichloroacetic acid

$TPMP^+$ = Triphenyl methylphosphonium

$\Delta\psi$ = Transmembrane electrical potential

1. INTRODUCTION AND LITERATURE REVIEW

1.1. General:

Nitrogen is one of the essential elements as cellular constituent of living organisms. Although, 78% of the earth's atmosphere consists of molecular nitrogen, most organisms cannot utilize this as nitrogen source. Instead, they require combined nitrogen. The commonest nitrogen source for plants and microbes is ammonia. In modern agriculture, for high yield, chemical fertilizers are used on a large scale to serve as nitrogen source for crop plants. Chemical fertilizer production requires enormous amounts of energy. In addition, considerable amounts of energy, time and labour is needed for distribution and application of these factory produced fertilizers at the field level. Due to this, prices of chemical fertilizers is constantly on increase with a widening gap between supply and demand. However, there are some prokaryotic microorganisms which can convert molecular nitrogen into ammonia under normal physiological conditions. A thorough understanding of the process of biological nitrogen fixation will help to devise strategies resulting in an alternative to the chemical nitrogen fertilizers. Hence, there is a world-wide attempt, by the scientific community, to study the physiological, biochemical and genetic aspects of N_2-fixing organisms. Among such organisms, cyanobacteria are of special interest because of their simple growth requirements, their diazotrophic nature, and their being capable of carrying out

oxygenic photosynthesis (Singh, 1961; Sprent, 1979; Stewart, 1980; Whitton & Carr, 1982; Gallon & Chaplin, 1987; Rai, 1990).

1.2. Cyanobacteria:

Cyanobacteria are widely distributed in both aquatic and terrestrial habitats (Fogg *et al.*, 1973). They occur freely (free-living) as well as in symbiotic associations (Stewart *et al.*, 1983; Gallon & Chaplin, 1987; Rai, 1990). They are photosynthetic prokaryotes with a higher plant type oxygenic photosynthesis and share characteristics of gram-negative bacteria (Stanier, 1977; Stanier & Cohen-Bazire, 1977; Stewart, 1980). They produce two types of metabolically active cells namely, vegetative cells and heterocysts. The third type of cells, akinetes, are metabolically less active and serve as perennating bodies (Nichols & Adams, 1982; Rai *et al.*, 1985; 1988). Various details of cyanobacteria have been extensively reviewed in the recent past (Stanier *et al.*, 1978; Rippka *et al.*, 1979; Bothe *et al.*, 1980; Gallon, 1980; Stewart, 1980; Stewart *et al.*, 1980; 1982; Bothe, 1982; Carr & Whitton, 1982; Gallon & Chaplin, 1987; Hallenbeck, 1987; Rai, 1990).

1.2.1. Vegetative cells:

The vegetative cells have photosynthetic machinery essentially like chloroplasts, which carry out higher plant type oxygenic photosynthesis using water as ultimate source of reductant (Doolittle, 1979; 1982; Ho & Krogmann, 1982). Photosynthetic components are located on a system of thylakoid

membranes which are topographically and functionally distinct from plasma membrane. They are usually distributed near periphery of the cell and often oriented parallel to the cell wall (Fogg *et al.*, 1973; Long & Whitton, 1973). Chlorophyll **a** is the principal light harvesting pigment, while phycobiliprotein complexes are accessory pigments of PS II. These pigments are located on thylakoid membrane (Cohen-Bazire & Bryant, 1982; Ho & Krogmann, 1982; Morschel & Rhiel, 1987). Under conditions of N-deficiency the accessory pigments are readily used as nitrogen sources (Allen & Smith, 1969; Stewart *et al.*, 1978; Cohen-Bazire & Bryant, 1982). Vegetative cells produce ATP by oxidative phosphorylation and via cyclic and non-cyclic photophosphorylation. Carbon-di-oxide fixation occurs through Calvin cycle (Stanier, 1977, Stewart, 1977; Allen, 1984).

The major reserve polymers present in cyanobacterial vegetative cells are polysaccharides (glycogen), cyanophycin (N-reserve) and polyphosphates which are mobilized when a need arises (Merrick, 1979; Smith, 1982). Polysaccharide reserves are located along the thylakoid membranes (Ris & Singh, 1961; Jost, 1965).

Vegetative cells of cyanobacteria also accumulate poly-hedral bodies which are store houses of Calvin cycle enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (Codd & Stewart, 1976; Allen, 1984).

In non-heterocystous N_2-fixing strains, the vegetative cells also contain nitrogenase (Gallon & Chaplin, 1988). In heterocys-tous forms; however, the nitrogenase is located in heterocysts which are non-photosynthetic and which receive fixed-carbon from neighbouring vegetative cells (Bothe *et al.*, 1984; Stewart *et al.*,

1985; Bergman *et al.*, 1986).

1.2.2. Heterocysts:

In inorganic growth medium devoid of combined nitrogen, 5-10% of the vegetative cells differentiate into specialized structures called heterocysts. Heterocysts are generally larger than vegetative cells and comparatively paler in colour. They are sites of dinitrogen fixation (Fay *et al.*, 1968; Stewart, 1980; Janaki & Wolk, 1982; Bergman *et al.*, 1986) and have undergone a number of structural, biochemical and genetic changes during their development (Wolk, 1982; Golden *et al.*, 1985; Haselkorn *et al.*, 1987). They possess thick envelope comprising of an inner laminated layer, a central homogeneous layer and an outer fibrous layer (Wolk, 1982). The thylakoid membranes are present but they lack PS II activity and have little or no phycobiliproteins (Reinman & Thornber, 1979; Alberte *et al.*, 1980; Stewart, 1980). They are unable to fix CO_2 due to lack of ribulose 1,5-bisphosphate carboxylase (Winkenbach & Wolk, 1973; Codd & Stewart, 1977; Codd *et al.*, 1980; Cossar *et al.*, 1985) and depend on adjacent vegetative cells for provision of carbohydrates (Wolk, 1968; Stewart, 1980; Bothe *et al.*, 1984; Stewart *et al.*, 1985).

The carbon dissimilation, in heterocysts, is mainly through oxidative pentose phosphate pathway (Smith, 1982). The key enzymes of hexose oxidizing pathway, glucose 6-phosphate NADP: oxidoreductase and 6-phosphogluconate NADP: oxidoreductase are present in heterocysts (Winkenbach & Wolk, 1973; Bohme, 1987) at far higher levels than in vegetative cells (Gallon & Chaplin, 1987). Recently, it has been reported that heterocysts contain all

glycolytic pathway enzymes and a low level of pyruvate ferredoxin oxidoreductase (Stewart *et al.*, 1985). Primary assimilation of nitrogenase-derived ammonia occurs in heterocysts. Glutamine is transported to vegetative cells. It is however, debatable whether GOGAT occurs in heterocysts (Hallenbeck, 1987).

Thioredoxins have been implicated in regulation of carbon metabolism in cyanobacteria. They are small proteins which are reduced in light by reduced ferredoxins. Reduced thioredoxin activates Calvin cycle enzymes and inactivates the oxidative pentose phosphate pathway enzyme, glucose 6-phosphate NADP: oxidoreductase. Thioredoxin has been reported to be absent in heterocysts (Rowell *et al.*, 1985a; Bohme, 1987). Thus, glucose 6-phosphate NADP: oxidoreductase remains active in heterocysts even during light.

Heterocysts generate ATP by cyclic photophosphorylation, oxidative phosphorylation (Stewart, 1980; Gallon & Chaplin, 1987) and oxyhydrogen reaction (Hallenbeck & Benemann, 1979; Bothe, 1982). Heterocyst production is inhibited by ammonium (Singh *et al.*, 1983a).

1.3. Nitrogen-metabolism in cyanobacteria:

The common sources of inorganic nitrogen for cyanobacteria are N_2, NO_3^- and NH_3. While all cyanobacteria are capable of utilizing NO_3^- and NH_3, N_2-utilization is limited to those cyanobacteria which are capable of nitrogen-fixation. These include all heterocystous and some non-heterocystous forms.

1.3.1.1. Nitrogen fixation:

The enzyme responsible for the reduction of dinitrogen to ammonia is nitrogenase (Gallon, 1980; Stewart, 1980; Hallenbeck, 1987; Smith *et al.*, 1987a). Some of the nitrogen-fixing cyanobacteria are listed in table 1.1.

Table: **1.1. Nitrogen-fixing cyanobacteria:**

Category	Name of the cyanobacteria
I. Filamentous, heterocystous forms.	*Anabaena* sp.
	Calothrix sp.
	Chlorogleopsis sp.
	Fischerella sp.
	Nodularia sp.
	Nostoc sp.
	Scytonema sp.
II. Filamentous, non-heterocystous forms.	*Oscillatoria* sp.
	Plectonema sp.
	Trichodesmium sp.
III. Unicellular, aerobic forms:	*Gloeothece* sp.
	Synechocystis sp.
IV. Unicellular, microaerobic forms.	*Dermocarpa* sp.
	Pleurocarpa sp.
	Xenococcus sp.

1.3.1.2. Nitrogenase:

There are at least two nitrogenase systems capable of

reducing molecular nitrogen to ammonia: the classical nitrogenase (Mo-nitrogenase) and vanadium-nitrogenase (Smith *et al.*, 1987a). Both enzymes consist of two oxygen-sensitive metalloproteins. While the iron-protein is common in both enzyme systems, the second metalloprotein varies: In case of classical nitrogenase it is a molybdenum-iron protein while in the case of vanadium nitrogenase it is a vanadium-iron protein. The vanadium nitrogenase has only recently been discovered in bacteria (*Azotobacter vinelandii*) and in cyanobacteria (*Anabaena variabilis*) hence only limited data on its nature and physiology are available (Hales *et al.*, 1985; Robson *et al.*, 1986a; 1986b; Gallon & Chaplin, 1987; Smith *et al.*, 1987a; Kentemich *et al.*, 1988).

1.3.1.3. Biochemistry of nitrogenase:

Nitrogenase consists of two iron-sulphur proteins, neither of which is active by itself. One of them is the iron protein which is a dimer of two identical subunits. It has molecular weight of 50,000 - 60,000 Da encoded by *nif* H gene. In general, it contains a single 4Fe-4S cluster, but in cyanobacteria 2Fe-2S cluster has also been reported (Haaker *et al.*, 1985; Howard *et al.*, 1985; Gallon & Chaplin, 1987; Smith *et al.*, 1987a). It has redox potential in the range of -0.24 V to -0.393 V, depending on the source (Smith *et al.*, 1987a).

Molybdenum-iron protein of nitrogenase is a tetramer $\alpha_2\beta_2$ of two different subunits: α subunit encoded by the gene *nif* D has molecular weight of 50,000 Da and β subunit encoded by the gene *nif* K has molecular weight of 60,000 Da (Gallon, 1980; Smith *et al.*, 1987a). It contains 2 Mo atoms, 30 Fe atoms and slightly lower number of sulphur atoms (Watt *et al.*, 1986). It also

possesses two FeMoco centers, four Fe-S clusters of 4Fe-4S type (P centers) and an additional 2 Fe atoms designated as 'S' centers (Orme-Johnson, 1985; Lowe *et al.*, 1985; Shah *et al.*, 1986).

Nitrogenase being a versatile catalyst, uses substrates as varied as N_2, C_2H_2, N_2O, CN^-, H_2, HCN, CH_3CN, C_2H_4 and a number of small triple bonded substrates (Bothe *et al.*, 1982; Lowe *et al.*, 1985; Jensen & Burris, 1986; Gallon & Chaplin, 1987; Smith *et al.*, 1987a).

1.3.1.4. Requirements for nitrogenase activity:

For its activity, the enzyme nitrogenase requires, reductant, ATP and a reducing atmosphere.

Nitrogenase is rapidly inactivated by exposure to oxygen (half life = 40 s in air) (Smith *et al.*, 1987b). In heterocystous cyanobacteria, the heterocysts provide suitable environment for nitrogen-fixation in aerobic conditions, because heterocysts lack photosynthetic oxygen evolution (Reinman & Thornber, 1979; Alberte *et al.*, 1980) and have high respiratory oxygen consumption rate (Haury & Wolk, 1978; Walsby, 1982; Jensen & Cox, 1983; Sprent *et al.*, 1987). Aerobic nitrogen-fixation in non-heterocystous cyanobacteria is made possible by temporal separation of photosynthesis and nitrogen-fixation (Mullineaux *et al.*, 1981). Other cyanobacteria fix nitrogen under microaerobic or anaerobic conditions.

1.3.1.4.1. Provision of reductant:

In most nitrogen-fixing organisms, including cyanobacteria, ferredoxin is believed to be the immediate electron donor to nitrogenase (Stewart, 1980; Bothe *et al.*, 1982; 1984; Schrautemeier & Bohme, 1985; Gallon & Chaplin, 1987). However, under

iron-deficient conditions flavodoxin substitutes for ferredoxin as electron donor (Smillie, 1965; Bothe, 1969; Hallenbeck, 1987; Smith *et al.*, 1987a).

Ferredoxins are iron-sulphur proteins containing one or more iron-sulphur clusters which are involved in electron transfer. Cyanobacteria contain typical soluble plant type ferredoxin with 2Fe-2S (acid-liable) clusters in the prosthetic group (Bothe 1969; Hallenbeck, 1987; Smith *et al.*, 1987a). Flavodoxins are the simplest flavoproteins containing one molecule of FMN in the prosthetic group per protein molecule. In catalysis, they act as electron carriers shuttling between the fully reduced and semi-quinone forms (Bothe, 1969; Klugkist *et al.*, 1986; Smith *et al.*, 1987a).

At present, the mechanism whereby ferredoxin is reduced is a controversial subject. However, several electron generating systems are reported. The possible reductant generating enzymic pathways are pyruvate: ferredoxin oxidoreductase (Leach & Carr, 1971; Smith *et al.*, 1987a), glucose 6-phosphate NADP: oxidoreductase, 6-phosphogluconate NADP: oxidoreductase and isocitrate NADP: oxidoreductase (Apte *et al.*, 1978; Schrautemeier & Bohme, 1984; 1985; Bohme, 1987). The enzymes glucose 6-phosphate NADP: oxidoreductase and isocitrate NADP: oxidoreductase are sensitive to high concentration of $NADPH_2$ and negatively regulated by light through thioredoxin (Duggan & Anderson, 1975; Grossman & McGowan, 1975; Schaeffer & Stanier, 1978; Cossar *et al.*, 1984). However, very low $NADPH_2$/NADP ratio (between 1 - 3) is reported in N_2-fixing organisms (Gallon & Chaplin, 1987). Under such conditions, reduction of ferredoxin (midpoint electrical potential -0.42 V) by $NADPH_2$ (midpoint electrical potential -0.32 V) is thermodinami-

cally unfavourable. Therefore, Haaker *et al* (1980) and Hawkesford *et al* (1981) suggested the involvement of $\Delta\psi$ component of proton motive force for transfer of electrons to nitrogenase by reversed electron flow (Stewart *et al*., 1982). In heterocystous cyanobacteria, hydrogen is another possible source of reductant for nitrogen fixation, through the action of uptake hydrogenase (Smith *et al*., 1987a). In the light, hydrogen may generate the reduced ferredoxins by donating electrons to photosynthetic electron transport chain prior to PS I (Bothe *et al*., 1984; Klugkist *et al*., 1986; Smith *et al*., 1987a).

1.3.1.4.2. Provision of ATP:

N_2-fixation is a highly ATP dependent process. Hydrolysis of ATP triggers electron transfer from iron protein to molybdenum-iron protein (Smith *et al*., 1987a; Cordewener *et al*., 1988). In general, 2 Mg-ATP molecules are required for transfer of one electron. However, recent studies indicate that, ATP/e^- ratio is close to 4 (Cordewener *et al*., 1988). In heterocysts, ATP is generated in the light via cyclic photophosphorylation and by the action of uptake hydrogenase, whereas, in the dark, it is produced via oxidative phosphorylation (Maryan *et al*., 1986) and substrate level phosphorylation (Bottomley & Stewart, 1976).

1.3.1.5. Mechanism of action:

A low redox potential molecule, reduced ferredoxin/flavodoxin (midpoint redox potential -0.44 to -0.45 V), donates electrons to iron protein component of nitrogenase (Bohme, 1987; Smith *et al*., 1987a). The iron protein of nitrogenase has two binding sites for Mg-ATP (Eady, 1986; Watt *et al*., 1986). Binding of Mg-ATP lowers the midpoint redox potential of iron protein from

about -0.3 V to -0.4 V or more making it a powerful reducing species and markedly alters the conformation of iron protein (Smith *et al.*, 1987a). The reduced iron protein complexes with the oxidized form of molybdenum-iron protein (Lowe *et al.*, 1985). The molybdenum-iron protein component has midpoint redox potential of 0.0 to -0.29 V when it binds with FeMoco (Watt *et al.*, 1986). It has two binding sites for iron protein (Postgate, 1982; Smith *et al.*, 1987a). The transfer of electrons from reduced iron protein to molybdenum-iron protein is rapid and irreversible (Lowe *et al.*, 1985). This reduction of molybdenum-iron protein is coupled with the simultaneous hydrolysis of Mg-ATP (Burgess, 1985; Mortenson *et al.*, 1985; Cordewener *et al.*, 1988). This hydrolysis of Mg-ATP occurs only when the iron-protein complexes with molybdenum-iron protein of nitrogenase in the presence or absence of external electron donor (Gallon & Chaplin, 1987). As soon as the electrons are transferred to molybdenum-iron protein, the nitrogenase complex dissociates (Burgess, 1985). Then the electron flow proceeds to substrate, which is thought to be reduced in three two-electron steps (Smith *et al.*, 1987a). On the whole, 25% of the available electrons reduce the protons and evolve hydrogen (Simson & Burris, 1984; Smith *et al.*, 1987a).

1.3.1.6. Regulation of nitrogenase by ammonia:

Nitrogenase activity, in all N_2-fixing organisms, is known to be affected by various combined-nitrogen sources and oxygen (Stewart & Lex, 1970; Neilson & Nordlund, 1975; Rippka & Stanier, 1978; Jones & Monty, 1979; Brill, 1980; Roberts & Brill, 1981; Bognar *et al.*, 1982; Thomas *et al.*, 1982; Paparao & Singh, 1983; Singh *et al.*, 1983b; Yoch *et al.*, 1983; Collins & Brill, 1985;

Bohme, 1986; Reich *et al.*, 1986; 1987; Stewart *et al.*, 1987).

Nitrogenase activity is quickly and reversibly inhibited by the addition of ammonia (Haaker *et al.*, 1980; Salminen, 1981; Ludden *et al.*, 1984). Different phenomena have been described with respect to regulation of nitrogenase activity *in vivo*. For example, in *Rhodospirillum rubrum* the inhibition is due to covalent modification of iron-protein component of nitrogenase (Pope *et al.*, 1985; Zumft, 1985; Hallenbeck, 1987). Whereas, in bacteriods of *Pisum sativum* (L) it is due to uncoupling effect (Salminen, 1981). In *A. vinelandii* Haaker *et al* (1980) have shown that inhibition of nitrogenase by ammonia is due to deenergization of the membrane by ammonium resulting in the inhibition of the supply of reductant for nitrogen-fixation.

In cyanobacteria however, such fast nitrogenase switch-off is not observed except at higher pH. At the alkaline environment ammonia enters in the cyanobacterium by diffusion and leads to an immediate inactivation of nitrogenase and this inactivation is thought to be due to uncoupling effect (Reich *et al.*, 1986; 1987).

In vivo nitrogenase synthesis is regulated, in various nitrogen-fixing bacteria and cyanobacteria, upon the addition of ammonia and/or a product of ammonia assimilation (Gallon & Chaplin, 1987; Hallenbeck, 1987). The mechanism is not same in all nitrogen-fixing organisms. For example, in *Klebsiella pneumoniae* and *Rhodopseudomonas capsulata*, *nif* L gene product acts as a repressor for other *nif* genes (Hallenbeck *et al.*, 1982; Collins & Brill, 1985; Hallenbeck, 1987; Smith *et al.*, 1987a). However, it appears that ammonia itself is not the repressor of nitrogenase synthesis; it must be assimilated at least to gluta-

mine. The evidence comes from the glutamine auxotroph of *R. capsulata* where derepression of nitrogenase is found in the presence of ammonia (Wall & Gest, 1979). Whereas, in *R. rubrum*, glutamine and asparagine repressed the nitrogenase synthesis (Neilson & Nordlund, 1975). However, in *K. pneumoniae* regulation of nitrogenase synthesis is thought to be related with glutamine synthetase. It is found that a mutation of structural gene for GS (*gln* A) often caused a repression of nitrogenase synthesis (Gallon & Chaplin, 1987). While in *Clostridium pasteurianum* ammonia degraded the mRNA responsible to code for nitrogenase (Daesch & Mortenson, 1972).

In cyanobacteria, repression of nitrogenase synthesis is known to be influenced by ammonia and/or its derivatives. Nitrogenase synthesis and heterocyst development is repressed by ammonia (Stewart, 1980). However, presence of MSX, an inhibitor of GS, prevents the repression of nitrogenase synthesis by ammonia (Stewart and Rowell, 1975). Thus, it appears that ammonia needs to be assimilated before nitrogenase synthesis can be repressed (Stewart *et al.*, 1985). In *Gloeocapsa*, a product of ammonia assimilation, is thought to be repressor of nitrogenase synthesis (Thomas *et al.*, 1982).

Recently, ammonia *per se* has been suggested to be involved in repression of nitrogenase synthesis in cyanobacteria (Singh *et al.*, 1983b; Turpin *et al.*, 1984; Mackerras and Smith, 1986).

1.3.2. Nitrate assimilation:

Virtually all cyanobacteria tested so far, are capable of

using nitrate as nitrogen source. Diazotrophic cyanobacteria prefer nitrate to N_2. Exogenous nitrate is first transported into the cell where it is reduced to ammonia by the action of nitrate reductase (NR) and nitrite reductase (NIR) enzyme systems. Such nitrate reduction in cyanobacteria is linked to photosynthesis (Manzano *et al.*, 1976; Lara *et al.*, 1987).

In general, nitrate uptake system in diazotrophic cyanobacteria is of two types: a) nitrate inducible/ammonium-repressible. Such a system is found in the cyanobacterium, *Anabaena* 7120, where nitrate uptake activity develops in nitrate medium only and presence of ammonium represses it (Meeks *et al.*, 1983; Rai & Bergman, 1986). b) ammonium repressible/derepressible. Such a system is exemplified by the cyanobacterium *Anabaena cycadeae*, where nitrate uptake activity is present both in N_2- and NO_3^--medium; presence of ammonia represses it (Bagchi *et al.*, 1985b).

A genetic link between glutamine synthetase and the nitrate uptake system has been reported in the cyanobacterium *A. cycadeae* (Singh *et al.*, 1985a). The glutamine auxotroph of *A. cycadeae* has been shown to posses several fold higher nitrate uptake level than the wild type strain (Singh *et al.*, 1985a).

The kinetics of the development of nitrate uptake system studies indicated that uptake system has a regulatory role in the development of nitrate reductase system in *A. cycadeae* (Bagchi *et al.*, 1985a). In this cyanobacterium it was found that nitrate uptake system development is independent to that of nitrate reductase system development.

In heterocystous filamentous cyanobacteria, the nitrate

uptake and reductase systems are found to be associated with vegetative cells only and have been shown to be absent in akinetes and in heterocysts (Rao *et al.*, 1984; Kumar *et al.*, 1985; Rai & Bergman, 1986). Absence of nitrate metabolism in heterocysts results in the abolition of competition for molybdenum cofactor and reductant between nitrogenase and nitrate reductase (Kumar *et al.*, 1985; Rai & Bergman, 1986).

In *Nostoc muscorum*, nitrate transport is energy-dependent; its ATP requirement has been shown Rai by *et al* (1981a). In *Anabaena* 7120, nitrate and nitrite share a common transport system (Rai & Bergman, 1986). Nitrate reductase in cyanobacteria is found to be ferredoxin dependent (Kumar *et al.*, 1985; Rai & Bergman, 1986). In general, nitrate reductase in cyanobacteria is of two types; a) nitrate inducible/ammonia repressible. In *Anabaena cylindrica* it is nitrate inducible while in *Anacystis nidulans* it is ammonia repressible (Ohmori & Hattori, 1970; Herrero *et al.*, 1981). b) ammonia repressible/derepressible. Such a regulatory system is found in *Anabaena* 7120: In this cyanobacterium the nitrate reductase apoprotein undergoes repression/derepression control while the Mo-cofactor is constitutive. The apoprotein synthesis is repressed in NH_4^+-medium whereas derepressed in N_2- and NO_3^--medium (Kumar *et al.*, 1985; Rai, 1990).

1.3.3. Ammonia assimilation:

The product of nitrogen-fixation and nitrate or nitrite assimilation is ammonia, which is subsequently assimilated into organic compounds via glutamine synthetase and glutamate synthase pathway (GS-GOGAT pathway) (Dharmawardene *et al.*, 1973; Wolk *et*

al., 1976; Miflin & Lea, 1976; Stewart, 1980; Orr & Haselkorn, 1982; Stewart *et al.*, 1983; 1987; Rai *et al.*, 1986a).

Heterocysts, the sites of ammonia production by nitrogen-fixation, have a GS concentration nearly two-fold higher than that in the vegetative cells (Bergman *et al.*, 1985). The cyanobacterial GS is a dodecameric enzyme consisting of 12 identical subunits. Each subunit has relative molecular mass of 50,000 (Sampaio *et al.*, 1979; Stewart *et al.*, 1987). GS activity is essentially irreversible under normal physiological conditions; it has a high affinity for ammonium (Km = 0.02 mM) (Miflin & Lea 1976), is inhibited by methionine sulphoximine (MSX) (Stewart & Rowell, 1975; Gallon, 1980; Stewart *et al.*, 1985; 1987) and hydroxylysine (Ladha *et al.*, 1978; Stewart *et al.*, 1987), and is deactivated by ammonia or darkness in cyanobacteria (Rowell *et al.*, 1979). In symbiosis, the cyanobionts contain low GS-GOGAT activities which may be responsible for liberation of nitrogenase derived ammonia (Rai *et al.*, 1980; 1981b; 1984; Stewart *et al.*, 1983; 1987; Joseph & Meeks, 1987).

Glutamate synthase (GOGAT) in cyanobacteria converts glutamine to glutamate and requires reduced ferredoxin as a reductant (Ohmori, 1981; Stewart *et al.*, 1983). GOGAT has been reported to be absent in heterocysts (Thomas *et al.*, 1977; Rai *et al.*, 1982) however, some workers suggest GOGAT to be present in heterocysts at a low activity (Gupta & Carr, 1981; Bothe *et al.*, 1984). Azaserine is an inhibitor of this enzyme (Hartman, 1973; Ohmori *et al.*, 1985).

1.4. Ammonium transport:

Besides molecular nitrogen, ammonia is the most wide spread nitrogenous compound on earth available for utilization by various organisms (Kleiner, 1981; 1985a). Among all inorganic nitrogen sources ammonia is a preferred nitrogen source for all nitrogen-fixing organisms including cyanobacteria (Stewart, 1980; Gibson, 1984; Mackerras & Smith, 1986). When it is available in excess, it represses nitrogenase as well as nitrate uptake, NR and ammonium transport (Zumft & Castillo, 1978; Bothe, 1982; Stewart *et al.*, 1983; 1987; Singh *et al.*, 1983b; Gibson, 1984; Avissar, 1985; Bagchi *et al.*, 1985b; Mackerras & Smith, 1986).

Ammonia is a weak base and occurs in two forms; ammonia (NH_3) and ammonium (NH_4^+) (Kleiner, 1981; 1985a). Ammonium has dissociation constant of 9.25. The neutral molecule, ammonia, is a weak base and protonates to give ammonium at physiological pH (Henderson, 1971; Kleiner, 1985a).

Biological membranes show little or no permeability for ammonium, suggesting a need for specific ammonium transport carrier. The existence of specific carrier for ammonium have been inferred from the following criteria:

1. Metabolism of any compound starts with its passage across bio-membranes. It is generally assumed that if a molecule exist as a neutral and ionic species- like weak acid and base, bio-membranes are rather permeable towards the neutral form but less for ions, which normally requires a carrier (Henderson, 1971; Tien, 1974; Kleiner, 1985a).
2. Biomembranes are rather highly permeable towards unprotonated form of ammonia. In such cases, no specific transport system

is likely to exist for ammonium because of rapid equilibrium between ammonia and ammonium. However, when a pH difference occurs across biomembranes, unequal distribution of non-permeating ions is observed, indicating occurrence of specific carrier system (Henderson, 1971).

3. In the absence of Δ pH, microorganisms show ammonium and methylammonium gradients across biomembranes at the expense of energy. Accordingly, such a membrane system should have reduced permeability towards ammonium in order to avoid futile cycle (Kleiner, 1985a).
4. Saturation kinetics.
5. Distinct pH profile studies.
6. Specific inhibitor studies.
7. Genetic and metabolic control studies and
8. Studies on transport-deficient mutants.

Thus, in summary, ammonium transport across biomembranes, is a carrier mediated process which equilibrate the ions across the membrane according to an electric field. This implies that ammonium and not ammonia is the transported species. However, it is yet to be discovered whether it is uniport channel or mobile carrier (Kleiner, 1985a). Ammonium transport system (ATS) has a role in the retention of internally generated ammonia from nitrogen fixation and nitrate reduction (Kleiner, 1985b; Kerby *et al.*, 1987).

1.4.1. Ammonium transport in bacteria:

Nitrogen-fixing bacteria provide a suitable model system to study ATS, because no enzyme system would be expected to be

repressible by the ubiquitous nitrogen source, N_2 (Kleiner, 1985a). ATS has been found to occur in several diazotrophic bacteria (Barnes & Zimniak, 1981; Alef & Kleiner, 1982a; 1982b; Hartman & Kleiner, 1982; Ivanovsky *et al.*, 1982; Gober & Kashket, 1983; Glenn & Dilworth, 1984; Genthner & Wall, 1985; Hara *et al.*, 1985; Holtel & Kleiner, 1985; Howitt *et al.*, 1986). In most of the bacteria, ATS has been characterized by using methylammonium as ammonium analogue. The apparent Km value for methylammonium uptake ranges from 2 to 140 μM (Alef & Kleiner, 1982a; 1982b; Hartman & Kleiner, 1982; Kleiner, 1982; Muzzucco & Benson, 1984; Genthner & Wall, 1985; Pargent & Kleiner, 1985; Howitt *et al.*, 1986) and is competitively inhibited by the addition of ammonium; Ki value ranges from 1 to 15 μM, indicating that both ammonium and methylammonium are transported by same carrier (Alef & Kleiner, 1982a; Kleiner, 1982; Gober & Kashket, 1983; Muzzucco & Benson, 1984; Kleiner, 1985a; Howitt *et al.*, 1986). In *Paracoccus denitrificans*, two different carriers, depending on growth conditions, have been reported for methylammonium uptake (Holtel & Kleiner, 1985; Kleiner, 1985a). Most bacteria have capacity to accumulate methylammonium upto 100 fold against a concentration gradient across the membranes. The intracellular ammonium pool ranges from 0.2 to 2.9 mM (Booth & Hamilton, 1980; Gordon & Moore, 1981; Kleiner, 1981; 1982; 1985a).

Ammonium transport show a distinct pH profile having optimum pH between 6.4 to 6.8 (Kleiner, 1982; Gober & Kashket, 1983; Genthner & Wall, 1985; Howitt *et al.*, 1986). Ammonium transport in all bacterial species, is found to be an energy dependent process. Most studies, dealing with energetics of ammonium

transport, have revealed that transmembrane electrical potential, is the driving force for ammonium transport (Barnes & Zimniak, 1981; Kleiner & Fitzke, 1981; Kleiner, 1982; 1985a). It is found that ammonium accumulation requires transmembrane electrical potential of -70 mV (Kleiner, 1982; 1985a). However, the involvement of ATP in ammonium accumulation is not ruled out in *A. vinelandii* (Gordon & Moore, 1981; Moore & Gordon, 1984).

In most species the synthesis of ammonium of carrier is subjected to nitrogen control, while the activity may be regulated either by glutamine or by ammonia (Kleiner, 1982; 1985a). High levels of ammonium in the medium repress the ammonium carrier in most of the organisms (Alef & Kleiner, 1982a; Hartman & Kleiner 1982; Bogdahn *et al.*, 1983; Muzzucco & Benson, 1984; Holtel & Kleiner, 1985). This nitrogen control of ammonium transport is thought to be mediated by the *ntr* regulatory system (Streicher *et al.*, 1974; Magasanik, 1982; Kleiner, 1985a). Recent studies on *Escherichia coli* strains containing different *ntr* gene mutants revealed that *ntr* A & C gene products are required to derepress the ammonium carrier, while, *ntr* B gene product plays a role in repression (Genthner & Wall, 1985). Addition of glutamine strongly decreases the methylammonium uptake (Ivanovsky *et al.*, 1982; Jayakumar & Barnes, 1984; Hara *et al.*, 1985) which is thought not to be regulated by intracellular glutamine pool (Kleiner & Castroph, 1982; Jayakumar & Barnes, 1984). MSX also inhibits the ammonium transport, which is probably due to its regulatory binding site at the carrier (Kleiner & Castroph, 1982; Kleiner *et al.*, 1983).

1.4.2. Ammonium transport in cyanobacteria:

The importance of ATS lies in the uptake of exogenous ammonium and for the retention of ammonium produced during nitrogen fixation (Kleiner, 1985b; Kerby *et al.*, 1987). Although, ATS in nitrogen fixing bacteria has been studied extensively (Silver, 1978; Kleiner, 1981; 1985a), in cyanobacteria, work on ATS commenced very recently. Rai *et al* (1984) were the first to characterize the ATS in *A. variabilis* and *Anabaena azollae*. Subsequently, it has been characterized in several cyanobacteria (Boussiba *et al.*, 1984a; Kashyap & Johar, 1984a; 1984b; Kashyap & Singh, 1985; Singh *et al.*, 1985b; 1986; 1987; Kerby *et al.*, 1986; 1987; Rai *et al.*, 1986a; 1986b; Stewart *et al.*, 1987).

In most studies, the ammonium analogue, methylammonium, has been used to characterize ATS in cyanobacteria, since methylammonium uses the same transport system as ammonium (Boussiba *et al.*, 1984b; Rai *et al.*, 1984; Singh *et al.*, 1985**b**; 1987; Kerby *et al.*, 1986; 1987). However, it has also been characterized by using ammonium (Kashyap & Johar, 1984a; Kashyap & Singh, 1985).

Ammonium transport, in most of the cyanobacteria so far tested, is found to be biphasic with a rapid initial phase followed by a relatively slower second phase. The first phase, lasting 2-3 minutes, is independent of methylammonium metabolism, while, the second phase is dependent on methylammonium metabolism via GS (Boussiba *et al.*, 1984b; Rai *et al.*, 1984; Singh *et al.*, 1985b; 1986; 1987; Kerby *et al.*, 1986; 1987; Boussiba & Gibson, 1987).

Ammonium uptake at pH 9.0 is much higher than pH 7.0; at this pH ammonia could diffuse into cells and be concentrated by protonation as a consequence of the pH difference (Δ pH) (Rai *et*

al., 1984; Kerby *et al.*, 1986; 1987). At pH 7.0, MSX showed an inhibitory effect on second uptake phase, which is thought to be due to MSX inhibition of GS (Rai *et al.*, 1984; Boussiba & Gibson, 1985). However, studies on a GS mutant, *A. cycadeae*, indicated that second phase of ammonium transport may not necessarily be linked to GS activity but may represent a separate ATS, suggesting two ATS in cyanobacteria. They are: an MSX-insensitive rapid ATS and an MSX-sensitive slower ATS (Singh *et al.*, 1985b). Studies on a MSX mutant of *Anabaena doliolum* have revealed that MSX has two inhibitory targets in cyanobacteria, one at the transport level and other at the GS activity level (Singh *et al.*, 1986). MSX does not show any inhibitory effect on ammonium transport at pH 9.0 (Turpin *et al.*, 1984).

Kinetics of concentration-dependent ammonium transport studies revealed two ATS in *N. muscorum*: a high affinity ATS at low external ammonium concentration ranging from 1 - 35 μM with a Km of 11 μM; and a low affinity ATS at the higher external ammonium concentration ranging from 35 - 300 μM with a Km of 66 μM. Both systems are inhibited by methylammonium (Kashyap & Johar, 1984a; Kashyap & Singh, 1985). The internal pool of free methylammonium is found to be 1.25 mM in *A. cycadeae* and 1.4 mM in *A. variabilis* (Rai *et al.*, 1984; 1986b).

Ammonium transport, in *A. variabilis* & *A. cycadeae*, is an active and energy dependent process. It is inhibited by the addition of CCCP & $TPMP^+$, indicating $\Delta\psi$ is the driving force for ammonium transport (Rai *et al.*, 1984; 1986a; Kerby *et al.*, 1987; Singh *et al.*, 1987). In *A. nidulans* ammonium transport has been shown to be ATP-dependent (Boussiba *et al.*, 1984a; Kashyap &

Singh, 1985).

Ammonium transport in *A. variabilis* and *A. cycadeae* is ammonium repressible (Rai *et al.*, 1986b; Boussiba & Gibson, 1987; Singh *et al.*, 1987). Repression of ammonium transport is caused by ammonium itself, whereas, derepression requires *de novo* protein synthesis (Rai *et al.*, 1986b; Singh *et al.*, 1987). Genetic control of ATS has been inferred from studies on the methylamine, streptomycin and MSX mutants of *N. muscorum* and *A. cycadeae* (Kashyap & Johar, 1984a; Singh *et al.*, 1985b). In symbiotic cyanobacterium *A. azollae*, both MSX-insensitive and MSX-sensitive ATS are present whereas, in the cyanobiont of cycad root nodules, only the MSX-insensitive rapid ATS is present (Rai *et al.*, 1984; 1986a).

1.4.3. Cyclic retention of ammonia:

When N_2-fixing bacteria or cyanobacteria are grown under N_2- and NO_3^--media a high internal and a low external ammonia level is observed (Booth & Hamilton, 1980; Gordon & Moore, 1981; Kleiner, 1981; 1985b; Rai *et al.*, 1984; 1986a). This concentration gradient must result in outward diffusion of ammonia (Henderson, 1971; Kleiner, 1981; 1985a). However, such a diffusion is not observed in N_2- and NO_3^--grown conditions, because of the derepression of ammonium carrier. This carrier is responsible for recovery of ammonia, which is diffused from an internal pool, as ammonium and is an energy requiring process (Rai *et al.*, 1984; 1986b; Kashyap & Singh, 1985; Singh *et al.*, 1987). This energy expenditure is to balance the energy dependent export of protons (Kleiner, 1985a; 1985b). Thus, a constant ammonia excretion/

absorption is found under ammonia limited conditions. This cyclic ammonia/ammonium retention is also evidenced from; a) repression of ammonium carrier by ammonia and b) ammonium transport mutants, which constantly release ammonia to the medium (Kashyap & Johar, 1984b; Kleiner, 1985a). It is assumed that every ammonia molecule passes the futile cycle about 6 times before it is being assimilated (Kleiner, 1985a; 1985b). Based on the average H^+/ATP stiochiometry (3), it is found that 2 ATP molecules are expended for cyclic retention of each ammonia molecule (Kleiner, 1985a; 1985b). An outline of the cyclic retention of ammonia, proposed by Kleiner (1985b), is given in Fig 1.1.

1.5. Glutamine & glutamate transport:

Glutamine and glutamate are products of ammonia assimilation which regulate the heterocyst differentiation and aerobic nitrogen fixation in cyanobacteria (Neilson & Nordlund, 1975; Stewart, 1980; Stewart *et al.*, 1983; 1987; Rai *et al.*, 1984; Rai, 1990).

Relatively, little is known about amino acid transport in cyanobacteria (Kleiner, 1985a). In unicellular cyanobacterium *A. nidulans* amino acid transport is an energy dependent process (Leekaden & Simonis, 1979; Labarre *et al.*, 1987). Chapman & Meeks (1983), working with filamentous, heterocystous cyanobacterium, *A. variabilis* showed that glutamate and glutamine are transported via two transport systems (a high affinity & a low affinity system) with Km values of 13.8 & 100 μM and 1.3 & 1.1 mM, respectively. However, glutamine transport studies in symbiotic *Nostoc* sp. of

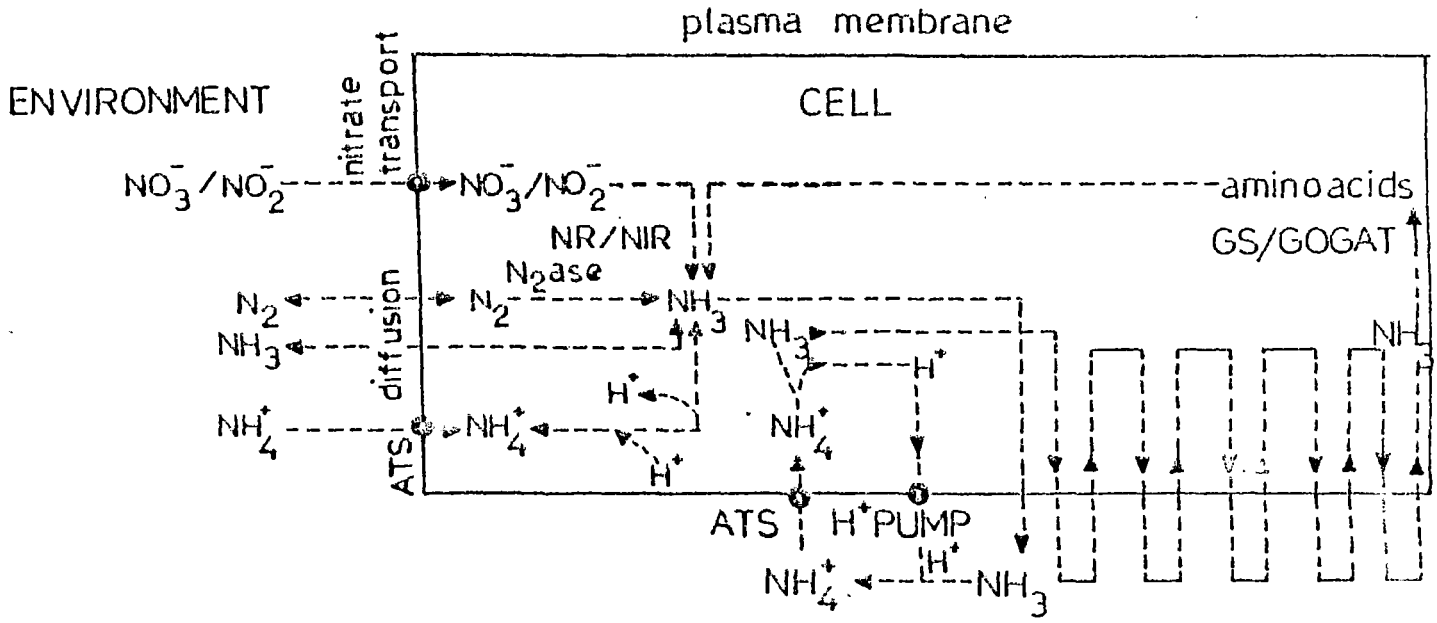

Fig 1.1 Proposed cyclic retention of ammonia, import of exogenous inorganic nitrogen and its fate. The idea of cyclic retention of ammonia is taken from bacterial systems (Kleiner, 1985b). ATS, ammonium transport system; GS, glutamine synthetase; GOGAT, glutamate synthase; NR, nitrate reductase, NIR, nitrite reductase; N_2ase, nitrogenase.

Geosiphon pyriforme indicated only one transport system with much higher affinity (Km = 0.1 to 1.3 μM) (Strasser & Falkner, 1986).

Comparative studies on glutamate & glutamine transport in *A. variabilis* and *A. cylindrica* indicated that glutamate transport in the latter is nearly 3-fold higher than that of glutamine transport. However, glutamine transport in both *Anabaena* spp. is similar (Rowell *et al.*, 1977). In symbiotic *Nostoc* sp. of *G. pyriforme*, asparatate and glutamate share a common transport carrier (Strasser & Falkner, 1986).

The inhibition studies of amino acid transport indicated that, glutamine & glutamate inhibit the transport of each other. Glutamine transport is competitively inhibited by glutamate and asparatate but glutamine showed mixed type of inhibition on glutamate transport (Chapman & Meeks, 1983; Strasser & Falkner, 1986).

1.6. Photobiological production of ammonia by cyanobacteria and their application in field:

Cyanobacteria have long been used as biofertilizers due to their dual function: Photosynthesis and nitrogen fixation (Singh, 1961; Jenkinson, 1977; Reynaud & Roger, 1978; Stewart *et al.*, 1979; 1987; Stewart, 1980). The input of fixed nitrogen by cyanobacteria ranges from a few Kg of $N.ha^{-1}.a^{-1}$ to 100 Kg of $N.ha^{-1}.a^{-1}$ (Fogg *et al.*, 1973; Stewart *et al.*, 1979; Venkatraman, 1980). However, the current use of cyanobacteria in rice-fields has serious limitations, because, normal cyanobacteria use fixed nitrogen present in the field, instead of fixing nitrogen, thus

becoming weed for the crop (Musgrave *et al.*, 1982; Stewart *et al.*, 1983; 1987). So, the strains which would liberate more ammonia and fix N_2 at higher rate would be better for biofertilizer use or for use in photobiological production of ammonia (Stewart *et al.*, 1987). Therefore, it is obvious that, we should produce suitably modified strains, maximizing nitrogen fixation, minimizing GS-GOGAT activities and/or abolishing ATS. Such modifications are well known in the cyanobacterial symbionts (Rai *et al.*, 1984; 1986b; Kleiner, 1985b; Nierzwicki-Bauer & Haselkorn, 1986). Free-living cyanobacteria have been used for ammonia production by inhibiting the GS enzyme either by MSX or hydroxylysine (Stewart & Rowell, 1975; Stewart & Rodgers, 1977; Ladha *et al.*, 1978; Stewart *et al.*, 1987) and immobilizing them in alginate (Musgrave *et al.*, 1982; 1983a; 1983b; Kerby *et al.*, 1983; Muallem *et al.*, 1983). Inhibition of GS enzyme by MSX makes the cyanobacterium a glutamine auxotroph which requires glutamine for growth (Kerby *et al.*, 1985; Rowell *et al.*, 1985b; Spiller *et al.*, 1986). However, suitable ammonia liberating strains can be obtained by partially inhibiting the GS activity (about 90 - 95%) or by manipulating the ATS so that most of the nitrogenase-derived ammonia is liberated and still the organism survives by assimilating part of the ammonia (Stewart *et al.*, 1987).

1.7. The present study:

Ammonium transport system is necessary for uptake of exogenous ammonium and retention of the nitrogenase derived ammonia (Kleiner, 1985b; Kerby *et al.*, 1987). Hence, modifi-

cation/abolition of this system would lead to leakage of nitrogenase derived ammonia and lack of intracellular accumulation of exogenous ammonia. This type of strains would be useful in production of ammonia without the need of GS-inhibitor, MSX, which is toxic and expensive. These can also be used in field where they would liberate much more of the fixed ammonia than normal cyanobacteria. Furthermore, ATS mutants would be much better than GS-mutants because:

1. GS mutants would be glutamine auxotrophs. Hence, their survival in field would be difficult and their maintenance in the laboratory would be expensive.
2. ATS mutants would fix nitrogen even in the presence of exogenous ammonium because nitrogenase will remain active. This is because exogenous ammonia would not accumulate in the cell in absence of ATS and hence nitrogenase will not be repressed.
3. ATS mutants will still assimilate enough ammonia to sustain themselves albeit at a slower growth rate. This is even more advantageous since, less fixed-C would be required for biomass and growth, diverting more photosynthate for nitrogen fixation.

However, before ATS can be manipulated a thorough knowledge of ATS in cyanobacteria is needed. With this in view the present study was undertaken to characterize the ATS in *Nostoc* ANTH and *Anabaena* 7120 and to understand its role in physiology and biochemistry of the nitrogen fixing cyanobacteria.

2. MATERIALS AND METHODS

2.1. Organisms:

Nostoc ANTH and *Anabaena* 7120 (ATCC 27893) parent and its Het^- Nif^- mutant were used as experimental materials. *Nostoc* ANTH was isolated from *Anthoceros punctatus* whereas, *Anabaena* 7120 was obtained from Dr. B.Bergman, University of Uppsala, Sweden. Both these strains were filamentous, heterocystous forms capable of aerobic N_2-fixation. Both the organisms form homogeneous suspension in liquid medium and produce little or no mucilage. Non heterocystous (Het^-) and non N_2-fixing (Nif^-) mutant of *Anabaena* 7120 (ATCC 27893) was obtained from Prof. H.N. Singh, University of Hyderabad, Hyderabad.

2.2. Culture methods:

2.2.1. Culture vessels:

'Corning' grade glasswares were used throughout the experimental study. Washing of glasswares were done by immersing them in 5% decon solution for 24 h followed by washing in running tap water. Finally, the glasswares were rinsed twice with double distilled water and then dried in hot air oven.

2.2.2. Sterilization:

Mineral nutrient solutions as well as the glasswares

sterilized by wet heat in an autoclave at 121°C (103.5 kPa steam pressure) for 15 min. However, the chemicals which are heatliable were sterilized by ultrafiltration using Whatman membrane filters (pore size 0.45 μm) and added to the medium whenever required.

2.2.3. Growth medium:

Cyanobacteria were routinely grown and maintained in $BG\text{-}11_o$ medium (Rippka *et al.*, 1979) unless otherwise indicated. The medium consisted of macro- and microelements. Stock solutions of both were made in 200 cm^3 stock bottles. From each stocks of macronutrients, 1 cm^3 was measured out and made to 1 dm^3 with double distilled water. To this 1 cm^3 of micronutrient solution was added. The pH of the medium was adjusted to 7.2 before sterilization.

The composition of the macro- & micro-elements in $BG\text{-}11_o$ stock solutions were as follows:

Macro elements	g/dm^3	Micro elements	g/dm^3
1. K_2HPO_4	40.00	H_3BO_3	28.60
2. $MgSO_4.7H_2O$	75.00	$MnCl_2.4H_2O$	18.10
3. $CaCl_2.2H_2O$	36.00	$ZnSO_4.7H_2O$	02.22
4. Citric acid	06.00	$Na_2MoO_4.2H_2O$	03.90
5. Ferric ammonium citrate	06.00	$CuSO_4.5H_2O$	00.79
6. EDTA (di Na^+ salt)	01.00	$Co(NO_3)_2.6H_2O$	00.494
7. Na_2CO_3	20.00		

The medium without combined nitrogen source is referred as nitrogen free or $BG\text{-}11_o$ medium. When KNO_3(5 $mol.dm^{-3}$) or NH_4Cl (1 $mol.dm^{-3}$) were added the medium is termed as nitrate or ammonium

medium, respectively. Photoheterotrophic and heterotrophic growth media were prepared by adding different carbohydrates, in light or dark, respectively. Carbohydrate solutions were filter sterilized and added to sterile medium under aseptic conditions and dark incubation was obtained by wrapping the flask with aluminium foil.

2.2.4. Culture conditions:

All cultures were grown in a culture room, which is maintained at 28 ± 1°C and illuminated with day light fluorescent tubes (photon fluence rate of 50 $\mu mol.m^{-2}.s^{-1}$ on the surface of the vessel). Fish tank aerators were used to aerate the medium through a sterile membrane filter (pore size 0.45 μm) to obtain aerated batch cultures.

2.3. Isolation, purification & maintenance of organisms:

2.3.1. Collection of *Anthoceros punctatus*:

A. punctatus gametophytes, bearing cyanobacterial colonies, were collected from North-Eastern Hill University campus (Shillong, India) during September.

2.3.2. Isolation of *Nostoc* ANTH:

After collection, *A. punctatus* thalli were brought into the lab and washed with sterile distilled water. After surface sterilization with sodium hypochlorite (0.5% for 2 min) algal colonies were carefully excised with sterile needles. The colonies were washed with sterilized distilled water and plated on

nitrogen free nutrient ($BG\text{-}11_0$ medium) agar (1%). Subsequently, the plates were incubated in the culture room under light (photon fluence rate of 50 $\mu mol.m^{-2}.s^{-1}$) at 28 ± 1°C. After 3 weeks individual cyanobacterial colonies were picked up and transferred to liquid medium in test tubes. After growth, the plating and selection were repeated till unialgal suspension was achieved.

2.3.3. Purification of *Nostoc* ANTH:

Test tubes containing 2 cm deep nutrient agar were sterilized and allowed to set. A cyanobacterial colony was plated on top of the agar. Then a 2 cm deep second layer of agar containing polymixin-B-sulphate (0.01 $mg.dm^{-3}$) and cycloheximide (0.1 $mg.dm^{-3}$) was poured on top of this layer. Another 2 cm deep nutrient agar layer was poured on top of this, followed by 5 cm^3 of liquid medium. The tubes were plugged with cotton and incubated in light at 28 ± 1°C. Cyanobacterial filaments migrated through the antibiotic agar layer and eventually appeared in the liquid medium at the top. These were then transferred to fresh liquid medium in flasks. The procedure was repeated till axenic cultures were obtained.

2.3.4. Maintenance:

The purified cyanobacteria were maintained on agar slants as well as in liquid medium with periodic checks for any contamination by plating on nutrient agar containing 1% glucose. However, for the regular experiments, the aerated batch cultures were maintained in the logarithmic phase by transferring them into fresh sterilized medium regularly.

2.4. Growth parameters:

Cyanobacterial growth was measured using following parameters.

2.4.1. Chlorophyll:

Chlorophyll **a** was measured as described by Mackinney (1941). A known amount of algal suspension (10 cm^3) was centrifuged and the pellet was suspended in 5 cm^3 of methanol. This was mixed thoroughly and incubated overnight at 4°C in dark. Supernatant was withdrawn after filtration. The process was repeated to extract whole pigment and the volume was adjusted to 10 cm^3. The absorbance was read at 663 nm using a Beckman DU-40 spectrophotometer. The concentration of Chl **a** was calculated as follows:

Chl **a** ($\mu g.cm^3$) = O.D. at 663 x 12.63.

2.4.2. Protein:

Protein content of cyanobacteria was measured according to Lowry *et al* (1951). **2.4.2.1. Extraction of protein:**

Five cm^3 cyanobacterial sample pellet was collected by centrifugation and suspended in 1 cm^3 of distilled water. The cyanobacterial cells were disrupted by ultrasonication using a MSE, Soniprep 150 fitted with 19 mm, at 16 amplitude microns for 5 min. After centrifugation for 5 min at 3000 rpm the supernatant was collected and used for protein determination.

2.4.2.2. Estimation of protein:

2.4.2.2.1. Reagents:

A. 2% Na_2CO_3 in 0.1N NaOH solution

B. 1% sodium potassium tartarate solution

C. 0.5% $CuSO_4$ solution

D. 100 cm^3 of reagent A mixed with 1 cm^3 each of reagent B & C (freshly prepared before use)

E. 1N Folin-cio-calteu's reagent

F. Standard protein solution: Bovine Serum Albumin solution was prepared in the range of 10 - 160 $\mu g.cm^{-3}$

2.4.2.2.2. Procedure:

To the 1 cm^3 of cyanobacterial protein solution, 5 cm^3 of reagent D was added and mixed well. This was incubated for 10 min at room temperature and then 0.5 cm^3 reagent E was added rapidly. After 30 min, the mixture was centrifuged and the absorbance of the supernatant read in Beckman DU-40 spectrophotometer at 650 nm. A calibration curve was prepared by using Bovine Serum Albumin and used for determination of cyanobacterial protein content.

2.5. Enzyme activities:

2.5.1. Nitrogenase:

Nitrogenase activity was measured using acetylene reduction assay (Stewart *et al.*, 1967). 5 cm^3 cyanobacterial culture was placed in 15 cm^3 serum vials. Acetylene gas was injected to a final concentration of 10% (v/v) of the air phase in the vial. After incubating the vials for the required time at 28 $\pm$ 1°C and at a photon fluence rate of 50 $\mu mol.m^{-2}.s^{-1}$, 1 cm^3 gas sample was analyzed, for estimating C_2H_4 produced, using a Tracor-540 gas chromatograph fitted with a flame ionization detector and Porapak T column (SS column 6'x 1/8' packed with Porapak T mesh size 80/100).

2.5.2. Glutamine synthetase (GS):

GS biosynthetic and transferase activities were measured according to Sampaio *et al* (1979).

2.5.2.1. Extraction of enzyme:

Cultures were harvested by centrifugation, washed twice in 50 mmol.dm^{-3} Tris-HCl buffer pH 7.5 (buffer A) and resuspended in buffer B (buffer A supplemented with 5 mmol.dm^{-3} $MgCl_2$, 10 mmol.dm^{-3} sodium glutamate, 5 mmol.dm^{-3} mercaptoethanol and 1 mmol.dm^{-3} EDTA). The cyanobacterial cells were disrupted by ultrasonication using a MSE, Soniprep 150 fitted with 19 mm, at 16 amplitude microns for 5 min. .Cell debris were removed by centrifugation at 35,000 g for 30 min. The supernatant was dialyzed overnight in buffer B (without sodium glutamate) at 4°C.

2.5.2.2. GS biosynthetic assay:

This was done by coupling the production of ADP to the oxidation of NADH. The reaction mixture contained, in a final volume of 3 cm^3, 1 cm^3 of enzyme extract, 150 µmol Tris-HCl buffer pH 7.5, 3 µmol ATP, 200 µmol NH_4Cl, 60 µmol sodium glutamate, 150 µmol $MgCl_2$, 150 µmol KCl, 0.45 µmol NADH, 0.5 µmol phosphoenol pyruvate, 20 units of lactate dehydrogenase and 8 units of pyruvate kinase. The reaction was initiated by addition of sodium glutamate and the rate of oxidation of NADH at 30°C was measured at 340 nm in a Jasco spectrophotometer (UVIDEC 610).

2.5.2.3. GS transferase activity:

This was estimated both in *in situ* and *in vitro* conditions. *In situ* assay was carried out by treating the cells with a detergent, cetyl trimethyl ammonium bromide (CTAB), which permeabilizes the cells. CTAB was added to a final concentration of

0.1% and cells were incubated for 10 minutes. Such permeabilized whole cells were used for *in situ* measurements of enzyme activity. For *in vitro* enzyme activity measurements, cell-free extracts were prepared by ultrasonication using a MSE soniprep 150 ultrasonicator followed by centrifugation.

The reaction mixture contained, in a final volume of 3 cm^3, 1 cm^3 enzyme extract, 40 µmol Tris-HCl buffer pH 7.0, 3 µmol $MnCl_2$, 20 µmol potassium arsenate, 0.4 µmol ADP (Na^+ salt), 60 µmol hydroxylamine and 30 µmol glutamine. The reaction mixture was incubated in the dark for 10 min at 30°C. The reaction was terminated by the addition of 2 cm^3 of stop mixture (4 cm^3 of 10% $FeCl_3$, 1 cm^3 of 24% TCA, 0.5 cm^3 of 6N HCl and 6.5 cm^3 of water). The absorbance was read at 540 nm after 10 min of centrifugation at 2,000 rpm. The concentration of γ-glutamylhydroxamate was estimated from a standard curve which was prepared in the range of 0 - 2 $\mu mol.cm^{-3}$.

2.5.3. Glutamate dehydrogenase activity (GDH):

This was estimated by following the oxidation of NADPH at 340 nm as described by Stewart and Rowell (1977).

The reaction mixture contained, in a final volume of 3 cm^3, 1 cm^3 of enzyme extract, 300 µmol Tris-HCl buffer pH 7.5, 0.3 µmol NADPH, 10 µmol 2-oxyglutarate, 400 µmol NH_4Cl. The cell-free extracts were prepared by ultrasonication using a MSE soniprep 150 ultrasonicator followed by centrifugation.

2.6. Transport studies:

For all transport/uptake studies radioactive chemicals were used. $^{14}CH_3NH_3^+$ was used as a probe to estimate the ammonium transport, whereas, ^{14}C-glutamine and ^{14}C-glutamate were used to determine the glutamine and glutamate transport, respectively.

2.6.1. Experimental set up:

2.6.1.1. Preparation of cyanobacterial culture:

Mid-log-phase cyanobacterial cells were harvested by centrifugation and washed twice in 10 mmol.dm^{-3} Hepes-NaOH buffer pH 7.0 or 10 mmol.dm^{-3} Tricine-NaOH buffer pH 9.0. The pellet was resuspended in the same buffer to a final density of 5 μg Chl **a**.cm^{-3}. This cyanobacterial suspension was equilibrated for 30 min at 28 ± 1°C and at a photon fluence rate of 50 μmol.m^{-2}.s^{-1}, and then used for different studies.

2.6.1.2. Preparation of oil microcentrifugation tubes:

Oil microcentrifugation tubes were prepared according to Scott & Nicholls (1980). In 1.5 cm^3 size eppendorf tubes 200 mm^3 of 15% (v/v) perchloric acid was taken. A second layer of 400 mm^3 of silicon oil MS 550/dinonylphthalate (45:55, v/v) was then added.

2.6.1.3. Composition of scintillant:

PPO (4.0 g) and POPOP (0.2 g) were dissolved in one dm^3 of sulphur free toluene. To this 500 cm^3 of Triton X-100 was added.

2.6.2. Ammonium transport:

^{14}C-labeled $CH_3NH_3^+$, an analogue of ammonium, was used to

estimate ammonium transport in cyanobacteria. To the cyanobacterial culture $^{14}CH_3NH_3^+$ was added to a final concentration of 50 $\mu mol.dm^{-3}$ (specific activity, 185 $kBq.\mu mol^{-1}$) except where indicated otherwise. At specific intervals 400 mm^3 of the sample was taken out in duplicates and placed on the top of the silicon oil/dinonylphthalate layer of the oil microcentrifugation tubes. Immediately, the cells were separated from medium by centrifugation in eppendorf centrifuge 54141 at 15,000 rpm. After centrifugation the eppendorf tubes contained 3 layers: top layer (medium), middle layer (Silicon oil/Dinonylphthalate), and bottom layer (cells in PCA). The top layer was removed and then the PCA layer containing the cells was taken out using a syringe through the Silicon oil/Dinonylphthalate layer. The sample in the syringe was then put into a scintillation vial, 5 cm^3 scintillant added, the vials counted in a Beckman LS 1801 Scintillation Spectrometer.

2.6.3. Glutamine transport:

^{14}C-glutamine was used to determine the glutamine transport. To the cyanobacterial culture, ^{14}C-glutamine (specific activity 182 $kBq.\mu mol^{-1}$) was added to a final concentration of 50 $\mu mol.dm^{-3}$. At specific intervals 400 mm^3 duplicate samples were withdrawn, subjected to oil microcentrifugation and ^{14}C level measured in the cells (bottom layer) as described above.

2.6.4. Glutamate transport:

^{14}C-glutamate was used to study the glutamate transport in cyanobacteria. ^{14}C-glutamate was added to the incubation mixture

to a final concentration of 50 $\mu mol.dm^{-3}$ (specific activity 185 $kBq.\mu mol^{-1}$). At regular time intervals 400 mm^3 cells were withdrawn, subjected to oil microcentrifugation and ^{14}C level measured in the cells (bottom layer).

2.6.5. Measurement of intracellular volume:

The internal cell volume of the cyanobacterial filaments was estimated by measuring relative distribution of ^3H-dextran and ^3H-water as described by Bakkar *et al* (1976). To the cyanobacterial filaments 1 $\mu mol.dm^{-3}$ of ^3H-dextran or tritiated water was added and 400 mm^3 cell suspension were separated after 5 minutes ^3H-dextran or 30 minutes ^3H-water incubation. Separation of the cells from bathing medium was achieved by silicon oil microcentrifugation and ^3H levels measured in the cells (bottom layer). Intracellular volume was measured based on the difference between the ^3H-water occupied volume minus ^3H-dextran occupied volume.

2.6.6.1. Estimation of transmembrane electrical potential difference:

Transmembrane electrical potential was estimated using the lipophilic cation triphenylmethyl phosphonium ($TPMP^+$), which accumulates within the cells in accordance with the Nernst equation (Lolkema *et al.*, 1982). Cell suspensions were incubated with 1 $\mu mol.dm^{-3}$ ^3H-$TPMP^+$ (specific activity 888 $kBq.\mu mol^{-1}$) for 30 min. Thereafter the cells were separated from their bathing medium using oil microcentrifugation. ^3H levels were then measured in the medium (top layer) and in the cells (bottom layer).

2.6.6.2. Calculation of $\Delta\psi$:

$\Delta\psi$ was calculated based on the $TPMP^+$ accumulation in accordance with Nernst equation (Lolkema *et al.*, 1982) which is as follows:

$$= \frac{R\ T}{z\ F} \cdot \ln \frac{(Co)}{(Ci)}$$

Where : R = universal gas constant (8.3143 $JK^1.mol^{-1}$)

T = Absolute temperature in °K (i.e. at 25°C

25 + 273.15°K = 298.15°K)

z = Algebraic valency (1 for $TPMP^+$)

F = The Faraday constant (96,500 $C.mol^{-1}$)

or

$$= 60 \log \frac{(Co)}{(Ci)} \quad mV$$

where : Co & Ci are amount of the $TPMP^+$ present outside and inside the cell.

2.6.7. Non-specific binding:

This was estimated by measuring incorporation of radioactive material ($^{14}CH_3NH_3^+$, ^{14}C-glutamine or ^{14}C-glutamate) in toluene-treated cells. 1% (v/v) toluene was added to cyanobacterial filaments and incubated for 20 minutes. After this, the cell suspension was centrifuged to remove toluene and resuspended in fresh buffer without toluene. Incorporation of the radioactive material was then measured under conditions similar to those described above.

2.7. Chemicals:

All the inorganic and organic chemicals used were 'Anlar' grade. Methylamine (MA), Glutamine, Glutamate, Methionine sulphoximine (MSX), Adenosine triphosphate (ATP), Adenosine diphosphate (ADP), Azaserine, Bis phenyloxazolyl benzene (POPOP), Bovine Serum Albumin (BSA), Carbomyl cyanide-m-chlorophenyl hydrazone (CCCP), Cycloheximide, Cetyl trimethyl ammonium bromide (CTAB), Diphenyloxazole (PPO), Hepes, Hydroxylamine, Lactate dehydrogenase (LDH), Mercaptoethanol, Nicotinamide adenine dinucleotide (NADH), Nigericin, Pyruvate kinase (PK), Potassium arsenate, Polymixin-B-sulphate, Phosphoenol pyruvate, Tricine and Tris were obtained from Sigma chemical company, USA. Silicon oil and Dinonylphthalate were obtained from Fluka AC, Buchs, Switzerland. ^{14}C-glutamine and ^{14}C-glutamate from BARC, Bombay, India and $^{14}CH_3NH_2Cl$, ^{3}H-dextran and ^{3}H-water from Amersham International Plc, Amersham, England. Acetylene and Ethylene gasses were obtained from Matheson gas products, USA and Cocktail for liquid scintillation Spectrometer from Spectrochem, India.

3. GROWTH AND METHYLAMMONIUM METABOLISM IN *ANABAENA* 7120 AND IN *NOSTOC* ANTH

3.1. INTRODUCTION:

A better understanding of nitrogen metabolism is prerequisite for enhancing the potential use of diazotrophic cyanobacteria as biofertilizer. Ammonium transport mechanism is one of the crucial aspects which needs a detailed study. This is because ammonium is the first product of N_2-fixation and it exerts regulatory effect on various processes of N-metabolism in diazotrophic cyanobacteria. For example, ammonium acts as repressor of nitrogenase synthesis, heterocyst differentiation and uptake and utilization of other nitrogenous compounds (Stewart, 1980; Whitton and Carr, 1982; Gibson, 1984; Bagchi *et al.*, 1985a; 1985b; Mackerras and Smith, 1986).

Occurrence of ammonium transport system (ATS) is well known in various prokaryotes (Kleiner, 1985a). Characterization of this system in cyanobacteria started in 1984 by Rai *et al* and considerable work has been done in this regard since then (Boussiba *et al*, 1984a; Kashyap and Johar, 1984a; Singh *et al.*, 1985b; 1986; 1987; Rai *et al.*, 1986a; Rai and Prakasham, 1989). Most of this work has been done using radioactive analogue of ammonium $^{14}CH_3NH_3^+$, as probe. $CH_3NH_3^+$ is a growth inhibitor of various organisms (Singh *et al.*, 1983a) however, it can be utilized as carbon and/or nitrogen source by some prokaryotes depending on the growth conditions (Holtel and Kleiner, 1985). Therefore, before

using it to characterize ATS in cyanobacteria, the effect of $CH_3NH_3^+$ on growth and the fate of intracellular $CH_3NH_3^+$ was studied, in the two strains of diazotrophic cyanobacteria--*Anabaena* 7120 (a free living form) and *Nostoc* ANTH (a cultured isolate from *Anthoceros punctatus*).

3.2. MATERIALS AND METHODS:

3.2.1. Organisms and growth conditions:

Axenic cultures of *Anabaena* 7120 and *Nostoc* ANTH were grown in $BG\text{-}11_0$ medium (Rippka *et al.*, 1979) at 28 ± 1°C, and at a photon fluence rate of 50 $\mu mol.m^{-2}.s^{-1}$. The growth medium was supplemented with 5 $mmol.dm^{-3}$ $CH_3NH_3^+$ and/or 10 $\mu mol.dm^{-3}$ dichlorophenyldimethylurea (DCMU), as and when required, and the medium buffered with hydroxymethylpiperazine ethane sulphonic acid (HEPES) pH 7.5 (10 $mmol.dm^{-3}$) before autoclaving.

3.2.2. Chlorophyll estimation:

The concentration of Chl **a** was estimated according to Mackinney (1941).

3.2.3. 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).

3.2.4. Calculation of heterocyst frequency:

This was calculated as percentage of total cells, by light microscope observation of the filaments.

3.2.5. Measurement of nitrogenase activity:

Nitrogenase activity was measured using acetylene reduction technique (Stewart *et al.*, 1967).

3.2.6. Extraction and estimation of amino acid pools:

$^{14}CH_3NH_3^+$ was added to a final concentration of 50 $\mu mol.dm^{-3}$ (Specific activity 185 $kBq.\mu mol^{-1}$), into log phase cultures of *Anabaena* 7120 and *Nostoc* ANTH. The $^{14}CH_3NH_3^+$ incubated cyanobacterial cells were centrifuged and resuspended in ethanol/water mixture (70/30; v/v) followed by incubation for 6 h at 4°C in the dark. Then the filtrate was subjected to rotary evaporation at 35°C using rotary vacuum evaporator (Rikakikai N-1; Tokyo). The residue was then dissolved in 2 cm^3 of 0.2 $mol.dm^{-3}$ sodium citrate buffer pH 2.2. The amino acids, in the extracts, were detected/separated using acidic cation exchange column (Shimapac ISC-07/S1504) and sodium citrate buffer system in a High Pressure Liquid Chromatograph (Shimadzu LC-4A). The fractions were collected at every min from the outlet of the column and ^{14}C-incorporation in each fraction/amino acid was measured by Liquid Scintillation

Spectrometer LS 1801 (Beckman).

3.2.7. Chemicals:

$^{14}CH_3NH_2Cl$ was obtained from Amersham International Plc, Amersham, U.K. All other chemicals used were purchased from Sigma Chemical Company, U.S.A.

3.3. RESULTS:

3.3.1. Growth of *Anabaena* 7120 and *Nostoc* ANTH in N_2- and $CH_3NH_3^+$ supplemented medium:

Table 3.1 shows the growth, heterocyst frequency and nitrogenase activity in *Anabaena* 7120 and *Nostoc* ANTH grown in the presence and absence of $CH_3NH_3^+$. Both organisms grew well at the expense of N_2 as nitrogen source (Fig 3.1 and Table 3.1). The observed specific growth rates in N_2-grown conditions, were 1.065 and 0.62 divisions per day for *Anabaena* 7120 and *Nostoc* ANTH, respectively. The data indicate that both cyanobacteria are able to utilize N_2 as nitrogen source. This conclusion was also supported by the observation that, both these organisms showed heterocyst differentiation and nitrogenase activity, in N_2-grown conditions (Table 3.1). The observed heterocyst frequencies of *Anabaena* 7120 and *Nostoc* ANTH were 5.4% and 6.5%, respectively. The corresponding nitrogenase activity in these organisms was 4.25 and 5.25 nmol C_2H_2 reduced.h^{-1}.μg^{-1} Chl **a**.

The growth pattern, however, in $CH_3NH_3^+$ supplemented medium was different in the two cyanobacteria (Fig 3.1). This medium did

Table 3.1:

Growth (specific growth rate.day^{-1}), heterocyst frequency (%) and nitrogenase activity (nmol C_2H_2 reduced.h^{-1}.μg^{-1} Chl a) in absence and presence of $CH_3NH_3^+$ and/or DCMU in *Nostoc* ANTH and *Anabaena* 7120 ($\pm$ SEM; ND = Not detectable).

Organism	$CH_3NH_3^+$	Growth		Heterocyst frequency	Nitrogenase activity
		+ DCMU	-DCMU		
Nostoc ANTH	+	0.00	0.76	0.5 $\pm$ 0.02	ND
	-	0.00	0.62	6.5 $\pm$ 0.30	5.25 $\pm$ 0.25
Anabaena 7120	+	0.00	0.00	0.00	0.00
	-	0.00	1.06	5.4 $\pm$ 0.20	4.25 $\pm$ 0.50

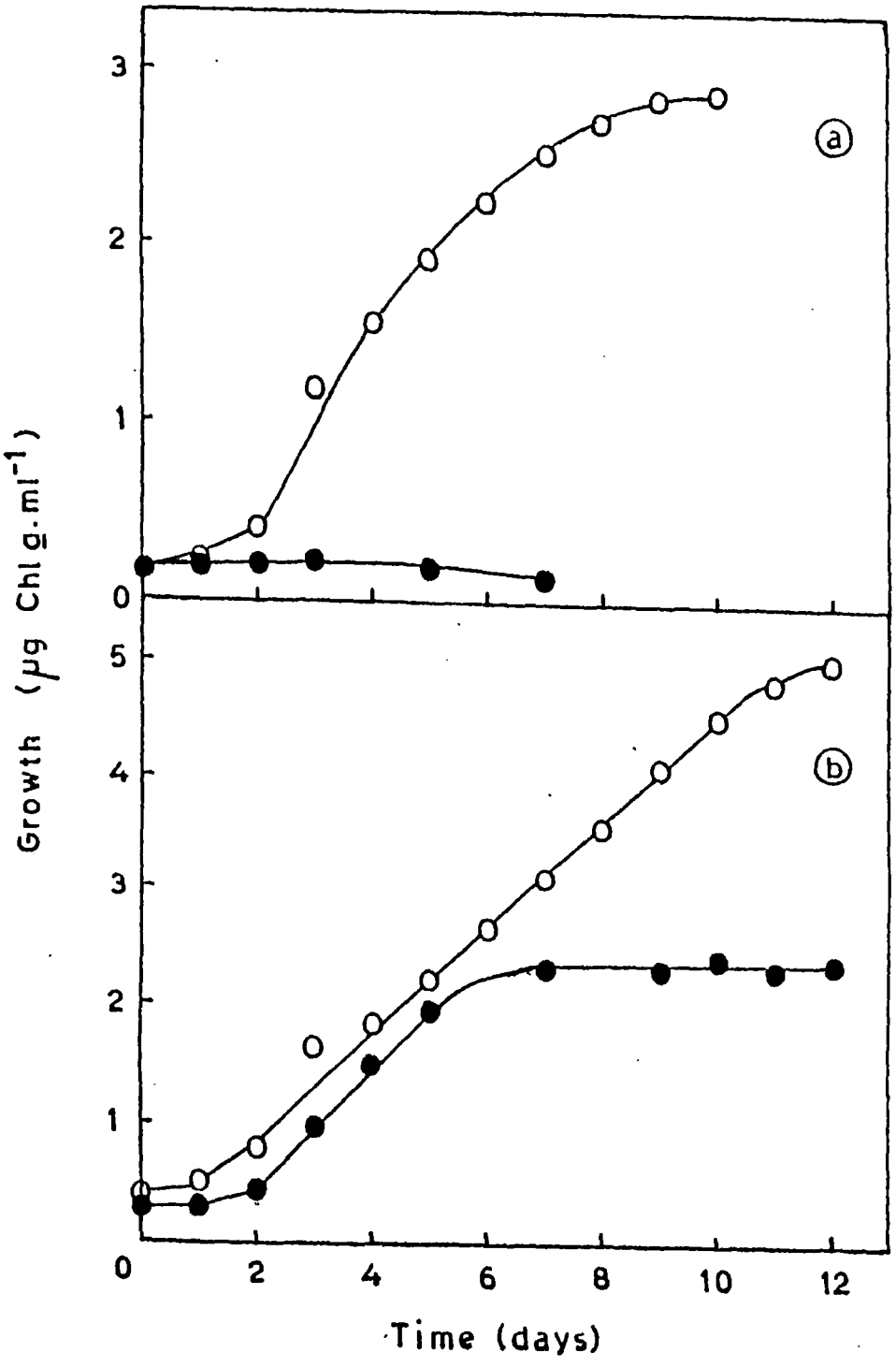

Fig 3.1 Growth of *Anabaena* 7120 (a) and *Nostoc* ANTH (b) in $BG\text{-}11_o$ medium in the presence (●) and absence (O) of 5 mmol.dm^{-3} $CH_3NH_3^+$.

not support the growth of *Anabaena* 7120. In contrast, *Nostoc* ANTH grew in $CH_3NH_3^+$ supplemented medium with a specific growth rate similar to that in control (Table 3.1) but the final growth was lower than that in the control (Fig 3.1). No nitrogenase activity was found in *Nostoc* ANTH cultures grown in $CH_3NH_3^+$ supplemented medium. The heterocyst frequency was also very low (0.5%) which may be accounted for by the presence of heterocysts in the inoculum used rather than new heterocyst differentiation. These results suggest that $CH_3NH_3^+$ was utilized as N-source by this cyanobacterium.

To know whether $CH_3NH_3^+$ can be utilized as C-source by this organism, the growth of *Nostoc* ANTH was measured in DCMU (to inhibit photosynthesis and subsequent carbon fixation) and $CH_3NH_3^+$ supplemented medium. Such medium did not support the growth of *Nostoc* ANTH (Table 3.1) indicating $CH_3NH_3^+$ is not metabolized as C-source.

3.3.2. $CH_3NH_3^+$ metabolism in *Anabaena* 7120 and *Nostoc* ANTH:

$CH_3NH_3^+$ metabolism was investigated by incubating the organism with $^{14}CH_3NH_3^+$ and at time intervals thereafter, following the ^{14}C-incorporation in ethanol soluble fractions. These fractions were analyzed for amino acids and then measured for ^{14}C-incorporation. First, the possible $CH_3NH_3^+$ metabolism in *Anabaena* 7120 was examined by measuring the ^{14}C-incorporation in amino acids in cells incubated with $^{14}CH_3NH_3^+$ upto 1 hour. In such samples radioactivity was detected solely in an amino acid peak eluted near glutamine peak, in addition to the methylamine peak (Fig 3.2a; data for only 1 hour $^{14}CH_3NH_3^+$ incubated cells is

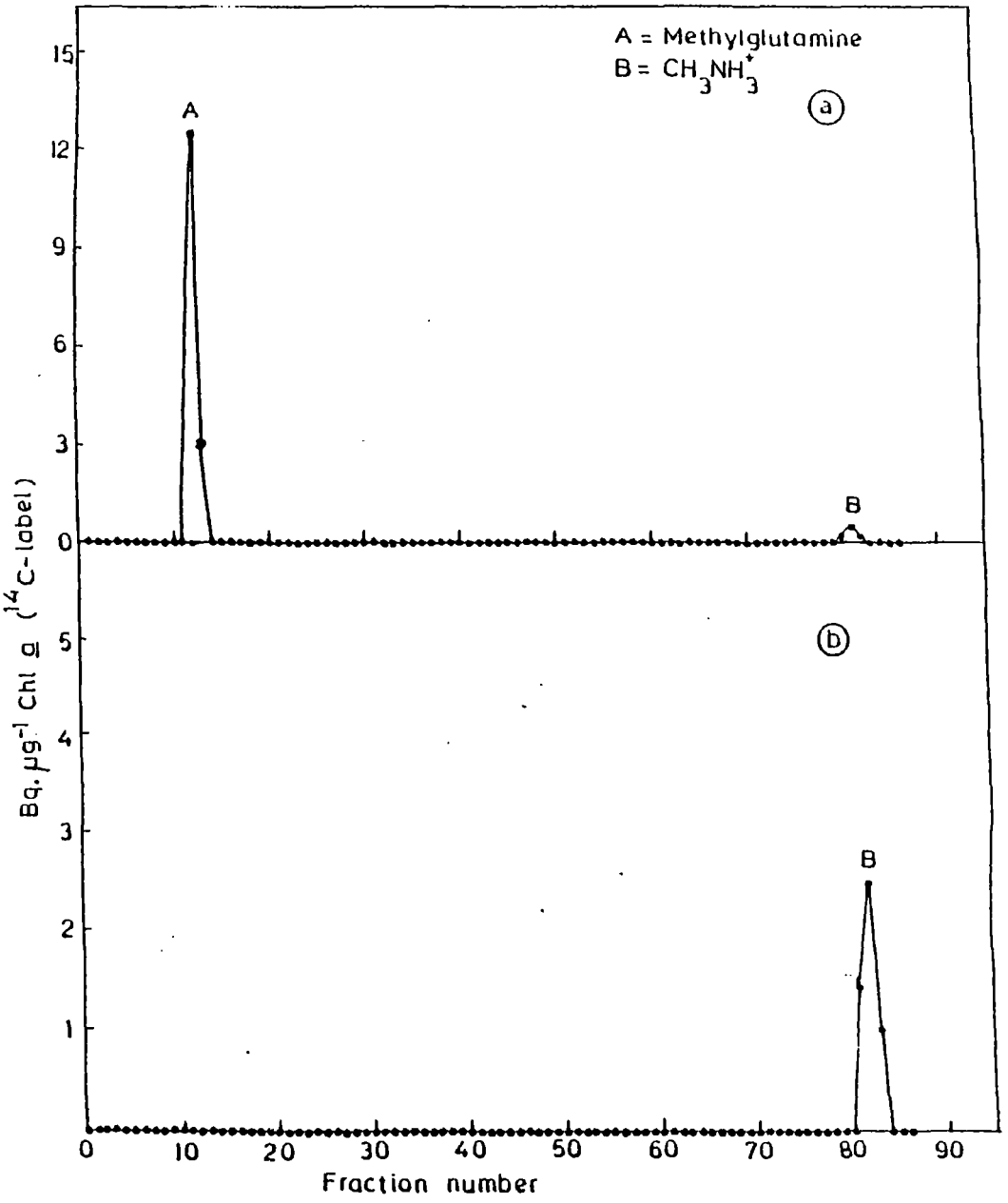

Fig 3.2 $^{14}CH_3NH_3^+$ metabolism by *Anabaena* 7120. Exponentially growing filaments (in this and next two experiments) were harvested and resuspended in HEPES pH 7 (10 mmol.dm^{-3}) buffer. $^{14}CH_3NH_3^+$ was then added to a final concentration of 50 µmol.dm^{-3} and after specified incubation times the cells were harvested, washed and then extracted for amino acid analysis (see materials and methods). a) Elution profile of ^{14}C-amino acids in *Anabaena* 7120 cells incubated with $^{14}CH_3NH_3^+$ for 60 min (fractions were collected at every min during amino acid seperation by HPLC and radioactivity in the fractions measured). b) Hydrolyzed ^{14}C-amino acid peak of Fig 3.2a.

given). This amino acid when analyzed for its composition, after hydrolysis, yielded $^{14}CH_3NH_3^+$ (Fig 3.2b) and unlabelled glutamate peak (data not shown) suggesting that this compound was methylglutamine. Such results are consistent with the results observed in other cyanobacteria indicating that methylammonium was metabolized only upto methylglutamine and no further (Rai *et al.*, 1984; Kerby *et al.*, 1986; 1987; Reglinski *et al.*, 1989).

Second, the $CH_3NH_3^+$ metabolism in *Nostoc* ANTH was studied. After 10 min incubation with $^{14}CH_3NH_3^+$ *Nostoc* ANTH showed ^{14}C-incorporation in three peaks corresponding to aspartic acid, glutamate/glutamine and alanine, apart from $^{14}CH_3NH_3^+$ peak (Fig 3.3). These data are consistent with $CH_3NH_3^+$ being metabolized as N-source by this cyanobacterium. Azaserine pretreated *Nostoc* ANTH cells showed ^{14}C-incorporation only in methylglutamine (Fig 3.4) similar to that of *Anabaena* 7120 (Fig 3.2a). These results suggest that $CH_3NH_3^+$ is assimilated, in *Nostoc* ANTH, via primary ammonia assimilating pathway (glutamine synthetase-glutamate synthase: GS - GOGAT) while in *Anabaena* 7120 $CH_3NH_3^+$ is metabolized only upto methylglutamine via glutamine synthetase (GS).

3.4. DISCUSSION:

The data presented here provide the information that the two strains differ in their capacity to utilize $CH_3NH_3^+$ as N-source.

Anabaena 7120 took up $CH_3NH_3^+$ but could not metabolize it beyond methylglutamine (Fig 3.2a). This may explain why $CH_3NH_3^+$ could not be used as N-source by this cyanobacterium. Further, $CH_3NH_3^+$ inhibition of growth (Fig 3.1a) indicated that it also

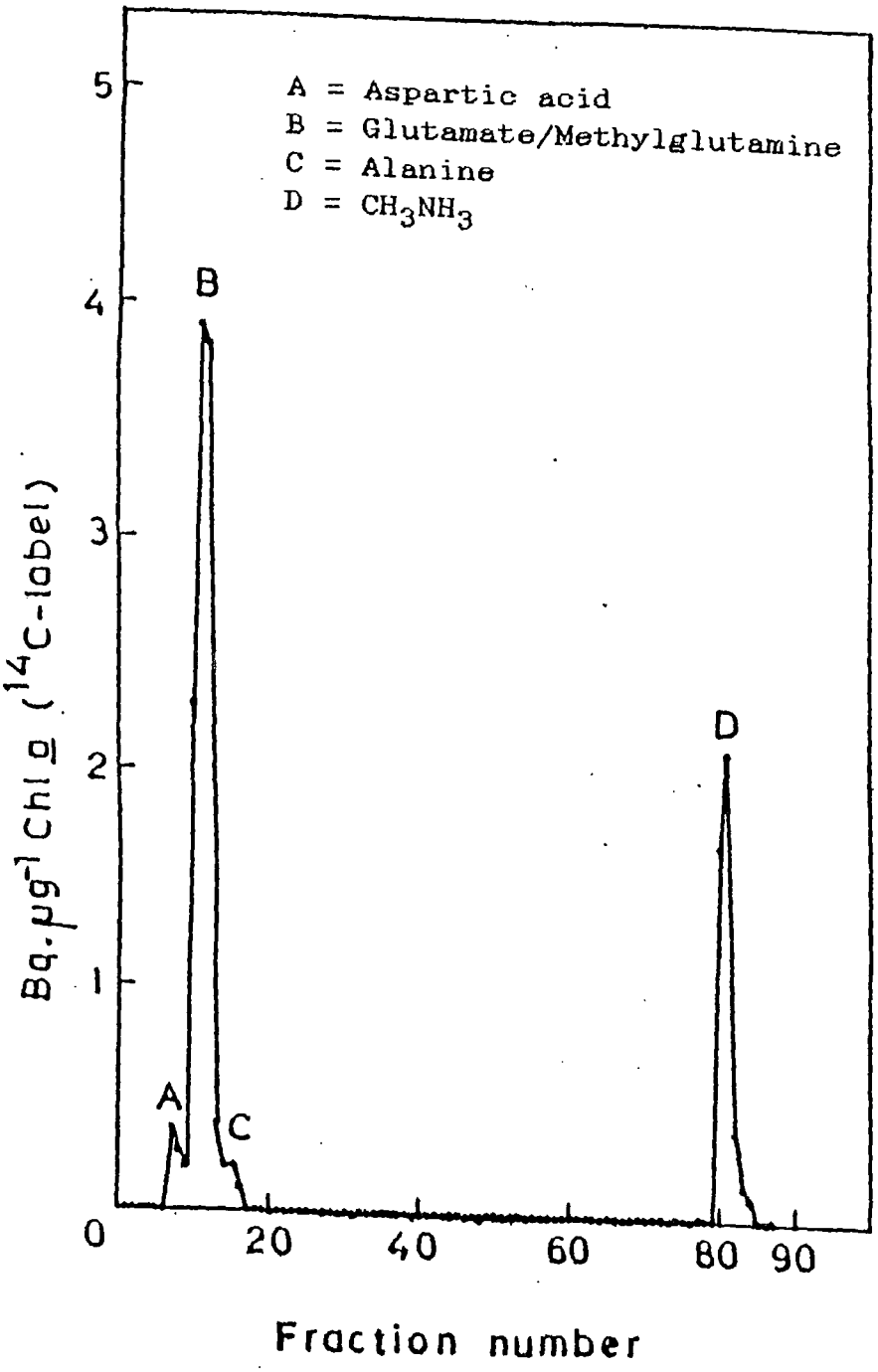

Fig 3.3 $^{14}CH_3NH_3^+$ metabolism in *Nostoc* ANTH. Elution profile of ^{14}C-amino acids (detected as in Fig 3.2) in cells incubated with $^{14}CH_3NH_3^+$ for 10 min.

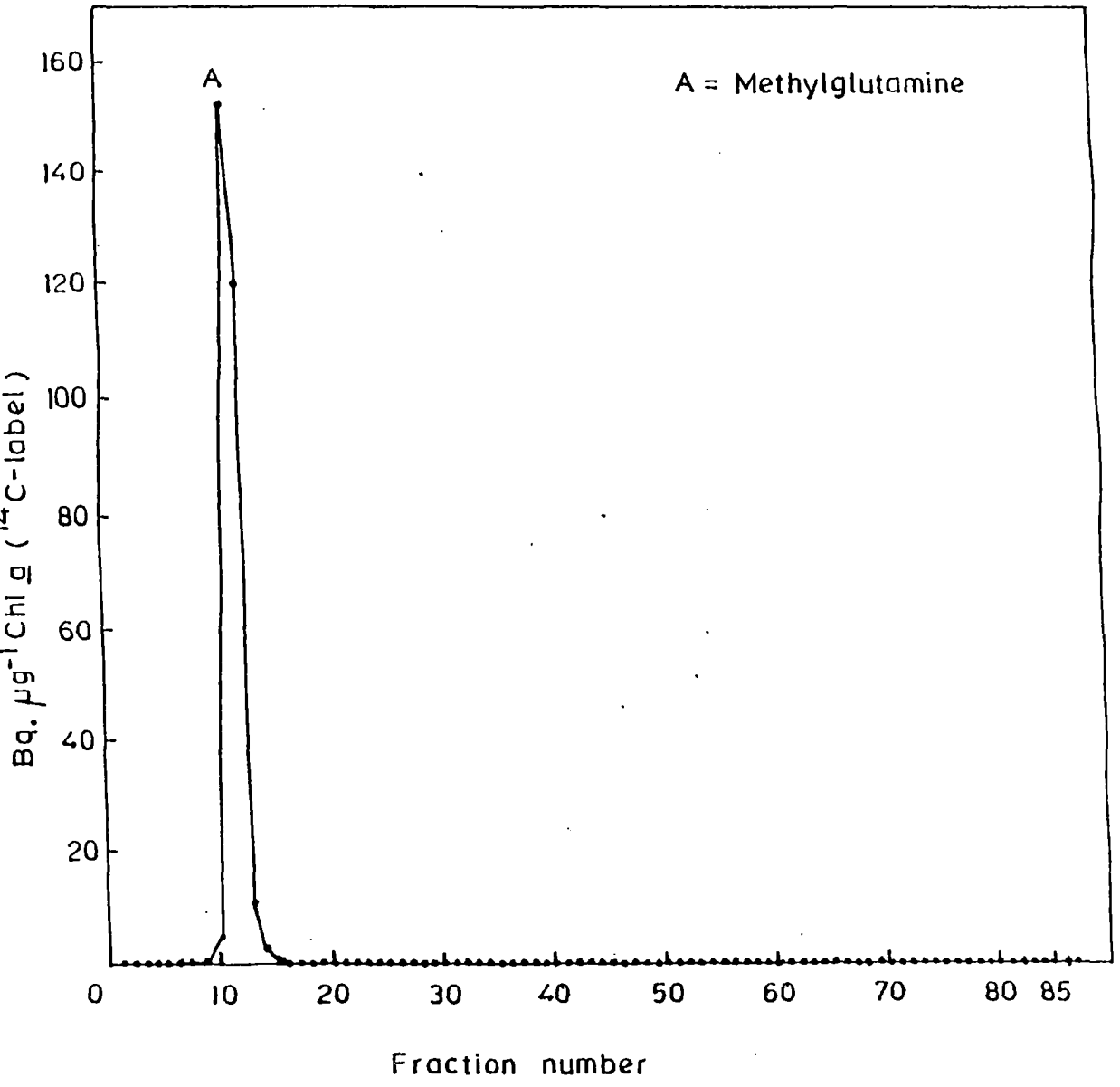

Fig 3.4 $^{14}CH_3NH_3^+$ metabolism in *Nostoc* ANTH cells pretreated with azaserine (100 µmol.dm^{-3}) for 60 min. The $^{14}CH_3NH_3^+$ incubation time was 60 min.

affected heterocyst differentiation and nitrogenase activity/ synthesis (Table 3.1) because of which organism could not use N_2 as N-source. $CH_3NH_3^+$ is indeed known to affect nitrogenase in cyanobacteria (Kerby *et al.*, 1987; Reglinski *et al.*, 1989).

Nostoc ANTH was able to metabolize CH_3NH_3 beyond methylglutamine (Fig 3.3). This explains the ability of *Nostoc* ANTH to utilize $CH_3NH_3^+$ as N-source. The growth of *Nostoc* ANTH in $CH_3NH_3^+$ medium, without having any nitrogenase activity (Table 3.1), further supports this view.

The low heterocyst frequency in $CH_3NH_3^+$-grown culture (Table 3.1) indicated repression of heterocyst differentiation by $CH_3NH_3^+$. The small number of heterocysts present may have been those present in the N_2-grown inoculum used for raising the $CH_3NH_3^+$-grown cultures. The total lack of any nitrogenase activity in $CH_3NH_3^+$-grown cultures indicated that even the small number of heterocysts which were present lacked functional nitrogenase. Thus, $CH_3NH_3^+$ was inhibitory to heterocyst differentiation as well as to nitrogenase activity/synthesis in pre-existing heterocysts. The fact that, in *Anabaena* 7120, where $CH_3NH_3^+$ was not metabolized beyond methylglutamine, nitrogenase activity and heterocyst differentiation was blocked indicated that the actual repressor of heterocyst differentiation and nitrogenase may be $CH_3NH_3^+$ or methylglutamine.

The growth pattern of *Nostoc* ANTH in $CH_3NH_3^+$ medium shows that although it was able to use $CH_3NH_3^+$ as N-source, the overall growth in the $CH_3NH_3^+$-medium was only about 50% of that in the control (Fig 3.1b). This was because in $CH_3NH_3^+$-grown *Nostoc* ANTH batch cultures, the growth paralleled to that of the control

cultures for the first 5 days only (Fig 3.1b). During this period the specific growth rate in both cultures was similar (Table 3.1). However, after 5th day the $CH_3NH_3^+$ grown cultures entered post lag phase while control cultures showed continued growth upto 10 days entering post lag phase thereafter (Fig 3.1b). It is not clear why in $CH_3NH_3^+$ medium the growth was arrested earlier than that in control. One possible explanation is that since $CH_3NH_3^+$ was used only as N-source, but not as C-source, there may be build up of the methyl-C in the cell which could not be used.

Overall, the data showed that in contrast to the *Anabaena* 7120 the *Nostoc* ANTH is capable of utilizing $CH_3NH_3^+$ as N-source. In cyanobacteria $CH_3NH_3^+$ has been shown to be an uncoupler of photophosphorylation (Singh *et al.*, 1983a). However, in *Nostoc* ANTH, this effect must be lacking as indicated by the fact that it grows in $CH_3NH_3^+$ medium. The precise mechanism by which *Nostoc* ANTH avoids the uncoupling effect of $CH_3NH_3^+$ is unknown at present.

4. AMMONIUM/METHYLAMMONIUM TRANSPORT IN *ANABAENA* 7120:

4.1. INTRODUCTION:

N_2-fixing cyanobacteria are O_2-evolving photosynthetic prokaryotes which have major application potential in agriculture and biotechnology (Stewart, 1982). Therefore, various aspects of cyanobacterial nitrogen-metabolism have attracted much research interest (Stewart, 1980; Carr and Whitton, 1982). Ammonia is a preferred source of inorganic nitrogen in such microbes and when available in excess, it represses nitrogenase (Stewart, 1980; Singh *et al.*, 1983b; Mackerras and Smith, 1986) as well as the uptake and utilization of other inorganic nitrogenous compounds (Guerrero *et al.*, 1981; Bagchi *et al.*, 1985b). Furthermore, ammonia is an obligate intermediate in N_2 and nitrate assimilation. Though much work has been done on ammonia assimilation and its impact on the regulation of nitrogen-metabolism in N_2-fixing cyanobacteria, the study of NH_4^+ transport started rather recently (Kleiner, 1985a). Detailed studies of NH_4^+ transport in N_2-fixing cyanobacteria are desirable for better understanding of the regulation of their nitrogen metabolism and for their efficient employment in agriculture or biotechnology.

At physiological pH, ammonia occurs predominantly as NH_4^+ (pKa of NH_3/NH_4^+ = 9.25) to which biological membranes show little or no permeability suggesting a need for specific NH_4^+ carrier (Henderson, 1971). Indeed, operation of a genetically controlled-

energy dependent ammonium transport system (ATS) has been shown in prokaryotes (Kleiner, 1981; 1985a; Kashyap and Singh, 1985; Singh *et al.*, 1985b; Stewart *et al.*, 1985b; Kerby *et al.*, 1986). Most studies have used the radioactive analogue of NH_4^+, $^{14}CH_3NH_3^+$, to characterize the ATS in cyanobacteria since it uses the same transport system as NH_4^+ in these organisms (Boussiba *et al.*, 1984b; Rai *et al.*, 1984; Singh *et al.*, 1985b; 1987; Kerby *et al.*, 1986; Reglinski *et al.*, 1989) and is a much more sensitive method than following NH_4^+ uptake. However, some significant differences have been noticed between $^{14}CH_3NH_3^+$ uptake and NH_4^+ uptake characteristics of the non N_2-fixing cyanobacterium *Anacystis nidulans* (Boussiba *et al.*, 1984a; 1984b). On the otherhand, a thorough understanding of ATS in cyanobacteria is unlikely to be achieved based solely on NH_4^+ uptake studies since in such studies a distinction between uptake and metabolism as well as the distinction between first and second phase of uptake, noticed in $^{14}CH_3NH_3^+$ experiments, can not be made (Boussiba *et al.*, 1984a; Kashyap and Johar, 1984a; Kashyap and Singh, 1985). A better understanding of ATS in cyanobacteria thus requires a comparative study of NH_4^+ and $^{14}CH_3NH_3^+$ transport characteristics. Such a study is lacking so far. Keeping this in view, study of $^{14}CH_3NH_3^+$ transport characteristics of *Anabaena* 7120, which has earlier been characterized with regard to its NH_4^+ uptake characteristics (Kashyap and Johar, 1984a; 1984b), was made in this chapter. The data provide significant additional information regarding ATS in *Anabaena* 7120, with relevance to ATS in other cyanobacteria. In addition, important differences in $^{14}CH_3NH_3^+$ and NH_4^+ uptake characteristics are reported and discussed.

4.2. MATERIALS AND METHODS

4.2.1. Organism and growth conditions:

Anabaena 7120 (ATCC 27893) was grown in axenic aerated batch cultures in $BG\text{-}11_0$ medium (Rippka *et al.*, 1979) at 28 ± 1°C and at a photon fluence rate of 50 $\mu mol.m^{-2}.s^{-1}$. When required, NH_4Cl or $NaNO_3$ was added to the medium to a final concentration of 1 & 5 $mmol.dm^{-3}$, respectively, and the medium buffered with 10 $mmol.dm^{-3}$ HEPES-NaOH (pH 7.5).

4.2.2. Chlorophyll and protein estimations:

Chl a and protein concentrations were determined according to Mackinney (1941) and Lowry *et al* (1951), respectively.

4.2.3. Measurement of intracellular volume and $CH_3NH_3^+$ concentration:

Internal cell volume was measured by differential distribution of ^3H-water (distributed throughout the cell) and ^3H-dextran (distributed only up to plasma membrane; does not enter the cell) (Bakker *et al.*, 1976). The difference between ^3H-water and ^3H-dextran occupied volumes gave the internal cell volume. Labeling of cells with ^3H-water and ^3H-dextran, separation of cells and scintillation counting was done as described below for $^{14}CH_3NH_3^+$. Internal $CH_3NH_3^+$ concentration was calculated by measuring intracellular free pool of $CH_3NH_3^+$ ($^{14}CH_3NH_3^+$ displaced by ammonium from preloaded cells) per unit internal cell volume.

4.2.4. Measurement of $^{14}CH_3NH_3^+$ uptake:

Exponentially growing cyanobacterial filaments were harvested by centrifugation, washed and resuspended in either 10 $mmol.dm^{-3}$ HEPES-NaOH buffer (pH 7) or 10 $mmol.dm^{-3}$ Tricine-NaOH buffer (pH 9) to a final density of 5 μg Chl $a.cm^{-3}$. The cells were then equilibrated for 30 min at 28 ± 1°C and at a photon fluence rate of 50 $\mu mol.m^{-2}.s^{-1}$. $^{14}CH_3NH_3^+$ was then added to the suspension to a final concentration of 50 $\mu mol.dm^{-3}$ (specific activity 240 $kBq.\mu mol^{-1}$), except where indicated otherwise, and at specific times cells from 400 mm^3 duplicate samples were separated from their bathing medium by centrifugation through silicon oil DC 550/dinonyl phthalate (40/60, v/v) into perchloric acid/water (15/85, v/v) (Scott and Nicholls, 1980). Samples of the medium and perchloric acid fractions were withdrawn and the ^{14}C-label determined using a Beckman LS 1801 Liquid Scintillation Spectrometer and a toluene-based scintillant.

4.2.5. Measurement of non-specific binding:

This was estimated by measuring incorporation of $^{14}CH_3NH_3^+$ into toluene treated cells. Cyanobacterial filaments were incubated for 15 min in either 10 $mmol.dm^{-3}$ HEPES-NaOH buffer (pH 7) or Tricine-NaOH buffer (pH 9) containing 1% (v/v) toluene. After toluene treatment the suspension was centrifuged, toluene removed and cells resuspended in fresh buffer without toluene. $^{14}CH_3NH_3^+$ incorporation was then measured as above under conditions similar to the respective control experiments.

4.2.6. Measurement of GS activity:

GS biosynthetic activity and transferase activity were measured in cell-free extracts following the method of Sampaio *et al* (1979).

4.2.7. Chemicals:

$^{14}CH_3NH_2Cl$, ^{3}H-dextran, ^{3}H-water were purchased from Amersham International plc, Amersham, U.K. Silicon oil DC 550 and dinonylphthalate were purchased from Fluka AC, Buchs, Switzerland. All other chemicals were from Sigma Chemical Company, St.Louis, U.S.A.

4.3. RESULTS:

4.3.1. $^{14}CH_3NH_3^+$ uptake by N_2-, NO_3^-- and NH_4^+-grown cells:

N_2-grown (air-grown) cells showed a biphasic pattern of $^{14}CH_3NH_3^+$ uptake consisting of an initial rapid phase, during the first 120 s, followed by a slower second phase which remained linear during the 12 min experimental period (Fig 4.1). These data are similar to those found in other cyanobacteria in which $^{14}CH_3NH_3^+$ transport has been studied (Boussiba *et al.*, 1984a; Rai *et al.*, 1984; Rowell *et al.*, 1985b; Singh *et al.*, 1985b; Stewart *et al.*, 1985b; Kerby *et al.*, 1986; Reglinski *et al.*, 1989). $^{14}CH_3NH_3^+$ uptake was also observed in NO_3^--grown cells with the uptake pattern and rates being similar to those in N_2-grown cells. In contrast, NH_4^+-grown cells showed no $^{14}CH_3NH_3^+$ uptake (Fig 4.2). Such observations are consistent with reports suggesting ATS

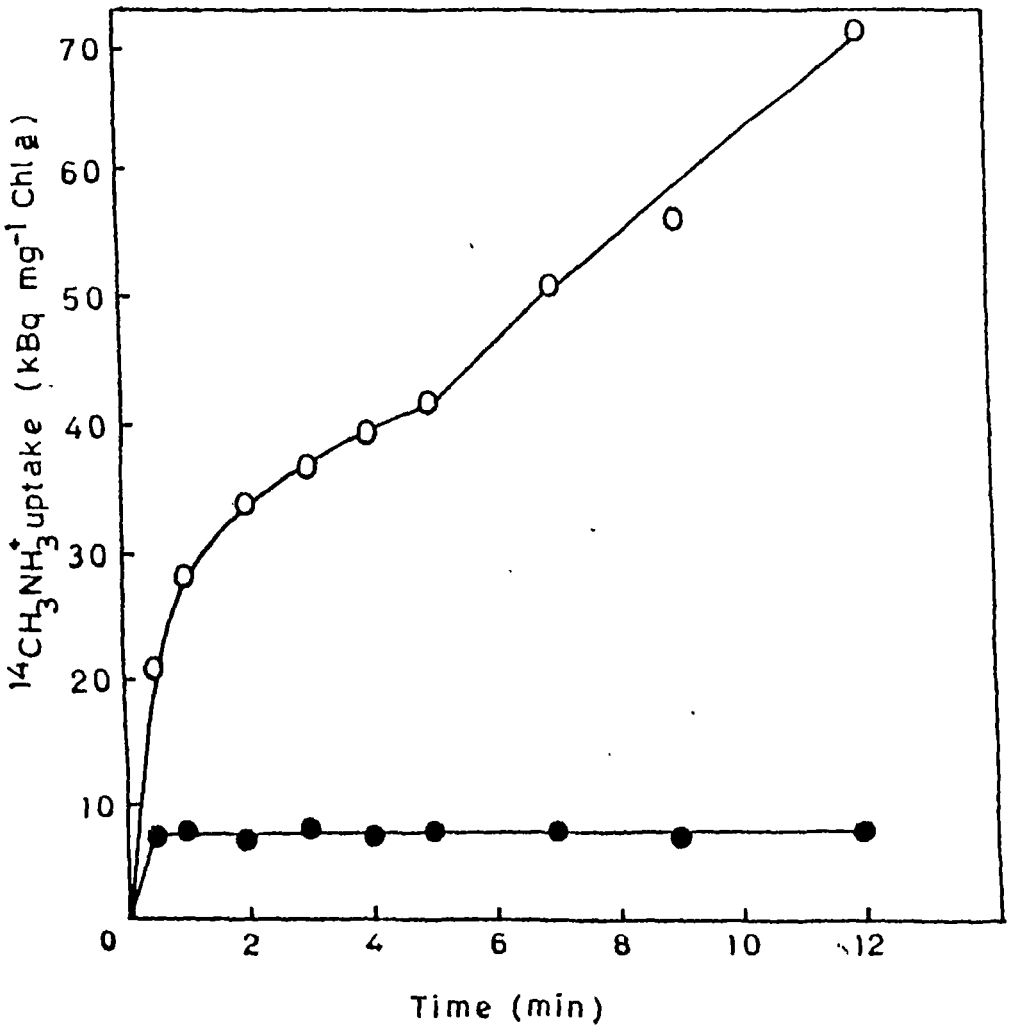

Fig 4.1 $^{14}CH_3NH_3^+$ uptake, at pH 7, by N_2-grown *Anabaena* 7120. O, control; ●, toluene treated cells. (In this and all other experiments the data are means of four replicates obtained from two repeat experiments. The variation range was between 5 - 10% from the average.

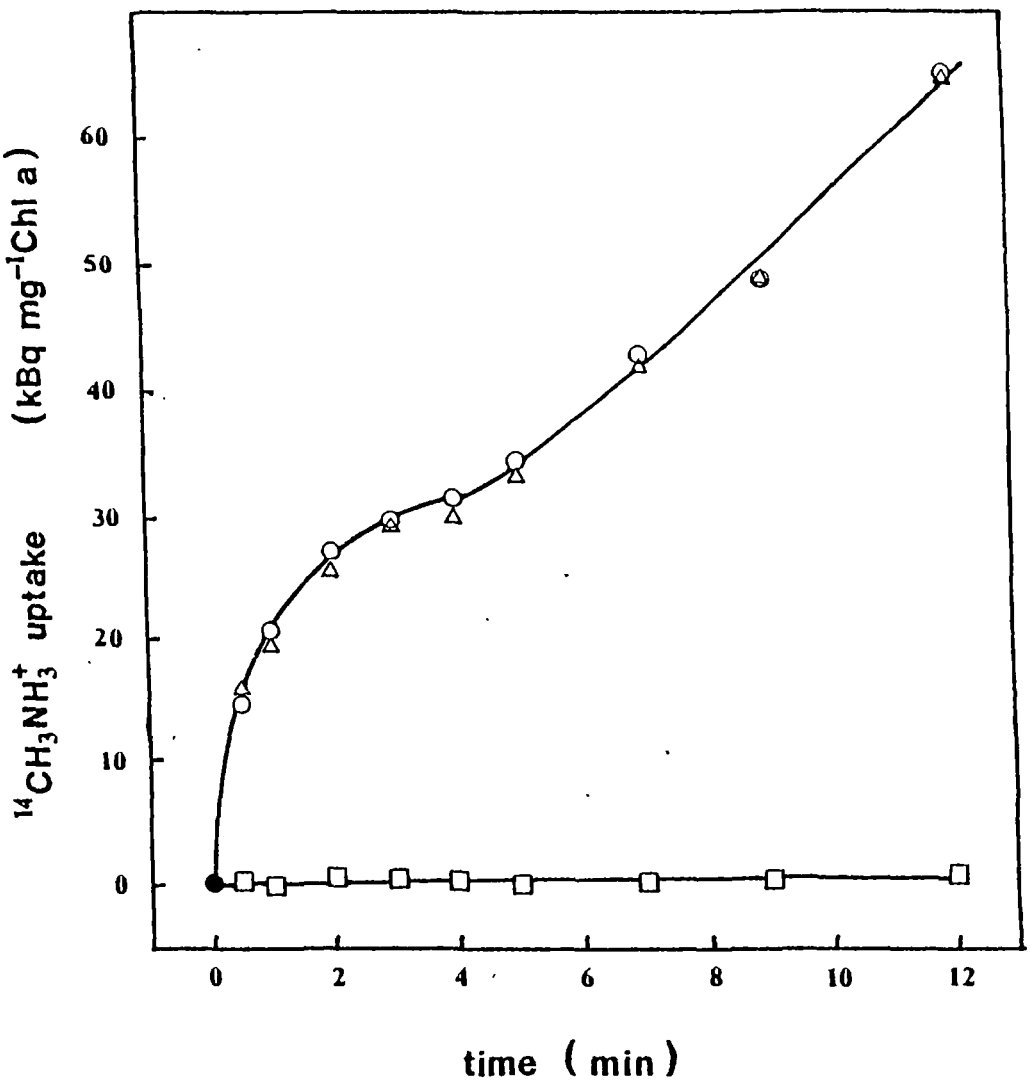

Fig 4.2 $^{14}CH_3NH_3^+$ uptake, at pH 7, by *Anabaena* 7120 filaments grown on N_2- (O), NO_3^-- (Δ) or NH_4^+- (□) medium.

repression in NH_4^+-grown bacterial and cyanobacterial cells (Kleiner, 1985a; Rai *et al.*, 1986b). In all the experiments data were corrected for nonspecific adsorption of $^{14}CH_3NH_3^+$ (see Methods and Legend to Figures). All further experiments were done on N_2-grown cells only.

4.3.2. Effect of NH_4Cl:

In various cyanobacteria, a common transport system for both $CH_3NH_3^+$ and NH_4^+ has been suggested by the observed inhibition of $CH_3NH_3^+$ uptake by ammonium (Kleiner, 1985a). Therefore, the effect of NH_4^+ addition on $^{14}CH_3NH_3^+$ uptake by *Anabaena* 7120 was investigated (Fig 4.3). Addition of 200 $\mu mol.dm^{-3}$ NH_4Cl, simultaneously with $^{14}CH_3NH_3^+$, caused a total inhibition of $^{14}CH_3NH_3^+$ uptake. When added after the addition of $^{14}CH_3NH_3^+$, it caused an efflux of the preaccumulated $^{14}CH_3NH_3^+$ from the cells into the medium. Such an effect was not observed with the addition of 5-100 $\mu mol.dm^{-3}$ NaCl (data not shown) suggesting that the effects observed with the addition of NH_4Cl were specific for NH_4^+. While the addition of NH_4Cl after 3 min of $^{14}CH_3NH_3^+$ addition resulted in efflux of most of the ^{14}C-label from the cells, with increase in time, more and more of the label remained in the cells which was not displaced by NH_4Cl; the NH_4^+-displaceable ^{14}C-label however, remained constant through out. This is not surprising since $CH_3NH_3^+$ is converted into γ-methylglutamine, by assimilation via GS (see chapter 3), which accumulates in the cells and is not displaced by ammonium (Boussiba *et al.*, 1984a; Rai *et al.*, 1984). Assuming that NH_4^+-displaceable ^{14}C-label from the cells represented the pool of free $^{14}CH_3NH_3^+$, such a pool seems to be built up

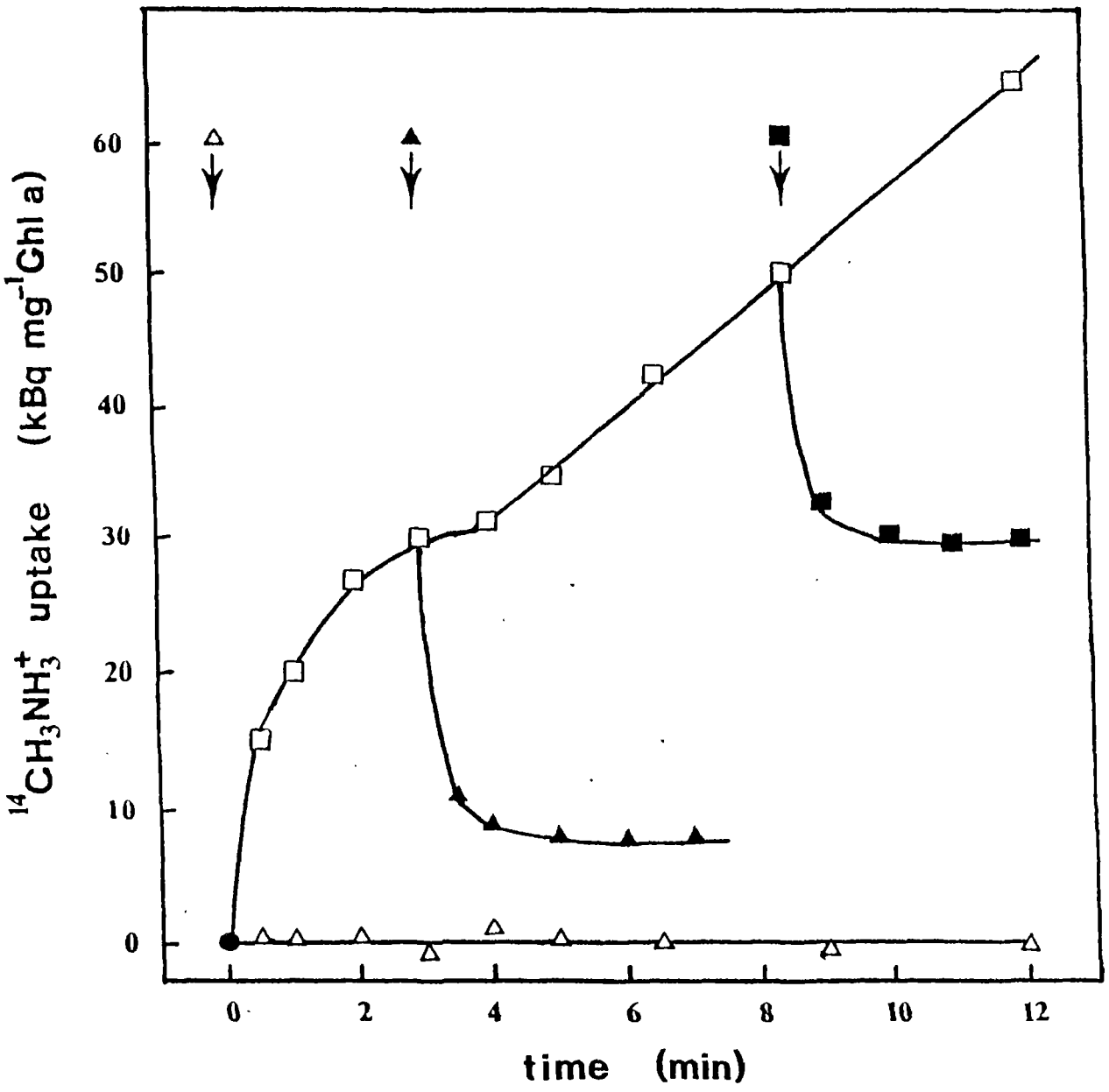

Fig 4.3 Effect of NH_4Cl on $^{14}CH_3NH_3^+$ uptake, at pH 7, by N_2-grown *Anabaena* 7120 filaments. NH_4Cl was added at times indicated (arrows) to a final concentration of 200 μmol.dm^{-3}. □, control ($^{14}CH_3NH_3^+$ only); △, NH_4^+ and $^{14}CH_3NH_3^+$ added simultaneously at zero time; ▲, NH_4^+ added 3 min after the addition of $^{14}CH_3NH_3^+$; ■, NH_4^+ added 8.5 min after the addition of $^{14}CH_3NH_3^+$.

within 120 s as indicated by the fact that a ^{14}C-label similar to that displaced by NH_4^+ was observed in cells within 120 s of $^{14}CH_3NH_3^+$ uptake (Fig 4.3). Overall, these data are consistent with observations on other cyanobacteria and suggest a common transport system for both $^{14}CH_3NH_3^+$ and NH_4^+ (see also Kleiner, 1985a).

4.3.3. Effect of external $CH_3NH_3^+$ concentration on the intracellular free $^{14}CH_3NH_3^+$ pool:

The level of intracellular $^{14}CH_3NH_3^+$ pool varied with increase in the external $^{14}CH_3NH_3^+$ concentration and showed a distinct biphasic pattern (Fig 4.4), a trend similar to that observed for ammonium (Kashyap and Johar, 1984a). At the external concentration range of 1 - 50 $\mu mol.dm^{-3}$, the maximum internal $^{14}CH_3NH_3^+$ concentration (2.5 $mmol.dm^{-3}$) was reached at 30 $\mu mol.dm^{-3}$ external concentration and remained constant thereafter upto 50 $\mu mol.dm^{-3}$ external concentration. However, further increase in external concentration resulted in an increase of internal $^{14}CH_3NH_3^+$ pool reaching a maximum intracellular concentration of 7.5 $mmol.dm^{-3}$ at 400 $\mu mol.dm^{-3}$ external concentration and remaining constant thereafter. This is probably the highest concentration of intracellular $CH_3NH_3^+$ which the transport system can maintain. An accumulation pattern similar to the one reported here was observed earlier in *Anabaena variabilis* at the external concentration range of 1 - 50 $\mu mol.dm^{-3}$ (Rai *et al.*, 1984). However, the capacity of the transport system in *A. variabilis* was probably underestimated since external concentrations beyond 50 $\mu mol.dm^{-3}$ were not investigated.

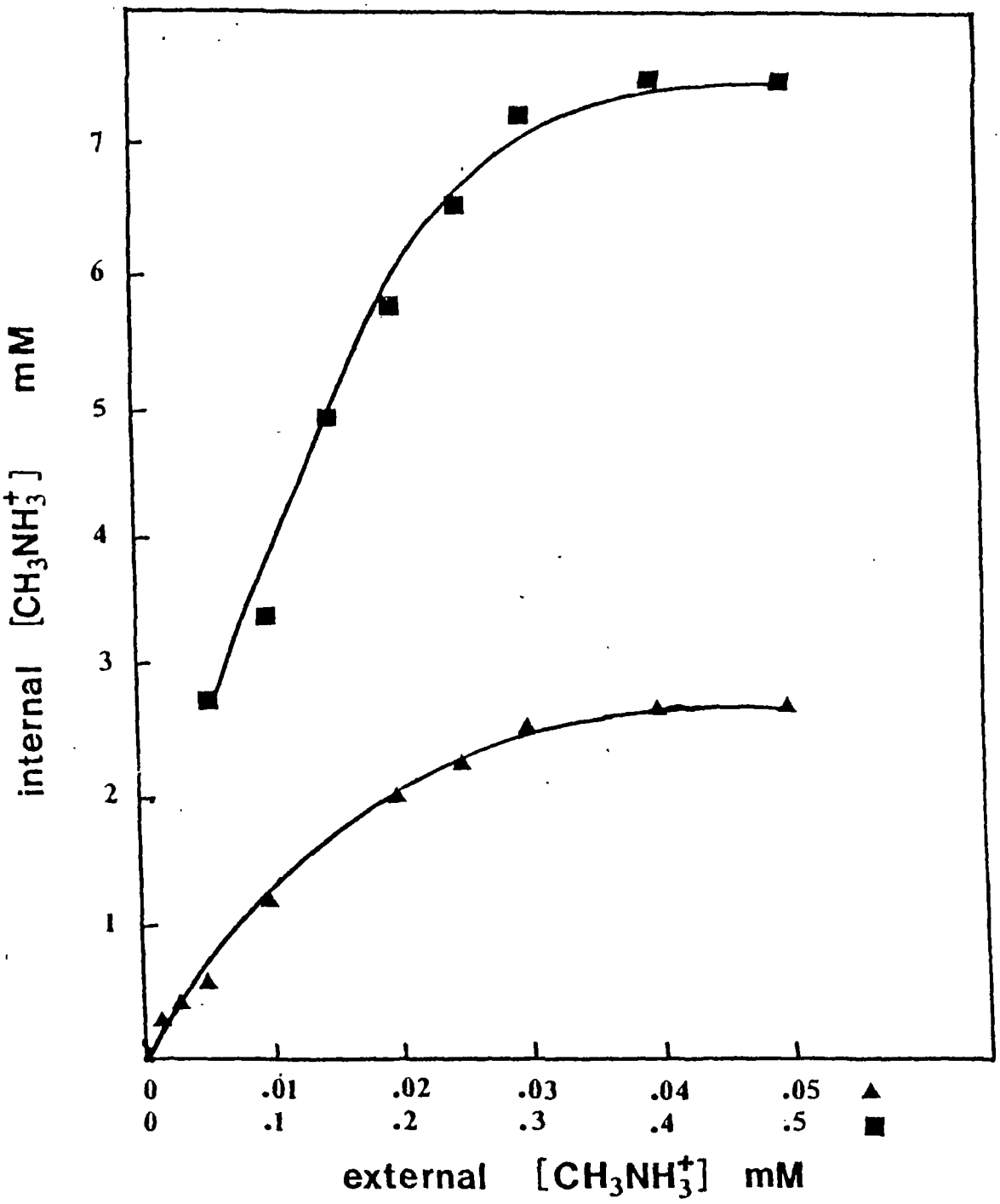

Fig 4.4 Effect of external $^{14}CH_3NH_3^+$ concentration on $^{14}CH_3NH_3^+$ accumulation, at pH 7, by N_2-grown *Anabaena* 7120 filaments. Internal $^{14}CH_3NH_3^+$ pools were calculated from NH_4^+-displaced ^{14}C label from $^{14}CH_3NH_3^+$-preloaded cells at various external $^{14}CH_3NH_3^+$ concentrations (▲, 1 - 50 $\mu mol.dm^{-3}$; ■, 50 - 500 $\mu mol.dm^{-3}$).

4.3.4. Effect of pH, $TPMP^+$ and CCCP:

At pH 7, CCCP (an uncoupler) and $TPMP^+$ (a lipophilic cation which collapses, $\Delta\psi$) caused a total inhibition of $^{14}CH_3NH_3^+$ uptake (Fig 4.5a). This suggested that at pH 7, the transport of $^{14}CH_3NH_3^+$ was energy dependent and driven by $\Delta\psi$, as in other prokaryotes (Kleiner, 1985a). In contrast, uptake at pH 9 showed a nearly four-fold higher rate during both phases of $^{14}CH_3NH_2$ uptake which were unaffected by CCCP and $TPMP^+$ (Fig 4.5b). This can be explained by $^{14}CH_3NH_2$ being present at a higher concentration in the external medium at pH 9 which can diffuse through the cell membrane without the need of carrier (Walker *et al.*, 1979). Carrier mediated uptake of the protonated species, at physiological pH, may thus be the limiting step in utilization of exogenous methylammonium/ammonium by cyanobacteria, as has been suggested for nitrate (Guerrero *et al.*, 1981).

4.3.5. Effect of MSX addition on $^{14}CH_3NH_3^+$:

MSX is a glutamate analogue and an irreversible inhibitor of GS, the primary ammonia assimilating enzyme in cyanobacteria (Stewart, 1980). A recent study on the GS-mutant of *A. cycadeae* suggests that the second phase of $^{14}CH_3NH_3^+$ uptake represents an MSX-sensitive ATS, quite distinct from the one responsible for the MSX-insensitive first phase (Singh *et al.*, 1985b). Unfortunately, this has not been possible to show in the case of normal cyanobacteria since, so far, the experiments have been done on MSX-preincubated cells where a distinction between the inhibitory effect of MSX at the transport level and its effect via GS-inactivation is not possible.

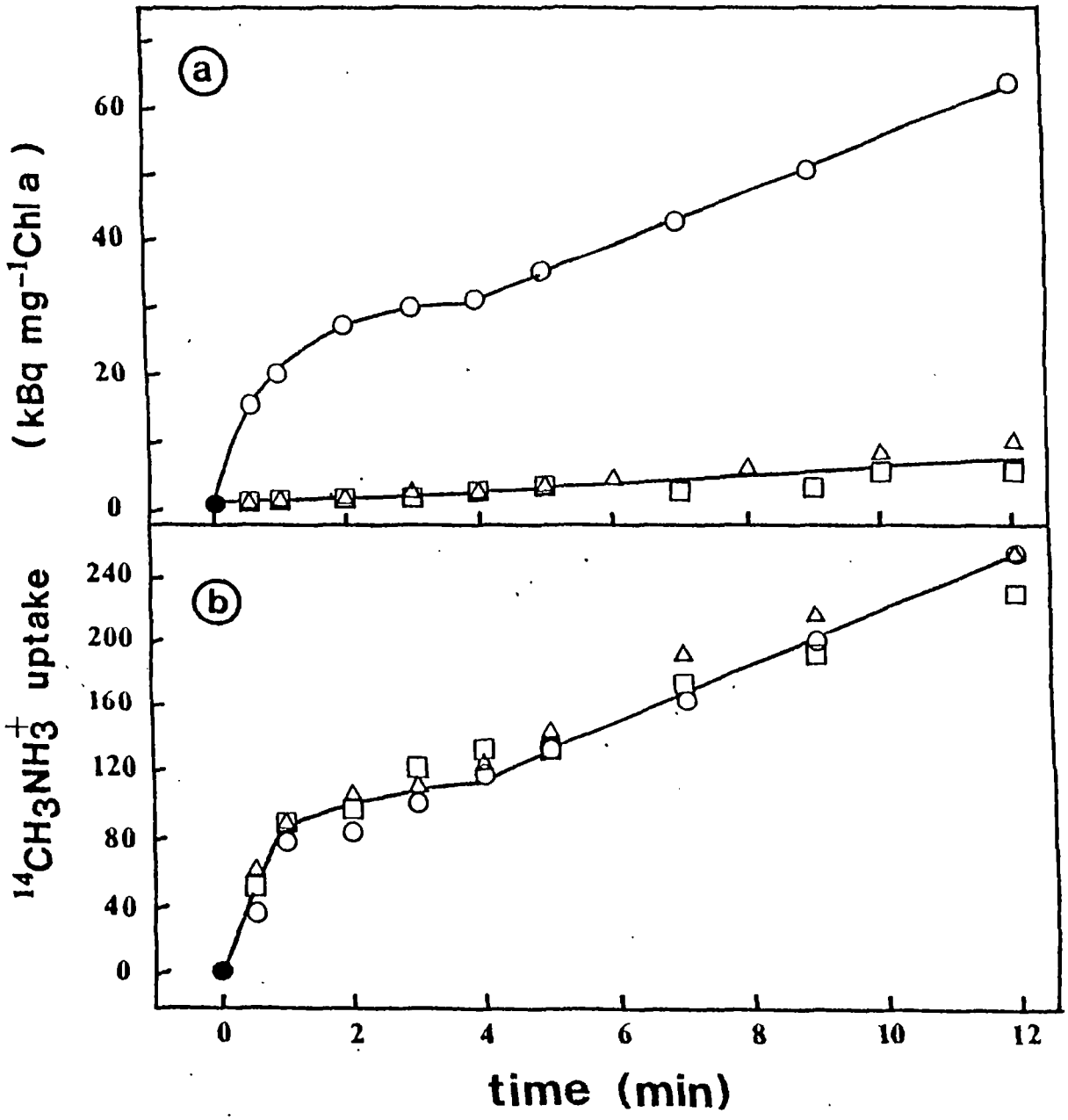

Fig 4.5 $^{14}CH_3NH_3^+/^{14}CH_3NH_2$ uptake at pH 7 (a) and 9 (b) by N_2-grown *Anabaena* 7120 filaments in the presence (△, □) or absence (○) of CCCP (△) and $TPMP^+$ (□). CCCP (10 μmol.dm^{-3}) and $TPMP^+$ (100 μmol.dm^{-3}) additions were made 30 min prior to the addition of $^{14}CH_3NH_3^+$.

Keeping this in view, the immediate effect of MSX addition on $^{14}CH_3NH_3^+$ uptake was studied in cells which have not been preincubated with MSX. Simultaneous addition of MSX and $^{14}CH_3NH_3^+$ to the cell suspension resulted in an initial $^{14}CH_3NH_3^+$ uptake phase similar to that observed in the absence of MSX but the second phase of uptake was totally inhibited by MSX (Fig 4.6). MSX additions subsequent to $^{14}CH_3NH_3^+$ additions, showed immediate and total inhibition of the second phase without any detectable ^{14}C-efflux in the short term (Fig 4.6). These immediate effects of MSX on the second phase of $^{14}CH_3NH_3^+$ uptake must be at the transport level since, at the concentrations used here (10 $\mu mol.dm^{-3}$) no effect of MSX was detectable on GS activity (Table 4.1) until after 20 min (see also Stewart and Rowell, 1975; Rai *et al.*, 1984; Kerby *et al.*, 1986). The second uptake phase also remained inhibited in cells which were preincubated with MSX for 5 min (full GS activity) and in which $^{14}CH_3NH_3^+$ uptake was studied subsequently in the absence of MSX. Such observations indicate an MSX-insensitive and an MSX-sensitive methylammonium/ammonium transport system to operate in N_2-grown *Anabaena* 7120 cells. This implies that there are two sites of MSX action, one at the transport level and another at the level of GS inactivation.

Further characteristics of these two transport systems were studied by investigating Km and Vmax for $^{14}CH_3NH_3^+$ uptake during the MSX-insensitive first phase and the MSX-sensitive second phase separately.

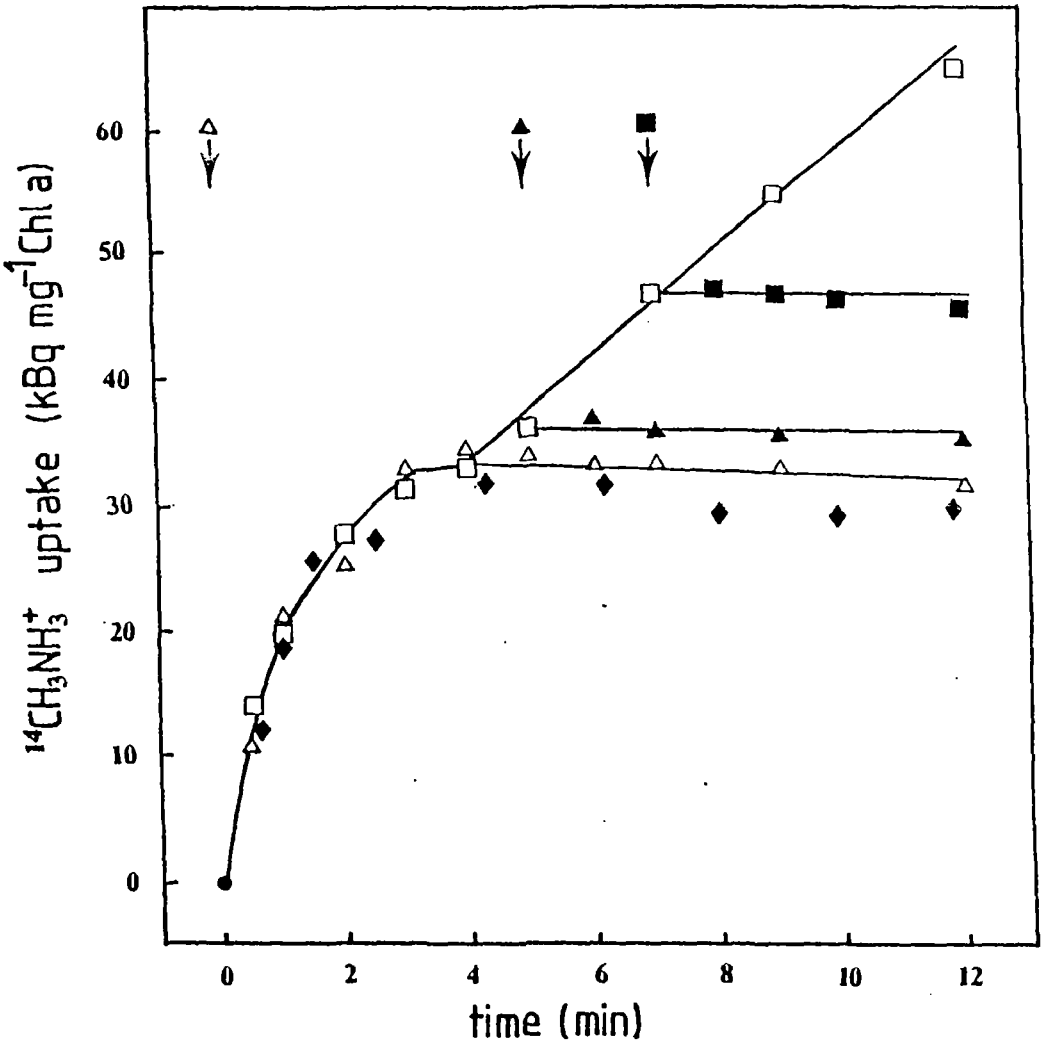

Fig4.6 Effect of MSX on $^{14}CH_3NH_3^+$ uptake, at pH 7, by N_2-grown *Anabaena* 7120 filaments. MSX was added at times indicated (arrows) to a final concentration of 10 μmol.dm^{-3}. □, control ($^{14}CH_3NH_3^+$ only); △, MSX and $^{14}CH_3NH_3^+$ added simultaneously at zero time; ▲, MSX added 5 min after the addition of $^{14}CH_3NH_3^+$; ■, MSX added 7 min after the addition of $^{14}CH_3NH_3^+$; ◆, cells preincubated with MSX for 5 min then washed and resuspended in fresh buffer and $^{14}CH_3NH_3^+$ uptake studied without MSX being present in the medium.

Table 4.1.

Effect of L-methionine-DL-sulphoximine (MSX) on glutamine synthetase (GS) activity of *Anabaena* 7120.

MSX was added to the *Anabaena* 7120 log phase cultures to a final concentration of 10 $\mu mol.dm^{-3}$. At time intervals indicated below cells were harvested, washed and GS activity measured in cell-free extracts (see Methods). The data are average of three independent assays done in duplicates (± SEM).

Time course of MSX incubation (min)	GS activity (nmol product formed.min^{-1}.mg^{-1} protein)	
	Biosynthetic	Transferase
0	45 ± 2.5	715 ± 10
10	43 ± 2.0	720 ± 08
20	46 ± 2.5	718 ± 08
30	40 ± 2.5	696 ± 10
45	33 ± 2.5	605 ± 7.5
60	24 ± 2.0	340 ± 7.5

4.3.6. Kinetics of concentration-dependent $^{14}CH_3NH_3^+$ uptake during the MSX-insensitive initial rapid phase and the subsequent MSX-sensitive slower second phase:

First, uptake rates via the MSX-insensitive ATS, at various external $CH_3NH_3^+$ concentrations (1 - 500 $\mu mol.dm^{-3}$), were studied by following $^{14}CH_3NH_3^+$ uptake at 15 s intervals during the first 2 min after the addition of $^{14}CH_3NH_3^+$ to the cell suspension at pH 7. Rates were calculated from the linear portions of the curves. A biphasic pattern of concentration-dependent increase in $^{14}CH_3NH_3^+$ uptake rates was observed with Vmax values of 1 and 7 $nmol.min^{-1}.mg^{-1}$ protein in the external concentration range of 1 - 25 $\mu mol.dm^{-3}$ and 25 - 500 $\mu mol.dm^{-3}$, respectively (Fig 4.7a; data beyond 400 $\mu mol.dm^{-3}$ not shown). The corresponding Km values, calculated from Lineweaver-Burk plots (Lineweaver and Burk, 1934), were 8 and 80 $\mu mol.dm^{-3}$, respectively (Fig 4.7b and 4.7c).

Next, $^{14}CH_3NH_3^+$ uptake rates via the MSX-sensitive ATS, was studied, at $CH_3NH_3^+$ concentration range and conditions similar to those above. The rates were calculated from the linear second phase of $^{14}CH_3NH_3^+$ uptake (between 5 and 12 min after the addition of $^{14}CH_3NH_3^+$ to the cell suspension). The pattern of concentration-dependent increase in $^{14}CH_3NH_3^+$ uptake rates via the MSX-sensitive ATS (Fig 4.8) was similar to the one via MSX-insensitive ATS (Fig 4.7). The observed Km values were 2.5 and 70 $\mu mol.dm^{-3}$ in the 1 - 10 and 10 - 500 $\mu mol.dm^{-3}$ external $^{14}CH_3NH_3^+$ concentration range, respectively (data beyond 200 $\mu mol.dm^{-3}$ external concentration is not shown). The corresponding Vmax values were 0.1 and 0.7 $nmol.min^{-1}.mg^{-1}$ protein, respectively (Fig 4.8b and 4.8c).

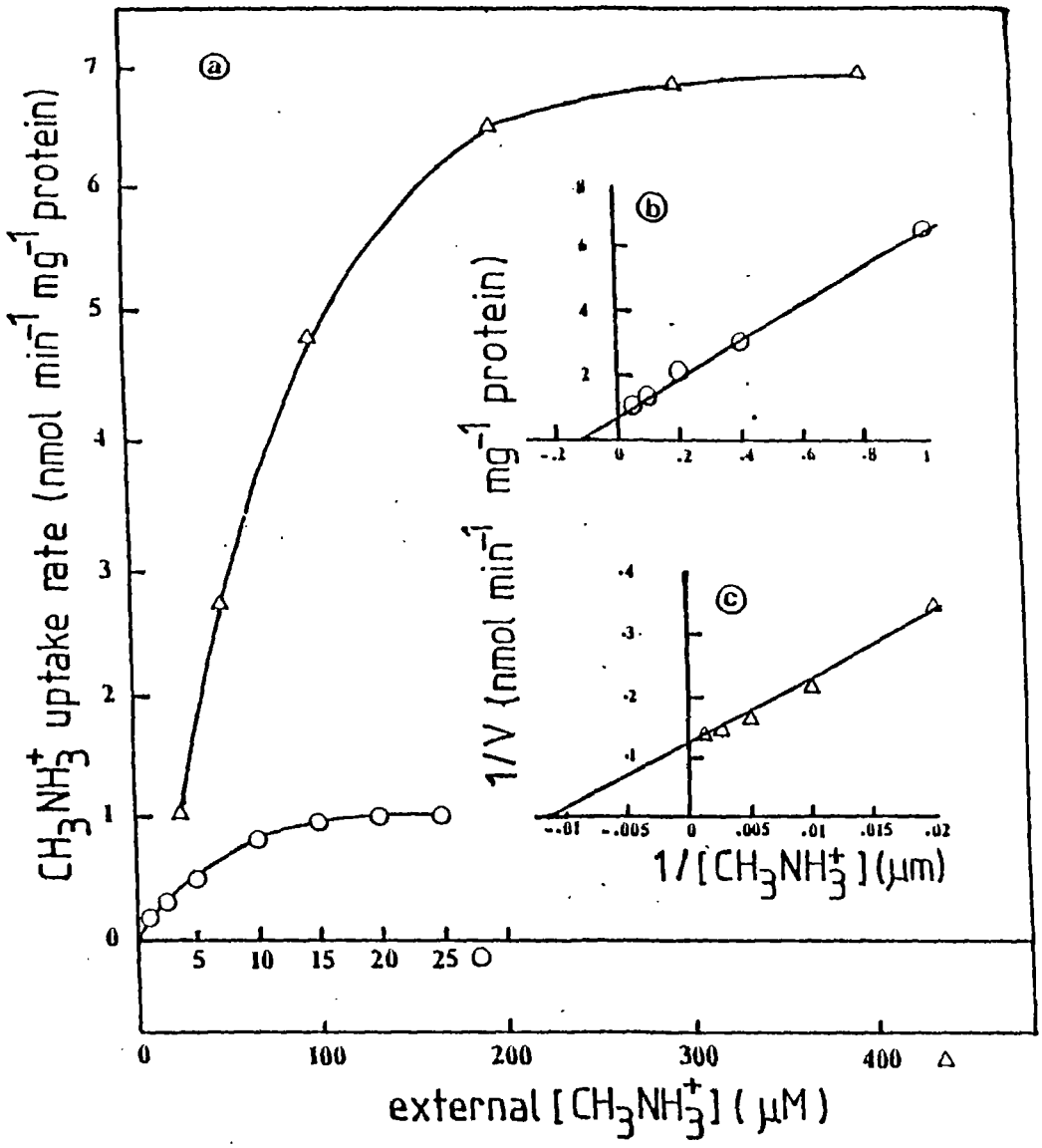

Fig 4.7 a) Concentration dependent $^{14}CH_3NH_3^+$ uptake rates, at pH 7, during the initial MSX-insensitive rapid phase, by N_2-grown *Anabaena* 7120 filaments showing isotherm Michaelis-Menten kinetics. O, uptake rates at external concentration range of 1 - 25 μmol.dm^{-3} $^{14}CH_3NH_3^+$; Δ, uptake rates at external concentration range of 25 - 400 μmol.dm^{-3} $^{14}CH_3NH_3^+$.

b and c) Lineweaver-Burk plots for $^{14}CH_3NH_3^+$ uptake during high affinity mode (1 - 25 μmol.dm^{-3} external $^{14}CH_3NH_3^+$ concentration, O) and low affinity mode (25 - 400 μmol.dm^{-3} external $^{14}CH_3NH_3^+$ concentration, Δ). Calculated from Fig 7a as before (Lineweaver and Burk, 1934).

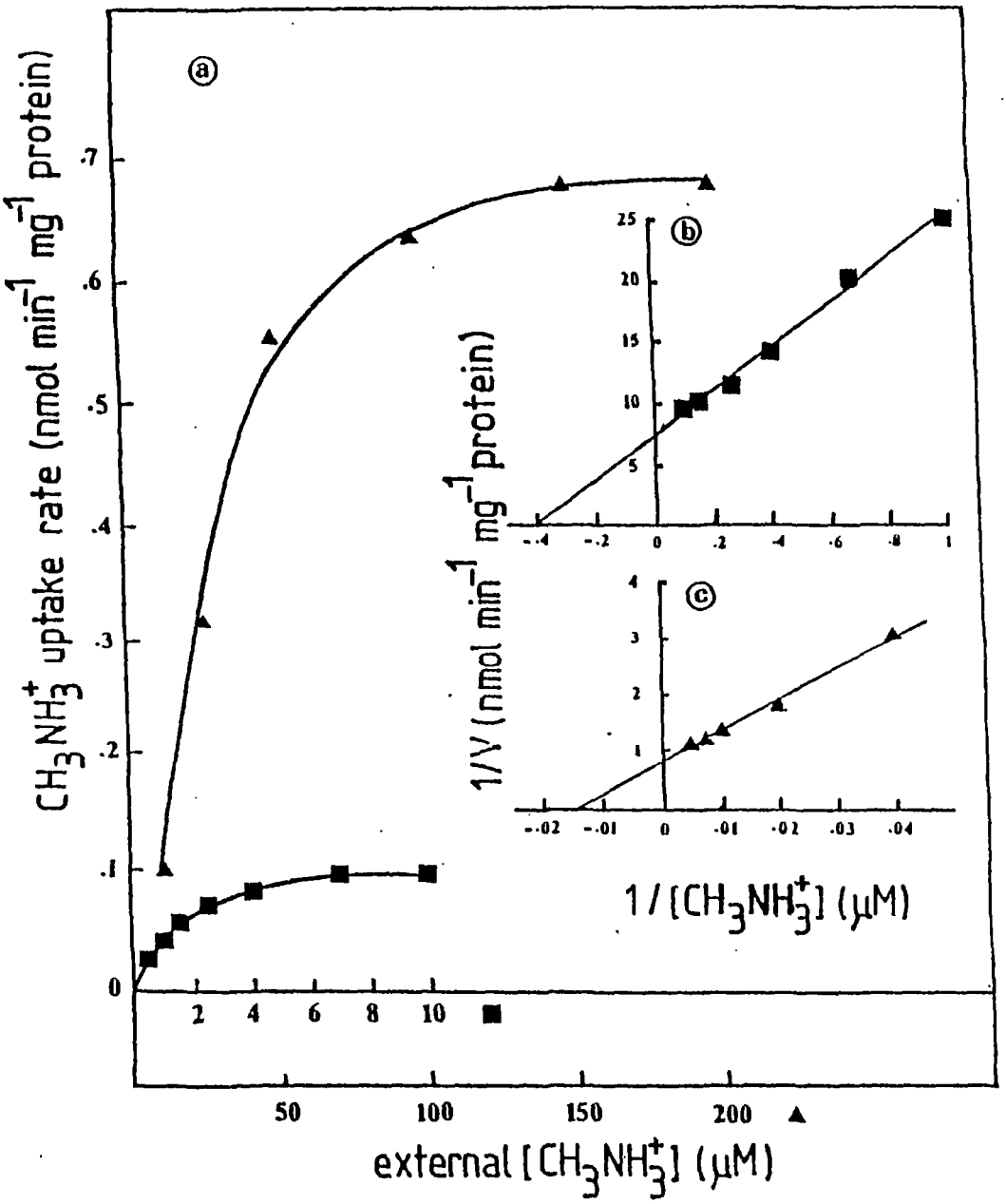

Fig 4.8a) Concentration dependent $^{14}CH_3NH_3^+$ uptake rates, at pH 7, during the subsequent MSX-sensitive slower phase, by N_2-grown *Anabaena* 7120 filaments showing dual isotherm Michaelis-Menten kinetics. ■, uptake rates at external concentration range of 1 - 10 μmol.dm^{-3} $^{14}CH_3NH_3^+$; ▲, uptake rates at external concentration range of 10 - 200 μmol.dm^{-3} $^{14}CH_3NH_3^+$.

b and c) Lineweaver-Burk plots for $^{14}CH_3NH_3^+$ uptake during high affinity mode (1 - 10 μmol.dm^{-3} external $^{14}CH_3NH_3^+$ concentration, ■) and low affinity mode (10 - 200 μmol.dm^{-3} external $^{14}CH_3NH_3^+$ concentration, ▲). Calculated from Fig 8a as before (Lineweaver and Burk, 1934).

Thus, both transport systems showed dual operation mode: a high affinity mode at low substrate concentrations and a low affinity mode at high substrate concentrations. During both these modes, the maximal rate of transport via the MSX-insensitive ATS was ten-fold higher than that via the MSX-sensitive ATS. The maximal rate of transport via each transport system was seven-fold higher during the low affinity mode as compared to that during the high affinity mode.

4.4. DISCUSSION:

The $^{14}CH_3NH_3^+$ studies presented here provide evidence for operation of two ATS in *Anabaena* 7120. An initial rapid phase and a slower second phase of $^{14}CH_3NH_3^+$ uptake was detectable in N_2- and NO_3^--grown cells (Fig 4.2). The first phase represented a $\Delta\psi$-dependent ATS as evidenced from the facts that *Anabaena* 7120 accumulated $^{14}CH_3NH_3^+$ against a concentration gradient, that NH_4^+ inhibited $^{14}CH_3NH_3^+$ accumulation, that accumulated $^{14}CH_3NH_3^+$ was released from the cells by subsequent addition of NH_4^+, and that $^{14}CH_3NH_3^+$ accumulation was inhibited by CCCP and $TPMP^+$. Since the first phase of $^{14}CH_3NH_3^+$ uptake was not inhibited by MSX it was termed as MSX-insensitive. Such findings are in keeping with the earlier reports on bacteria (Kleiner, 1985a) and cyanobacteria (Boussiba *et al.*, 1984a; Rai *et al.*, 1984; Kerby *et al.*, 1986).

In addition to the above mentioned MSX-insensitive ATS responsible for $^{14}CH_3NH_3^+$ uptake during the first phase, existence of an MSX-sensitive $\Delta\psi$-dependent ATS responsible for $^{14}CH_3NH_3^+$ uptake during the second phase has been shown. Evidence for this

comes from the fact that there was an immediate inhibition of the second phase of $^{14}CH_3NH_3^+$ uptake on addition of MSX, and that NH_4^+, CCCP and $TPMP^+$ inhibited the second phase of uptake as in the case of the first phase. The effect of MSX on $^{14}CH_3NH_3^+$ uptake during the second phase was concluded to be on transport level because no GS inhibition by MSX was detectable before 20 min (see also, for other cyanobacteria Rai *et al.*, 1984; Kerby *et al.*, 1986). while the inhibition of $^{14}CH_3NH_3^+$ uptake was immediate (within 1 min, the shortest time measured). Such data agree with the observations on the GS-mutant of *A. cycadeae* where an MSX-sensitive and an MSX-insensitive ATS have been reported (Singh *et al.*, 1985b). Two transport systems have also been reported for glutamine and for glutamate in cyanobacteria (Chapman and Meeks, 1983; Labarre *et al.*, 1987).

The build up of $^{14}CH_3NH_3^+$ pool during the MSX-insensitive first phase, the NH_4^+-displaceable $^{14}CH_3NH_3^+$ being constant throughout the second phase, and the absence of the second phase in the presence of MSX while GS remains active, suggest that the MSX-insensitive first ATS plays a role in build up and maintenance of a free pool of intracellular ammonium which is not available for assimilation via GS (probably located in the thylakoids). The second ATS on the otherhand, plays a role in uptake of exogenous ammonium into a pool which is available to GS (probably located in the cytoplasm). Evidence for this comes from the fact that in the presence of MSX, no $^{14}CH_3NH_3^+$ uptake occurred beyond the first phase. If the $^{14}CH_3NH_3^+$ transported by the first ATS were available for assimilation, a continued uptake of $^{14}CH_3NH_3^+$ would be expected till the GS was inactivated; i.e., the pattern of

$^{14}CH_3NH_3^+$ uptake should not have changed on addition of MSX during the 12 min experimental period since the GS inactivation took a longer time. Such data indicate the necessity of the second ATS for utilization of exogenous $CH_3NH_3^+/NH_4^+$. Thus, the slow rate of the second phase as compared to the first, may indicate that the rate limiting step in the utilization of exogenous $CH_3NH_3^+/NH_4^+$ is the rate of transport via second ATS, rather than a limitation at the GS activity level as suggested earlier (Rai *et al.*, 1984). This is further supported by the fact that:

1. The maximum rate of $CH_3NH_3^+$ transport via the second ATS (0.7 nmol.min^{-1}.mg^{-1} protein) and the maximum rate of ammonium transport (1.25 nmol.min^{-1}.mg^{-1} protein) (Kashyap and Johar, 1984a) both are much lower than the GS activity (50 - 60 nmol product formed.min^{-1}.mg^{-1} protein);
2. At pH 9, when methylamine enters the cell at a faster rate through diffusion, there is a corresponding increase in the rate of its metabolism via GS (Kerby *et al.*, 1986); and
3. In the cyanobiont of cycad coralloid roots, absence of the second ATS results in the inability of the cyanobiont to utilize exogenous NH_4^+ despite the presence of fully active GS (Rai *et al.*, 1986a).

Additional evidence regarding control of NH_4^+ utilization at the transport level comes from the inhibition/repression of both ATS in NH_4^+-grown cells, as indicated by the absence of both the phases of $^{14}CH_3NH_3^+$ uptake in such cells (Fig 4.2), and from the observed changes in the affinity of the ATS, from high affinity to low affinity, at higher substrate concentrations (Fig 4.7 and 4.8). Similar observations have been made in bacteria where

synthesis of NH_4^+ carriers has been shown to be repressed at high NH_4^+ concentrations and under such conditions diffusible NH_3 serves the cellular requirements (Kleiner, 1985a). Since cyanobacteria generally grow at alkaline pH, sufficient NH_3 may exist, at high ammonium concentrations, to satisfy their growth requirements without the need of energy demanding transport systems.

Both ATS showed dual affinity modes with uptake rates through the first, MSX-insensitive ATS, being ten-fold faster, during both the affinity modes, than those through the second, MSX-sensitive ATS. An earlier study (Kashyap and Johar, 1984a) has shown a dual Michaelis-Menten kinetics for the concentration-dependent NH_4^+ uptake in *Anabaena* 7120. This was explained by suggesting that either there are two ATS in *Anabaena* 7120 or there is a single ATS with variable affinity. In view of our present study we suggest that in Kashyap and Johar's experiments (Kashyap and Johar, 1984a) the Michaelis-Menten kinetics observed was due to the second ATS showing dual affinity. This is because their experiments were done by following disappearance of NH_4^+ from the medium at 10 min intervals and therefore, the first ATS could not have been detected since it is detectable only during the initial 1 - 2 min. A comparision of the rates of NH_4^+ uptake (Kashyap and Johar, 1984a) and that of $CH_3NH_3^+$ (presented here) shows that Vmax values of NH_4^+ and $CH_3NH_3^+$ are 6 - 7 times higher during the low affinity mode than those during the high affinity mode, that rates of NH_4^+ uptake during both high and low affinity modes are nearly twice as fast as that of $CH_3NH_3^+$ uptake during the respective affinity modes, and that the maximum level of intracellular NH_4^+ pool is nearly double of the maximum intracellular free $^{14}CH_3NH_3^+$

pool. The differences in NH_4^+ and $CH_3NH_3^+$ uptake rates and accumulation levels may reflect the fact that NH_4^+ is the natural substrate for ATS. However, certain limitations should be pointed out: first, the intracellular NH_4^+ concentrations reported by Kashyap and Johar (1984a) may contain a significant margin of error since the intracellular volume was calculated 'assuming that aquatic prokaryotes contain about 80% water'; second, their experiments were done in $BG\text{-}11_o$ medium where the presence of various other nutrient ions may have some effect on the uptake rates.

A comparison of the intracellular free $^{14}CH_3NH_3^+$ pools of *Anabaena* 7120 with those of *A. variabilis* reveals that upto the external $CH_3NH_3^+$ concentration of 50 $\mu mol.dm^{-3}$ the trend of accumulation is similar in both cases (see Fig 4.4 and Rai *et al.*, 1984) although the level of accumulation in *Anabaena* 7120 is higher. Intracellular $CH_3NH_3^+$ pools beyond 50 $\mu mol.dm^{-3}$ external $^{14}CH_3NH_3^+$ concentrations have not been checked in the case of *A. variabilis* (Rai *et al.*, 1984) but it has been concluded that ATS of *A. variabilis* can maintain a maximum of 1.4 $mmol.dm^{-3}$ intracellular $CH_3NH_3^+$ pool. In view of the observed increase in $CH_3NH_3^+$ pools of *Anabaena* 7120 at external concentration range of 50 - 500 $\mu mol.dm^{-3}$, it is likely that the capacity of ATS in *A. variabilis* was underestimated.

Overall, our data suggest that in *Anabaena* 7120, two energy-dependent ATS exist, that one of the ATS is MSX-sensitive, and that both these ATS exhibit dual affinity modes. We suggest that regulation of ATS may be a key step in controlling rate of exogenous NH_4^+ utilization by cyanobacteria.

5. AMMONIUM/METHYLAMMONIUM TRANSPORT IN *NOSTOC* ANTH

5.1. INTRODUCTION:

N_2-fixing cyanobacteria enter into symbiotic associations with plants ranging from algae to angiosperms (see Stewart *et al.*, 1983; Smith and Douglas, 1987). In symbiosis, the cyanobiont undergoes extensive structural and metabolic alterations which lead to a higher rate of N_2-fixation and transfer of fixed-N from cyanobiont to the eukaryotic partner (Stewart *et al.*, 1983; Rai *et al.*, 1989; Rai, 1988; 1989).

Among the bryophyte-cyanobacterial symbiosis, the hornwort *Anthoceros-Nostoc* symbiosis is better characterized. N_2-fixing *Nostoc* sp. develops in cavities on the undersurface of the *Anthoceros*-gametophyte thallus. The cyanobiont shows a high heterocyst frequency, is photosynthetically inactive and liberates nearly 90% of the nitrogenase derived ammonia to the eukaryotic partner (see Stewart *et al.*, 1983; Meeks *et al.*, 1985; Rai *et al.*, 1989). This liberation of ammonia by the cyanobiont has been suggested to be due to low GS activity (Stewart *et al.*, 1983; Meeks *et al.*, 1985).

The cyanobiont of *Anthoceros punctatus* was isolated and cultured in free-living state. It showed a marked difference in methylammonium metabolism compared to other cyanobacteria (see chapter 3). In the present chapter, characteristics of ATS in the cultured (free-living) cyanobiont of *A. punctatus* (*Nostoc* ANTH) was investigated to see if ATS in this cyanobacteria has any

unique features. A study of ATS in the cyanobiont of *A. punctatus*, directly isolated from the thallus, could not be done because of the problem of isolating enough clean cells from the thallus.

5.2. MATERIALS AND METHODS:

5.2.1. Organism and growth conditions:

Nostoc ANTH, an isolate from *A. punctatus*, was grown in axenic aerated batch cultures in BG-11_0 medium (Rippka *et al.*, 1979), at 28 ± 1°C and at a photon fluence rate of 50 $\mu mol.m^{-2}.s^{-1}$ as described in chapter 2. When required NH_4Cl (1 $mmol.dm^{-3}$), glutamine (1 $mmol.dm^{-3}$), glucose (50 $mmol.dm^{-3}$) or CH_3NH_2Cl (5 $mmol.dm^{-3}$) was added to the medium and buffered with 10 $mmol.dm^{-3}$ HEPES-NaOH (pH 7.5).

5.2.2. Chlorophyll and protein determinations:

Chl **a** and protein content of cyanobacteria were measured according to Mackinney (1941) and Lowry *et al.* (1951), respectively.

5.2.3. Measurement of $^{14}CH_3NH_3^+$ uptake:

$^{14}CH_3NH_3^+$ uptake was assayed at 28°C and at a photon fluence rate of 50 $\mu mol.m^{-2}.s^{-1}$., unless otherwise indicated, and non-specific binding of $^{14}CH_3NH_3^+$ by cell membranes was measured by using toluene treated cells. (Details given in Chapter 2).

5.2.4. Measurement of GS activity:

GS biosynthetic and transferase activities were measured according to Sampaio *et al.* (1979).

5.2.5. Chemicals:

$^{14}CH_3NH_2Cl$, ^{3}H-dextran and ^{3}H-water were purchased from Amersham International plc, Amersham, U.K. Silicon DC 550 and dinonylphthalate were purchased from Fluka AC, Buchs, Switzerland. All other chemicals were obtained from Sigma chemical company, U.S.A.

5.3. RESULTS:

5.3.1. $^{14}CH_3NH_3^+$ uptake in N_2-grown *Nostoc* ANTH cells at pH 7:

5.3.1.1: $^{14}CH_3NH_3^+$ uptake:

The ammonium transport in *Nostoc* ANTH cells was studied using $^{14}CH_3NH_3^+$ as probe. N_2-grown *Nostoc* ANTH cells, at pH 7 and external $^{14}CH_3NH_3^+$ concentration of 50 $\mu mol.dm^{-3}$, showed a biphasic pattern of $^{14}CH_3NH_3^+$ uptake with an initial rapid phase during first 60 s followed by a slower second phase which remained linear over the 12 min experimental period. The uptake rates during initial rapid and slower second phase were found to be 0.119 and 0.0156 $nmol.min^{-1}.\mu g^{-1}$ Chl a, respectively (Fig 5.1). The toluene treated cells showed a much lower level of $^{14}CH_3NH_3^+$ incorporation during first 30 s which remained constant over the 12 min experimental period (Fig 5.1). This incorporation was found to be 6.16 $kBq.mg^{-1}$ Chl a representing about 22% of the total uptake by

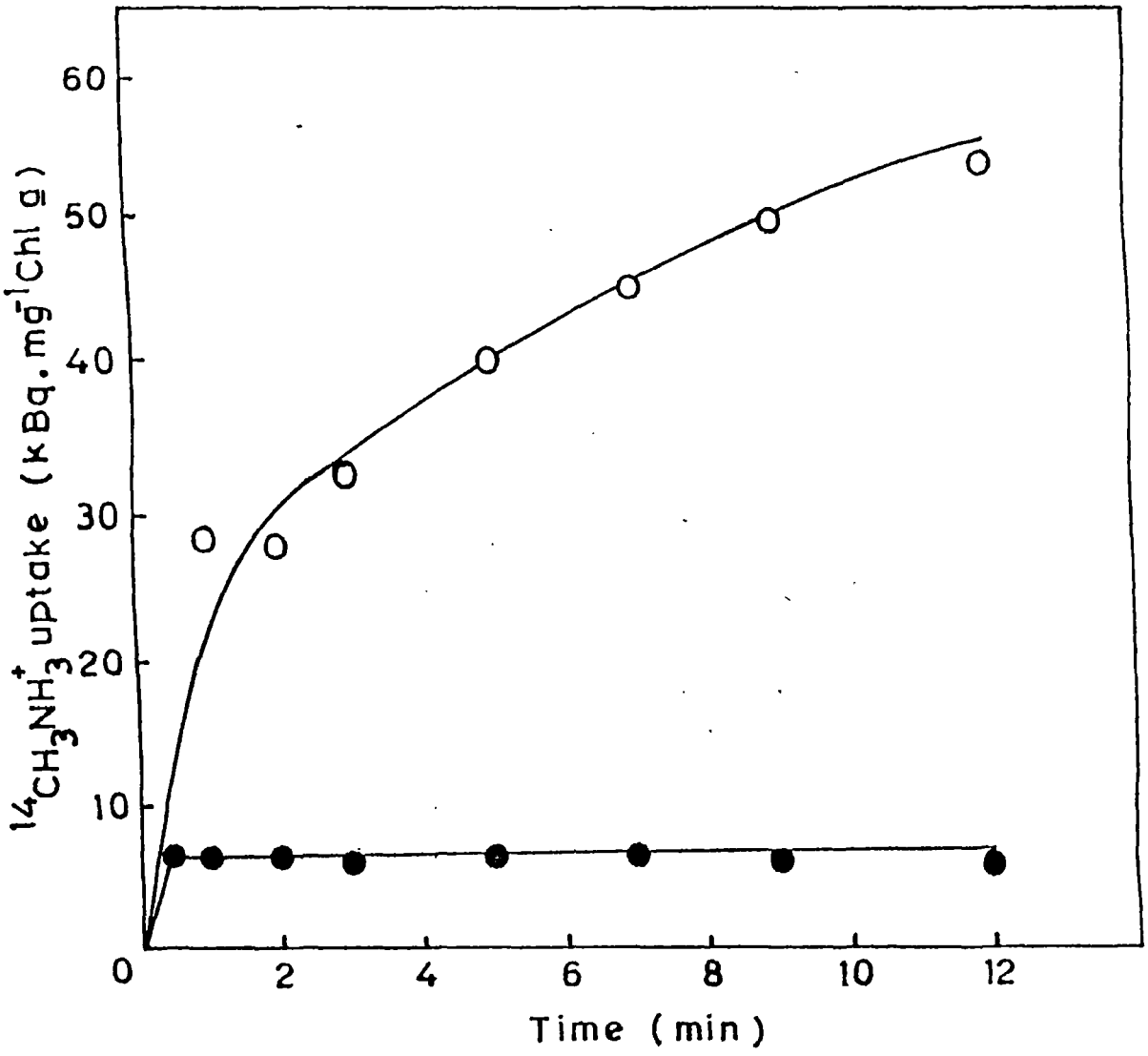

Fig 5.1 $^{14}CH_3NH_3^+$ uptake, at pH 7, by *Nostoc* ANTH filaments grown on N_2-medium. O, control; ●, toluene treated cells. (In this and all other experiments the data are means of four replicates obtained from two repeat experiments. The variation range was between 5 - 10% from the average.

untreated cells after 60 s (first phase). These values were considered to be non-specific adsorption of $^{14}CH_3NH_3^+$ by cell membranes and substracted from the respective values in untreated cells, before data were plotted in all other experiments which follow.

5.3.1.2. Effect of NH_4Cl:

Simultaneous addition of NH_4Cl and $^{14}CH_3NH_3^+$ resulted in total inhibition of $^{14}CH_3NH_3^+$ uptake, during both phases, in N_2-grown cells, at pH 7, indicating a common transport for NH_4Cl and $^{14}CH_3NH_3^+$ (Fig 5.2). This suggested that, as in the case of *Anabaena* 7120, $^{14}CH_3NH_3^+$ can be used as a probe to study the NH_4^+ uptake in *Nostoc* ANTH. When NH_4Cl was added subsequent to the addition of $^{14}CH_3NH_3^+$, it showed two effects: a) a sudden efflux of preaccumulated ^{14}C-label from the cells into the cell suspension, and b) complete inhibition of further $^{14}CH_3NH_3^+$ uptake by cells (Fig 5.2). The NH_4^+-displaceable ^{14}C-label remained constant over the experimental period and equaled to the ^{14}C-label which was transported during initial rapid phase. This indicated existence of a free $^{14}CH_3NH_3^+$ pool inside the cells which built up during initial phase of $^{14}CH_3NH_3^+$ uptake. Considering that the NH_4^+-displaceable $^{14}CH_3NH_3^+$ represented the internal free $^{14}CH_3NH_3^+$ pool, the latter was calculated to be 4.9 $mmol.dm^{-3}$. Thus, at an external $^{14}CH_3NH_3^+$ concentration of 50 $\mu mol.dm^{-3}$, *Nostoc* ANTH cells showed a nearly 100 fold accumulation of $^{14}CH_3NH_3^+$.

While $^{14}CH_3NH_3^+$ displaced by NH_4^+ remained constant, with time more and more ^{14}C-label remained inside the cells. This is not surprising since $^{14}CH_3NH_3^+$ is metabolized in *Nostoc* ANTH cells by

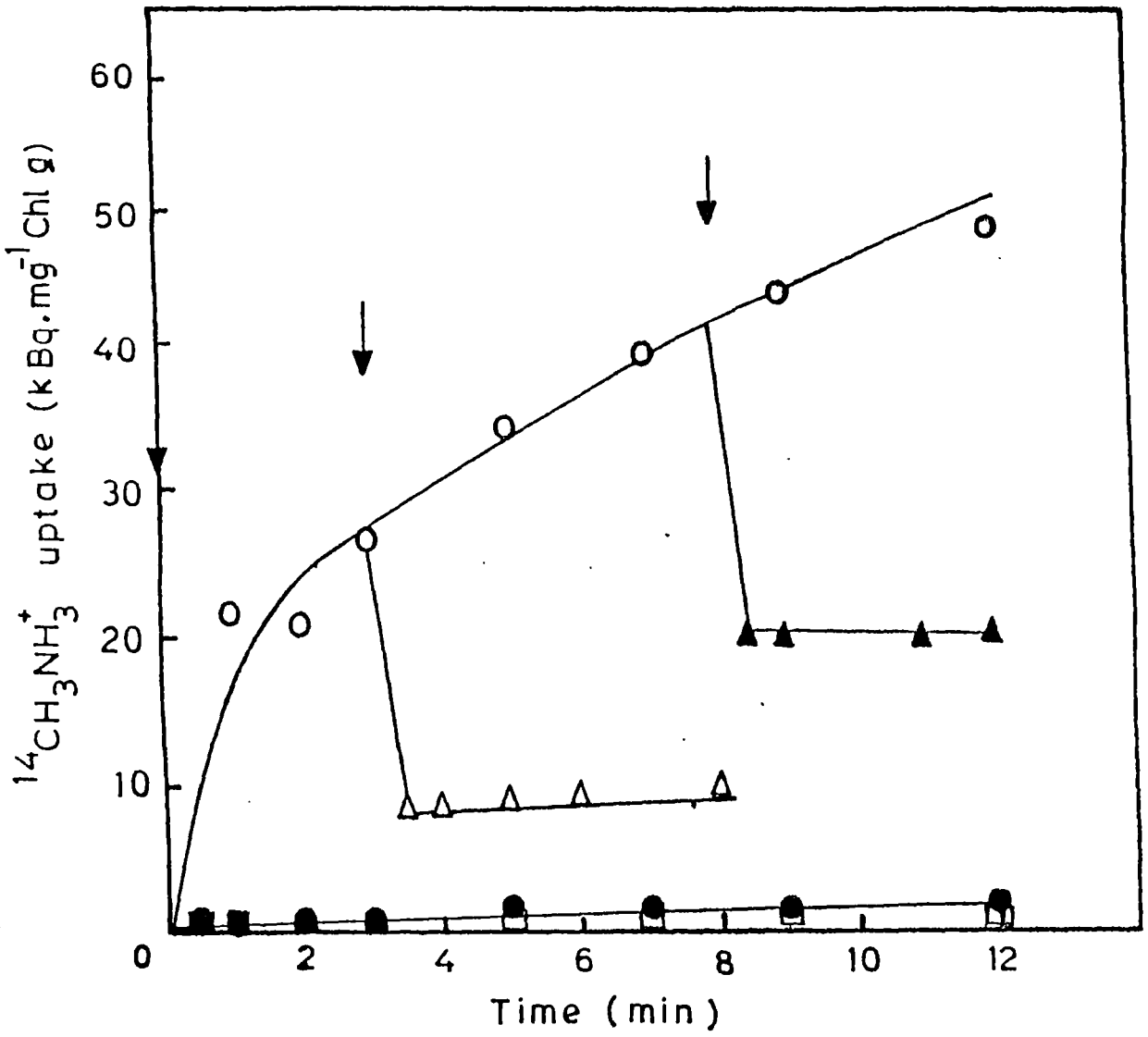

Fig 5.2 Effect of NH_4Cl on $^{14}CH_3NH_3^+$ uptake, at pH, 7, by N_2-grown *Nostoc* ANTH filaments. NH_4Cl was added at times indicated (arrows) to a final concentration of 200 μmol.dm^{-3}. O, control ($^{14}CH_3NH_3^+$ only); ●, NH_4Cl and $^{14}CH_3NH_3^+$ added simultaneously at zero time; Δ, NH_4Cl added 3 min after $^{14}CH_3NH_3^+$ addition; ▲, NH_4Cl added 8 min after $^{14}CH_3NH_3^+$ addition; □, $^{14}CH_3NH_3^+$ uptake in NH_4Cl (1 mmol.dm^{-3})-grown cells.

GS-GOGAT pathway (see chapter 3). Such data indicate that the second phase of $^{14}CH_3NH_3^+$ uptake represents metabolism of the transported $^{14}CH_3NH_3^+$.

In NH_4^+-grown cells no $^{14}CH_3NH_3^+$ accumulation/incorporation was observed (Fig 5.2). In these cells the $^{14}CH_3NH_3^+$ uptake pattern was similar to that observed in toluene-treated cells. These data are consistent with earlier findings on *Anabaena variabilis* (Rai *et al.*, 1986a), *Anabaena* 7120 (see Rai and Prakasham, 1989), and other bacteria (Kleiner, 1985a) showing repression of ATS in NH_4^+-grown cells.

5.3.1.3. Effect of MSX:

In one set of experiments cells were preincubated with MSX (10 μmol.dm^{-3}) for 1 hour to inactivate GS and then $^{14}CH_3NH_3^+$ uptake was measured subsequently in absence of MSX. In such experiments only the first phase of $^{14}CH_3NH_3^+$ uptake was detectable (Fig 5.3); the second phase was absent. This indicated that the first phase was MSX insensitive. The lack of second phase of uptake in such cells may be due to blockage of $^{14}CH_3NH_3^+$ metabolism in the cells since GS was inhibited. It was also possible, however, that MSX may have affected $^{14}CH_3NH_3^+$ uptake during the second phase at transport level as found in a GS mutant of *Anabaena cycadeae* (singh *et al.*, 1985b). In an attempt to distinguish the effect of MSX at uptake level from that at the level of GS inhibition, further experiments were done to see the effect of MSX on $^{14}CH_3NH_3^+$ uptake in cells where GS remained unaffected during the experimental period. In these experiments no preincubation with MSX was done. MSX (10 μmol.dm^{-3}) was added together with $^{14}CH_3NH_3^+$ at zero

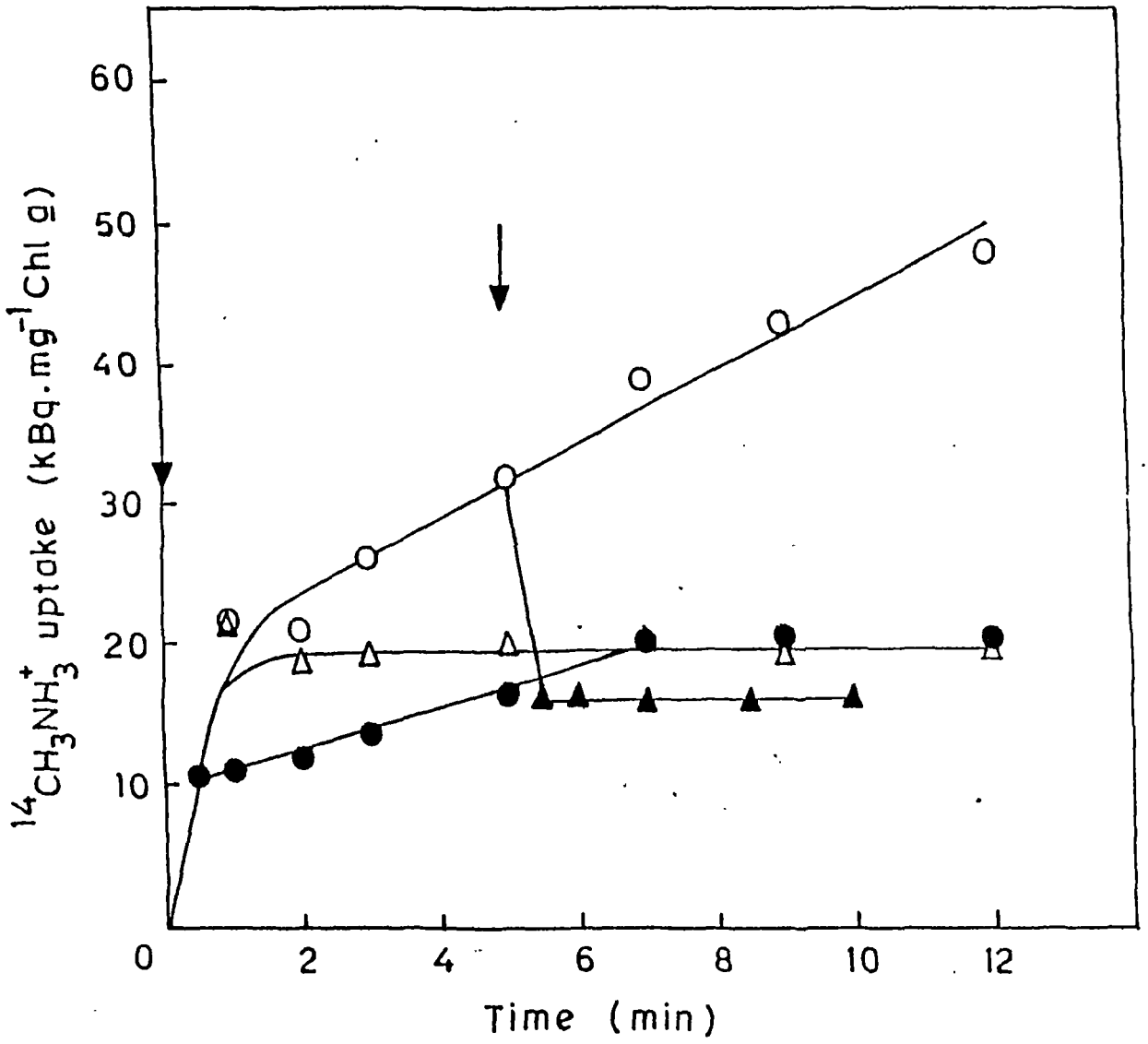

Fig 5.3 Effect of MSX on $^{14}CH_3NH_3^+$ uptake, at pH 7, by N_2-grown *Nostoc* ANTH filaments. MSX was added at times indicated (arrows) to a final concentration of 10 μmol.dm^{-3}. O, control ($^{14}CH_3NH_3^+$ only); ●, MSX and $^{14}CH_3NH_3^+$ added simultaneously at zero time; ▲, MSX added 5 min after $^{14}CH_3NH_3^+$ addition; △, $^{14}CH_3NH_3^+$ uptake in MSX-preincubated cells (cells were incubated with 10 μmol.dm^{-3} MSX for 1 h then washed and resuspended in fresh buffer and uptake was studied without MSX being present in the medium.

time or 5 min after the addition of $^{14}CH_3NH_3^+$ and $^{14}CH_3NH_3^+$ uptake measured during the next 12 min in the presence of MSX (during this period GS remained fully active; see Rai and Prakasham, 1989). When MSX and $^{14}CH_3NH_3^+$ were added simultaneously, $^{14}CH_3NH_3^+$ uptake occurred at a rate similar to that in the control for the first 30 s after which the uptake continued at a slower rate till the $^{14}CH_3NH_3^+$ in cell reached a level similar to that at the end of first phase of $^{14}CH_3NH_3^+$ uptake in control cells (Fig 5.3). Such data indicated that although MSX partially affected the rate of $^{14}CH_3NH_3^+$ uptake during first phase it did not affect the overall pool size built up during this phase. On the otherhand, it totally blocked the second phase of $^{14}CH_3NH_3^+$ uptake (Fig 5.3). Since GS activity was not affected during this period it was concluded that this effect of MSX was at the level of $^{14}CH_3NH_3^+$ uptake. Thus, as in *Anabaena* 7120 (Rai and Prakasham, 1989), *Nostoc* ANTH also has two uptake systems for $NH_4^+/CH_3NH_3^+$; one MSX-insensitive and the other MSX-sensitive. These observations are consistent with MSX having two targets of inhibitory action; one at GS level and the other at transport level, as found in other cyanobacteria (see Singh *et al.*, 1985b; Rai and Prakasham, 1989).

Addition of MSX to the cell suspension during the second phase of $^{14}CH_3NH_3^+$ uptake caused an immediate inhibition of $^{14}CH_3NH_3^+$ uptake and an efflux (15 Bq.μg^{-1} Chl a) of preaccumulated ^{14}C-label from the cells (Fig 5.3). The former observation is similar to the effect of MSX found in *A. variabilis* (Rai *et al.*, 1984), *Anabaena* 7120 (Rai and Prakasham, 1989) and *A. cycadeae* (Singh *et al.*, 1985b). However, the efflux of ^{14}C-label caused by MSX contrasts with the observations in other cyanobacteria (Rai

al., 1984; Singh *et al.*, 1985b; Rai and Prakasham, 1989) where no ^{14}C-efflux was caused by MSX. It is probable that MSX caused partial efflux of some intracellular $^{14}CH_3NH_3^+$. It should be noted here that MSX did partially affect, transiently, the build up of internal free pool of $^{14}CH_3NH_3^+$ during the first phase of $^{14}CH_3NH_3^+$ uptake. Alternatively, the ^{14}C-efflux may have been due to displacement by MSX of an internal pool of some metabolized product of $CH_3NH_3^+$. (Unlike other cyanobacteria, *Nostoc* ANTH does *metabolize* $CH_3NH_3^+$ as N-source; chapter 3).

5.3.1.4. Kinetics of concentration-dependent $^{14}CH_3NH_3^+$ uptake via the two ATS:

$^{14}CH_3NH_3^+$ uptake rates, in *Nostoc* ANTH, at pH 7, via the two ATS at various external $^{14}CH_3NH_3^+$ concentrations (1-500 $\mu mol.dm^{-3}$) were studied (Fig 5.4a - 5.4c) as in *Anabaena* 7120 (see chapter 4). First, the $^{14}CH_3NH_3^+$ uptake rates via the MSX-insensitive ATS were studied by following $^{14}CH_3NH_3^+$ uptake during the first phase (initial 60 s) at 15 s intervals after $^{14}CH_3NH_3^+$ addition to the cell suspension. Rates were calculated from the linear portions of the curve. A biphasic pattern of concentration-dependent $^{14}CH_3NH_3^+$ uptake rate, similar to that in *Anabaena* 7120 (see chapter 4), was observed (Fig 5.4a) with Vmax values of 0.125 and 0.225 $nmol.min^{-1}.\mu g^{-1}$ Chl a, in the external concentration range of 1 - 15 and 15 - 500 $\mu mol.dm^{-3}$, respectively (Fig 5.4a; data not shown beyond 150 $\mu mol.dm^{-3}$). The corresponding Km values (calculated from Lineweaver-Burk plots) were 3 and 45 $\mu mol.dm^{-3}$, respectively (Fig 5.4b & 5.4C).

Next, $^{14}CH_3NH_3^+$ rates, via the second ATS were studied, at

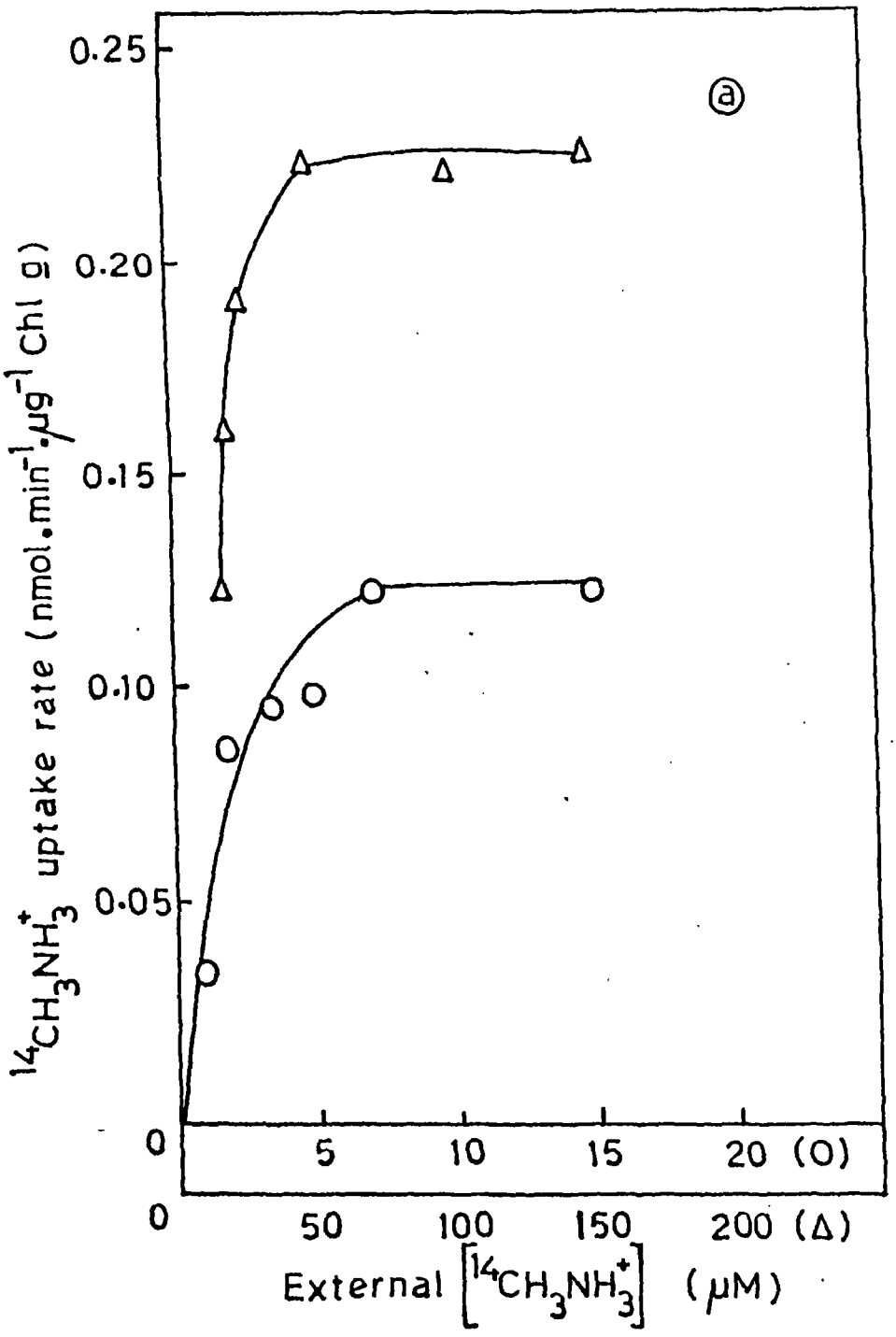

Fig 54a Concentration-dependent $^{14}CH_3NH_3^+$ uptake rates, at pH 7, during initial MSX-insensitive rapid phase by N_2-grown *Nostoc* ANTH filaments showing dual isotherm Michaelis-Menten Kinetics. O, uptake rates at an external concentration range of 1 - 15 μmol.dm^{-3} $^{14}CH_3NH_3^+$; Δ, uptake rates at an external concentration range of 15 - 150 μmol.dm^{-3} $^{14}CH_3NH_3^+$.

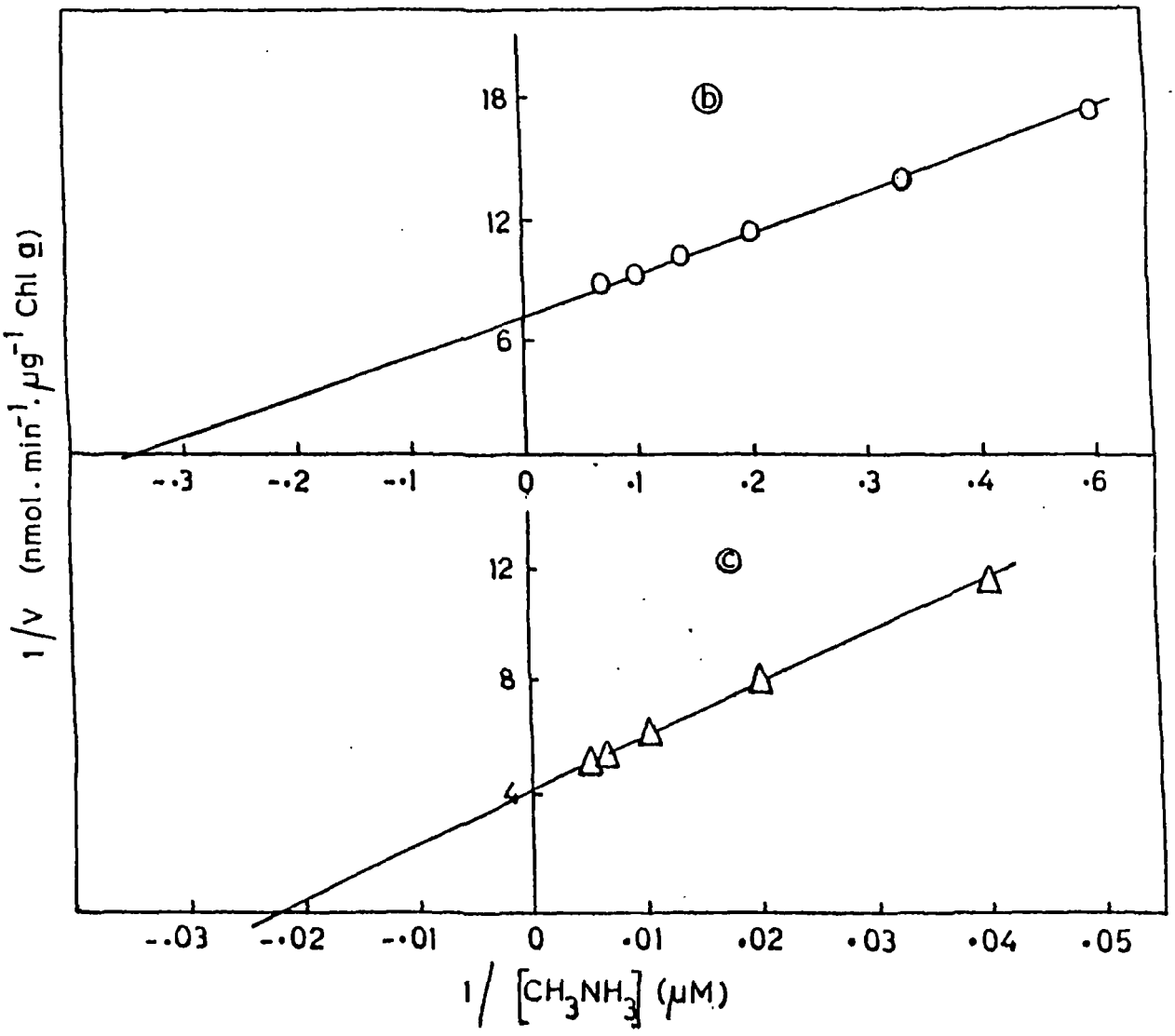

Fig 5.4 b&c Lineweaver-Burk plots for $^{14}CH_3NH_3^+$ uptake during high affinity mode (1- 15 μmol.dm^{-3} external $^{14}CH_3NH_3^+$ concentration, O) and low affinity mode (15 - 150 μmol.dm^{-3} external $^{14}CH_3NH_3^+$ concentration, Δ) calculated from Fig 4a as before (Lineweaver and Burk, 1934).

$^{14}CH_3NH_3^+$ concentration range and conditions similar to the above. The rates were calculated from linear second phase of $^{14}CH_3NH_3^+$ uptake (between 5 and 12 min after $^{14}CH_3NH_3^+$ addition to cell suspension). The observed Vmax values were 0.028 and 0.0205 $nmol.min^{-1}.\mu g^{-1}$ Chl a, in the external concentration range of 1 - 20 and 50 - 400 $\mu mol.dm^{-3}$, respectively (Fig 5.5a). The corresponding Km values were 4.6 and 135 $\mu mol.dm^{-3}$, respectively (Fig 5.5b & 5.5c; calculated from Lineweaver-Burk plots).

Thus, as in *Anabaena* 7120 (see chapter 4), in *Nostoc* ANTH also both ATS showed dual affinity mode: a high affinity mode at low substrate concentration and a low affinity mode at high substrate concentration. However, the pattern of change in $^{14}CH_3NH_3^+$ uptake rates, via the second ATS, in response to external $^{14}CH_3NH_3^+$ concentration, differed in the two organisms (see Fig 4.8a of chapter 4 and Fig 5.5a of this chapter). In *Anabaena* 7120, the $^{14}CH_3NH_3^+$ uptake rate via the second ATS saturated below 10 $\mu mol.dm^{-3}$. When external $^{14}CH_3NH_3^+$ concentration was increased beyond 10 $\mu mol.dm^{-3}$ a further increase in the rate of uptake occurred which saturated at 150 $\mu mol.dm^{-3}$. Further increase in external substrate concentration did not result in any increase in uptake rate (see chapter 4 Fig 4.8a). In *Nostoc* ANTH, however, the uptake rate increased in response to increase in external substrate concentration upto 20 $\mu mol.dm^{-3}$. Further increase in external $^{14}CH_3NH_3^+$ concentration resulted in a transient decrease in $^{14}CH_3NH_3^+$ uptake rate. However, when external $^{14}CH_3NH_3^+$ concentration was raised beyond 50 $\mu mol.dm^{-3}$, an increase in uptake rate was observed. This increase continued upto external substrate concentration of 300 $\mu mol.dm^{-3}$. Further increase in

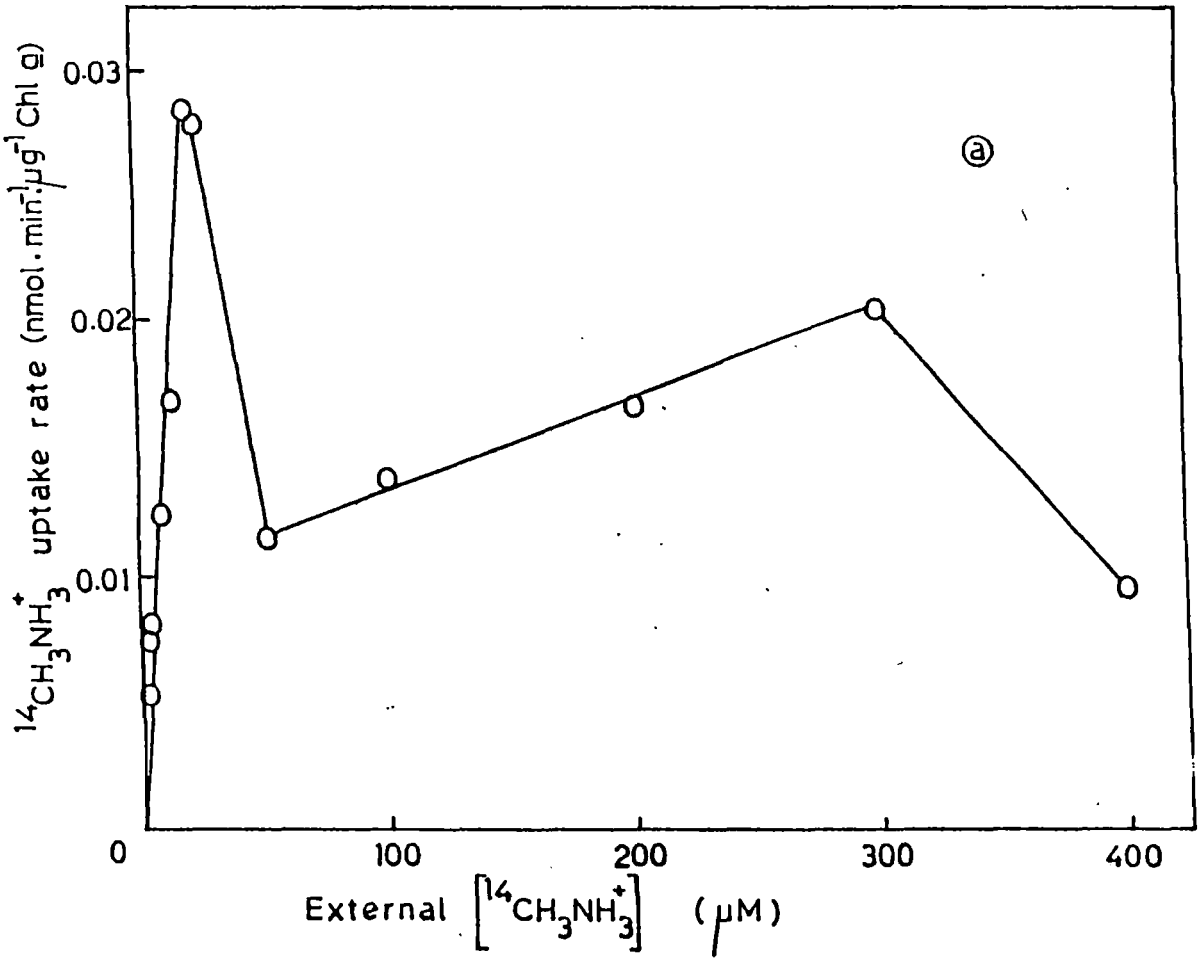

Fig 5.5a Concentration-dependent $^{14}CH_3NH_3^+$ uptake rates, at pH 7, during the subsequent MSX-sensitive slower phase by N_2-grown *Nostoc* ANTH filaments showing dual isotherm Michaelis - Menten kinetics. O, uptake rates at an external concentration range of 1 - 400 µmol.dm^{-3} $^{14}CH_3NH_3^+$.

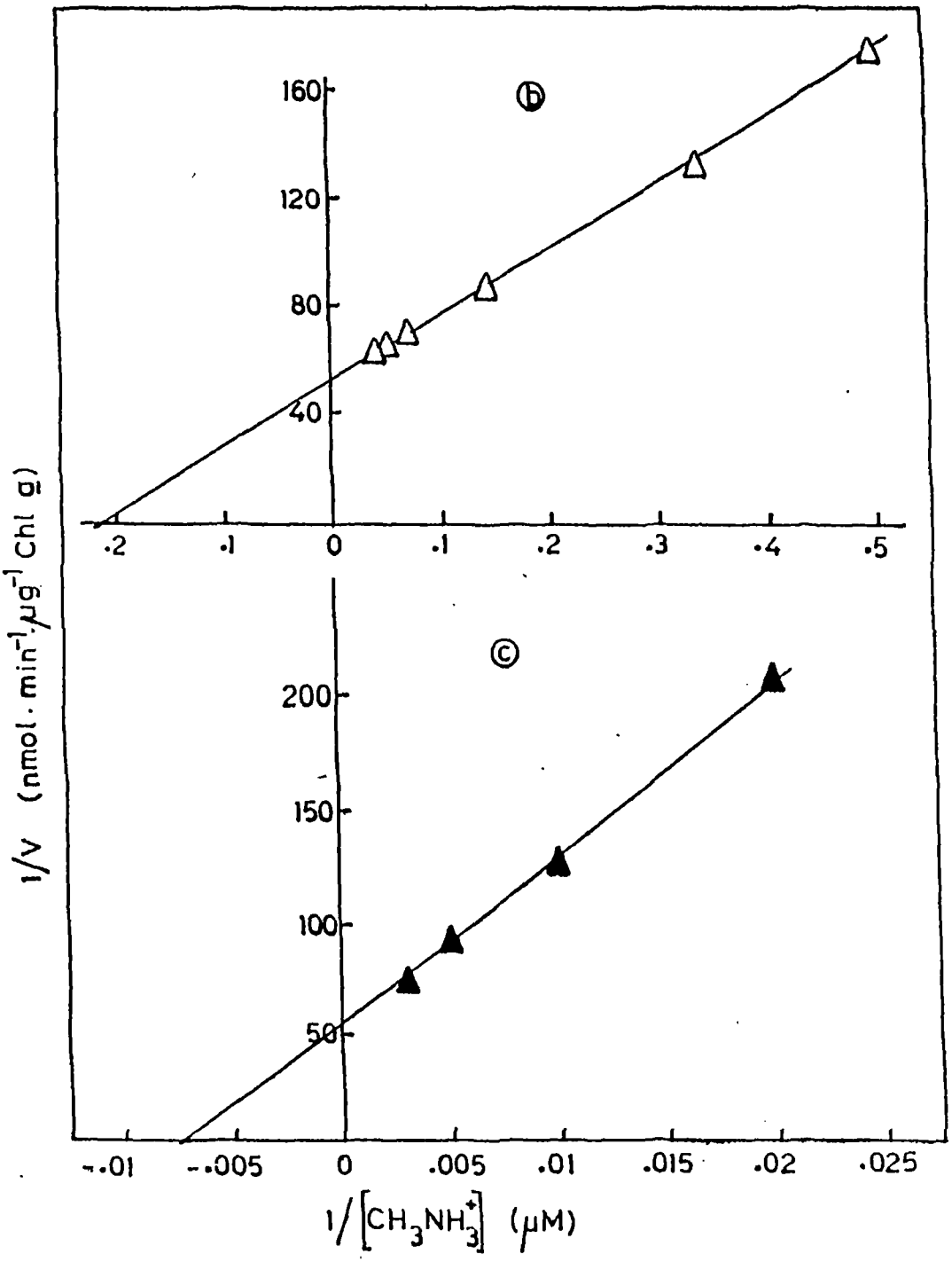

Fig 5.5 b &c Lineweaver-Burk plots for $^{14}CH_3NH_3^+$ uptake during high affinity mode (1 - 25 µmol.dm^{-3} external $^{14}CH_3NH_3^+$ concentration, △) and low affinity mode (50 - 300 µmol.dm^{-3} external substrate concentration, ▲) calculated from Fig 5a as before (Lineweaver and Burk, 1934).

external $^{14}CH_3NH_3^+$ concentration again resulted in a decrease in uptake rate (Fig 5.5a). Furthermore, in *Nostoc* ANTH, the Vmax value decreased during the shift from high to low affinity mode whereas, in *Anabaena* 7120 the Vmax value increased (see Fig 5.5a of this chapter and Fig 4.8a of chapter 4).

5.3.1.5. Effect of CCCP and $TPMP^+$:

Ammonium transport in various prokaryotes, so far tested, is found to be active and energy-dependent and driven by transmembrane electrical potential (Kleiner, 1981; 1985a; Rai *et al.*, 1984; 1986a; Singh *et al.*, 1985b; 1987; Rai and Prakasham, 1989). Therefore, the effect of CCCP and $TPMP^+$ on $^{14}CH_3NH_3^+$ uptake, in this cyanobacterium, at pH 7, was studied. CCCP caused complete elimination of $^{14}CH_3NH_3^+$ uptake during both phases (Fig 5.6) suggesting that $^{14}CH_3NH_3^+$ uptake in *Nostoc* ANTH also is an active and energy-dependent process like that in *Anabaena* 7120 (Rai and Prakasham, 1989). $TPMP^+$ treated cells showed a markedly lower level of $^{14}CH_3NH_3^+$ uptake (Fig 5.6). In $TPMP^+$ treated cells the uptake rate during first and second phase was 0.06 and 0.005 $nmol.min^{-1}.\mu g^{-1}$ Chl **a**, respectively. This represented 50 and 29.5% of the corresponding values in control cells, respectively. These results indicate that while transmembrane electrical potential is necessary for optimum rates of uptake during both phases of $^{14}CH_3NH_3^+$ uptake, $^{14}CH_3NH_3^+$ uptake can still occur in absence of transmembrane electrical potential. This contrasts with the results in *Anabaena* 7120 (Rai and Prakasham, 1989) and *A. variabilis* (Rai *et al.*, 1984) where $TPMP^+$ treated cells showed total lack of $^{14}CH_3NH_3^+$ uptake.

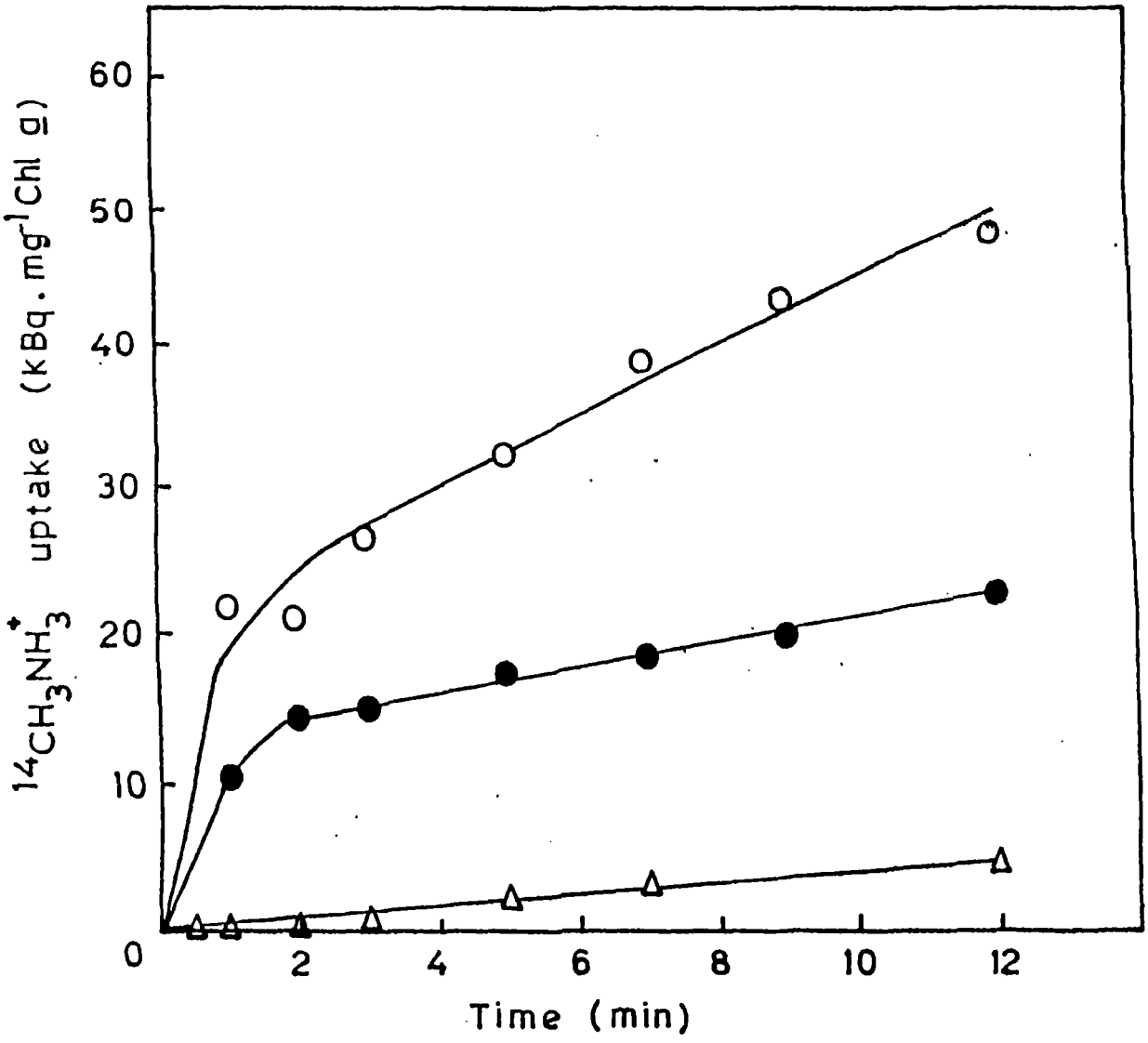

Fig 5.6 $^{14}CH_3NH_3^+$ uptake, at pH 7, by N_2-grown *Nostoc* ANTH filaments in the presence (●, Δ) or absence (O) of CCCP (Δ) and $TPMP^+$ (●). CCCP (10 μmol.dm^{-3}) and $TPMP^+$ (100 μmol.dm^{-3}) were added 30 min before $^{14}CH_3NH_3^+$ addition.

5.3.1.6. Effect of glutamine and glutamate:

Glutamine and glutamate are the initial products of primary ammonia assimilation in cyanobacteria (Stewart, 1980). Hence, effects of these compounds on ATS, in this cyanobacterium was studied.

$^{14}CH_3NH_3^+$ uptake, in glutamine (1 mmol.dm^{-3}) grown *Nostoc* ANTH cells, at pH 7, is shown in fig 5.7a. The uptake pattern was found to be similar in N_2- and glutamine-grown cells. The rate of $^{14}CH_3NH_3^+$ uptake during the first phase was similar in glutamine- and N_2-grown cells, during the second phase $^{14}CH_3NH_3^+$ uptake was slightly higher in glutamine-grown cells. Such results suggest that glutamine is not a repressor of $^{14}CH_3NH_3^+$ uptake in *Nostoc* ANTH cells. This contrasts with the finding of Singh *et al.* (1987) who have shown that $^{14}CH_3NH_3^+$ uptake was repressed in glutamine grown cells of *A. cycadeae*.

The effect of various external glutamine concentration on $^{14}CH_3NH_3^+$ uptake, at pH 7, in N_2-grown *Nostoc* ANTH cells is shown in Fig 5.7b. In the presence of 200 μmol.dm^{-3} glutamine, $^{14}CH_3NH_3^+$ uptake during the first phase was severely inhibited with little or no effect on the rate of uptake during the second phase. Increasing concentration of glutamine caused progressively more inhibitory effect, however the uptake did resume after 5 min (i.e. the second phase). Thus, glutamine seems to inhibit the MSX-insensitive ATS while having little or no affect on the MSX-sensitive ATS.

Nostoc ANTH did not grow in glutamate containing medium. Therefore, for studying effect of glutamate on ATS, experiments were performed in N_2-grown cells only. As shown in fig 5.8,

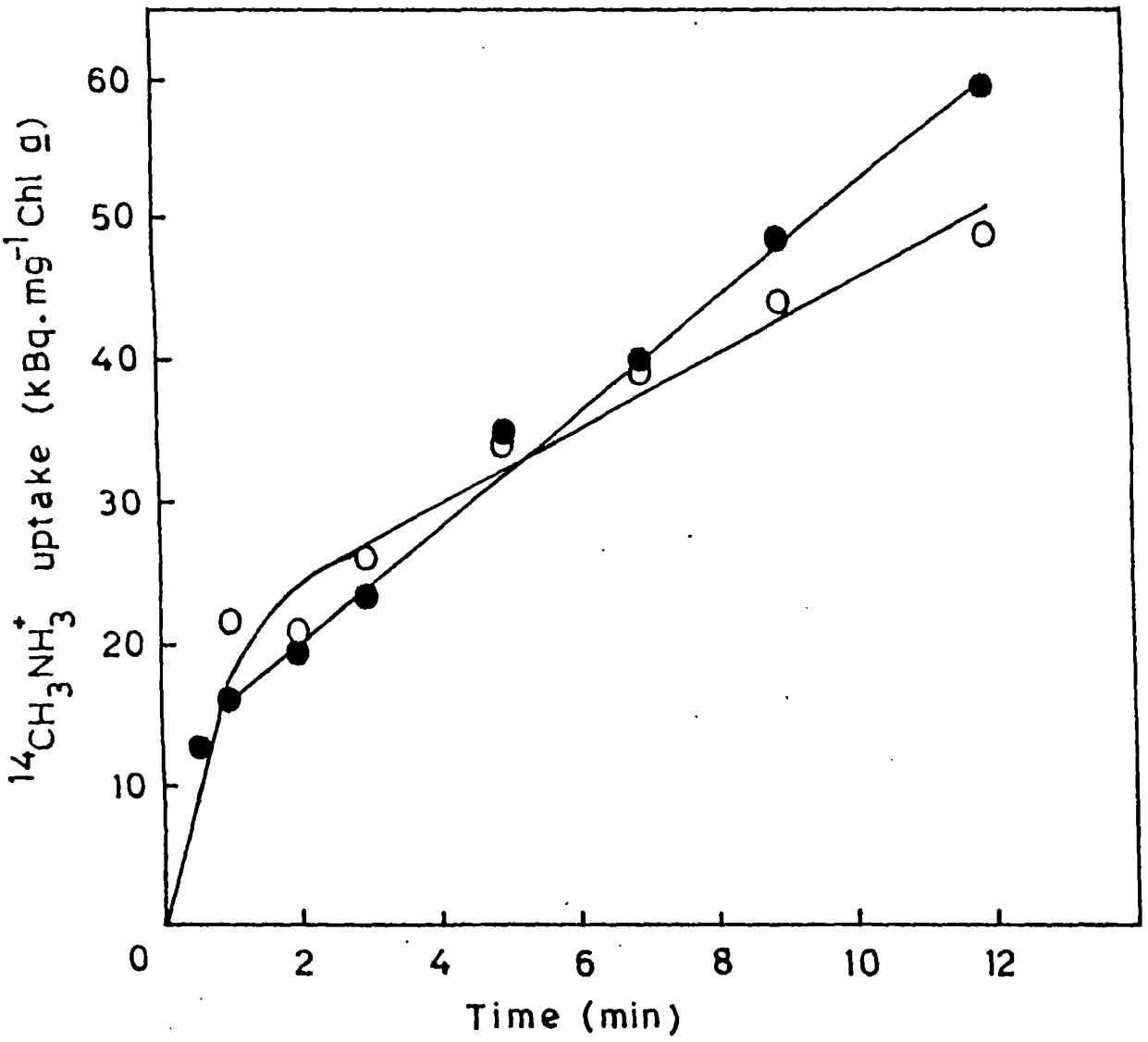

Fig 5.7a $^{14}CH_3NH_3^+$ uptake, at pH 7, by N_2-grown (O) and glutamine (1 mmol.dm^{-3})-grown (●) *Nostoc* ANTH filaments.

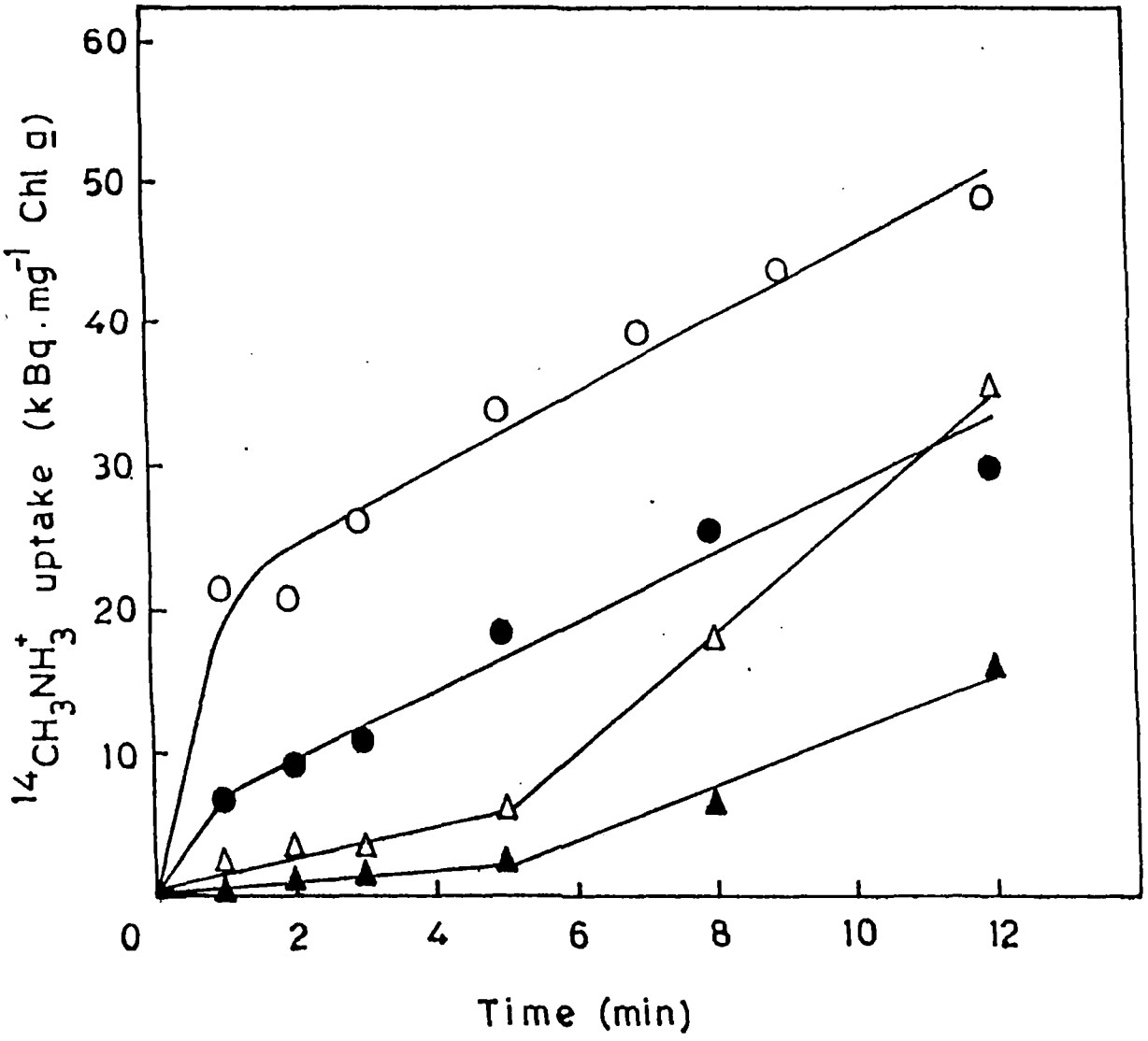

Fig 5.7b Effect of glutamine addition on $^{14}CH_3NH_3^+$ uptake, at pH 7, by N_2-grown *Nostoc* ANTH filaments. Glutamine was added along with $^{14}CH_3NH_3^+$ at zero time. O, control ($^{14}CH_3NH_3^+$ only); ●, 200 μmol.dm^{-3} glutamine; Δ, 500 μmol.dm^{-3} glutamine; ▲, 1000 μmol.dm^{-3} glutamine.

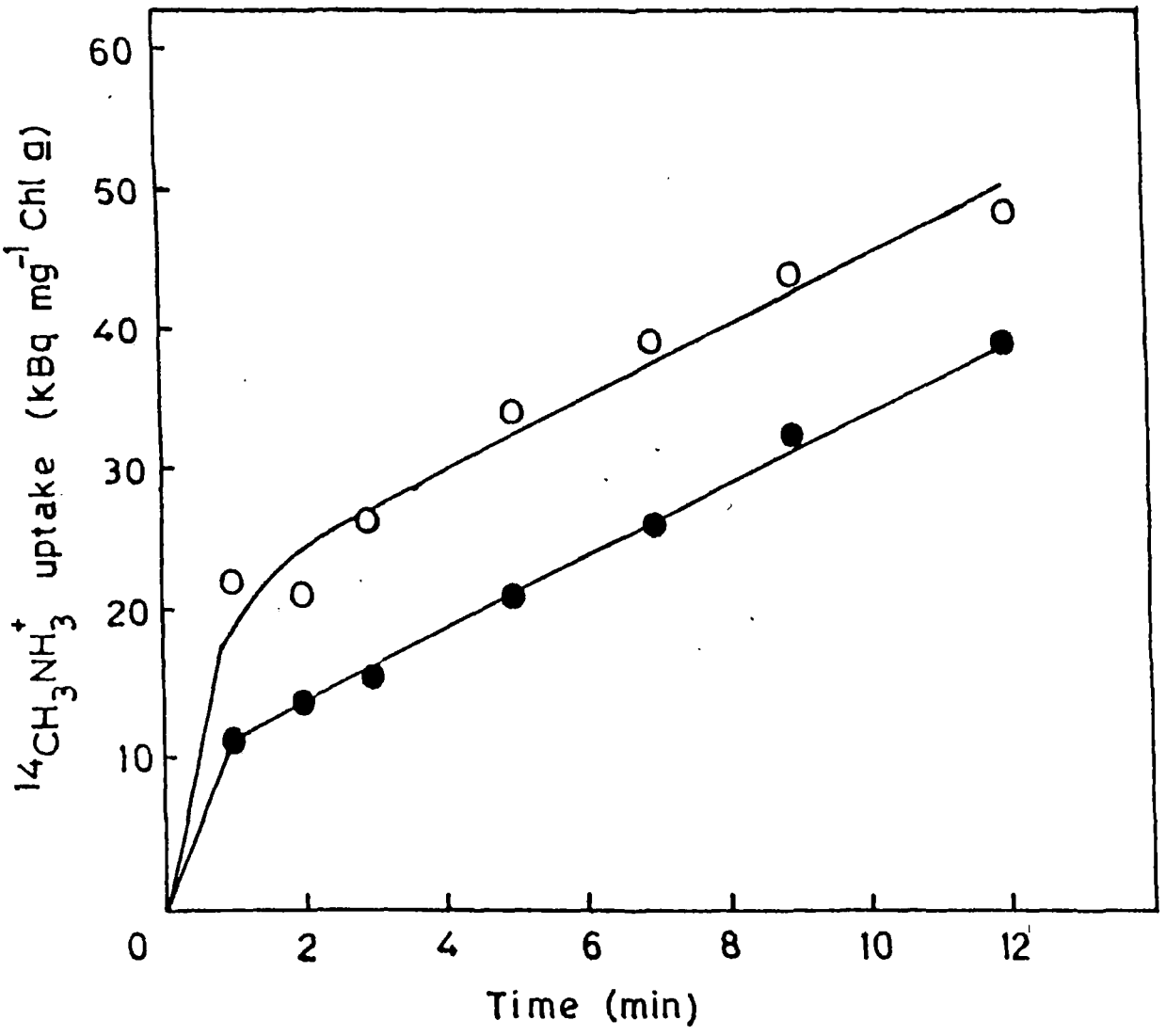

Fig 5.8 Effect of glutamate addition on $^{14}CH_3NH_3^+$ uptake, at pH 7, by N_2-grown *Nostoc* ANTH filaments. Glutamate was added along with NH_4Cl at zero time. O, control ($^{14}CH_3NH_3^+$ only); ●, 200 μmol.dm^{-3} glutamate.

presence of 200 μmol.dm^{-3} glutamate affected the first phase of $^{14}CH_3NH_3^+$ uptake but not the second phase. This was similar to the effect of glutamine on $^{14}CH_3NH_3^+$ uptake in N_2-grown cells (Fig 5.7b) and indicated that, like glutamine, glutamate affected only the MSX-insensitive ATS.

5.3.2. $^{14}CH_3NH_3^+$ uptake in glucose-grown cells:

A comparative study of $^{14}CH_3NH_3^+$ uptake in autotrophically-grown, photoheterotrophically-grown and heterotrophically-grown *Nostoc* ANTH cells is presented in fig 5.9. The $^{14}CH_3NH_3^+$ uptake pattern in photoheterotrophically-grown cells (cells grown in N_2-medium in light + 50 mmol.dm^{-3} glucose) and heterotrophically-grown cells (cells grown in N_2-medium in dark + glucose) was similar to that in the control (autotrophically-grown cells; cells grown in N_2-medium in light). However, both photoheterotrophically-grown and heterotrophically-grown cells accumulated less $^{14}CH_3NH_3^+$ than autotrophically-grown cells.

The rate of $^{14}CH_3NH_3^+$ uptake during the second phase (representing $^{14}CH_3NH_3^+$ metabolism) in autotrophically-grown and heterotrophically-grown cells was essentially identical. However, the rate in photoheterotrophically-grown cells was 57% higher indicating a higher rate of $^{14}CH_3NH_3^+$ metabolism. This may reflect the fact that compared to the autotrophically- and heterotrophically-grown cells, there was higher availability of C-skeleton and energy, for $^{14}CH_3NH_3^+$ metabolism, in photohetrotrophically-grown cells.

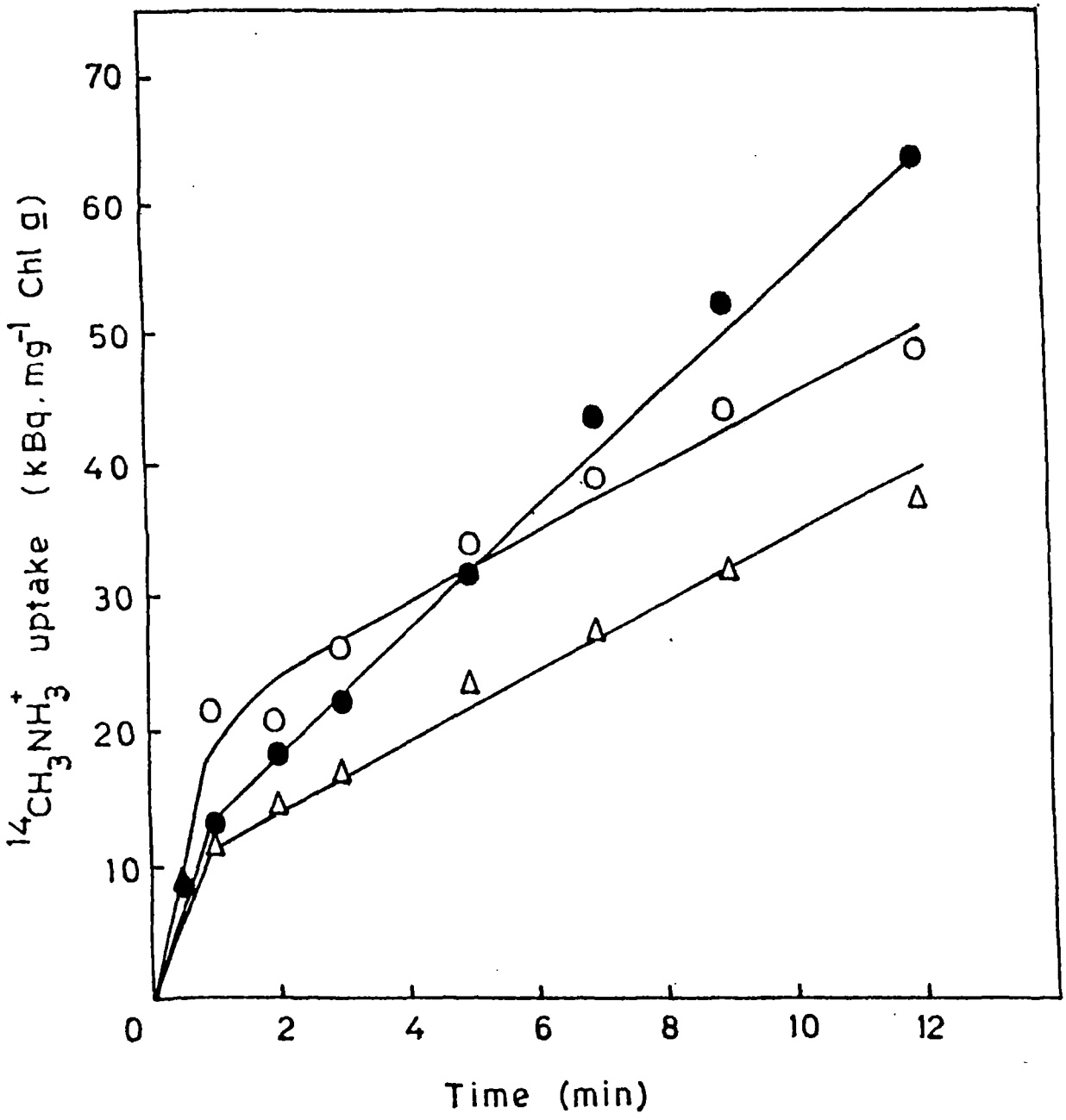

Fig 5.9 $^{14}CH_3NH_3^+$ uptake, at pH 7, by *Nostoc* ANTH filaments grown autotrophically, photoheterotrophically, and heterotrophically. O, cells grown in N_2-medium in light; ●, cells grown in N_2-medium in light + 50 mmol.dm^{-3} glucose; Δ, cells grown in N_2-medium in dark + 50 mmol.dm^{-3} glucose.

5.3.3. $^{14}CH_3NH_3^+$ uptake in $CH_3NH_3^+$-grown *Nostoc* ANTH cells:

5.3.3.1. $^{14}CH_3NH_3^+$ uptake:

Nostoc ANTH has an ability to metabolize $CH_3NH_3^+$ as N-source (see chapter 3). Since, in NH_4^+-grown *Nostoc* ANTH cells the methylammonium/ammonium transport system was repressed (Fig 5.2), it would be interesting to know whether such a transport system is operative in $CH_3NH_3^+$-grown cells. Therefore, $^{14}CH_3NH_3^+$ uptake in $CH_3NH_3^+$-grown *Nostoc* ANTH cells was investigated (Fig 5.10). In contrast to the NH_4^+-grown cells where no $^{14}CH_3NH_3^+$ uptake occurred, $CH_3NH_3^+$-grown cells showed $^{14}CH_3NH_3^+$ uptake indicating that despite $CH_3NH_3^+$ being used as N-source by *Nostoc* ANTH, $CH_3NH_3^+$ transport system was not repressed by $CH_3NH_3^+$.

The uptake pattern in $CH_3NH_3^+$-grown cells was different than that in N_2-grown cells. Unlike in N_2-grown cells, $^{14}CH_3NH_3^+$ uptake in $CH_3NH_3^+$-grown cells did not show as distinct a biphasic pattern as in N_2-grown cells.

5.3.3.2. Effect of NH_4Cl:

As seen in fig 11, addition of NH_4Cl at zero time caused a progressive inhibition of the $^{14}CH_3NH_3^+$ uptake. However, for the initial 2 - 3 min uptake was similar to that in control. Similar effect was observed when NH_4Cl was added subsequent to $^{14}CH_3NH_3^+$ addition. Furthermore, NH_4^+ did not cause any efflux of ^{14}C-label from cells into the medium (Fig 5.11). This is in contrast to the finding in N_2-grown cells where addition of NH_4^+ caused immediate and complete inhibition of $^{14}CH_3NH_3^+$ uptake and NH_4^+ addition subsequent to $^{14}CH_3NH_3^+$ addition caused efflux of preaccumulated $^{14}CH_3NH_3^+$ (see Fig 5.2). These data thus, indicate that the

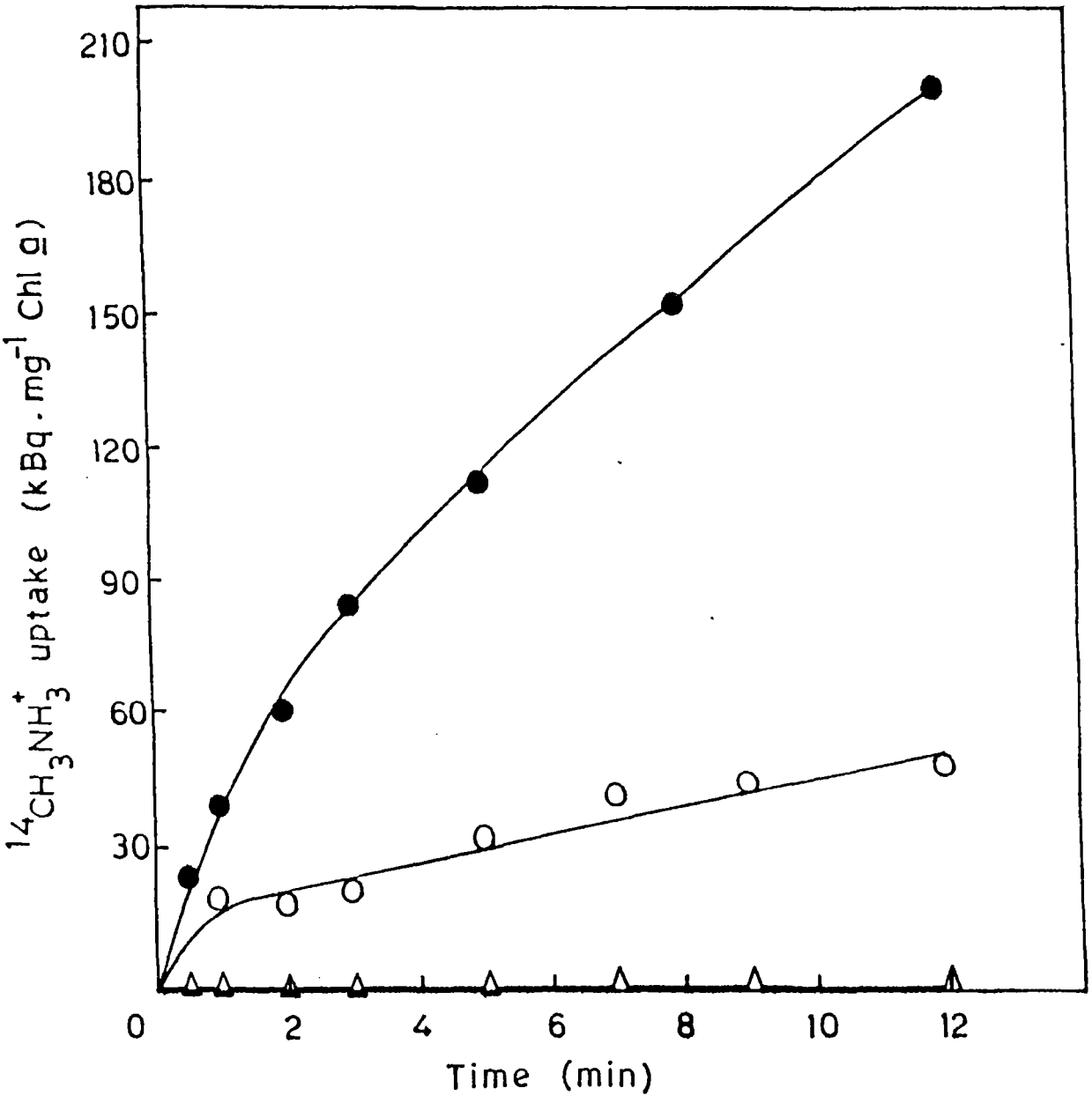

Fig 5.10 $^{14}CH_3NH_3^+$ uptake, at pH 7, by N_2-grown (O), $CH_3NH_3^+$ (5 mmol.dm^{-3})-grown (●), and NH_4^+ (1 mmol.dm^{-3})-grown (Δ) *Nostoc* ANTH filaments.

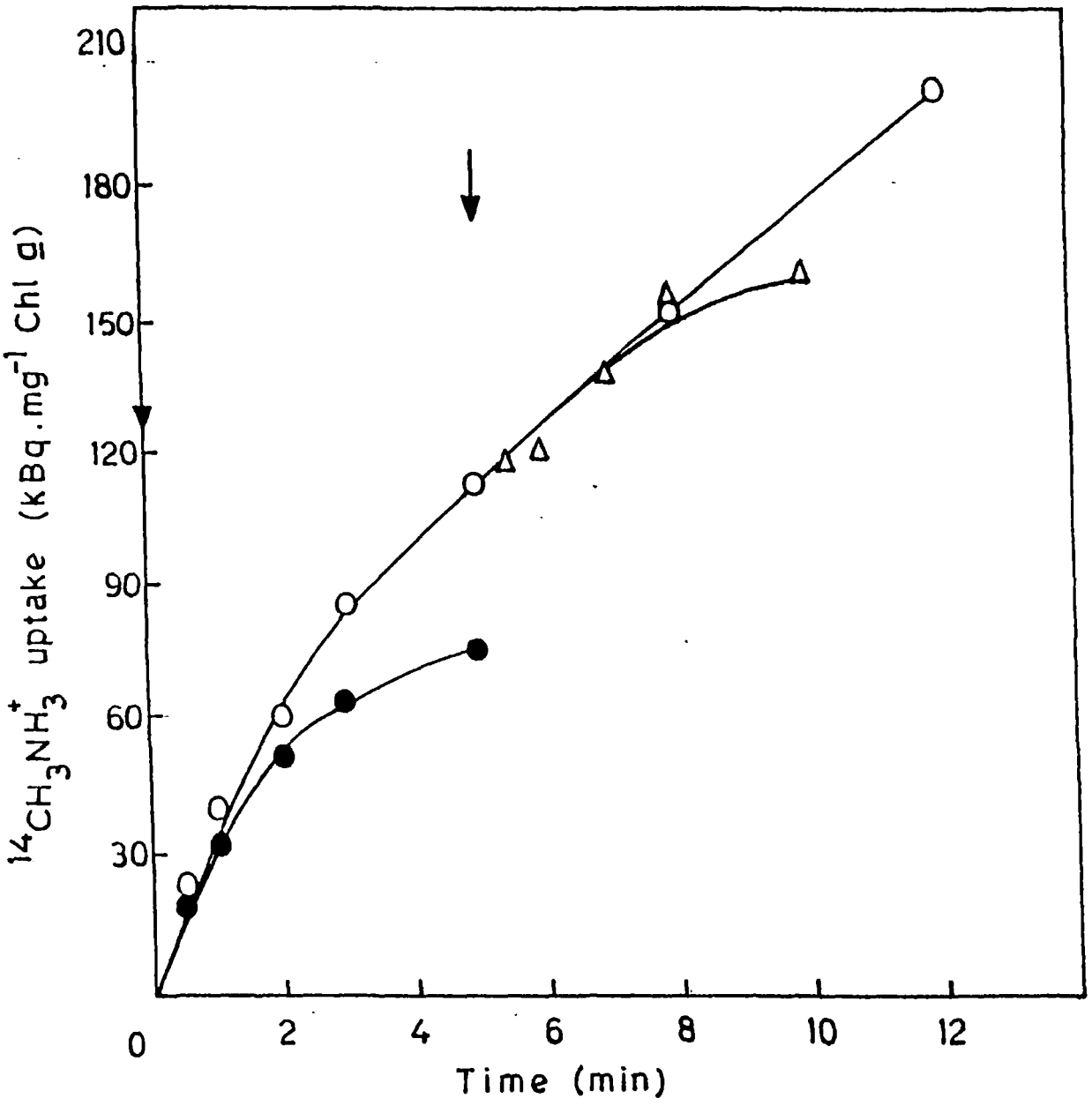

Fig 5.11 Effect of NH_4Cl on $^{14}CH_3NH_3^+$ uptake, at pH 7, by $CH_3NH_3^+$-grown *Nostoc* ANTH filaments. NH_4Cl (200 μmol.dm^{-3}) was added at times (arrows) indicated. O, control ($^{14}CH_3NH_3^+$ only); ●, NH_4Cl and $^{14}CH_3NH_3^+$ added simultaneously at zero time; Δ, NH_4Cl added 5 min after $^{14}CH_3NH_3^+$ addition.

carrier of $^{14}CH_3NH_3^+$ in $CH_3NH_3^+$-grown cells is a specific $CH_3NH_3^+$ carrier distinct from the $NH_4^+/CH_3NH_3^+$ carrier in N_2-grown cells.

The inhibition of $^{14}CH_3NH_3^+$ uptake by NH_4^+ after the initial 2 - 3 min may reflect the fact that NH_4^+ did not affect the $^{14}CH_3NH_3^+$ accumulation in $CH_3NH_3^+$-grown cells but affected the metabolism of the internal $^{14}CH_3NH_3^+$ because of it being the natural substrate for GS. This conclusion is also supported by the fact that: a) the $^{14}CH_3NH_3^+$ uptake in various cyanobacteria after 60 s is dependent on the metabolism of transported species (Rai *et al.*, 1984; Singh *et al.*, 1985b; 1986; 1987; Reglinski *et al.*, 1989), b) $CH_3NH_3^+$ and NH_4^+ are assimilated by the same enzyme in cyanobacteria (see chapter 3) and c) GS has a higher affinity for NH_4^+ than $CH_3NH_3^+$ because of the former being the natural substrate (see Kerby *et al.*, 1987). Such overall results suggested existence of a specific transport system for $CH_3NH_3^+$ in $CH_3NH_3^+$-grown *Nostoc* ANTH cells. These observations are similar to those observed in the $CH_3NH_3^+$ resistant cyanobacterium *A. variabilis* (Reglinski *et al.*, 1989).

5.3.3.3. Effect of MSX:

MSX was found to be an inhibitor of the second phase of $^{14}CH_3NH_3^+$ uptake in N_2-grown *Nostoc* ANTH (see Fig 5.3) and in *Anabaena* 7120 (see chapter 4). Therefore, the effect of MSX during $^{14}CH_3NH_3^+$ uptake in $CH_3NH_3^+$-grown *Nostoc* ANTH cells, at pH 7, was also investigated (Fig 5.12). MSX is a known inhibitor of GS (Stewart, 1980). To avoid MSX inhibition of $CH_3NH_3^+$ uptake via inhibition of GS and to ensure that the observed effects of MSX on $^{14}CH_3NH_3^+$ uptake, if any, were at the level of uptake, the experi-

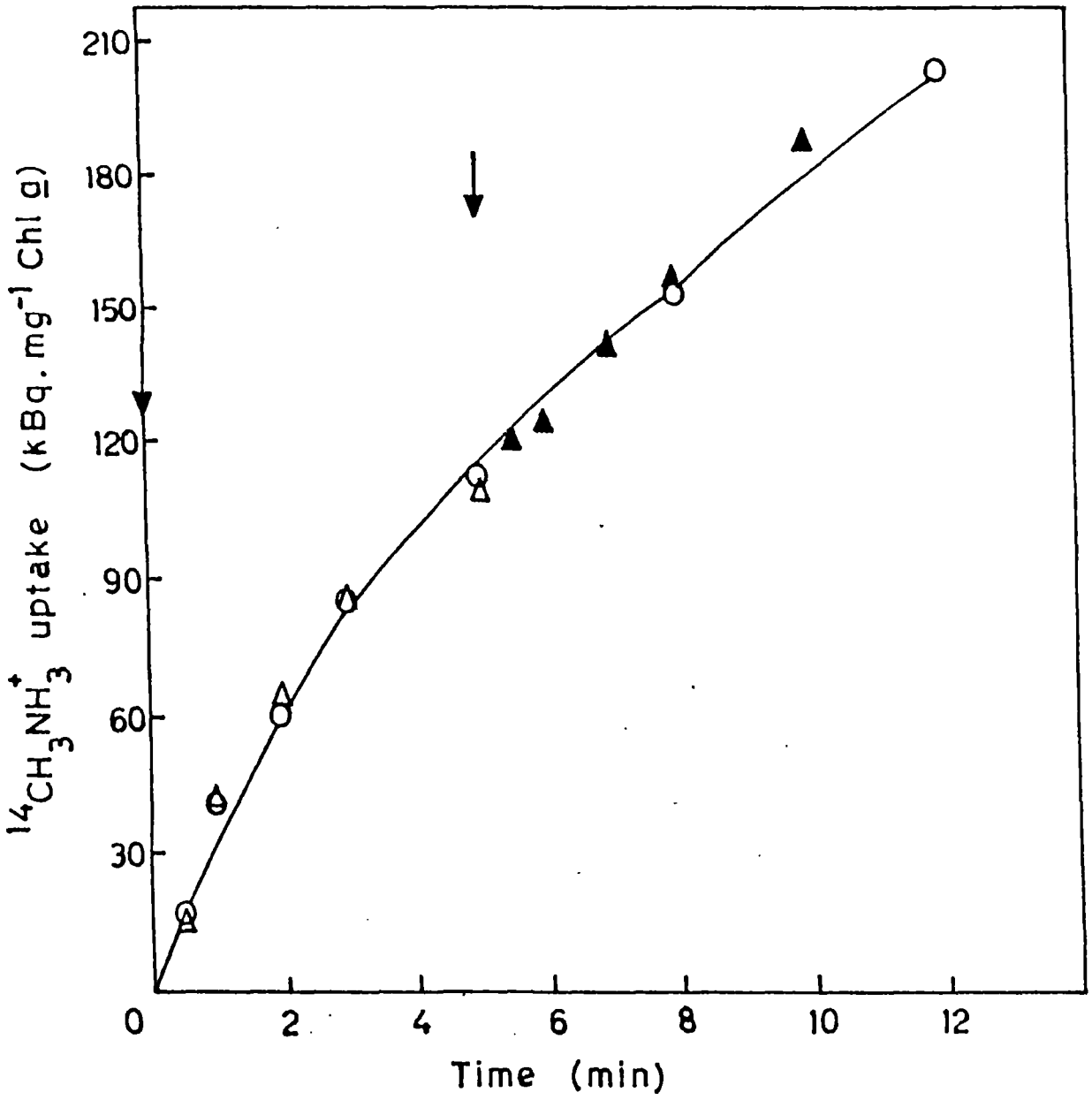

Fig 5.12 Effect of MSX on $^{14}CH_3NH_3^+$ uptake, at pH 7, by $CH_3NH_3^+$-grown *Nostoc* ANTH filaments. MSX (10 μmol.dm^{-3}) was added at times (arrows) indicated. O, control ($^{14}CH_3NH_3^+$ only); Δ, MSX and $^{14}CH_3NH_3^+$ added simultaneously at zero time; ▲, MSX added 5 min after $^{14}CH_3NH_3^+$ addition.

ments were conducted only for 12 min (inhibition of GS by MSX at a concentration of 10 $\mu mol.dm^{-3}$ was undetectable before 30 min).

Addition of 10 $\mu mol.dm^{-3}$ MSX at zero time did not affect $^{14}CH_3NH_3^+$ uptake in $CH_3NH_3^+$-grown cells. Addition of MSX, 5 min after $^{14}CH_3NH_3^+$ addition also gave similar results i.e. no inhibition of $^{14}CH_3NH_3^+$ uptake. This is in contrast to the findings in N_2-grown cells where MSX inhibited the second methylammonium/ammonium transport system.

These results further suggest that the $CH_3NH_3^+$ transport system in $CH_3NH_3^+$-grown cells is different from the methylammonium/ammonium transport systems in N_2-grown cells.

5.3.3.4. Effect of CCCP and $TPMP^+$:

Addition of CCCP caused a severe inhibition of $^{14}CH_3NH_3^+$ uptake in $CH_3NH_3^+$-grown cells (Fig 5.13). This is consistent with the finding in N_2-grown cells and indicate that the process is energy-dependent. $TPMP^+$ also caused a similar inhibition indicating that the process is dependent on transmembrane electrical potential.

5.3.4. $^{14}CH_3NH_3^+$ uptake at pH 9 in N_2-grown *Nostoc* ANTH cells:

Fig 5.14 show $^{14}CH_3NH_3^+$ uptake, at pH 9, in N_2-grown *Nostoc* ANTH. As at pH 7, a biphasic pattern of $^{14}CH_3NH_3^+$ uptake was also observed at pH 9. However, the $^{14}CH_3NH_3^+$ accumulation (first phase) was over 2 fold higher at pH 9 (40 $Bq.\mu g^{-1}$ Chl a higher than at pH 7; see Fig 5.14 and Fig 5.1). This may be explained by the fact that at pH 9 more methylamine occurs as uncharged species (CH_3NH_2) which can diffuse without need of the transport system

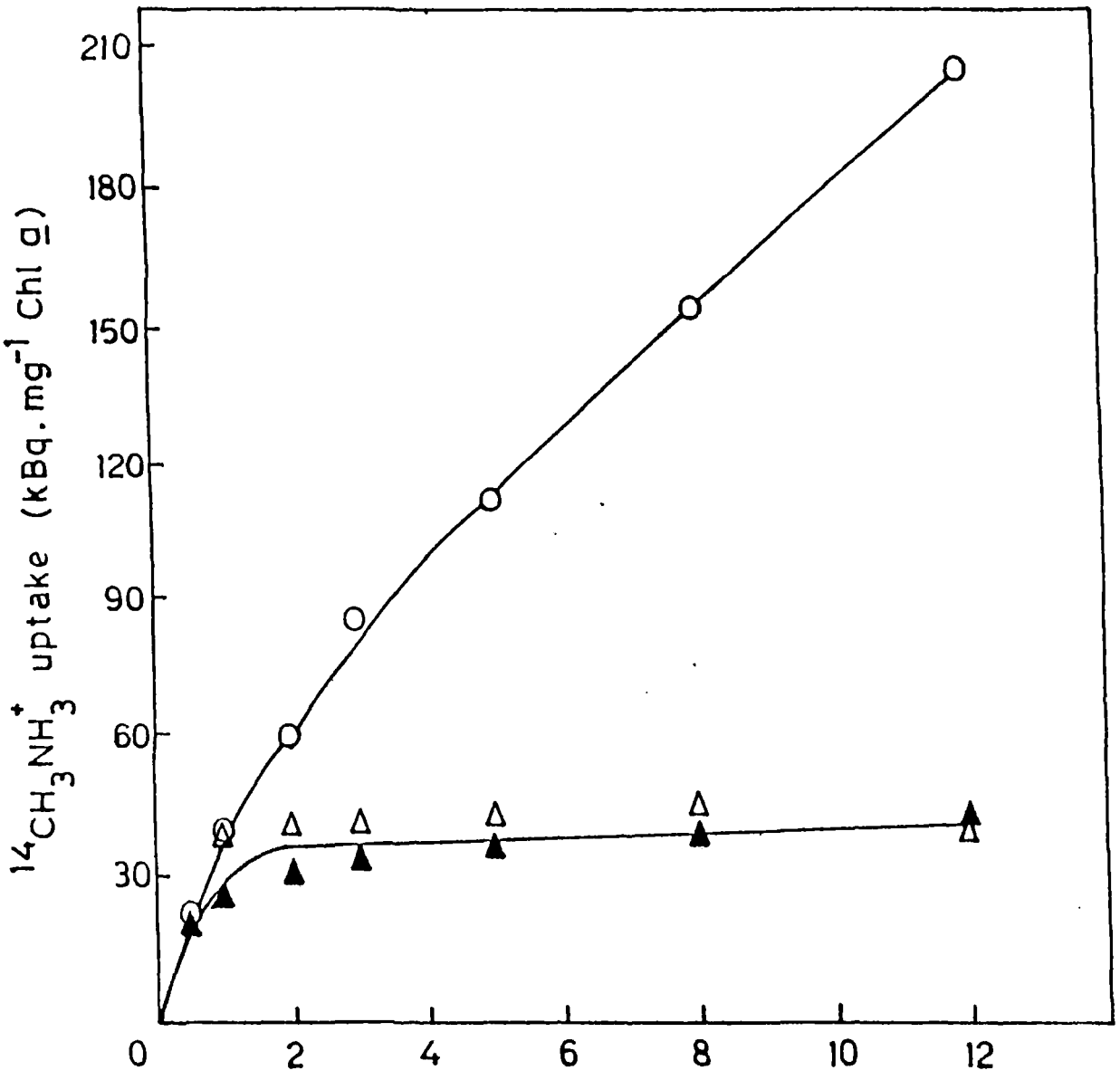

Fig 5.13 $^{14}CH_3NH_3^+$ uptake, at pH 7, by $CH_3NH_3^+$-grown *Nostoc* ANTH filaments in the presence (Δ, ▲) or absence (O) of CCCP (▲) and $TPMP^+$ (Δ). CCCP (10 μmol.dm^{-3}) and $TPMP^+$ (100 μmol.dm^{-3}) were added 30 min before $^{14}CH_3NH_3^+$ addition.

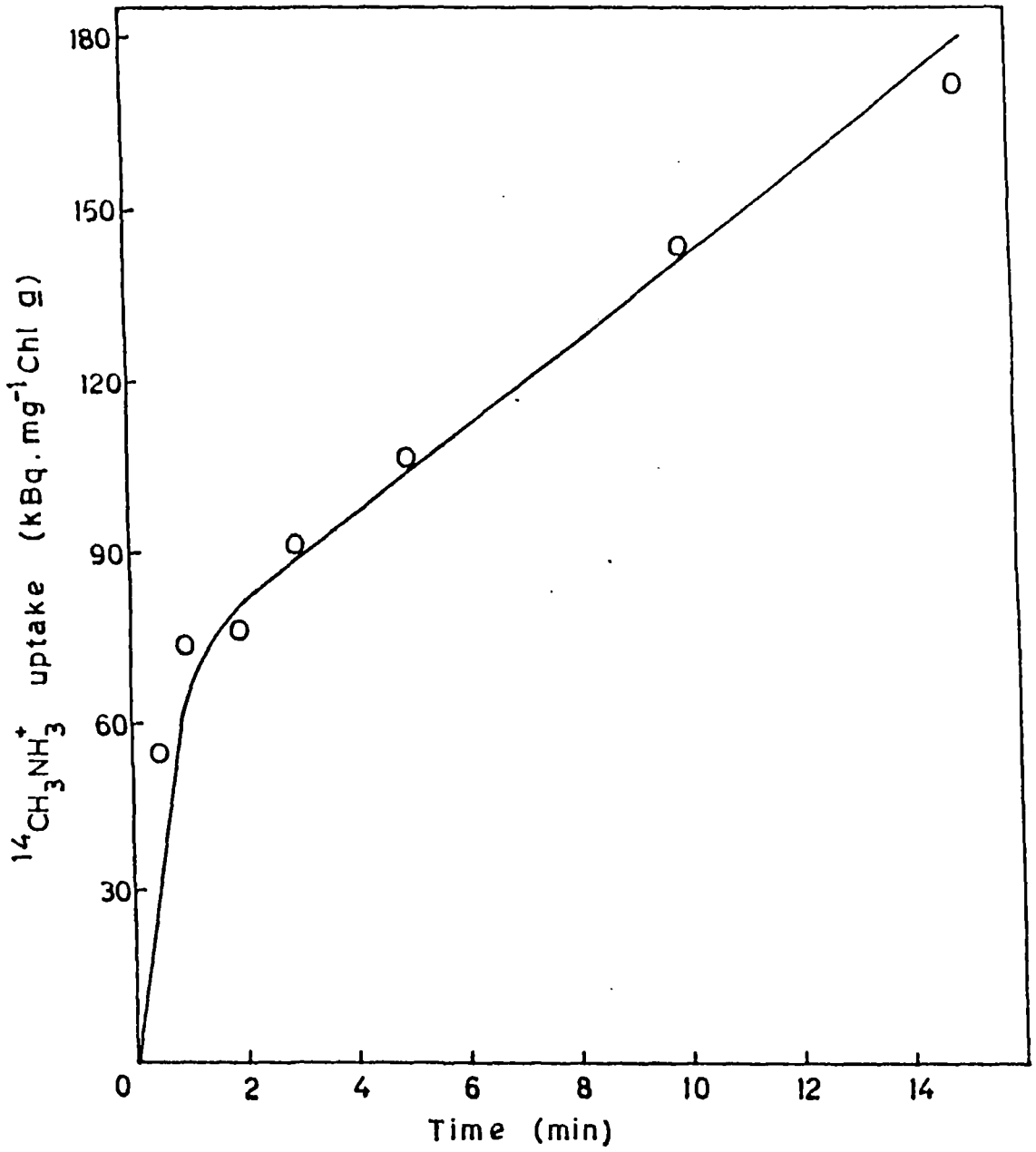

Fig 5.14. $^{14}CH_3NH_3^+/^{14}CH_3NH_2$ uptake, at pH 9, by N_2-grown *Nostoc* ANTH filaments.

and get trapped in the cell by protonation. The uptake during the second phase, at pH 9, was also higher than that at pH 7. This may reflect a higher level of metabolism of the intracellular $CH_3NH_3^+$ via GS due to higher level of $CH_3NH_3^+$ entry into the cells.

Addition of NH_4^+ during $^{14}CH_3NH_3^+$ uptake at pH 9 caused an efflux of preaccumulated $^{14}CH_3NH_3^+$ (Fig 5.15). However, the amount effluxed was far less than the $^{14}CH_3NH_3^+$ accumulated during the first phase. This contrasts with the finding at pH 7 where NH_4^+ caused total efflux of the preaccumulated $^{14}CH_3NH_3^+$ during the first phase (Fig 5.2). When a pH shift, from pH 9 to pH 7, was induced simultaneously with addition of NH_4^+ there was a total efflux of preaccumulated $^{14}CH_3NH_3^+$ from the cells. Probably, the intracellular $CH_3NH_3^+$ pool was in two compartments, one of which was displaced by NH_4Cl while the other by the pH shift.

NH_4^+ at pH 9, as well as after the shift from pH 9 to 7, apart from causing efflux of ^{14}C-label from the cells, also caused an inhibition of further $^{14}CH_3NH_3^+$ uptake (Fig 5.14 & 5.15). Inhibition of $^{14}CH_3NH_3^+$ uptake in the presence of NH_4^+ can be easily explained by the fact that NH_4^+ is the natural substrate and therefore preferred over $CH_3NH_3^+$.

5.3.4.1. **Effect of MSX:**

Addition of 10 $\mu mol.dm^{-3}$ MSX after $^{14}CH_3NH_3^+$ addition resulted in a transient efflux of ^{14}C-label from the cells followed by continued $^{14}CH_3NH_3^+$ uptake at a rate similar to that in control cells (Fig 5.16). This ^{14}C-displacement by MSX was similar to that found at pH 7 (Fig 5.3). However, the continued uptake of $^{14}CH_3NH_3^+$ at pH 9 in presence of MSX was in contrast to

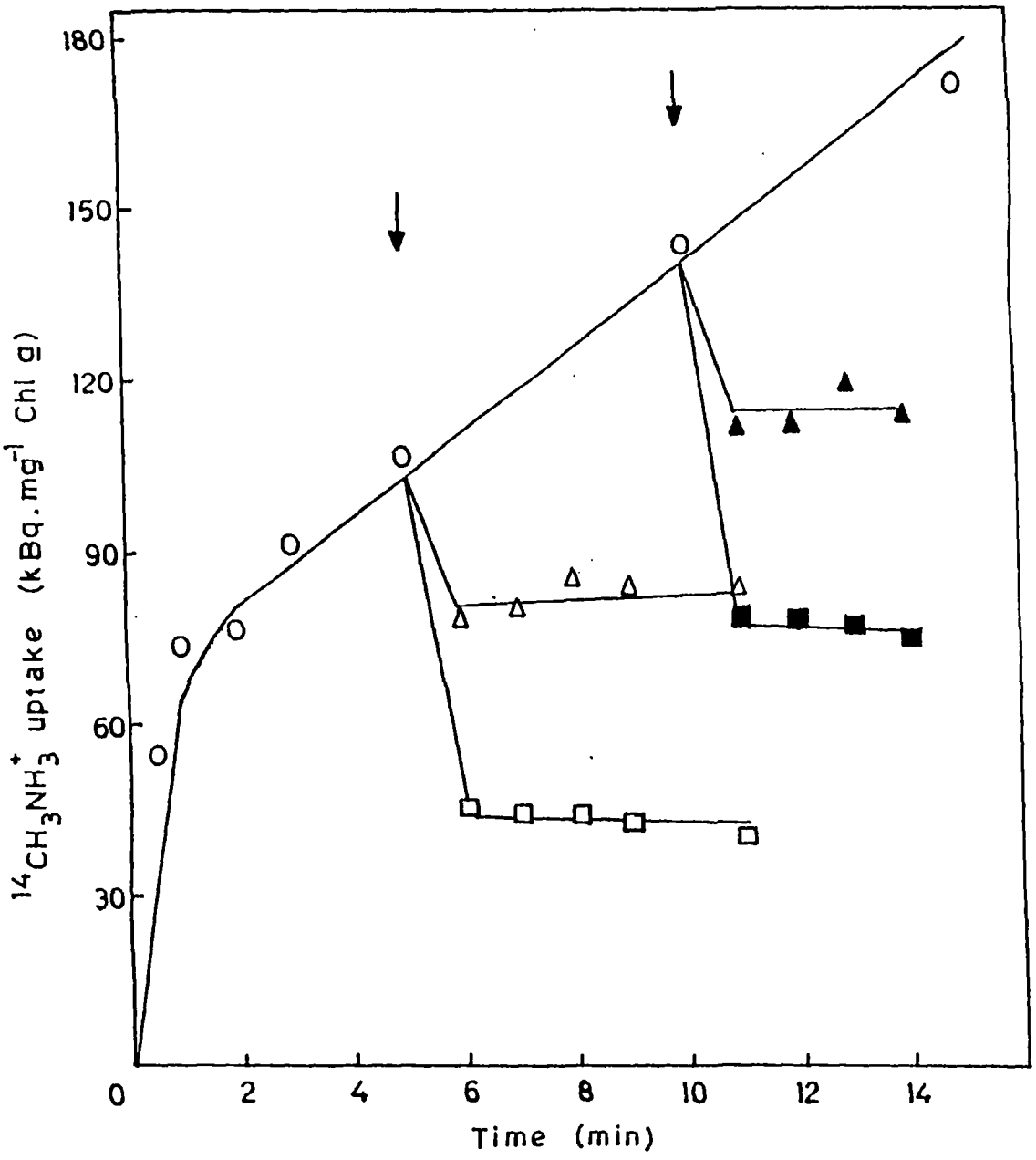

Fig 5.15 Effect of NH_4Cl and NH_4Cl + pH shift (from pH 9 to 7) on $^{14}CH_3NH_3^+/^{14}CH_3NH_2$ uptake, at pH 9, by N_2-grown *Nostoc* ANTH filaments. NH_4Cl and was added at times indicated (arrows) to a final concentration of 200 $\mu mol.dm^{-3}$. pH shift was achieved by addition of 4.5 mm^3 of HCl to 1 cm^3 of cell suspension at pH 9 to bring it down to pH 7. O, control ($^{14}CH_3NH_3$ only); Δ, NH_4Cl added 5 min after $^{14}CH_3NH_3^+$ addition; ▲, NH_4Cl added 10 min after $^{14}CH_3NH_3^+$ addition; □, NH_4Cl + HCl added 5 min after $^{14}CH_3NH_3^+$ addition; ■, NH_4Cl + HCl added 10 min after $^{14}CH_3NH_3^+$ addition.

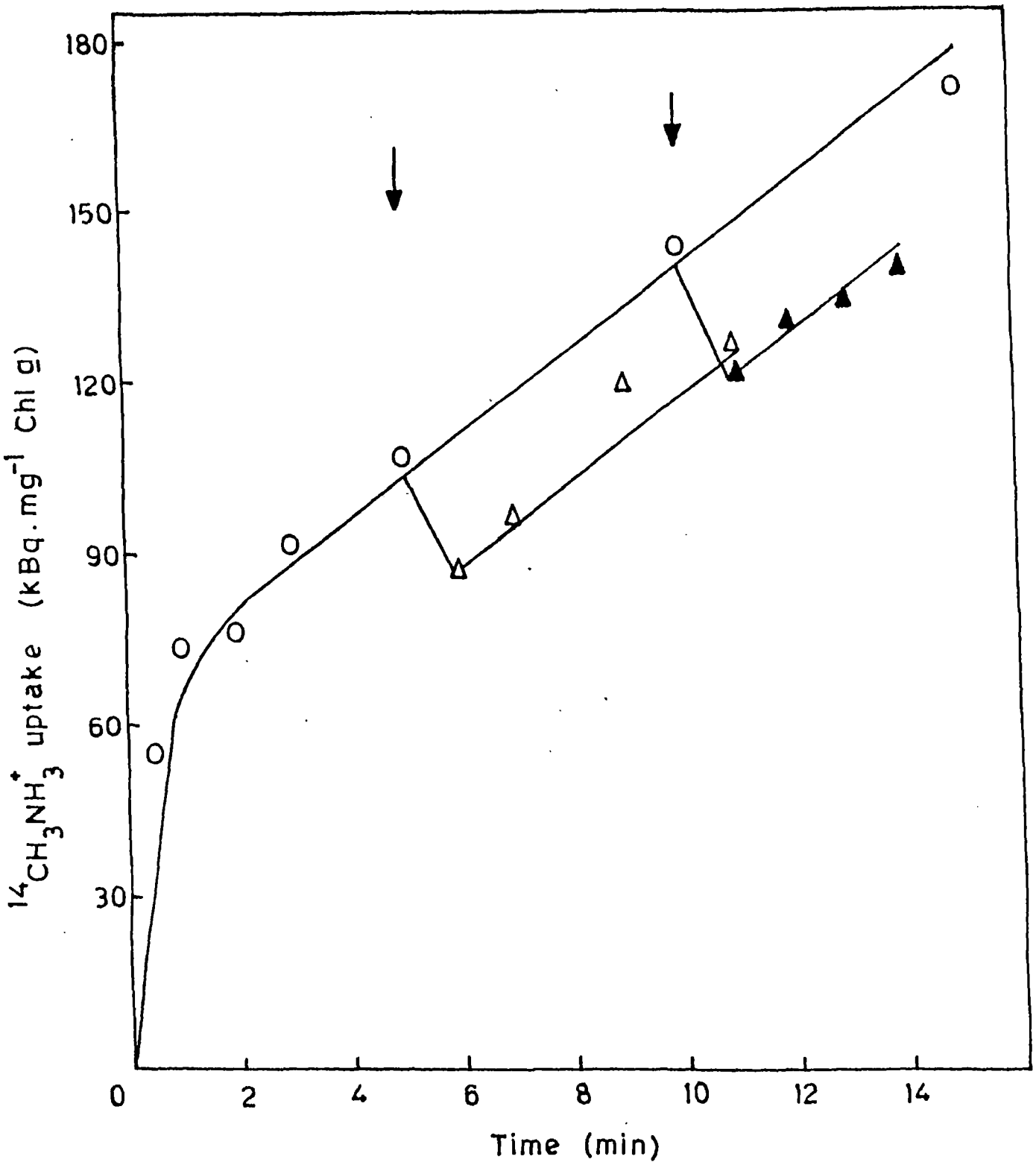

Fig 5.16 Effect of MSX on $^{14}CH_3NH_3^+/^{14}CH_3NH_2$ uptake, at pH 9, by N_2-grown *Nostoc* ANTH filaments. MSX (10 μmol.dm^{-3}) was added at time (arrows) indicated. O, control ($^{14}CH_3NH_3^+$ only); Δ, MSX added 5 min after $^{14}CH_3NH_3^+$ addition. ▲, MSX added 10 min after $^{14}CH_3NH_3^+$ addition.

the finding at pH 7 where MSX inhibited $^{14}CH_3NH_3^+$ uptake at the transport level (Fig 5.3). This may again reflect the fact that unlike at pH 7 where the $^{14}CH_3NH_3^+$ uptake was carrier mediated, at pH 9 much of the uptake occurred via diffusion. The diffused CH_3NH_2 was metabolized in the cells since MSX, during the short experimental period, did not affect GS.

5.3.4.2. Effect of CCCP and $TPMP^+$:

Addition of CCCP or $TPMP^+$ to N_2-grown *Nostoc* ANTH cells, at pH 9 did not change the $^{14}CH_3NH_3^+$ uptake pattern and accumulation rates (Fig 5.17). Such results indicate that the $^{14}CH_3NH_3^+$ uptake at pH 9, in this cyanobacterium is an energy-independent process. These results are similar to those observed in other cyanobacteria (Kerby *et al.*, 1986; Boussiba, 1989; Reglinski *et al.*, 1989; Rai and Prakasham, 1989).

5.4. DISCUSSION:

The $^{14}CH_3NH_3^+$ uptake studies presented in this chapter show that, as in *Anabaena* 7120 (see chapter 4; Rai and Prakasham, 1989), N_2-grown cells of *Nostoc* ANTH possess two methylammonium/ ammonium transport systems (one MSX-insensitive fast ATS and the other MSX-sensitive slower ATS). The arguments for existence of these two ATS are the same as those discussed in chapter 4. In addition, the differential effects of glutamine, glutamate and mode of C-nutrition (Fig 5.7, 5.8 and 5.9) on the two phases of $^{14}CH_3NH_3^+$ uptake also suggest that the biphasic $^{14}CH_3NH_3^+$ uptake pattern observed is a reflection of two distinct methylammonium/

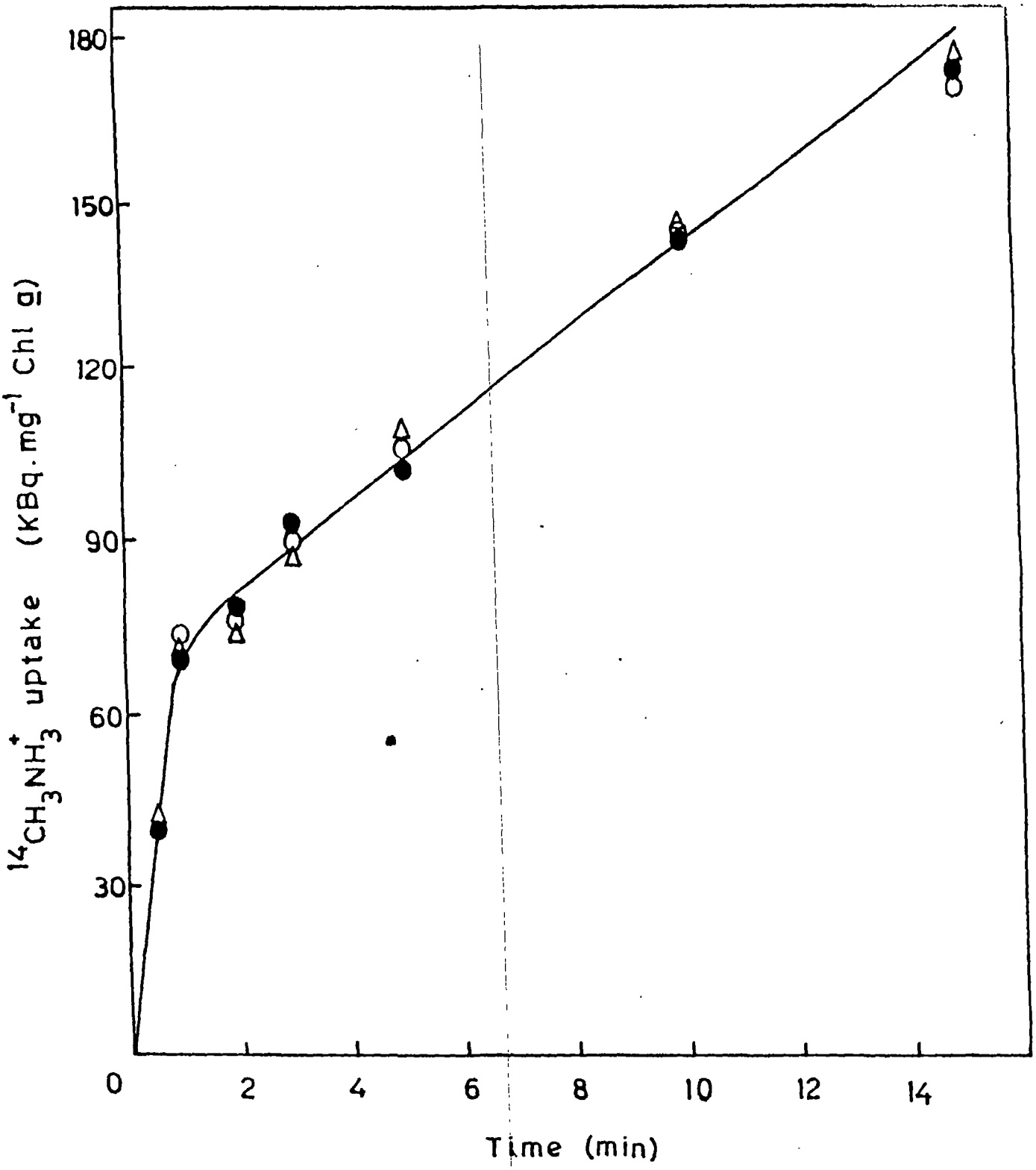

Fig 5.17 $^{14}CH_3NH_3^+/^{14}CH_3NH_2$ uptake, at pH 9, by N_2-grown *Nostoc* ANTH filaments in the presence (Δ , ▲) or absence (O) of $TPMP^+$ (Δ) and CCCP (▲). $TPMP^+$ (100 μmol.dm^{-3}) and CCCP (10 μmol.dm^{-3}) were added 30 min before $^{14}CH_3NH_3^+$ addition.

ammonium transport systems. Most characteristics of these ATS are similar to those of *Anabaena* 7120. However, some differences were also evident. First, unlike the case in *Anabaena* 7120 where $TPMP^+$ totally blocked $^{14}CH_3NH_3^+$ transport, in *Nostoc* ANTH cells only a partial inhibition of $^{14}CH_3NH_3^+$ uptake was observed in the presence of $TPMP^+$ (Fig 5.6). Thus, while $^{14}CH_3NH_3^+$ transport in *Anabaena* 7120 was wholly dependent on transmembrane electrical potential, the uptake in *Nostoc* ANTH was only partially dependent on transmembrane electrical potential. Second, while MSX did not cause any efflux of ^{14}C-label from *Anabaena* 7120 cells, in *Nostoc* ANTH cells MSX did cause such an efflux either by displacing the intracellular $^{14}CH_3NH_3^+$ pool or a metabolized product of $CH_3NH_3^+$. Third, in *Anabaena* 7120 the shift to lower affinity mode showed an increase in Vmax values for the second ATS while in *Nostoc* Vmax decreased.

Another distinctive feature of methylammonium/ammonium transport system in *Nostoc* ANTH was the fact that glutamine-grown cells still possessed the two methylammonium/ammonium transport systems. That is glutamine was not a repressor for methylammonium/ammonium transport system in *Nostoc* ANTH. In contrast glutamine has been reported to be a repressor of methylammonium/ammonium transport system in *A. cycadeae* (Singh *et al.*, 1987).

In *Anabaena* 7120 existence of two intracellular $CH_3NH_3^+$ pools was argued based on the $^{14}CH_3NH_3^+$ uptake and metabolism studies (see chapter 4; Rai and Prakasham, 1989). The same arguments apply here too. In addition, the results of $^{14}CH_3NH_3^+$ uptake studies at pH 9 further strengthen the arguments for two intracellular pools of $CH_3NH_3^+$ (see Fig 5.15). At pH 9 NH_4^+ could only partially

displace the intracellular pool of $CH_3NH_3^+$. However, NH_4^+ together with a pH shift from 9 to 7 resulted in a total efflux of the intracellular free $CH_3NH_3^+$ (see Fig 5.15 and the relevant result section).

In addition to the methylammonium/ammonium transport systems, a specific methylammonium transport system was found in $CH_3NH_3^+$-grown *Nostoc* ANTH cells. That this methylammonium transport system was specific for $CH_3NH_3^+$ and that it did not transport NH_4^+ was concluded from the fact that:

1) Unlike the $CH_3NH_3^+$ accumulation by methylammonium/ammonium transport system (Fig 5.2), the $CH_3NH_3^+$ accumulation by the methylammonium transport system was not inhibited by addition of ammonium (Fig 5.11), and
2) In contrast to the observations in N_2-grown cells (Fig 5.2), addition of NH_4^+ did not cause efflux of the preaccumulated $CH_3NH_3^+$ from $CH_3NH_3^+$-grown cells (Fig 5.11).

The methylammonium transport system in $CH_3NH_3^+$-grown cells had the following characteristics:

1) $CH_3NH_3^+$ transport through methylammonium transport system was an energy-dependent process, driven by transmembrane electrical potential as shown by inhibition of $CH_3NH_3^+$ uptake by CCCP (an uncoupler) and $TPMP^+$ (an agent causing collapse of transmembrane electrical potential) (Fig 5.13).
2) Unlike the observation in N_2-grown cells where MSX inhibited the Methylammonium/ammonium uptake during the second phase, $CH_3NH_3^+$ uptake by methylammonium transport system was unaffected by MSX (Fig 5.12). That is, this methylammonium transport system was MSX-insensitive.

3) While methylammonium/ammonium transport system are known to be repressed by excess availability of its own substrate: ammonium (Rai *et al.*, 1986b; Rai and Prakasham, 1989; see also Fig 5.2), methylammonium transport system was not repressed in cells grown on $CH_3NH_3^+$.

The methylammonium transport system seems to be an inducible system since it developed only in $CH_3NH_3^+$-grown cells. This is supported by the fact that:

1) In NH_4^+-grown cells no $CH_3NH_3^+$ uptake occurred, and
2) In N_2-grown cells $CH_3NH_3^+$ uptake occurred only through methylammonium/ammonium transport systems. If the specific methylammonium transport system was operative in N_2-grown cells then NH_4^+ should not have caused total inhibition of $CH_3NH_3^+$ uptake and total efflux of pre-accumulated $CH_3NH_3^+$ in such cells.

Overall, the data show that like other cyanobacteria, N_2-grown cells of *Nostoc* ANTH possess two energy-dependent methylammonium/ammonium transport systems. Both these methylammonium/ammonium transport systems show affinity modulation in response to external substrate concentration. A specific methylammonium transport system was found to be induced in $CH_3NH_3^+$-grown cells of *Nostoc* ANTH. This is the first report of existence of a specific methylammonium transport system in cyanobacteria so far, except in a $CH_3NH_3^+$-resistant mutant strain of *A. variabilis* (Reglinski *et al.*, 1989).

6. GLUTAMATE UPTAKE AND METABOLISM IN *ANABAENA* 7120

6.1. INTRODUCTION:

Glutamate and glutamine are known to serve as nitrogen donors for biosynthetic reactions leading to the production of almost all cellular nitrogenous compounds. Enteric bacteria generate glutamine by glutamine synthetase and glutamate by glutamate dehydrogenase or glutamate synthase (Ninfa *et al.*, 1986). Modulation of enteric bacterial N-metabolism as a function of N-source is known to operate under the well defined control system of *ntr* genes (Reitzer and Magasanik, 1986). In heterocystous cyanobacteria glutamate is produced by glutamate synthase and glutamine by glutamine synthetase (Haselkorn, 1978; Stewart, 1980). Repression-derepression or induction system of control is one known type of nitrogen control functioning in regulation of cyanobacterial nitrogen assimilation (Bagchi *et al.*, 1985a; 1985b). The other known type of nitrogen control involved in modulating nitrogen dependent expression of *gln* A gene in *Anabaena* 7120 is mediated by a novel cyanobacterial RNA-polymerase sigma factor (Tumer *et al.*, 1983). Much more studies on the possible range of cyanobacterial nitrogen sources are needed to understand clearly the nature and types of molecular control regulating cyanobacterial nitrogen assimilation.

There are very few studies on amino acids as sole N- sources and the findings are variable as well as conflicting (Neilson and Larson, 1980; Vaishampayan, 1982). Glutamate has been found growth

toxic in *Anabaena variabilis* (Chapman and Meeks, 1983) and growth stimulatory in *Anabaena cylindrica* PCC 7122 (Rawson, 1985). Glutamine synthetase mutants of *Anabaena cycadeae* (glutamine auxotrophs) are capable of utilizing glutamine as sole N-source (Singh *et al.*, 1985b). In the present chapter, using a class of non N_2-fixing (Nif^-), non heterocystous (Het^-) mutant of *Anabaena* **7120** and its parent, the role of glutamine and glutamate as sole N-source was studied. The evidences show that glutamate is not metabolized like a N-source, but acts as an inhibitor of *in vivo* nitrogenase activity and diazotrophic growth, that NO_3^- availability eliminates growth inhibition by glutamate by inhibiting glutamate uptake, and that glutamine is utilized like a fixed N-source. Association of glutamate toxicity with nitrogenase activity leading to the inhibition of diazotrophic growth suggests this to be a possible reason for vegetative cell localization, and not heterocyst localization, of glutamate synthase, the enzyme catalyzing glutamate production during growth with N_2 as N-source.

6.2. MATERIALS AND METHODS:

6.2.1. Organisms and growth conditions:

Axenic cultures of *Anabaena* **7120** parent (Het^+, Nif^+) and Het^- Nif^- mutant were grown in 5 $mmol.dm^{-3}$ NO_3^- supplemented $BG\text{-}11_o$ medium (Rippka *et al.*, 1979), unless otherwise stated, at 28 ± 1°C and at a photon fluence rate of 50 $\mu mol.m^{-2}.s^{-1}$. Growth was measured by measuring O.D. at 663 nm.

6.2.2. Estimation of chlorophyll and nitrogenase activity:

Chl **a** concentration was measured according to Mackinney (1941). Nitrogenase activity was measured using acetylene reduction assay (Stewart *et al.*, 1967).

6.2.3. Measurement of glutamate uptake:

Exponentially growing cells were centrifuged, washed and resuspended in 10 $mmol.dm^{-3}$ HEPES-NaOH buffer, pH 7, and equilibrated for 30 min at 28°C and at a photon fluence rate of 50 $\mu mol.m^{-2}.s^{-1}$. ^{14}C-labelled glutamate was then added to a final concentration of 50 $\mu mol.dm^{-3}$ (specific activity 185 $kBq.\mu mol^{-1}$) and at time intervals 400 mm^3 samples were taken and cells separated from their bathing medium using oil microcentrifugation technique (Scott and Nicholls, 1980). ^{14}C-incorporation was determined using Beckman Liquid Scintillation Spectrometer LS 1801. When needed NO_3^- was added to a final concentration of 5 $mmol.dm^{-3}$ in the assay medium. Non-specific binding of ^{14}C-glutamate was determined by measuring its incorporation in toluene treated cells as described by Rai *et al.* (1984).

6.2.4. Calculation heterocyst frequency:

Heterocyst frequency was calculated as percentage of total cells, by light microscope observation of the filaments of *Anabaena* 7120 .

6.2.5. Estimation of glutamate dehydrogenase activity:

Glutamate dehydrogenase activity was measured as described by Stewart and Rowell (1977).

6.2.6. Estimation of protein concentration:

Protein was estimated by Lowry method (Lowry *et al.*, 1951).

6.2.7. Chemicals:

^{14}C-glutamate was purchased from BARC, Bombay, India. Silicon oil DC 550 and dinonylphthalate were obtained from Fluka AC, Buchs, Switzerland. All other chemicals were obtained from Sigma Chemical Company, U.S.A.

6.3. RESULTS:

As shown in Table 6.1 parent strain produced heterocyst and nitrogenase activity in N_2-medium and also grew reasonably well at the expense of N_2 as sole N-source, while its mutant strain neither produced heterocyst and nitrogenase activity nor grew at the expense of N_2 under such conditions. Growth of parent strain in NO_3^--medium was accompanied by absence of heterocyst and nitrogenase activity. The mutant strain grew nearly as well as its parent in NO_3^--medium, thus suggesting the two strains to be almost similar in using nitrate as N-source. Both strains also grew almost equally well in glutamine-medium where the parental strain produced non heterocystous, non N_2-fixing filaments as it did in NO_3^--medium. The ability of Het^- Nif^- mutant strain to grow in glutamine-medium provides evidence for glutamine to be utilized as a sole N-source for the cyanobacterial growth. The growth of the parent in glutamine-medium without producing heterocyst and nitrogenase is a further evidence for utilization of glutamine as N-source in *Anabaena* 7120. In contrast, glutamate failed to

Table 6.1.

Growth (O.D. at 663 nm after 6 days of inoculation), heterocyst frequency (%) and nitrogenase activity (nmol C_2H_2 reduced.h^{-1}.μg^{-1} Chl **a**) of parent (Het^+ Nif^+) and mutant (Het^- Nif^-) strains of **Anabaena** 7120 in different nitrogen media.

Nitrogen medium	Parent strain			Mutant strain		
	Growth	Heterocyst frequency	Nitrogenase activity	Growth	Heterocyst frequency	Nitrogenase activity
N_2 medium ($BG\text{-}11_o$)	0.68	5-6	12.6	0.00	0.00	0.00
$BG\text{-}11_o$ + 5 mmol.dm^{-3} KNO_3	0.85	0.0	00.0	0.82	0.00	0.00
$BG\text{-}11_o$ + 2 mmol.dm^{-3} glutamine	0.75	0.0	00.0	0.78	0.00	0.00
$BG\text{-}11_o$ + 1 mmol.dm^{-3} glutamate	0.00	0.0	00.0	0.00	0.00	0.00
$BG\text{-}11_o$ + 5 mmol.dm^{-3} KNO_3 + 1 mmol.dm^{-3} glutamate	0.72	0.0	00.0	0.68	0.00	0.00

support the growth of Het$^-$ Nif$^-$ mutants thereby suggesting lack of the ability in the mutant strain to metabolize glutamate as N-source. Addition of NO_3^- to the glutamate-medium resulted in recovery of the cyanobacterial mutant growth to as good a level as that obtained in glutamine-medium. This further suggested that absence of growth of the mutant strain in the glutamate-medium was due to the cyanobacterial inability to metabolize glutamate as N-source. The implication of this observation is that cyanobacterial mutant strain lacks the catabolic glutamate dehydrogenase which is essential for utilization of glutamate as N-source. Indeed both parent and mutant had very low NADH-dependent glutamate dehydrogenase activity (<0.1 nmol product formed.min^{-1}. mg^{-1} protein).

Parent strain showed inhibition of nitrogenase activity and diazotrophic growth in glutamate-medium however, availability of NO_3^- in the glutamate-medium helped the recovery of parental growth like that of mutant growth. Evidently glutamate inhibition of diazotrophic growth seems to result from glutamate inhibition of nitrogenase activity. The occurrence of parental growth in glutamate containing NO_3^--medium suggest that NO_3^- relief of glutamate toxicity is the result of either NO_3^- mediated elimination of glutamate sensitive nitrogenase activity or NO_3^- inhibition of glutamate uptake or both. To check whether NO_3^- effects the glutamate uptake, ^{14}C-glutamate uptake was studied in N_2- and NO_3^--grown cultures. As shown in Fig 6.1 both the parent and mutant strain showed active biphasic system of glutamate uptake under N-limited/starved growth conditions. In the N_2-grown parent strain the ^{14}C-glutamate uptake rates were 0.075 and 0.030

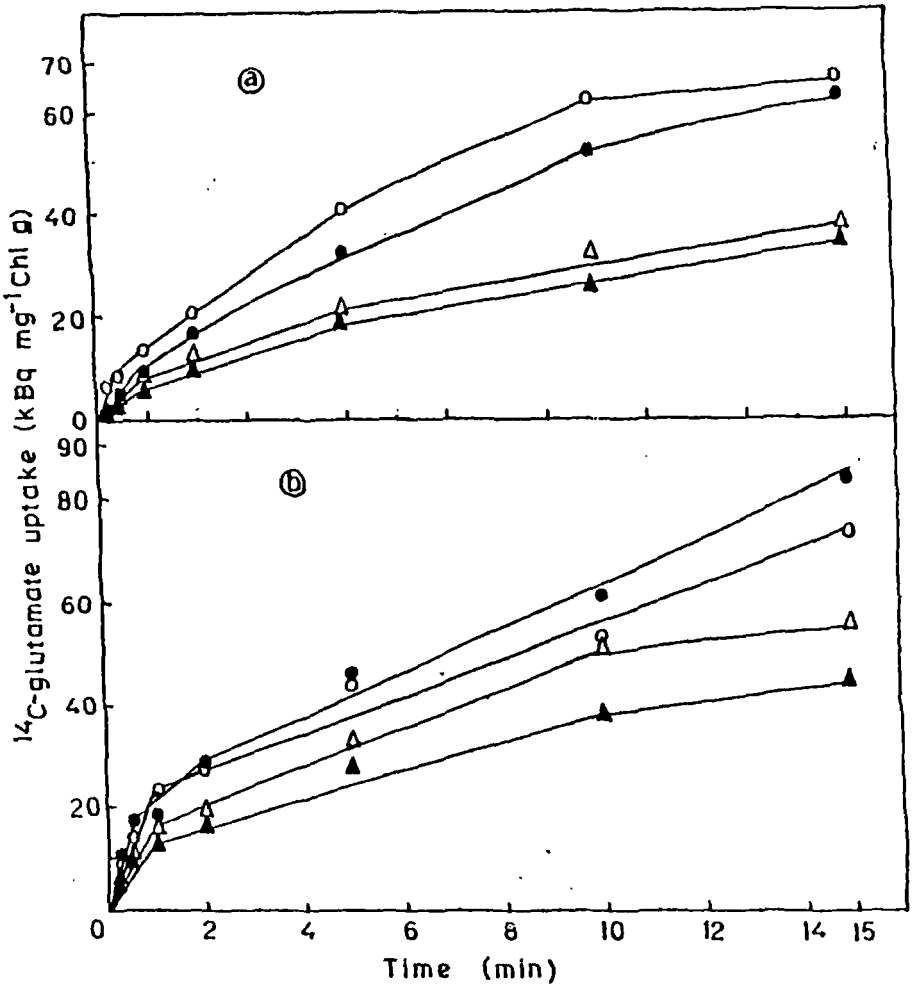

Fig 6.1 Glutamate uptake in *Anabaena* 7120(a) and its Het$^-$ Nif$^-$ mutant strain (b). O, N_2-grown cells, ^{14}C-glutamate uptake in absence of 5 mmol.dm^{-3} KNO_3; ●, N_2-grown cells, ^{14}C-glutamate uptake in presence of 5 mmol.dm^{-3} KNO_3; △, NO_3-grown cells, ^{14}C-glutamate uptake in absence of 5 mmol.dm^{-3} KNO_3; ▲, NO_3-grown cells, ^{14}C-glutamate uptake in presence of 5 mmol.dm^{-3} KNO_3.

In Fig 1b, the rates of ^{14}C-glutamate uptake in "N_2-grown cells" refers to the nitrate-grown cells which were subjected to N-starvation for 24 h before measuring ^{14}C-glutamate uptake; this is beoause the mutant does not grow in N_2-medium.

nmol.min^{-1}.μg^{-1} Chl **a** during the initial- and second phase, respectively. Since the mutant strain did not grow in N_2-medium, for a comparable study, the NO_3^--grown cultures of the mutant were N-starved for 24 hours and then ^{14}C-glutamate uptake was measured. The ^{14}C-glutamate uptake in such cells was 0.12 and 0.02 nmol. min^{-1}.μg^{-1} Chl **a**, during the first- and second phase of uptake, respectively. The observed ^{14}C-glutamate uptake rates, in NO_3^--grown cultures, were 0.0459 and 0.0115 nmol.min^{-1}.μg^{-1} Chl **a** in the parent; and 0.0864 and 0.0154 nmol.min^{-1}.μg^{-1} Chl **a** in the mutant, during first- and second phase, respectively. Thus, NO_3^--grown cultures of both strains showed almost 50% reduction in the level of glutamate uptake. Presence of NO_3^- in the reaction mixture did not inhibit significantly the process of glutamate uptake (Fig 6.1). It is therefore, concluded that the inhibitor of glutamate uptake is not NO_3^- itself but a metabolic product of it. Since the inhibitor of heterocyst formation and nitrogenase activity is also a product of NO_3^- metabolism (Bagchi and Singh, 1984) one is tempted to think of some physiological connection between NO_3^- mediated inhibition of heterocyst and nitrogenase and NO_3^- mediated inhibition of glutamate uptake. The glutamate toxicity to N_2-fixing culture is certainly further suggestive of non utilization of this amino acid as N-source. It is thus concluded that glutamate can not serve as N-source in this cyanobacterium and glutamate inhibition of nitrogenase activity is possibly the consequence of the sensitivity of the cyanobacterial N_2-fixing process. NO_3^- relief of glutamate inhibition appears to be the result of NO_3^- inhibition of glutamate uptake.

6.4. DISCUSSION:

During diazotrophic growth, heterocystous cyanobacteria assimilate N_2 into glutamine by the sequential action of nitrogenase and glutamine synthetase within the heterocyst and the glutamine thus produced in heterocyst is then transported to adjacent vegetative cells where it is utilized for the synthesis of glutamate by glutamate synthase (Thomas *et al.*, 1977; Rai *et al.*, 1984). The physiological significance of localization of glutamate synthase in vegetative cells, and not in heterocysts, has so far remained largely unexplained. The present finding of glutamate inhibiting heterocyst located cyanobacterial nitrogenase activity leading to inhibition of diazotrophic growth suggests that localization of glutamate forming enzyme in vegetative cells away from heterocyst, the site of N_2-fixation, is the biological strategy involved to avoid glutamate inhibition of N_2-fixation in heterocystous forms. Cyanobacterial glutamine synthetase has two functions, one in assimilation of ammonia as N-source and the other associated with production of glutamine which has been found to function both as a sole N-source as well as a source of glutamine for protein formation (Bagchi and Singh, 1984). Growth of parent *Anabaena* **7120** in glutamine medium without producing heterocysts and nitrogenase activity and of Het^- Nif^- mutant strain clearly suggest that *Anabaena* **7120** can assimilate glutamine as sole N-source. Glutamate can be expected to serve as N-source provided it is degraded to ammonia by catabolic glutamate dehydrogenase needed for synthesis of glutamine by glutamine synthetase. Lack of this activity would preclude utilization of glutamate as N-source. These results suggest

possible lack of catabolic glutamate dehydrogenase to be the reason for non utilization of glutamate as N-source.

Since nitrogenase activity is the glutamate sensitive target and since NO_3^- assimilatory cultures do not show nitrogenase activity, NO_3^- inhibition of nitrogenase activity appears to be one reason for the observed NO_3^- elimination of glutamate toxicity. But since NO_3^- metabolism also results in 50% reduction in glutamate uptake, it could as well be that inhibition of glutamate toxicity in the mutant is the result of reduction in glutamate uptake by NO_3^- metabolizing cultures. It could also be possible that NO_3^- inhibition of nitrogenase and NO_3^- inhibition of glutamate uptake both contribute to the observed NO_3^- elimination of glutamate toxicity in this cyanobacterium.

7. GLUTAMINE UPTAKE IN *ANABAENA* 7120 AND *NOSTOC* ANTH

7.1. INTRODUCTION:

In diazotrophic cyanobacteria, glutamine is an assimilatory product of ammonia which is produced by the action of glutamine synthetase enzyme (Stewart, 1980). When present in excess, it affects heterocyst differentiation and N_2-fixation as well as some other N-metabolic activities in diazotrophs (Stewart *et al.*, 1983; 1987; Reitzer and Magasanik, 1986).

Though amino acid transport systems are thoroughly characterized in various bacteria (Rosen and Kashket, 1978; Booth and Hamilton, 1980; Ames, 1986; Antonucci and Oxdender, 1986; Drissen *et al.*, 1988; Speelmans *et al.*, 1989), relatively little is known about these systems in cyanobacteria. Presently, two studies exist on the glutamine transport in heterocystous cyanobacteria (Chapman and Meeks, 1983; Flores and Muro-Pastor, 1988). In the present chapter, glutamine uptake characteristics are studied in *Nostoc* ANTH, in *Anabaena* 7120, and in a Het^- Nif^- mutant of *Anabaena* 7120.

7.2. MATERIALS AND METHODS:

7.2.1. Organisms and growth conditions:

Axenic cultures of *Anabaena* 7120 and *Nostoc* ANTH were grown in $BG\text{-}11_o$ medium (Rippka *et al.*, 1979), at 28 ± 1°C and at a

photon fluence rate of 50 $\mu mol.m^{-2}.s^{-1}$. The Het^- Nif^- mutant was grown in NO_3^- supplemented $BG\text{-}11_o$ medium ($BG\text{-}11_o$ + 5 $mmol.dm^{-3}$ KNO_3). Where needed $BG\text{-}11_o$ medium was also supplemented with 2 $mmol.dm^{-3}$ glutamine (glutamine-medium) or 1 $mmol.dm^{-3}$ NH_4Cl (ammonium-medium).

7.2.2. Estimation of chlorophyll:

Chl a concentration was determined according to Mackinney (1941).

7.2.3. Measurement of glutamine uptake:

Exponentially growing cells were centrifuged, washed and resuspended in 10 $mmol.dm^{-3}$ HEPES-NaOH buffer pH 7, and equilibrated for 30 min, at 28 ± 1°C and at a photon fluence rate of 50 $\mu mol.m^{-2}.s^{-1}$. ^{14}C-glutamine was added to a final concentration of 50 $\mu mol.dm^{-3}$ (specific activity 185 $kBq.\mu mol^{-1}$) and at time intervals 400 mm^3 samples were taken and cells separated from their bathing medium using oil microcentrifugation technique (Scott and Nicholls, 1980). ^{14}C-incorporation was determined using Beckman Liquid Scintillation Spectrometer LS 1801. Non-specific binding of ^{14}C-glutamine was determined by measuring its incorporation in toluene treated cells. These values were substracted before plotting the data (for details see fig 7.1. legend).

7.2.4. Chemicals:

^{14}C-glutamine was obtained from BARC, Bombay, Silicon oil DC 550 and Dinonylphthalate were purchased from Fluka AC, Buchs,

Switzerland and all other chemicals were obtained from Sigma Chemical Company, U.S.A.

7.3. RESULTS:

7.3.1. ^{14}C-glutamine uptake by N_2-, NO_3^--, NH_4^+- and glutamine-grown *Anabaena* 7120 and its Het^- Nif^- mutant:

N_2-grown parent strain showed a biphasic pattern of ^{14}C-glutamine uptake with an initial rapid phase for first 60 s followed by a slower second phase which was linear over 15 min experimental period (Fig 7.1a). The observed uptake rates during first- and second phase were 0.086 and 0.0523 $nmol.min^{-1}.\mu g^{-1}$ Chl a, respectively.

Since Het^- Nif^- mutant does not grow in N_2-medium, NO_3^--grown cultures of this mutant were transferred to NO_3^--free medium and after 24 h used for uptake studies. Such N-starved/limited cells also showed a biphasic pattern of ^{14}C-glutamine uptake with uptake rate being higher than that in the parent (Fig 7.1b).

Similar biphasic pattern of ^{14}C-glutamine uptake has been observed by Chapman and Meeks (1983) in *Anabaena variabilis*.

^{14}C-glutamine uptake was also present in NO_3^--, NH_4^+- and glutamine-grown cultures of *Anabaena* 7120 and its Het^- Nif^- mutant (Fig 7.1). As in the case of N_2-grown cultures, the uptake pattern was biphasic in NO_3^-- and glutamine grown cultures of both the parent and the mutant.

The observed uptake rates in NO_3^--grown cultures, during the first- and second phase, were 0.167 and 0.0509 $nmol.min^{-1}.\mu g^{-1}$ Chl

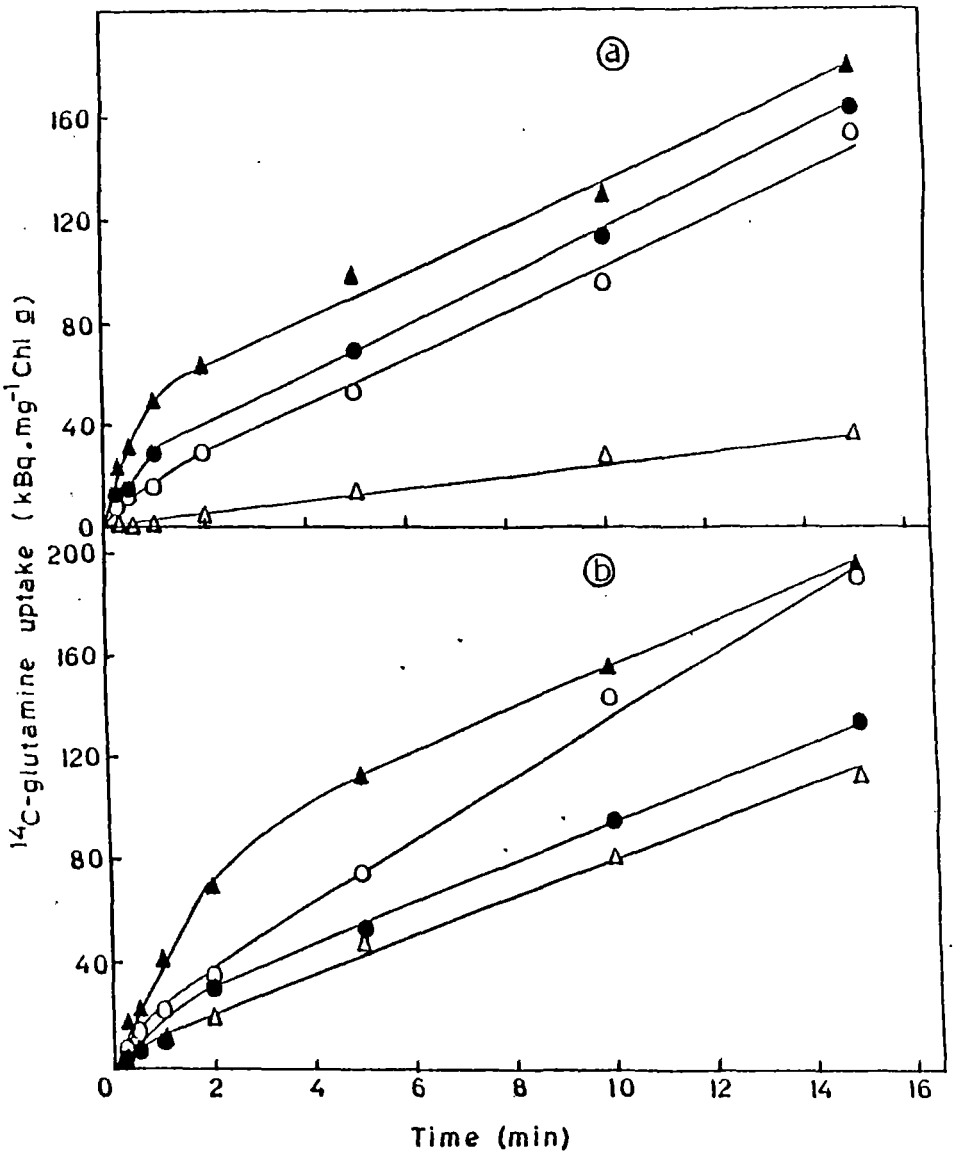

Fig 7.1 ^{14}C-glutamine uptake in *Anabaena* 7120 (a) and its Het$^-$ Nif$^-$ mutant (b) grown on N_2- (O); NO_3^-- (●); NH_4^+- (Δ) and glutamine- (▲) medium. Since mutant cells do not grow in N_2-medium, NO_3^-- grown cells were N-starved for 24 h in N_2-medium and then ^{14}C-glutamine uptake was measured. In this and all other experiments (Fig 7.2 to 7.7) the data are means of four replicates obtained from two repeat experiments. The variation range was between 5 - 10% from the average. All the data were calculated and plotted, in this and other figures (Fig 7.2 to 7.7), after substracting the respective values from toluene-treated cells to eliminate background due to non-specific binding.

a in the parent; and 0.081 and 0.0419 nmol.min^{-1}.μg^{-1} Chl a in the mutant, respectively.

The observed uptake rates in glutamine grown cultures, during the first- and second phase, were 0.27 and 0.0478 nmol.min^{-1}.μg^{-1} Chl a in the parent and 0.189 and 0.567 nmol.min^{-1}.μg^{-1} Chl a in the mutant, respectively.

^{14}C-glutamine uptake was also found in NH_4^+-grown cultures of *Anabaena* 7120 (parent) and its Het^- Nif^- mutant (Fig 7.1). But the uptake pattern and rates differed. The parent strain showed linear ^{14}C-glutamine uptake with a uptake rate lower than that in other cultures (0.0124 nmol.min^{-1}.μg^{-1} Chl a). The mutant strain showed a biphasic uptake pattern with uptake rates of 0.0756 and 0.0425 nmol.min^{-1}.μg^{-1} Chl a, during first- and second phase, respectively.

These data suggest that the glutamine uptake system is not under nitrogen control, but may be regulated by the nitrogen status of the cell. Since, the Het^- Nif^- mutant strain is similar to the parent strain with respect to ^{14}C-glutamine uptake, the above data also suggest that the uptake system is not controlled by a common regulatory system of heterocyst production and aerobic N_2-fixation in *Anabaena* 7120. These findings are consistent with the observations of Flores and Muro-Pastor (1988) on the cyanobacterium *Anabaena* 7120. Furthermore, the data showing presence of ^{14}C-glutamine uptake activity in N_2-, NO_3^--, NH_4^+- and glutamine-grown cells indicate that, unlike the ATS which is repressed by excess of NH_4^+, glutamine uptake system is not repressed by its substrate glutamine.

7.3.2. ^{14}C-glutamine uptake by *Nostoc* ANTH:

7.3.2.1. ^{14}C-glutamine uptake in N_2- and glutamine grown *Nostoc* ANTH:

^{14}C-glutamine uptake, in N_2-grown *Nostoc* ANTH cells, was found to be biphasic with an initial rapid phase for first 60 s followed by a second slower phase which was linear over the 15 min experimental period (Fig 7.2). The uptake rates during the initial- and second phase were 0.0405 and 0.0135 $nmol.min^{-1}.\mu g^{-1}$ Chl a, respectively. Thus the ^{14}C-glutamine uptake rate during the first phase was 3-fold higher than that during the second phase.

A similar pattern of ^{14}C-glutamine uptake was found in glutamine-grown *Nostoc* ANTH filaments (Fig 7.2). However, the uptake rates were different. The ^{14}C-glutamine uptake rates, in glutamine-grown cells, were 0.03405and 0.02644 $nmol.min^{-1}.\mu g^{-1}$ Chl a, during initial- and second phase, respectively. It is apparent that the difference in uptake rates, during first- and second phase, is higher in glutamine-grown cultures as compared to that in N_2-grown cultures. This is mainly due to a higher rate of ^{14}C-glutamine uptake and metabolism by glutamine-grown cells during the second phase.

7.3.2.2. Effect of NH_4Cl on ^{14}C-glutamine uptake by N_2-grown *Nostoc* ANTH:

Simultaneous addition of 200 $\mu mol.dm^{-3}$ NH_4Cl and ^{14}C-glutamine did not alter the ^{14}C-glutamine uptake pattern and rates in N_2-grown *Nostoc* ANTH filaments (Fig 7.3). Addition of NH_4Cl subsequent to ^{14}C-glutamine addition also did not affect the ^{14}C-glutamine uptake. These data suggest that transport of ammonium and glutamine are mediated by different carriers. It is

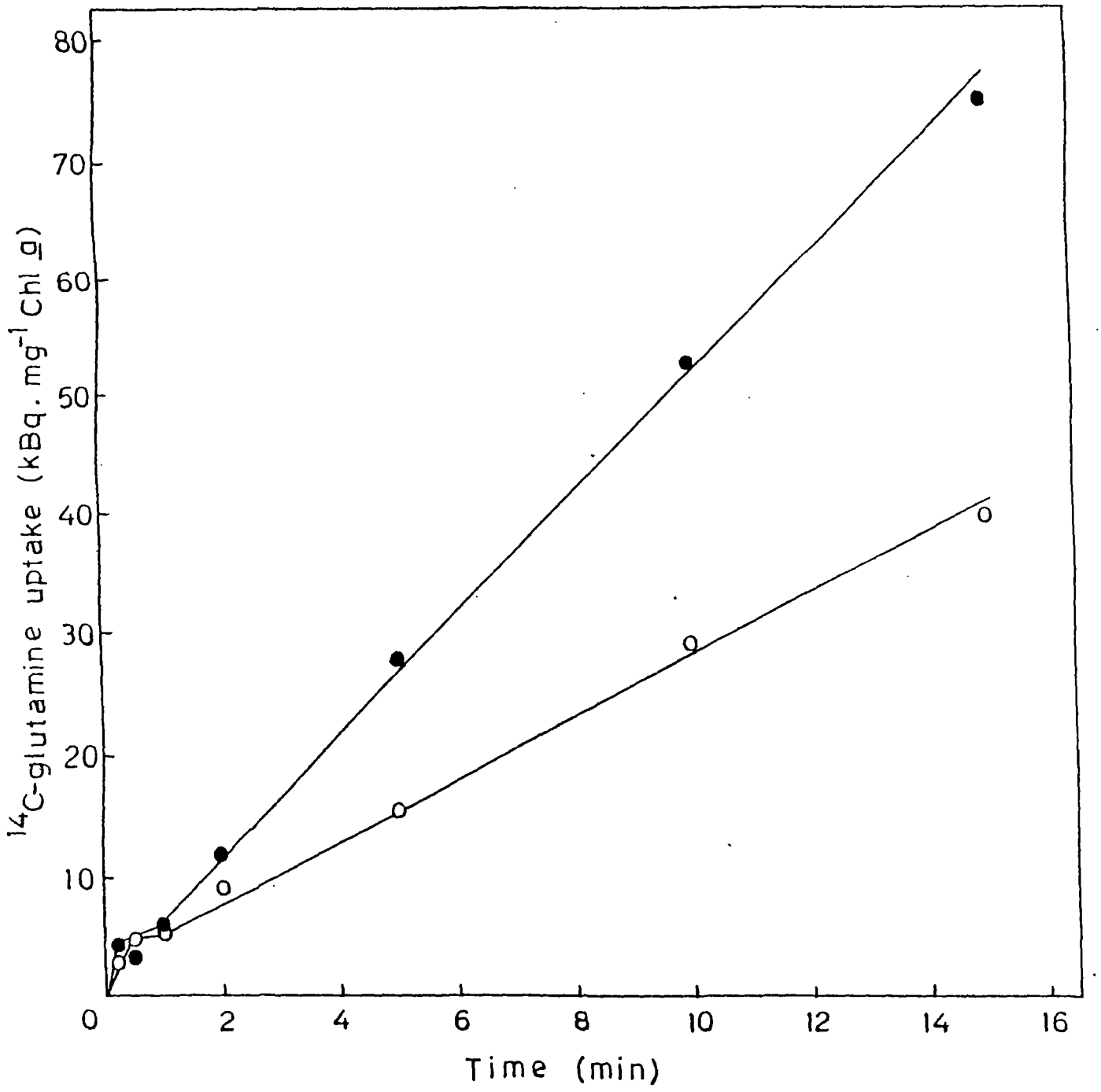

Fig 7.2 ^{14}C-glutamine uptake by N_2- (O) and glutamine- (●) grown *Nostoc* ANTH filaments.

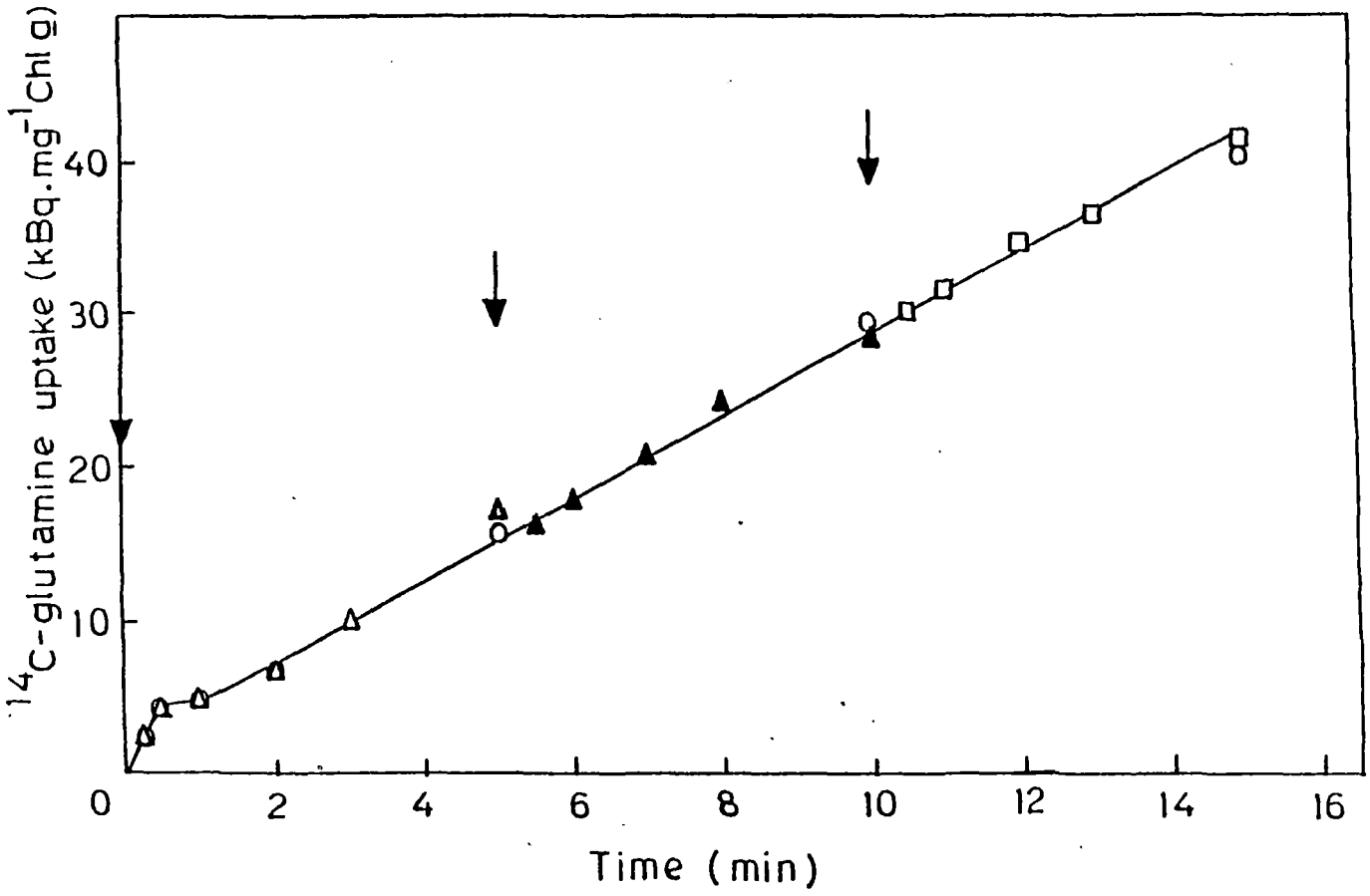

Fig 7.3 Effect of NH_4Cl addition on ^{14}C-glutamine uptake by N_2-grown *Nostoc* ANTH filaments. NH_4Cl was added at times indicated (arrows) to a final concentration of 200 $\mu mol.dm^{-3}$ O, control (^{14}C-glutamine only); Δ, NH_4Cl and ^{14}C-glutamine added simultaneously at zero time; ▲, NH_4Cl added 5 min after ^{14}C-glutamine addition; □, NH_4Cl added 10 min after ^{14}C-glutamine addition.

interesting to note that while glutamine affects ammonium transport (see chapter 5), ammonium does not affect glutamine transport.

7.3.2.3. Effect of glutamate on ^{14}C-glutamine uptake by N_2-grown *Nostoc* ANTH:

Based on the observation that glutamine inhibited glutamate uptake and vice versa, a common transport system for these two amino acids was suggested in some cyanobacteria (Lee-Kaden and Simonis, 1982; Chapman and Meeks, 1983; Flores and Muro-Pastor, 1988). Therefore, the effect of glutamate addition on ^{14}C-glutamine uptake, in N_2-grown *Nostoc* ANTH cells, at pH 7, was investigated. Simultaneous addition of glutamate (200 $\mu mol.dm^{-3}$) and ^{14}C-glutamine showed biphasic pattern of ^{14}C-glutamine uptake similar to that observed in control (Fig 7.4). These results are in contrast to earlier findings and indicate that different carriers are involved in uptake of exogenous glutamine and glutamate in *Nostoc* ANTH. These results are consistent with the results observed in symbiotic *Nostoc* sp. of *Geosiphon pyriforme* where a specific transport system for glutamate was observed (Strasser and Falkner, 1986). The above results, however, are not consistent to those results observed in *A. variabilis* and *Anabaena* sp. PCC 7120, where a common transport system for glutamine and glutamate was observed (Chapman and Meeks, 1983; Flores and Muro-Pastor, 1988).

Addition of glutamate after ^{14}C-glutamine addition resulted in a higher rate of ^{14}C-glutamine uptake (Fig 7.4). This higher glutamine uptake rate may be due to the higher glutamine assimilation via glutamate synthase (GOGAT).

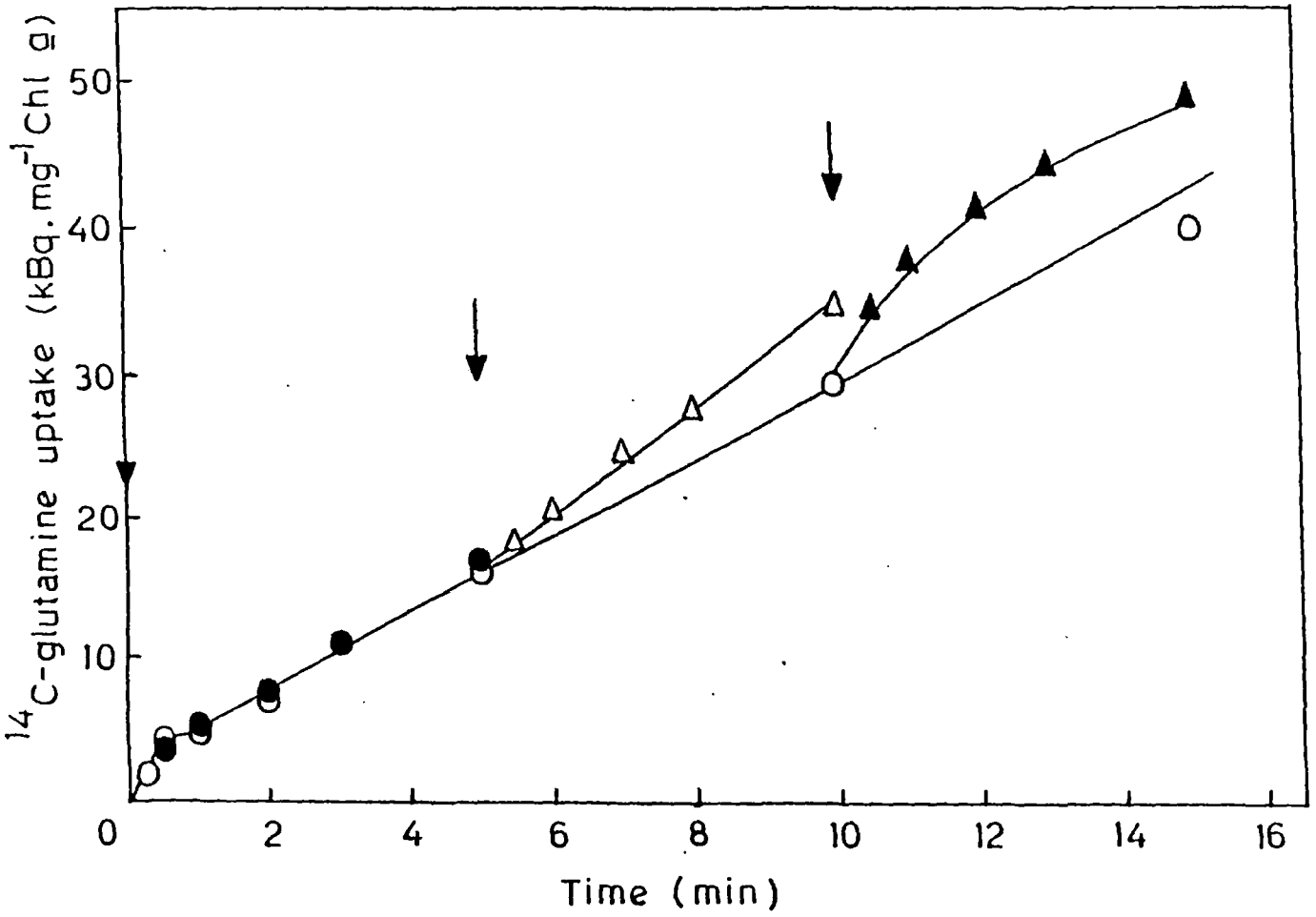

Fig 7.4 Effect of glutamate addition on ^{14}C-glutamine uptake by N_2-grown *Nostoc* ANTH filaments. Glutamate was added at times indicated (arrows) to a final concentration of 200 μmol.dm^{-3}. O, control (^{14}C-glutamine only); ●, glutamate and ^{14}C-glutamine added simultaneously at zero time; △, glutamate added 5 min after ^{14}C-glutamine addition; ▲, glutamate added 10 min after ^{14}C-glutamine addition.

7.3.2.4. Effect of MSX on ^{14}C-glutamine uptake by N_2-grown *Nostoc* ANTH:

Addition of MSX (10 μmol.dm^{-3}) and ^{14}C-glutamine to N_2-grown *Nostoc* ANTH filaments, at pH 7, at zero time, resulted in a biphasic pattern of ^{14}C-glutamine uptake similar to that observed in control (Fig 7.5). The ^{14}C-glutamine uptake rates during both phases also showed no significant changes in the presence of MSX. Addition of MSX after 5 and 10 min of ^{14}C-glutamine addition also showed similar results (Fig 7.5). Such observations indicated that the ^{14}C-glutamine transport system is not inhibited by MSX in *Nostoc* ANTH. This is in contrast to the finding in *A. variabilis* where MSX caused inhibition of glutamine uptake (Chapman and Meeks, 1983). It should be remembered here that, only one glutamine transport system (high affinity transport system), in *A. variabilis*, is eliminated by MSX but not the low affinity transport system (Chapman and Meeks, 1983). Based on the observed results in *Nostoc* ANTH, it is clear that the *Nostoc* ANTH possesses only one glutamine transport system unlike two in *A. variabilis* (Chapman and Meeks, 1983) and such a system is not affected by the presence of MSX. If both transport system were operative/present in N_2-grown *Nostoc* ANTH filaments, MSX would have inhibited the ^{14}C-glutamine uptake partially.

7.3.2.5. Effect of azaserine on ^{14}C-glutamine uptake by N_2-grown *Nostoc* ANTH:

Azaserine is an inhibitor of glutamate synthase (GOGAT), a glutamine assimilatory enzyme, in cyanobacteria (Hartman, 1973; Ohmori *et al.*, 1985). Therefore, the effect of azaserine on glutamine uptake in N_2-grown *Nostoc* ANTH filaments was studied.

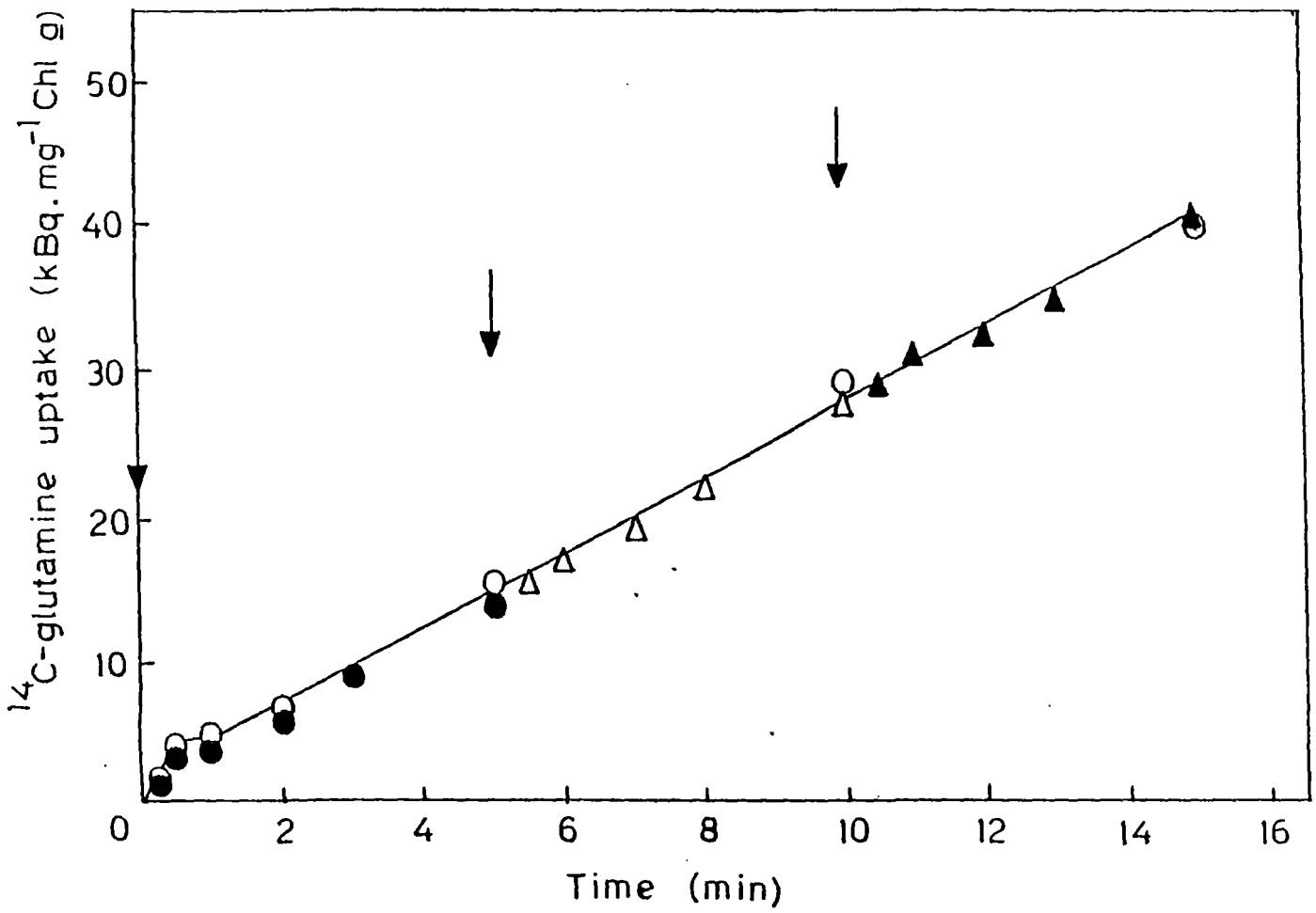

Fig 7.5 Effect of MSX addition on ^{14}C-glutamine uptake by N_2-grown *Nostoc* ANTH filaments. MSX was added at times indicated (arrows) to a final concentration of 10 μmol.dm^{-3}. O, control (^{14}C-glutamine only); ●, MSX and ^{14}C-glutamine added simultaneously at zero time; Δ, MSX added 5 min after ^{14}C-glutamine addition; ▲, MSX added 10 min after ^{14}C-glutamine addition.

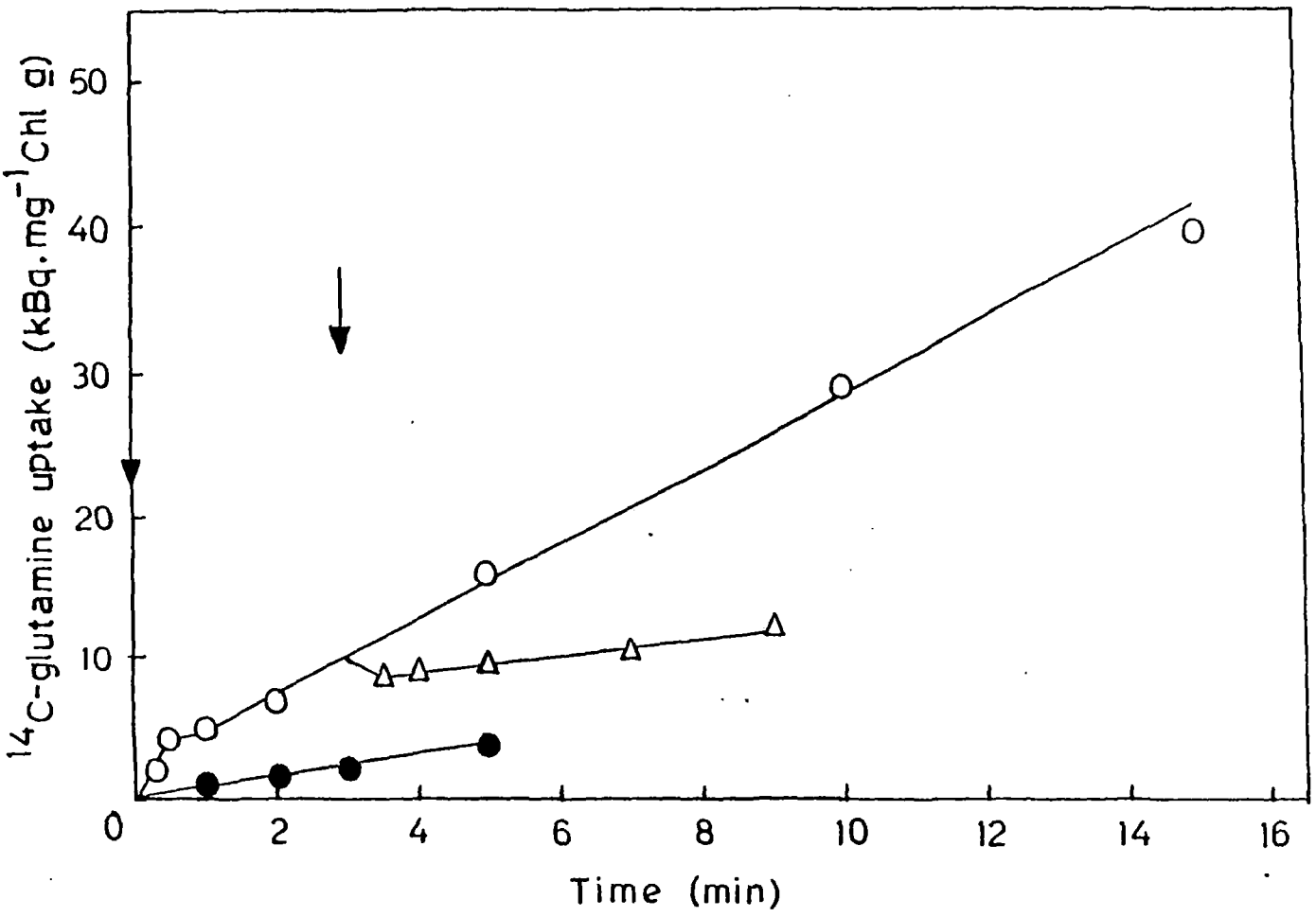

Fig 7.6 Effect of azaserine addition on ^{14}C-glutamine uptake by N_2-grown *Nostoc* ANTH filaments. Azaserine was added at times indicated (arrows) to a final concentration of 100 μmol.dm^{-3}. O, control (^{14}C-glutamine only); ●, azaserine and ^{14}C-glutamine added simultaneously at zero time; Δ, azaserine was added 3 min after ^{14}C-glutamine addition.

Simultaneous addition of both azaserine (100 μmol.dm^{-3}) and ^{14}C-glutamine resulted in a reduced rate of ^{14}C-glutamine uptake (Fig 7.6). This reduction in ^{14}C-glutamine uptake in the presence of azaserine was concluded to be due to the inhibition of glutamine uptake at the transport level because the azaserine effect was immediate (Fig 7.6). Addition of azaserine after 3 min addition of ^{14}C-glutamine also showed a similar effect (Fig 7.6). Such over all results suggest that azaserine has an inhibitory effect on ^{14}C-glutamine uptake at the transport level in *Nostoc* ANTH filaments.

7.3.2.6. Effect of CCCP, TPMP$^+$ and darkness on ^{14}C-glutamine uptake by N_2-grown *Nostoc* ANTH:

Amino acid uptake, in various prokaryotes, is found to be an energy-dependent process (Rosen and Kashket, 1978; Booth and Hamilton, 1980; Lee-Kaden and Simonis, 1982; Kleiner, 1985a). The source of energy for amino acid uptake is different in different organisms. In heterotrophic and photosynthetic bacteria amino acid transport processes are reported to be light-dependent (Guffanti *et al.*, 1979; Rosen, 1971). However, in cyanobacteria it is not well understood (Lee-Kaden and Simonis, 1982). Therefore, the effect of CCCP, TPMP$^+$ and darkness on ^{14}C-glutamine uptake, in N_2-grown *Nostoc* ANTH filaments was investigated.

CCCP, an energy uncoupler, treated cells showed negligible amount of ^{14}C-glutamine uptake (Fig 7.7). This suggested that the glutamine uptake process is active and energy-dependent in *Nostoc* ANTH filaments. The ^{14}C-glutamine uptake pattern in TPMP$^+$ and dark incubated filaments was, however, found to be similar to that in untreated cells (Fig 7.7). However, rate of ^{14}C-glutamine

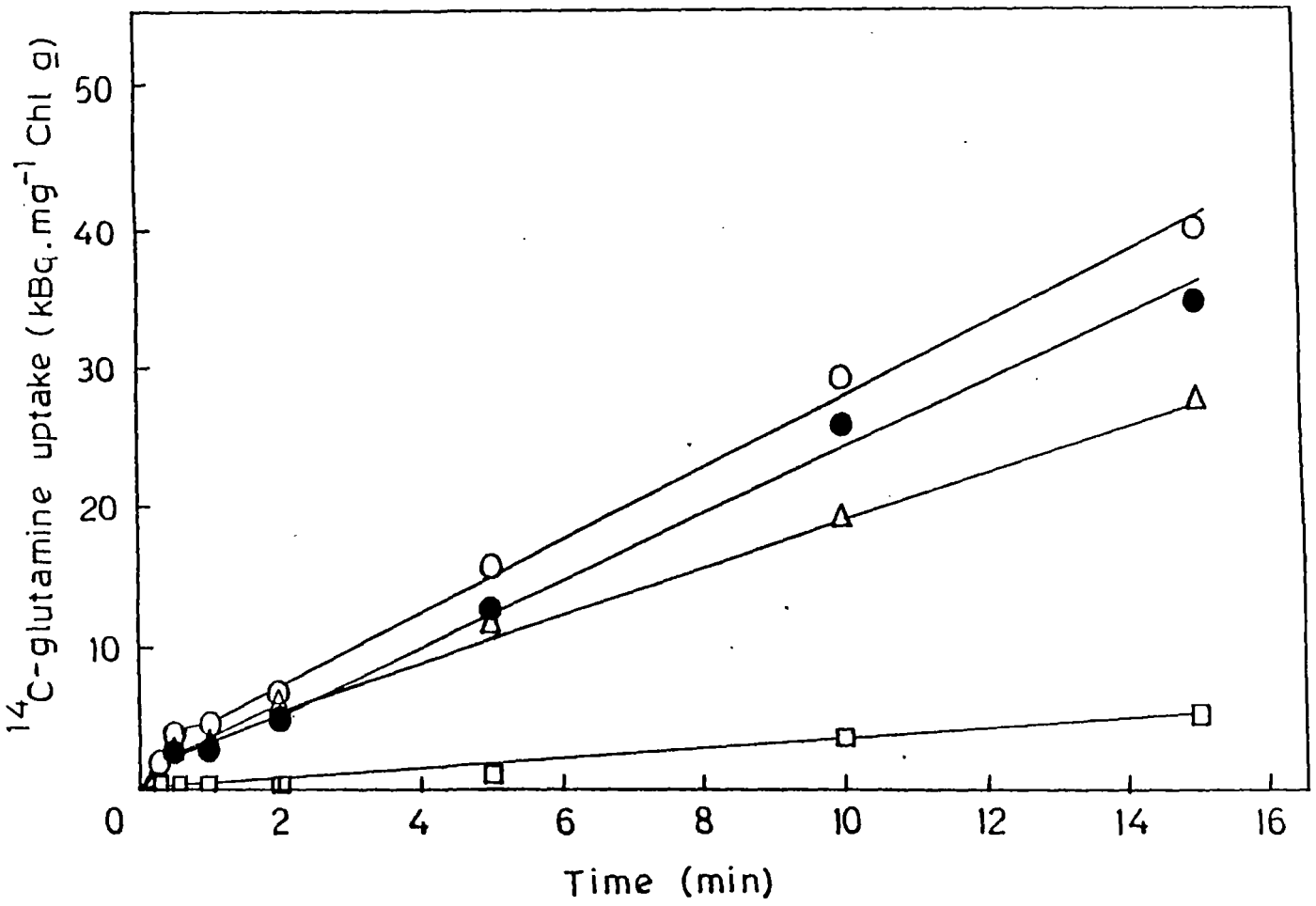

Fig 7.7 ^{14}C-glutamine uptake by N_2-grown *Nostoc* ANTH filaments in the presence (□, Δ) and absence (O) of CCCP (□) and $TPMP^+$ (Δ). CCCP (10 μmol.dm^{-3}) and $TPMP^+$ (100 μmol.dm^{-3}) were added 30 min prior to the addition of ^{14}C-glutamine. ●, ^{14}C-glutamine uptake by N_2-grown cells preincubated in dark for 30 min and the uptake measured in darkness.

uptake was 33% lower in $TPMP^+$ treated cells. Such data indicated that, the glutamine uptake process is not totally dependent on transmembrane electrical potential as reported in *Anacystis nidulans* (Lee-Kaden and Simonis, 1982). Since ^{14}C-glutamine uptake was unaffected in dark incubated cells, it was concluded that, the ^{14}C-glutamine uptake, in this cyanobacterium, is independent of light-dependent reactions.

7.4. DISCUSSION:

A biphasic pattern of ^{14}C-glutamine uptake was found in both the strains studied here: *Anabaena* 7120 and *Nostoc* ANTH. Similar uptake pattern has been found in other cyanobacteria (Chapman and Meeks, 1983; Flores and Muro-Pastor, 1988).

The apparent lack of total inhibition of ^{14}C-glutamine uptake in NO_3^-- and NH_4^+-grown cells, and the lack of any adverse affect of glutamate, NH_4^+ and MSX on ^{14}C-glutamine uptake by N_2-grown cells suggested that this uptake process is not under nitrogen control in these cyanobacteria. However, it is dependent on the metabolism of the transported species because NH_4^+-grown *Anabaena* 7120 showed reduced ^{14}C-glutamine uptake (Fig 7.1) while addition of NH_4Cl during ^{14}C-glutamine uptake in N_2-grown *Nostoc* ANTH filaments did not change the uptake pattern and rates (Fig 7.3).

The ^{14}C-glutamine uptake in *Nostoc* ANTH is active and energy-dependent process. This was concluded based on the facts that CCCP caused complete inhibition of ^{14}C-glutamine uptake. The fact that $TPMP^+$ did not cause a total inhibition of ^{14}C-glutamine

uptake and that ^{14}C-glutamine uptake occurred during dark, indicated that the uptake process is light-independent and only partially dependent on transmembrane electrical potential. Glutamine transport has also been shown to be energy dependent in *A. nidulans* (Lee-Kaden and Simonis, 1982) and *Synechocystis* sp. Strain 6803 (Labarre *et al.*, 1987).

In *Nostoc* ANTH, the glutamine transport system is specific for glutamine only and it is not shared by glutamate or MSX. This was concluded based on the evidences that ^{14}C-glutamine uptake was not affected by glutamate (Fig 7.4) or MSX (Fig 7.5). The above conclusion, in *Nostoc* ANTH, is consistent with the observed specific glutamine transport system in *Synechocystis* sp. Strain 6803 (Labarre *et al.*, 1987). However, these findings are in contrast to the observation made in *A. variabilis* (Chapman and Meeks, 1983) where a common transport system for glutamate, glutamine and MSX was reported. Chapman and Meeks (1983) reported that there are at least two glutamine transport systems in *A. variabilis* and that the high affinity system was shared by MSX and glutamate. The fact that, glutamate and MSX did not show any effect on *Nostoc* ANTH glutamine transport indicates that *Nostoc* ANTH has only one glutamine transport system and that it lacks the high affinity glutamine transport system shared by glutamine, glutamate and MSX.

Overall, the data indicate that a specific energy-dependent glutamine uptake system is operative in *Nostoc* ANTH and *Anabaena* 7120, and that, unlike *A. variabilis*, there is no common transport system for glutamine, glutamate and MSX in these two strains.

8. GENERAL DISCUSSION: NITROGENASE REGULATION AND TRANSPORT OF AMMONIUM, GLUTAMINE AND GLUTAMATE

8.1. Ammonium transport:

Ammonium is a preferred source of inorganic nitrogen in cyanobacteria. It can be taken up by the cell through a transport system. The ammonium transport system (ATS) has been characterized in various bacteria and cyanobacteria using an ammonium analogue, $^{14}CH_3NH_3^+$, as a probe (Boussiba *et al.*, 1984b; Rai *et al.*, 1984; 1986b; Kleiner, 1985a; Singh *et al.*, 1987; Boussiba, 1988). This is mainly due to the fact that, $CH_3NH_3^+$ uses the same transport system as NH_4^+ in these organisms. The $NH_4^+/CH_3NH_3^+$ uptake studies presented in the chapters 4 and 5 in *Anabaena* 7120 and *Nostoc* ANTH also indicated that $CH_3NH_3^+$ can be used as a probe to characterize ATS.

The ATS studies in *Anabaena* 7120 and *Nostoc* ANTH indicated that two energy-dependent transport systems are involved in uptake of external ammonium into the cells. One of the transport systems is MSX-insensitive while the other is MSX-sensitive. The former operates at a faster rate than the latter. Both these transport systems are NH_4^+-repressible and are derepressed in N_2- and NO_3^--grown cells (see chapter 4 and 5). Since ATS is repressed in ammonium grown cells, the N-needs of the cell is served by the diffusible NH_3 (Kleiner, 1985b). The two cyanobacterial strains examined indicate existence of two intracellular ammonium pools -- one in thylakoids and the other in cytoplasm (see chapter 4 and 5;

discussion section). The MSX-insensitive ATS serves the thylakoid pool while the MSX-sensitive ATS serves the cytoplasmic pool. The latter is assimilated by the cell via GS. Because of these characteristics of the two ATS, the pattern of $NH_4^+/CH_3NH_3^+$ uptake in these cells is biphasic: a fast initial phase, lasting about 60 s, due to MSX-insensitive ATS and a slower second phase due to MSX-sensitive ATS. Hence, the second phase of $NH_4^+/CH_3NH_3^+$ uptake is sensitive to MSX. MSX causes immediate inhibition of the second phase of ATS and hence the second phase of uptake. In longer term MSX also inhibits GS thereby blocking $NH_4^+/CH_3NH_3^+$ assimilation.

Both the ATS reported here show affinity modulation in response to external $CH_3NH_3^+/NH_4^+$ concentration (see chapter 4 and 5). At high external concentration of $NH_4^+/CH_3NH_3^+$, the transport systems show low affinity while at low external substrate concentration the affinity increases. This provides a novel mechanism to control ammonium metabolism in cyanobacteria by regulating its entry into the cell.

In addition, *Nostoc* ANTH shows a capability to utilize $CH_3NH_3^+$ as N-source (see chapter 3). For this a distinct $CH_3NH_3^+$ transport system was found to be induced only in $CH_3NH_3^+$- grown *Nostoc* ANTH cells (see chapter 5). This transport system was not shared by NH_4^+, was insensitive to MSX and specific for $CH_3NH_3^+$.

8.2. Ammonium transport and nitrogenase regulation:

In all free living diazotrophs nitrogenase synthesis/ activity is regulated by NH_4^+ and other combined nitrogen compounds

(Stewart and Lex, 1970; Rippka and Stanier, 1978; Jones and Monty, 1979; Brill, 1980; Stewart, 1980; Thomas *et al.*, 1982; Singh *et al.*, 1983b; Turpin *et al.*, 1984; Reich *et al.*, 1986; 1987; Stewart *et al.*, 1987). Ammonium, has been shown to cause two types of effects on nitrogenase in diazotrophs: a short term effect (within minutes) on nitrogenase activity and a long term effect on nitrogenase synthesis (Haaker *et al.*, 1980; Stewart, 1980; Hallenbeck, 1987).

In *Rhizobium leguminosarum* and *Azotobacter vinelandii*, Haaker *et al.* (1980) have shown that a minimum of -100 mV $\Delta\psi$ is needed for optimum nitrogenase activity. $\Delta\psi$ is involved in reverse electron flow from NADPH to ferredoxin. The latter is the e^- donor for nitrogenase. At $\Delta\psi$ values below -90 mV, reverse e^- flow is blocked. NH_4^+ uptake causes $\Delta\psi$ to collapse and therefore nitrogenase activity is severely inhibited, within minutes, due to blockage of e^- flow to nitrogenase. In cyanobacteria also it has been shown that $\Delta\psi$ and nitrogenase activity are directly correlated. A minimum of -70 mV $\Delta\psi$ is necessary for optimum nitrogenase activity in cyanobacteria (Hawkesford *et al.*, 1980; 1981). However, at physiological pH, NH_4^+ does not cause short term inhibition of nitrogenase (fig 8.1). As shown in fig 8.2 NH_4^+ addition to N_2- fixing cultures of *Nostoc* ANTH, at pH 7 lowered $\Delta\psi$ from -110 mV to -97.5 mV. However, it is clear that the residual $\Delta\psi$ was still above the value required for optimum nitrogenase activity in cyanobacteria (-70 mV). This may explain why NH_4^+ does not cause quick inhibition of nitrogenase activity in cyanobacteria. Short term effect of NH_4^+ on nitrogenase has been noted at high pH 10 (Reich *et al.*, 1986; 1987). However, this may

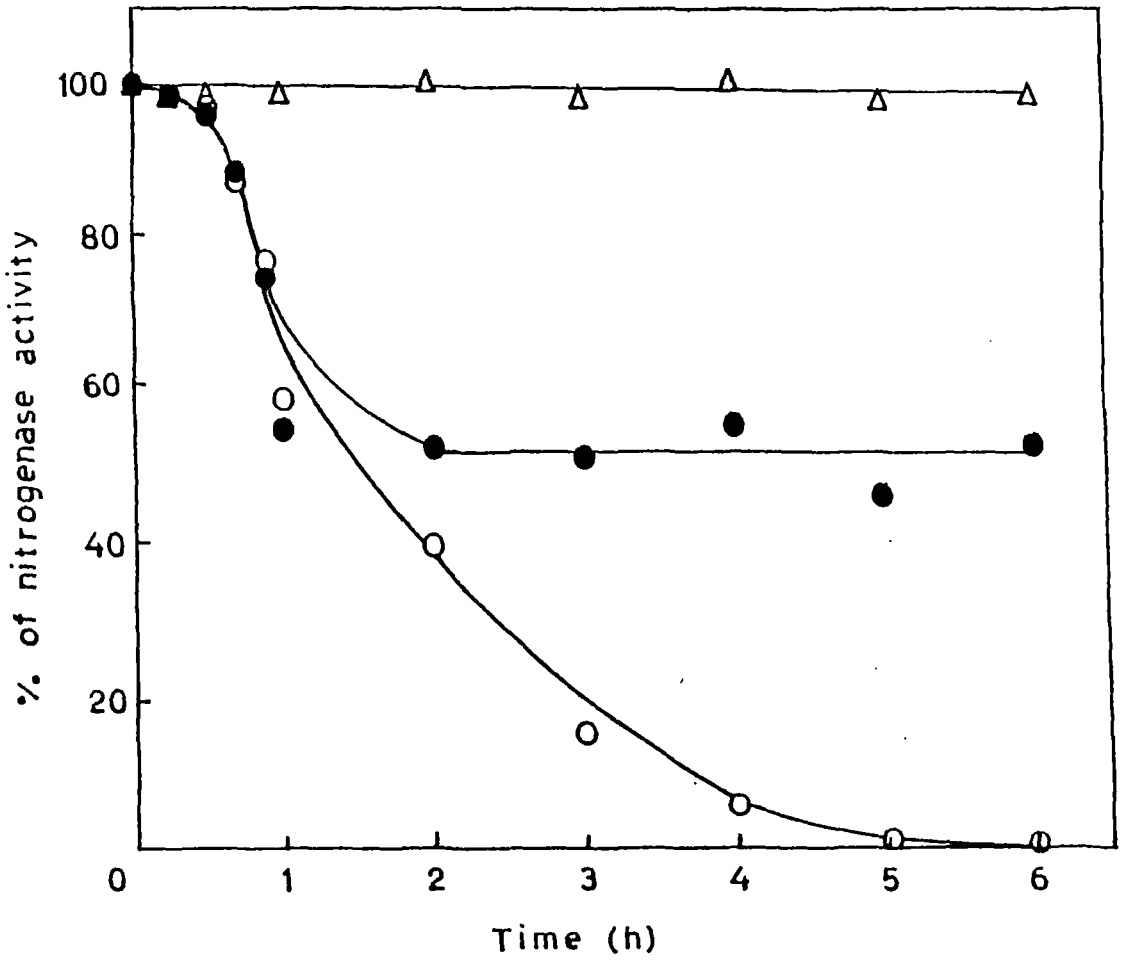

Fig 8.1 Effect of NH_4Cl and MSX on nitrogenase activity in N_2-grown *Nostoc* ANTH filaments, at pH 7. 100% nitrogenase activity = 5.83 nmol C_2H_2 reduced.h^{-1}.μg^{-1} Chl a. O, NH_4Cl (3 mmol.dm^{-3}) added at zero time; ●, NH_4Cl (3 mmol.dm^{-3}) + MSX (10 μmol.dm^{-3}) added at zero time; Δ, NH_4Cl (3 mmol.dm^{-3}) added at zero time to cultures preincubated with 10 μmol.dm^{-3} MSX for 2 h.

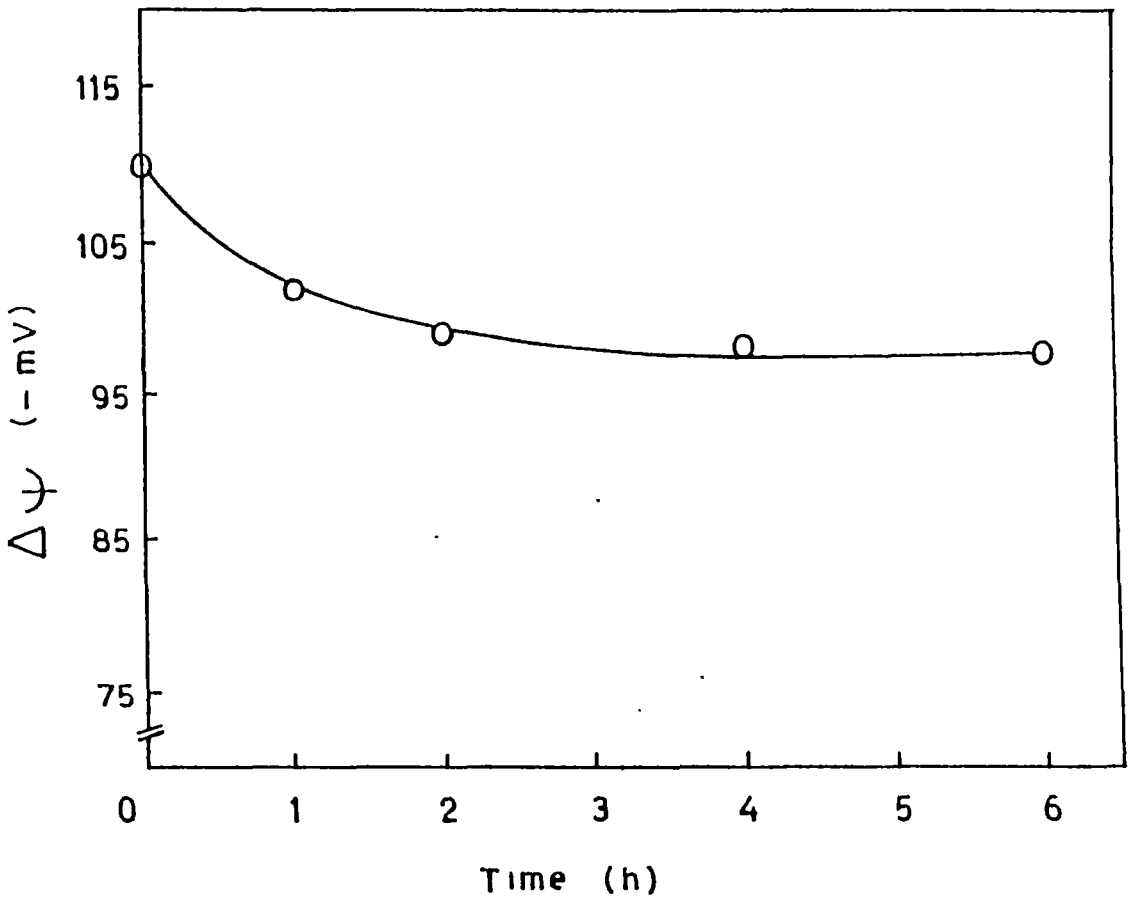

Fig 8.2 Effect of NH_4Cl (3 $mmol.dm^{-3}$) on $\Delta\psi$ in N_2- grown *Nostoc* ANTH filaments, at pH 7. $\Delta\psi$ values were measured, at pH 7, as described in chapter 2.

be due to uncoupling effect of NH_4^+ because at high pH excessive intracellular ammonia accumulation would occur by diffusion of external NH_3 and its entrapment in the cell by protonation. This would be consistent with the ammonium transport studies at pH 9 (see chapter 4 and 5).

NH_4^+ has been shown to cause nitrogenase repression in cyanobacteria (Stewart, 1980; Hallenbeck, 1987). This is exemplified by a slow inhibition of *in vivo* nitrogenase activity on ammonium addition (Fig 8.1). NH_4^+ caused total inhibition of nitrogenase activity over a period of 4 h. In MSX preincubated cells, however, NH_4^+ did not cause such an inhibition. Stewart and Rowell (1975), from similar experiments on *Anabaena cylindrica*, concluded that since MSX inhibits GS and thereby ammonium assimilation, the results may be taken to indicate that a product of ammonia assimilation, rather than NH_4^+ *per se*, was the actual repressor of nitrogenase in cyanobacteria. However, subsequently it was challenged. Singh *et al*. (1983b) and Turpin *et al*. (1984) suggested that MSX may also cause inhibition of ammonium uptake and therefore lack of nitrogenase inhibition in MSX preincubated cells may not necessarily be due to blockage of ammonia assimilation. It may be equally possible that NH_4^+ *per se* was the repressor but since MSX blocked entry of ammonium in the cells, the NH_4^+ effect on nitrogenase was not observed. Indeed our studies on ATS (chapters 4 and 5) show that MSX does block the second ATS which serves the cytoplasmic pool used by GS. However, if the above reasoning of Singh *et al*. (1983b) and Turpin *et al*. (1984) was correct, then NH_4^+ should have affected nitrogenase activity in the cells having been preincubated with MSX as well as

in the cells where MSX was added simultaneously with NH_4^+. As seen in fig 8.1 this is not so. In MSX-preincubated cells where GS was fully inhibited NH_4^+ did not cause nitrogenase inhibition but in cells where MSX was added together with NH_4^+, nitrogenase inhibition occurred during the first 1 h, after which no further inhibition was noted. It should be noted here that MSX (10 $\mu mol.dm^{-3}$) causes immediate inhibition of the second ATS while the GS inhibition is progressive and takes about 1 h for total inhibition in *Nostoc* ANTH (data not shown). Thus, the partial inhibitory effect of NH_4^+, in presence of MSX, noted in fig 8.1 correlates with GS activity rather than inhibition of second ATS by MSX. The results are consistent with the view that nitrogenase repression by ammonia is caused by a product of ammonia assimilation. The fact that $CH_3NH_3^+$ is not metabolized beyond methylglutamine in *A. variabilis* and *Anabaena* 7120 (*Nostoc muscorum*) (Rai *et al.*, 1984; Rai and Prakasham, 1989; and also see chapter 3) and yet it represses nitrogenase activity (Singh *et al.*, 1983a; and see chapter 3) suggests that, most likely, the repressor of nitrogenase is glutamine.

It should be further emphasized that MSX does not affect the first ATS. If NH_4^+ *per se* was the repressor then in the presence of MSX, nitrogenase inhibition should have continued rather than stop after 1 h when GS activity is fully inhibited. In addition, the fact that NH_4^+ represses ATS in the long term (Rai *et al.*, 1986a; see also chapter 4 and 5) and yet nitrogenase is repressed by assimilation of diffusible NH_3 species, argues against ATS inhibition by MSX being the explanation for MSX alleviating NH_4^+ repression of nitrogenase.

8.3. Glutamine & glutamate transport and regulation of nitrogenase

Addition of glutamine to N_2-fixing *Nostoc* ANTH filaments caused a complete inhibition of nitrogenase activity within 6 h (Fig 8.3). Such results are similar to those observed in other N_2-fixing organisms where nitrogenase activity inhibition is observed in the presence of glutamine (Arp and Zumft, 1983) In the presence of MSX, glutamine, however, caused only a partial inhibition of nitrogenase activity during the first 1 h; thereafter no further inhibition was observed (Fig 8.3). The lack of glutamine effect on nitrogenase, beyond 1 h, in presence of MSX may be either due to the inhibition of glutamine uptake by MSX or MSX, being structurally similar to glutamine, may compete for the glutamine binding site thereby preventing nitrogenase inhibition/ repression. The former possibility is ruled out since glutamine uptake studies in this cyanobacterium, presented in chapter 7, showed that MSX does not inhibit glutamine transport. Arp and Zumft (1983) have given a similar argument for observations on *Rhodopseudomonas palustris*.

Glutamate (see chapter 6) caused inhibition of diazotrophic growth and was toxic to the cyanobacteria studied (*Anabaena* 7120 and its Het^- Nif^- strain). This toxicity was related to the mode of N-nutrition; where NO_3^- was provided as N-source, glutamate toxicity was not observed. This was found to be due to partial inhibition of glutamate uptake by NO_3^-.

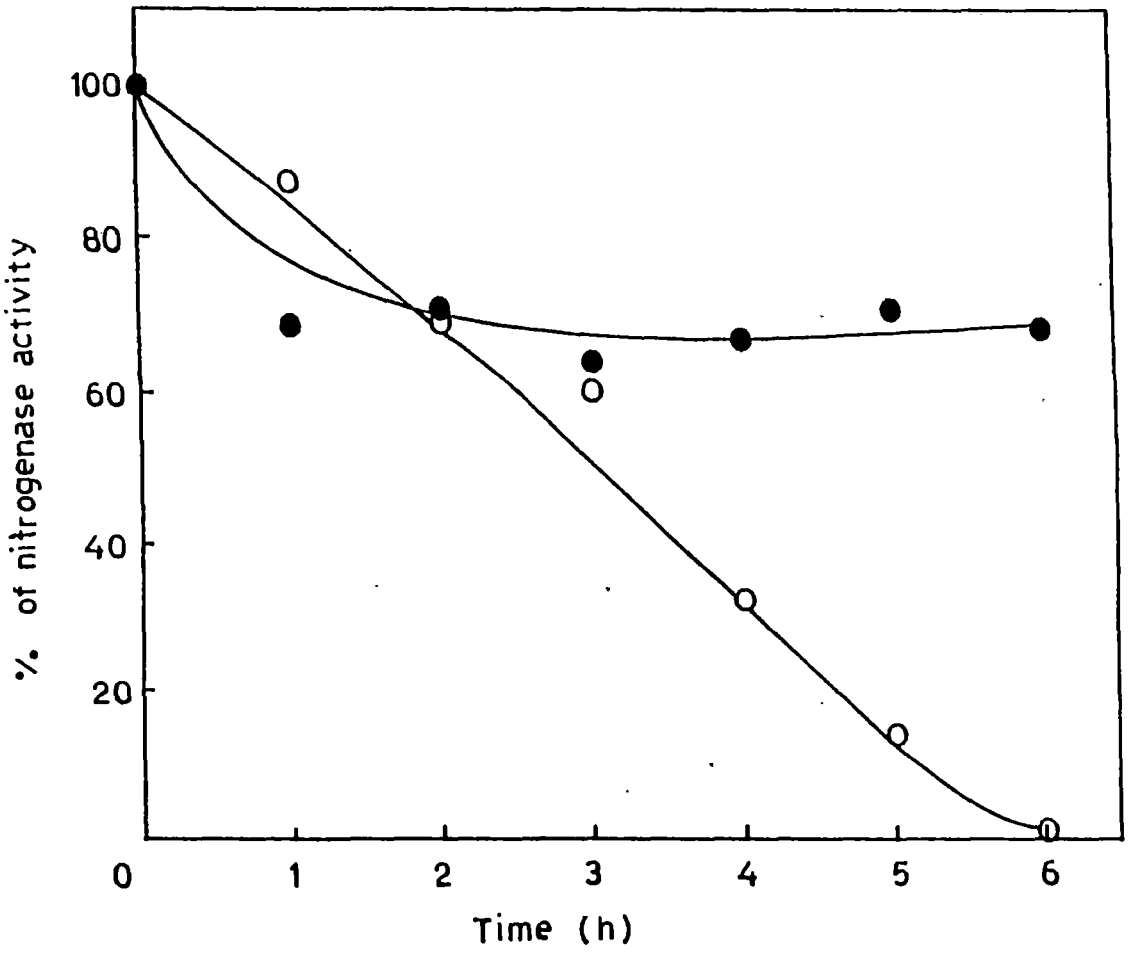

Fig 8.3 Effect of glutamine and MSX on nitrogenase activity in N_2-grown *Nostoc* ANTH filaments, at pH 7. 100% nitrogenase activity = 5.83 nmol C_2H_2 reduced.h^{-1}.μg^{-1} Chl a. O, glutamine (2 mmol.dm^{-3}) added at zero time; ●, glutamine (2 mmol.dm^{-3}) + MSX (10 μmol.dm^{-3}) added at zero time.

8.4. Some biotechnological implications:

Cyanobacteria have long been recognized as having enormous potential for use in biotechnology, especially in agriculture. In fact, use of cyanobacteria in rice fields is being popularized at present. This is mainly because,

1. Cyanobacteria are simple photosynthetic prokaryotes which have simple growth requirements and which use a cheap source of reductant, i.e. water. This gives them an edge over other photosynthetic bacteria.
2. Many cyanobacteria combine photosynthesis and N_2-fixation. This gives them an edge over other eukaryotic photosynthetic organisms.

Current use of cyanobacteria in rice fields (Singh, 1961; Venkatraman, 1980; Stewart *et al.*, 1987) has serious limitations. Normal cyanobacteria use much of their fixed-N for their own growth, releasing only a small amount in the field. Furthermore, presence of nitrogen fertilizers in the field adversely affects N_2-fixation and cyanobacteria, instead of fixing N_2, start using the nitrogen available in the field. Thus, they become like weed for the crop. Despite these problems cyanobacterial inoculation in the field has been shown to be beneficial for rice crops, saving considerable amount of expenditure which has to be incurred on chemical fertilizers. It is obvious therefore, that if we produce suitably modified strains the benefits could be increased several fold. To achieve this goal some of the strategies are discussed below:

First thing, of course, is to maximize N_2-fixation. For this, a modification of cyanobacterial metabolism resulting in

diversion of more energy for N_2-fixation, and less for ammonia assimilation and biomass growth, is necessary. Some of the target points for this have been shown in fig 8.4. The best modification point, in the above scheme, is the level of GS. It should be emphasized that these mutants should have 5-10% of GS activity left otherwise the mutant would become a glutamine auxotroph and its survival would require glutamine supply from outside. Reduction in GS level would restrict utilization of ATP, reductant and carbon skeletons for ammonia assimilation and biomass growth thus, leading to diversion of more energy for N_2-fixation. It may also lead to an increase in heterocyst frequency as in the case of symbiotic cyanobacteria (Rai, 1990). Reduction in GS level would also lead to accumulation of ammonia since ammonia assimilation would be restricted. This would lead to ammonia release, at larger scale than in normal cyanobacteria, since ATS would be unable to cope with the recycling requirements over and above the maintenance of normal internal concentration (Rai and Prakasham, 1989). At the same time, the residual GS activity would ensure some ammonia assimilation necessary for the survival of the cell.

An alternative strategy for generating suitable cyanobacterial strains for ammonia liberation is to manipulate its ATS. ATS plays an important role in cyclic retention of ammonia within the cell (Kleiner, 1985b). It has been calculated that nitrogenase derived ammonia can diffuse across the plasma membrane in bacteria six times before being captured by GS (Kleiner, 1985b; see Fig 1.1). So, if ATS is abolished, or made inefficient, much of the nitrogenase derived ammonia would escape out since cyclic retention by ATS will not take place or would take place at a

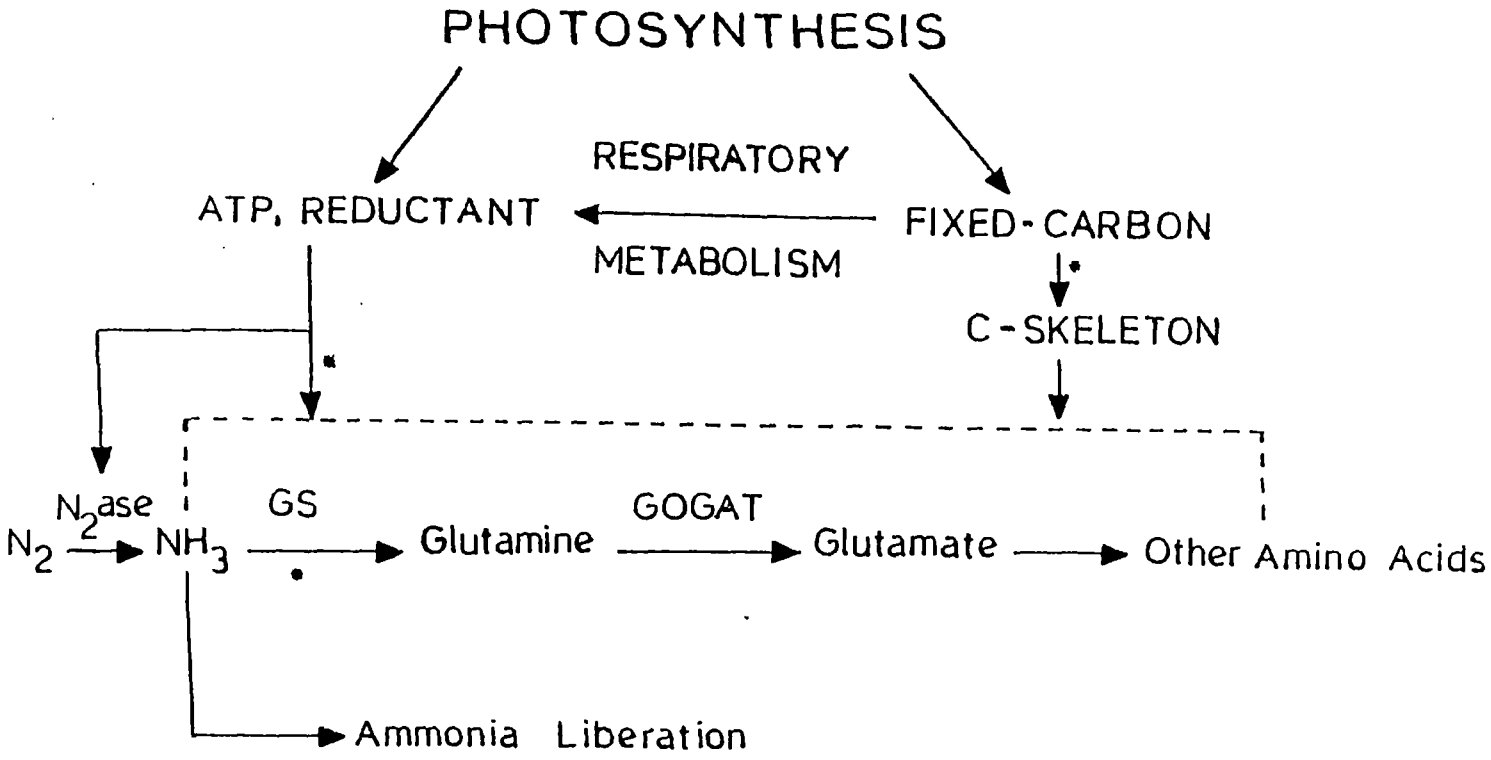

Fig 8.4 Targets for modification of cyanobacterial cellular metabolism for photobiological production of ammonia. Expected outcome of such modifications include: 1) diversion of more photosynthate for nitrogen fixation and less for biomass production; 2) increased nitrogen fixation and reduced ammonia utilization. *, targets of modification.

slower rate (Fig 1.1). Secondly, in such a strain nitrogenase will not be repressed by the presence of ammonium or nitrate fertilizers in the field since these exogenous sources will not cause ammonium accumulation in the cell in absence of ATS. This will not only ensure continued N_2-fixation but also ensure that cyanobacteria do not use of the nitrogen fertilizer meant for the crop. Thirdly, absence of ATS will result in less assimilation of nitrogenase-derived ammonia by the cyanobacterium (since much of it would escape). Therefore, less energy would be utilized in ammonia assimilation and biomass growth; i.e. more energy would be available for N_2-fixation. Some of the consequences of the absence of ATS are depicted in fig 8.5.

The ATS-deficient or the GS-deficient strains of the kind discussed above are obviously better suited for rice field application since they would liberate more ammonia and would fix N_2 at a higher rate. In addition, these strains can also be used in laboratory for photobiological production of ammonia. Currently, normal cyanobacteria, after immobilization, are being used for such a purpose (Stewart *et al.*, 1983; 1987) by inhibiting endogenous ammonia assimilation using MSX (a GS inhibitor). Such a system has serious drawback since total inhibition of GS renders the cyanobacterium totally dependent on provision of exogenous glutamine for its survival. Moreover, the MSX is poisonous and expensive. Therefore, the use of ATS-deficient or GS-deficient strains would be far superior for such an application.

The ATS-deficient and GS-deficient strains can also be used for establishment of artificial symbiosis with crop plants. Earlier attempts in this direction have not been successful but

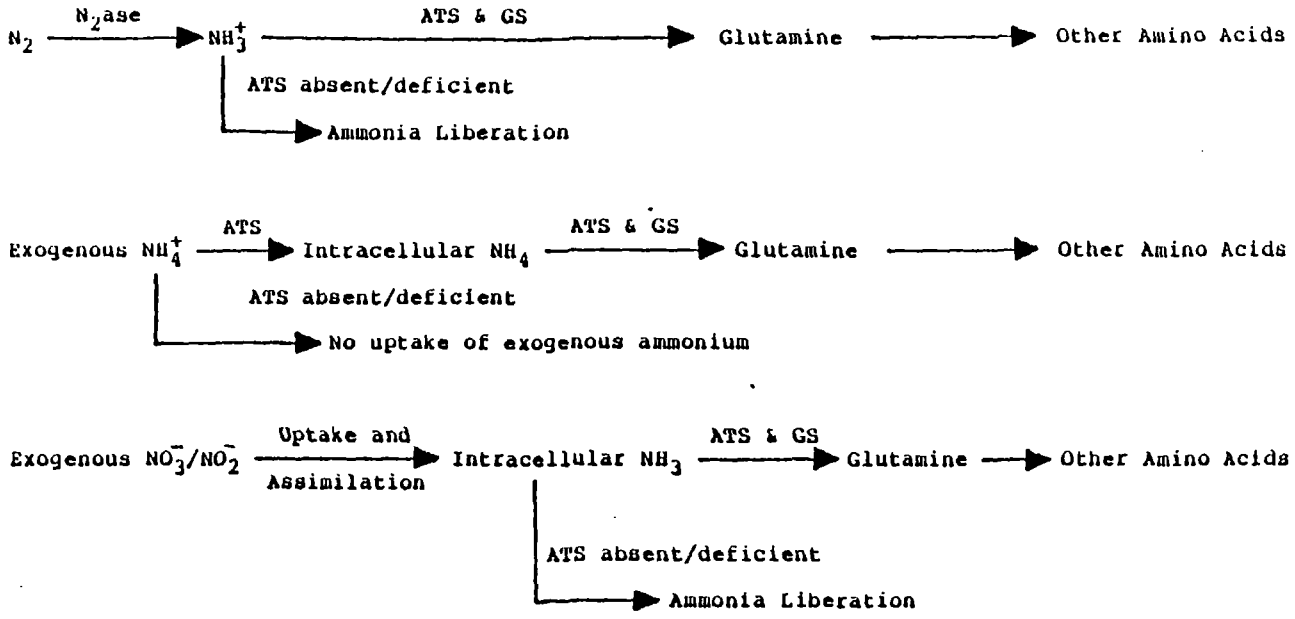

Fig 8.5 Modification of ammonium transport system: Consequences for a diazotrophic cyanobacterium. Expected outcome of such a modification includes inefficient nitrogen-assimilation, slow growth, less photosynthate for biomass growth, higher energy availability for nitrogen-fixation and inefficient nitrogen control of nitrogenase.

with the increased knowledge about the symbiotic cyanobacteria it is worthwhile to continue the attempt. Recent work of Gusev and his colleagues have shown encouraging results (Gusev and Korzhenevskaya, 1990).

At present, the production of various amino acids or other compounds are obtained from cyanobacteria by increasing the permeability of plasmamembrane (Fukui and Ishida, 1972; Clement *et al.*, 1984) or by making a transitory loss of plasmalemma integrity (Reed *et al.*, 1986). This is achieved by using specific detergents. The constant use of detergents causes damage to the cellular metabolic mechanism and consequently growth is affected (Reed *et al.*, 1986). Moreover, separation of liberated compounds from the detergent is another difficult problem. Kleiner (1985a) has suggested that amino acid transport systems may have a role in maintaining intracellular pools of amino acids and that abolition of these transport systems may cause liberation of amino acids from the cells. Indeed the two cyanobacterial strains studied here do possess specific transport systems for glutamine and glutamate (see chapters 6 and 7). Further detailed studies on amino acid transport systems followed by their manipulation may yield amino acid transport-defective mutants which can be used for photobiological production of amino acids. Such strains would have an advantage over detergent affected strains in production of amino acids without disturbing the cellular integrity and/or metabolism. They also show a constant growth as well as diversion of more energy to produce that particular compound.

9. SUMMARY

Anabaena 7120 (a free-living strain) and *Nostoc* ANTH (an isolate from *Anthoceros punctatus*) were studied with regards to transport and metabolism of NH_4^+, glutamine and glutamate. The implications of these findings for nitrogenase regulation were also explored. For ammonium transport studies $^{14}CH_3NH_3^+$, the radioactive analogue of ammonium, was used as probe. The findings are summarized below.

I. Studies on $CH_3NH_3^+$ metabolism in *Anabaena* 7120 and *Nostoc* ANTH indicated:

1. that $CH_3NH_3^+$ is assimilated by glutamine synthetase (GS) in both the strains used. However, *Anabaena* 7120 could not metabolize $CH_3NH_3^+$ as N-source since it could not assimilate $CH_3NH_3^+$ beyond methylglutamine. In contrast, *Nostoc* ANTH was able to use $CH_3NH_3^+$ as N-source for growth.
2. that none of the strains used were able to utilize $CH_3NH_3^+$ as C-source.

II. The ammonium/methylammonium transport studies in *Anabaena* 7120 showed:

1. that the pattern of intracellular accumulation of free $^{14}CH_3NH_3^+$ is biphasic. A maximum intracellular concentration of 2.5 and 7.5 $mmol.dm^{-3}$ was reached in the external $^{14}CH_3NH_3^+$ concentration range of 1-50 and 50-500 $\mu mol.dm^{-3}$, respectively.

2. that, at pH 7, two energy-dependent ammonium/methylammonium transport systems (ATS) operate in N_2- and NO_3^-- grown cells. One of the transport systems is fast, methionine sulphoximine (MSX)-insensitive and responsible for the first phase of $CH_3NH_3^+$ uptake. The other transport system is slower, MSX-sensitive and is responsible for the second phase of $CH_3NH_3^+$ uptake. Both these transport systems are repressed in ammonium- grown cells.
3. that both transport systems undergo affinity modulation (a low- and a high- affinity mode of operation) depending on the external substrate concentration.
4. that the MSX-insensitive ATS has Km values of 8 and 80 $\mu mol.dm^{-3}$ (corresponding Vmax values are 1 and 7 $nmol.min^{-1}.mg^{-1}$ protein) in the external $^{14}CH_3NH_3^+$ concentration range of 1-25 and 25-500 $\mu mol.dm^{-3}$, respectively.
5. that the MSX-sensitive ATS has Km values of 2.5 and 70 $\mu mol.dm^{-3}$ (corresponding Vmax values are 0.1 and 0.7 $nmol.min^{-1}.mg^{-1}$ protein) in the external $^{14}CH_3NH_3^+$ concentration range of 1-10 and 10-500 $\mu mol.dm^{-3}$, respectively.
6. that, there are two ammonium/methylammonium pools in the cells---one in thylakoid spaces, served by the MSX-insensitive ATS, and the other in cytoplasm, served by MSX-sensitive ATS. The latter pool is used by GS.

III. The ammonium/methylammonium transport studies in *Nostoc* ANTH, indicated:

1. that, at pH 7, two energy-dependent transport systems operate in N_2-, glutamine- and glucose- grown cells; but in

NH_4^+- grown cells, both these transport systems are repressed.

2. that both the transport systems undergo affinity modulation (a high- and a low- affinity mode of operation) depending on the external substrate concentration.
3. that one of the transport system is insensitive to MSX but affected by glutamine and glutamate; hence called MSX-insensitive ATS.
4. that the other transport system is sensitive to MSX (hence called MSX-sensitive ATS) but not affected by glutamine and glutamate.
5. that the MSX-insensitive .ATS has Km values of 3 and 45 $\mu mol.dm^{-3}$ (corresponding Vmax values are 0.125 and 0.225 $nmol.min^{-1}.\mu g^{-1}$ Chl **a**) in the external $^{14}CH_3NH_3^+$ concentration range of 1 - 15 and 15 - 500 $\mu mol.dm^{-3}$, respectively.
6. that the MSX-sensitive ATS has Km values of 4.6 and 135 $\mu mol.dm^{-3}$ (corresponding Vmax values are 0.028 and 0.0205 $nmol.min^{-1}.\mu g^{-1}$ Chl **a**) in the external $^{14}CH_3NH_3^+$ concentration range of 1 - 20 and 20 - 500 $\mu mol.dm^{-3}$, respectively.
7. that a specific methylammonium transport system is expressed in $CH_3NH_3^+$-grown *Nostoc* ANTH filaments, which is neither affected by NH_4Cl nor by MSX.

IV. In contrast to the earlier assumption that MSX is only a specific irreversible inhibitor of glutamine synthetase, MSX was found to have two sites of action in both strains studied:

1. inhibition of GS.
2. inhibition of the one of the ammonium/methylammonium transport systems (the slower second ATS).

In view of the above finding, the interpretations of future experiments involving MSX, have to take into account its effect on ammonium transport level also.

V. Glutamate uptake and metabolism studies in *Anabaena* 7120 and its Het^- Nif^- mutant revealed:

1. that glutamate is not assimilated as N-source by any of the strains.
2. that the glutamate acts as an inhibitor of heterocyst differentiation and nitrogenase activity.
3. that the nitrate moderates glutamate toxicity by inhibiting the glutamate uptake.

VI. The glutamine transport studies in *Anabaena* 7120, its Het^- Nif^- mutant and *Nostoc* ANTH indicated:

1. that a biphasic pattern of glutamine uptake occurs in N_2-, NO_3^--, NH_4^+- and glutamine- grown *Anabaena* 7120 and its Het^- Nif^- mutant filaments.
2. that an energy-dependent glutamine uptake system was present in N_2- and glutamine- grown *Nostoc* ANTH filaments.
3. that the glutamine transport system in *Nostoc* ANTH filaments is not affected by NH_4Cl, glutamate and MSX, indicating it is specific for glutamine only.

VII. No short term effect of ammonium, on nitrogenase activity, occurred at physiological pH. In bacteria, NH_4^+ has been shown to collapse $\Delta\psi$ thereby inhibiting nitrogenase activity by adversely affecting e^- donation to nitrogenase within minutes.

However, in the cyanobacterial strain studied here, NH_4^+ affected $\Delta\psi$ only partially. The residual $\Delta\psi$ level still remained above the level required for optimum nitrogenase activity (> -70 mV). Over a longer period, ammonium caused repression of nitrogenase. The data show that under physiological conditions a product of ammonia assimilation rather than ammonia *per se* is the repressor for nitrogenase in heterocystous cyanobacteria.

VIII. The present study indicated that repression of ATS may provide a novel method for generation of cyanobacterial strains which are leaky for ammonia and which have permanently derepressed nitrogenase. Such strains can be employed for photobiological production of ammonia or can be used in rice fields to supplement chemical fertilizers without having any adverse effect on nitrogenase.

10. REFERENCES

Alberte RJ, Tel-Or E, Packer L and Thornber JP (1980): Functional organization of the photosynthetic apparatus in heterocysts of nitrogen-fixing cyanobacteria. **Nature 284; 481-483.**

Alef K and Kleiner D (1982a): Evidence for an ammonium transport system in the N_2-fixing phototrophic bacterium *Rhodospirillum rubrum*. **Arch. Microbiol. 132; 79-81.**

Alef K and Kleiner D (1982b): Regulatory aspects of inorganic nitrogen metabolism in the *Rhodospirilliaceae*. **Arch. Microbiol. 133; 239-241.**

Allen MM (1984): Cyanobacterial cell inclusions. **Ann. Rev. Microbiol. 38; 1-25.**

Allen MM and Smith AJ (1969): Nitrogen chlorosis in blue-green algae. **Arch. Microbiol. 69; 114-120.**

Ames GF-L (1986): Bacterial periplasmic transport systems: structure, mechanism and evolution. **Ann. Rev. Biochem. 55; 397-425.**

Antonucci TK and Oxender DO (1986): The molecular biology of amino acid transport in bacteria. **Adv. Microbiol. Physiol. 28; 146-180.**

Apte SK, Rowell P and Stewart WDP (1978): Electron donation to ferredoxin in heterocysts of the N_2-fixing alga *Anabaena cylindrica*. **Proc. R. Soc. Lond. B. 200; 1-25.**

Arp DJ and Zumft WG (1983): L-Methionine-SR-Sulfoximine as a probe for the role of glutamine synthetase in nitrogenase switch-off by ammonia and glutamine in *Rhodopseudomonas palustris*. **Arch. Micriobiol. 134; 17-22.**

Avissar YJ (1985): Induction of nitrate assimilation in the cyanobacterium *Anabaena variabilis*. **Physiol. Plant 63; 105-108.**

Bagchi SN, Rai UN, Rai AN and Singh HN (1985b): Nitrate metabolism in cyanobacterium *Anabaena cycadeae*: Regulation of nitrate uptake & reductase by ammonium. **Physiol. Plant. 63; 322-326.**

Bagchi SN, Rai AN and Singh HN (1985a): Regulation of nitrate reductase in cyanobacteria: Repression derepression control of nitrate reductase apoprotein in the cyanobacterium *Nostoc muscorum*. **Biochim. Biophys. Acta 338; 370-373.**

Bagchi SN and Singh HN (1984): Genetic control of nitrate reduction in the cyanobacterium *Nostoc muscorum*. **Mol. Gen. Genet. 193; 82-84.**

Bakker EP, Rottenberg H, and Caplan SR (1976): An estimation of the light induced electrochemical potential difference of protons across the membrane of *Halobacterium halobium*. **Biochim. Biophys. Acta 440; 557-572.**

Barnes EM and Zimniak P (1981): Transport of ammonium and methylammonium ions by *Azotobacter vinelandii*. **J. Bacteriol. 146; 512-516.**

Bergman B, Lindblad P, Peterson A, Renstrom E and Tiberg E (1985): Immunogold localization of glutamine synthetase in a nitrogen fixing cyanobacterium *Anabaena cylindrica*. **Planta 166; 329-334.**

Bergman B, Lindblad P and Rai AN (1986): Nitrogenase in free living and symbiotic cyanobacteria: Immunoelectron microscope localization. **FEMS Microbiol. Letts. 35; 75-78.**

Bogdahn M, Andreesen JR and Kleiner D (1983): Pathways and regulation of N_2-ammonium and glutamate assimilation by *Clostridium* sp. **Arch. Microbiol. 134; 167-169.**

Bognar A, Desrosiers L, Libman M and Newman EB (1982): Control of nitrogenase in a photosynthetic autotrophic bacterium, *Ectothiorliodospira* sp. **J. Bacteriol. 152; 706-713.**

Bohme H (1986): Inhibition of N_2-fixation by nitrite in *Anabaena variabilis*. **Arch. Microbiol 146; 99-103.**

Bohme H (1987): Regulation of electron flow to nitrogenase in a cell free system from heterocysts of *Anabaena variabilis*. **Biochim. Biophys. Acta 891; 121-128.**

Bohme H and Schrautemeier B (1987): Electron donation to nitrogenase in a cell free system from heterocysts of *Anabaena variabilis*. **Biochim. Biochem. Acta 891; 115-120.**

Booth IR and Hamilton WA (1980): Energetics of bacterial amino acid transport in microorganisms and nitrogen sources (Payne JW ed.) **pp 171-207, Wiley, Chichester.**

Bothe H (1969): The role of phytoflavin in photosynthetic reaction. In: Progress in Photosynthesis Research (Metzner H ed.) Vol III. **pp 1483-1491, University of Tubingen, Germany.**

Bothe H (1982): Hydrogen production by algae. **Experientia 38; 59-64.**

Bothe H, Nelles H, Hager KP, Papen H and Neuer G (1984): Physiology and biochemistry of N_2-fixation by cyanobacteria. In: Advances in nitrogen fixation research (Veeger C & Newton WE eds.) **pp 199-210, The Hugue and Wageningen, Nijhoff, Junk, Pudoc.**

Bothe H, Neuer G, Kalbe I and Eisbrenner G (1980): Electron donors and hydrogenase in nitrogen fixing organisms. In: Nitrogen fixation (Stewart WDP & Gallon JR eds.) **pp 83-112, Academic Press, London & New York.**

Bothe H, Yates MG and Cannon FC (1982): Nitrogen fixation. In: Encyclopedia of plant physiology (Lauchli A & Bieleski R eds.) **New series, Springer, Berlin.**

Bottomley PJ and Stewart WDP (1976): ATP pools and transients in the blue green alga, *Anabaena cylindrica*. **Arch. Microbiol. 108; 249-258.**

Boussiba S (1989): Ammonium uptake in the alkalophillic cyanobacterium *Spirulina platensis*. **Plant Cell Physiol. 30; 303-308.**

Boussiba S, Dilling W and Gibson J (1984a): Methylammonium transport in *Anacystis nidulans* R-2. **J. Bacteriol. 160; 204-210.**

Boussiba S and Gibson J (1985): The role of glutamine synthetase activity in ammonium and methylammonium transport in *Anacystis nidulans* R-2. **FEBS Letts. 180; 13-16.**

Boussiba S and Gibson J (1987): Regulation of methylammonium/ ammonium transport in the unicellular cyanobacterium *Synechococcus* R-2 (PCC 7942). **FEMS Microbiol. Letts. 43; 289-293.**

Boussiba S, Resch CM and Gibson J (1984b): Ammonia uptake and retention in some cyanobacteria. **Arch. Microbiol. 138; 287-292.**

Brill WJ (1980): Biochemical genetics of nitrogen fixation. **Microbiol. Rev. 44; 449-467.**

Burgess BK (1985): Nitrogenase mechanisms - an overview. In: Nitrogen fixation research progress (Evans HJ, Bottomley PJ & Newton WE eds.) **pp 543-549, Dordrecht, Nijhoff.**

Carr NG and Whitton BA (1982): In: Biology of cyanobacteria (Carr NG & Whitton BA eds.) **Blackwell Scientific Publications, Oxford.**

Chapman JS and Meeks JC (1983): Glutamine and Glutamate transport by *Anabaena variabilis*. **J. Bacteriol. 156; 122-129.**

Clement Y, Escoffier B, Trombe MC and Laneelle G (1984): Is glutamate excreted by its uptake system in *Corynebacterium glutamicum*? A working hypothesis. **J. Gen. Microbiol. 130; 2589-2594.**

Codd GA, Okabe K and Stewart WDP (1980): Cellular compartmentation of photosynthetic and photorespiratory enzymes in the heterocystous cyanobacterium *Anabaena cylindrica*. **Arch. Microbiol. 124; 149-154.**

Codd GA and Stewart WDP (1976): Polyhedral bodies and ribulose-1,5-diphosphate carboxylase of the blue-green alga *Anabaena cylindrica*. **Planta . 130; 323-326.**

Codd GA and Stewart WDP (1977): D-ribulose-1,5-diphosphate carboxylase from the blue-green alga *Aphanocapsa* 6308. **Arch. Microbiol. 113; 105-110.**

Cohen-Bazire G and Bryant DA (1982): Phycobilisomes: Composition and structure. In: The Biology of Cyanobacteria (Carr NG & Whitton BA eds.) **pp 143-190, Blackwell Scientific Publications, Oxford.**

Collins JJ and Brill WJ (1985): Control of *Klebsiella nif* mRNA synthesis. **J. Bacteriol. 162; 1186-1190.**

Cordewener J, Kruse-Wolters M, Wassink H, Haaker H and Veeger C (1988): The role of MgATP hydrolysis in nitrogenase catalysis. **Eur. J. Biochem. 172; 739-745.**

Cossar JD, Rowell P, Darling AJ, Murry S, Codd GA and Stewart WDP (1985): Localization of ribulose-1,5-bisphosphate carboxylase/ oxygenase in the nitrogen fixing cyanobacterium *Anabaena cylindrica*. **FEMS Microbiol. Letts. 28; 65-68.**

Cossar JD, Rowell P and Stewart WDP (1984): Thioredoxin as a modulator of glucose-6-phosphate dehydrogenase in a N_2-fixing cyanobacteria. **J. Gen. Microbiol. 130; 991-998.**

Daesch G and Mortenson LE (1972): Effect of ammonia on the synthesis and function of the N_2-fixing enzyme system in *Clostridium pasteurianum*. **J. Bacteriol. 110; 103-109.**

Darmawardene MWN, Haystead A and Stewart WDP (1973): Glutamine synthetase of the nitrogen-fixing alga *Anabaena cylindrica*. **Arch. Microbiol. 90; 281-295.**

Doolittle WE (1979): The cyanobacterial genome and its expression and the control of that expression. **Adv. Microbiol. Physiol. 20; 1-102.**

Doolittle WE (1982): Molecular evolution. In: The Biology of Cyanobacteria (Carr NG & Whitton BA eds.) **pp 307-331, Blackwell Scientific Publications, Oxford.**

Driessen AJM, Ubbink-Kok T and Konings NW (1988): Amino acid transport by membrane vesicles of an obligate anaerobic bacterium *Clostridium acetobutylicum*. **J. Bacteriol. 170; 817-820.**

Duggan JX and Anderson LE (1975): Light regulation of enzyme activity in *Anacystis nidulans* (Richt). **Planta 122; 293-297.**

Eady RR (1986): Enzymology in free living diazotrophs. In: Nitrogen fixation Vol 4 (molecular biology) (Broughton WJ & Puhler A eds.) **pp 1-49, Clarender Press, Oxford.**

Fay P, Stewart WDP, Walsby AE and Fogg GE (1968): Is the heterocyst the site of nitrogen fixation in blue green algae? **Nature 220; 810-812.**

Flores E and Muro-Pastor MI (1988): Uptake of glutamine and glutamate by the dinitrogen fixing cyanobacterium *Anabaena* sp. PCC 7120. **FEMS Microbiol. Letts. 56; 127-130.**

Fogg GE, Stewart WDP, Fay P and Walsby AE (1973): The blue green algae. **Academic Press, London & New York.**

Fukui S and Ishida M (1982): Biomembrane permeability and the accumulation of amino acids. In: The microbial production of amino acids (Yamada K, Kinoshita S, Tsunoda T & Aida K eds.) **pp 123-237, John Wiley, New York.**

Gallon JR (1980): Nitrogen fixation by photoautotrophs. In: Nitrogen fixation (Stewart WDP & Gallon JR eds.) **pp 197-238, Academic press, London & New York.**

Gallon JR and Chaplin AE (1987): An Introduction to nitrogen fixation **Cassel Publications, Great Britain.**

Gallon JR and Chaplin AE (1988): Recent studies on N_2-fixation by non-heterocystous cyanobacteria. In: Nitrogen fixation: Hundred years after (Bothe H, deBruijn FJ & Newton E eds.) **pp 183-188, Gustav Fisher, New York.**

Genthner BRS and Wall JD (1985): Ammonium uptake in *Rhodopseudomonas capsulata*. **Arch. Microbiol. 141; 219-224.**

Gibson J (1984): Nutrient transport by anoxygenic and oxygenic photosynthetic bacteria. **Ann. Rev. Microbiol. 38; 135-159.**

Glenn AR and Dilworth MJ (1984): Methylamine and ammonium transport systems in *Rhizobium leguminosarum* MNF 3841. **J. Gen. Microbiol. 130; 1961-1968.**

Gober JW and Kashket EF (1983): Methylammonium uptake by *Rhizobium* sp. 32H1. **J. Bacteriol. 153; 1196-1201.**

Golden JW, Robinson SJ and Haselkorn R (1985): Rearrangement of nitrogen fixation genes during heterocyst differentiation in the cyanobacterium *Anabaena*. **Nature 314; 419-423.**

Gordon JK and Moore RA (1981): Ammonium and methylammonium transport by the nitrogen fixing bacterium *Azotobacter vinelandii*. **J. Bacteriol. 148; 435-442.**

Grossman A and McGowan RE (1975): Regulation of glucose-6-phosphate in blue-green algae. **Pl. Physiol. Lancaster. 55; 658-662.**

Guerrero MG, Vega JM and Losada M (1981): The assimilatory nitrate reducing system and its regulation. **Ann. Rev. Pl. Physiol. 32; 169-204.**

Guffanti AA, Blanco R and Krulwich TA (1979): A requirement for ATP for galactoside transport by *Bacillus alcalophilus*. **J. Biol. Chem. 254; 1033-1037.**

Guillard RL (1973): Division rates. In: Handbook of phycological methods - culture methods and growth measurements (Stein JR ed.) **pp 289-311, Cambridge Univ. Press, England.**

Gupta M & Carr NG (1981): Detection of glutamate synthase in heterocysts of *Anabaena* sp. strain 7120. **J. Bacteriol. 148; 980-982.**

Gusev MV and Korzhenevskaya TG (1990): Artificial associations. In: Handbook of symbiotic cyanobacteria (Rai AN ed.) **pp 173-230, CRC Press Inc., Boca Raton, Florida.**

Haaker H, Cordewener J, Tenasbroek A, Wassink H, Eady R and Veeger C (1985): The role of Fe-protein in nitrogenase catalysis. In: Nitrogen fixation research progress (Evans HJ, Bottomley PJ & Newton WE eds.) **pp 567-576, Dordrecht, Nijhoff.**

Haaker H, Laane C and Veeger C (1980): Dinitrogen fixation and the proton motive force. In: Nitrogen fixation (Stewart WDP & Gallon JR eds.) **pp 113-138, Academic press, London.**

Hales BJ, Case EE and Langosch D (1985): Nitrogen fixation in *nif* HDK deletion strains of *Azotobacter vinelandii*. In: Nitrogen fixation research progress (Evans JH, Bottomley PJ and Newton WE eds.) **p. 612, Dordrecht, Martinus, Nijhoff.**

Hallenbeck PC (1987): Molecular aspects of nitrogen-fixation by photosynthetic prokaryotes. **CRC Critical Rev. 14; 1-49.**

Hallenbeck PC and Benemann JR (1979): Hydrogen from algae. In: Photosynthesis in relation to model systems (Barber J ed.) **pp 331-364, Elsevier/North Holland Biomedical Press, Amsterdam.**

Hallenbeck PC, Meyer CM and Vignais PM (1982): Regulation of nitrogenase in the photosynthetic bacterium *Rhodopseudomonas capsulata* as studied by two dimentional electrophoresis. **J. Bacteriol. 151, 1612-**

O'Hara GW, Riley IT, Glenn AR and Dilworth MJ (1985): The ammonium permease of *Rhizobium leguminosarum* MN 3841. **J. Gen. Microbiol. 131, 757-764.**

Hartman SC (1973): Relationships between glutamine amidotransferases and glutaminases. In: The enzymes of glutamine metabolism (Prusiner S & Stadtman ER eds.) **pp 319-330, Academic Press, New York.**

Hartman A and Kleiner D (1982): Ammonium (methylammonium) transport by *Azospirillum* sp. **FEMS Microbiol. Letts. 15; 65-67.**

Haselkorn R (1978): Heterocyst. **Ann. Rev. Pl. Physiol. 29; 319-344.**

Haselkorn R, Golden JW, Lammers PJ and Mulligam ME (1987): Rearrangement of *nif* genes during cyanobacterial heterocyst differentiation. **Phil. Trans. R. Soc. Lond. B. 317; 173-181.**

Haury JF and Wolk CP (1978): Classes of *Anabaena variabilis* mutants with oxygen sensitive nitrogenase activity. **J. Bacteriol. 136; 688-692.**

Hawkesford MJ, Reed RH, Rowell P and Stewart WDP (1981): Nitrogenase activity and membrane electrogenesis in the cyanobacterium *Anabaena variabilis* Kutz. **Eur. J. Biochem. 115; 519-523.**

Henderson PJE (1971): Ion transport by energy conserving biological membranes. **Ann. Rev. Microbiol. 25; 393-248.**

Herrero A, Flores E and Guerrero MG (1981): Regulation of nitrate reductase levels in the cyanobacteria *Anacystis nidulans*, *Anabaena* sp. strain 7119 and *Nostoc* sp. strain 6719. **J. Bacteriol. 145; 175-180.**

Ho KK and Krogmann DW (1982): Photosynthesis. In: The Biology of cyanobacteria (Carr NG & Whitton BA eds.) **pp 191-214, Blackwell Scientific publications, Oxford.**

Holtel A and Kleiner D (1985): Regulation of methylammonium transport in *Paracoccus denitrificans*. **Arch. Microbiol. 142; 285-288.**

Howard JB, Deits TL, Anderson GL, Moroney M Que L and Hausinger RP (1985): Mechanisms and structure of nitrogenase Fe-protein. In: Nitrogen fixation and CO_2 metabolism (Ludden PW & Burris JE eds.) **pp 153-162, Elsevier, New York.**

Howitt SM, Udvardi MK, Day DA and Gresshoff PM (1986): Ammonia transport in free-living and symbiotic *Rhizobium* sp. ANU 289. **J. Gen. Microbiol. 132; 257-261.**

Ivanovsky RN, Vakulenko VP and Kondratieva EN (1982): Uptake of ammonium and methylammonium by *Rhodopseudomonas spheroides* 2R. **Abstr. 4th Int. Symp. Photosynth. Prokaryotes Bombannes (France) B31.**

Janaki S and Wolk CP (1982): Synthesis of nitrogenase in isolated heterocysts. **Biochim. Biophys. Acta 696; 187-192.**

Jayakumar A and Barnes EM (1984): The role of glutamine in regulation of ammonium transport in *Azotobacter vinelandii*. **Arch. Biochem. Biophys. 231; 95-101.**

Jenkinson DS (1977): The nitrogen economy of the Baardblack experiments. I. Nitrogen balance in the experiments. In: Rothamsted Experimental station. **Ann. Report for 1976 part 2 pp 103-110.**

Jensen BB and Burris RH (1986): N_2O as a substrate and as a competitive inhibitor of nitrogenase. **Biochem. 25; 1083-1088.**

Jensen BB and Cox RP (1983): Effect of oxygen concentration on dark nitrogen fixation and respiration in cyanobacteria. **Arch. Microbiol. 135; 287-292.**

Jones BJ and Monty KT (1979): Glutamine as a feedback inhibitor of the *Rhodopseudomonas sphaeroides* nitrogenase system. **J. Bacteriol. 139; 1007-1013.**

Joseph CM and Meeks JC (1987): Regulation and expression of glutamine synthetase in a symbiotic *Nostoc* strain associated with *Anthoceros punctatus*. **J. Bacteriol. 169; 2471-2475.**

Jost M (1965): Die ultrastruktur Von *Oscillatoria rubescens* DC. **Arch. Mikrobiol. 50; 211-245.**

Kashyap AK and Johar G (1984a): Ammonium transport in N_2 fixing cyanobacterium *Nostoc muscorum*: Interaction with copper and sulfhydryl agents. **J. Gen. Appl. Microbiol. 30; 267-275.**

Kashyap AK and Johar G (1984b): Genetic control of ammonium transport in nitrogen fixing cyanobacterium *Nostoc muscorum*. **Mol. Gen. Gent. 197; 509-512.**

Kashyap AK and Singh DP (1985): Ammonium transport in unicellular cyanobacterium *Anacystis nidulans*. **J. Pl. Physiol. 127; 319-330.**

Kentemich T, Danneberg G, Hundeshagen B and Bothe H (1988): The occurrance of alternative nitrogenase in the cyanobacterium *Anabaena variabilis*. In: Nitrogen fixation: Hundred years after (Bothe H, deBruijn FJ & Newton WE eds.) **p 89, Gustov Fischer, New York.**

Kerby NW, Musgrave SC, Codd GA, Rowell P and Stewart WDP (1983): Photoproduction of ammonia by immobilized cyanobacteria. In: Proceedings of Biotech'83. **pp 1029-1036, Online publications, Northwood, UK.**

Kerby NW, Rowell P and Stewart WDP (1985): Ethylene diamine uptake and metabolism in the cyanobacterium *Anabaena variabilis*. **Arch. Microbiol. 141; 244-248.**

Kerby NW, Rowell P and Stewart WDP (1986): The uptake and metabolism of methylamine by nitrogen fixing cyanobacteria. **Arch. Microbiol. 143; 353-358.**

Kerby NW, Rowell P and Stewart WDP (1987): Cyanobacterial ammonium transport, ammonium assimilation and nitrogenase regulation. **New Zealand J. Marine & Freshwater Res. 21; 447-455.**

Kleiner D (1981): The transport of NH_3 and NH_4^+ across biological membranes. **Biochim. Biophys. Acta 639; 41-52.**

Kleiner D (1982): Ammonium (methylammonium) transport by *Klebsiella pneumoniae*. **Biochim. Biophys. Acta 688; 702-708.**

Kleiner D (1985a): Bacterial ammonium transport. **FEMS Microbiol. Reviews 32; 87-100.**

Kleiner D (1985b): Energy expenditure for cyclic retention of NH_3/NH_4^+ during N_2-fixation by *Klebsiella pneumoniae*. **FEBS Letts. 187; 237-239.**

Kleiner D, Alef K and Hartmann A (1983): Uptake of methionine sulfoximine by some N_2-fixing bacteria and its effect on ammonium transport. **FEBS Letts. 164; 121-123.**

Kleiner D and Castroph H (1982): Inhibition of ammonium (methylammonium) transport in *Klebsiella pneumoniae* by glutamine and glutamine analogue. **FEBS Letts. 146; 201-203.**

Kleiner D and Fitzke E (1981): Some properties of a new electrogenic transport system: The ammonium (methylammonium) carrier from *Clostridium pasteurianum*. **Biochim. Biophys. Acta 641; 138-147.**

Klugkist J, Haaker H and Veeger C (1986): Studies on the mechanism of electron transport to nitrogenase in *Azotobacter vinelandii*. **Eur. J. Biochem. 155; 41-46.**

Kumar AP, Rai AN and Singh HN (1985): Nitrate reductase activity in isolated heterocysts of the cyanobacterium *Nostoc muscorum*. **FEBS Letts. 179; 125-128.**

Labarre J, Thuriaux P and Chauvat F (1987): Genetic analysis of amino acid transport in the facultatively heterotrophic cyanobacterium *Synechocystis* sp. strain 6803. **J. Bacteriol. 169; 4668-4673.**

Ladha JK, Rowell P and Stewart WDP (1978): Effects of 5-hydroxylysine on acetylene reduction and ammonia assimilation in the cyanobacterium *Anabaena cylindrica*. **Biophys. Biochem. Res. Commun. 83; 688-696.**

Lara C, Romero JM and Guerrero MG (1987): Regulated nitrate transport in the cyanobacterium *Anacystis nidulans*. **J. Bacteriol. 169; 4376-4378.**

Leach CK and Carr NG (1971): Pyruvate:ferredoxin oxido-reductase and its activation by ATP in the blue-green alga *Anabaena variabilis*. **Biochim. Biophys. Acta 245; 165-174.**

Lee-Kaden J and Simonis W (1979): Photosystem I & photophosphorylation-dependent leucine incorporation in the blue-green alga *Anacystis nidulans*. **Plant & Cell Physiol. 20; 1179-1190.**

Lee-Kaden J and Simonis W (1982): Amino acid uptake and energy coupling dependent on photosynthesis in *Anacystis nidulans*. **J. Bacteriol. 151; 229-236.**

Lineweaver H and Burk D (1934): The determination of enzyme dissociation constants. **J. Am. Chem. Soc. 56; 658-666.**

Lolkema JS, Hellingwerf KJ and Konings WN (1982): The effect of probe binding on the quantitative determination of the proton motive force. **Biochim. Biophys. Acta 681; 85-94.**

Long NJ and Whitton BA (1973): In: The biology of blue-green algae. (Carr NG & Whitton BA eds.) **pp 66-79, Blackwell Scientific publications, Oxford.**

Lowe DJ, Thornely RNF and Smith BE (1985): Nitrogenase. In: Metalloproteins part I (metal proteins with rodox roles) (Harrison PM ed.) **pp 207-249, MacMillan Press, London.**

Lowry OH, Rosebrough NJ, Farr AL and Randall RL (1951): Protein measurements with the Folin phenol reagent. **J. Biol. Chem. 193; 265-275.**

Ludden PS, Murrell SA, Pope M, Kanemoto R, Dowling TE, Saari LL and Triplett E (1984): Regulation of nitrogen-fixation in photosynthetic bacteria. In: Advances in nitrogen-fixation research (Veeger C & Newton NW eds.) **pp 181-187, The Hauge & Wageningen, Nijhoff, Junk, Pudoc.**

Mackerras AH and Smith GD (1986): Evidence for direct repression of nitrogenase by ammonia in the cyanobacterium *Anabaena cylindrica*. **Biophys. Biochem. Res. Commun. 134; 835-844.**

Mackinney G (1941): Absorption of light by chlorophyll solutions. **J. Biol. Chem. 140; 315-322.**

Magasanik B (1982): Genetic control of nitrogen assimilation in bacteria. **Ann. Rev. Genet. 16; 135-168.**

Manzano C, Candau P, Gomez-Moreno C, Relimpio AM and Lasada M (1976): Ferredoxin dependent photosynthetic reduction of nitrate and nitrite by particles of *Anacystis nidulans*. **Mol. Cell. Biochem. 10; 161-169.**

Maryan PS, Eady RR, Chaplin AE and Gallon JR (1986): Nitrogen fixation by *Gloeothece* sp. PCC (6909): Respiration and not photosynthesis supports nitrogenase activity in the light. **J. Gen. Microbiol. 132; 789-796.**

Meeks JC, Enderlin, CS, Joseph CM, Chapman JS and Lollar MWL (1985): Fixation of $[^{13}N]N_2$ and transfer of fixed nitrogen in the *Anthoceros-Nostoc* symbiotic association. **Planta 164; 406-414.**

Meeks JC, Wycoff KL, Chapman J and Enderlin CS (1983): Regulation of expression of nitrate and dinitrogen assimilation by *Anabaena* species. **Appl. and Environ. Microbiol. 45; 1351-1359.**

Merrick JM (1979): Metabolism of reserve materials. In: The photosynthetic bacteria (Clyton RK & Sistrom WR eds.) **pp 191-219, Plenum Press, New York.**

Miflin BJ and Lea PJ (1976): The pathway of nitrogen assimilation in plants. **Phytochem. 15; 873-885.**

Moore RA and Gordon JK (1984): Metabolism of methylammonium by *Azotobacter vinelandii*. **Arch. Microbiol. 140; 193-197.**

Morschel E and Rhiel E (1987): Phycobilisomes and thylakoids: The light harvesting system of cyanobacteria and red algae. In: Electron microscopy of protein (Harris JR & Horne RW eds.) **pp 209-254, Vol 6, Academic Press, London.**

Mortenson LE, Webb M, Bare R, Cramer SP and Morgan TV (1985): Studies on the structure and function of the Mo-Fe-protein of nitrogenase. In: Nitrogen fixation research progress (Evans HJ, Bottomley PJ & Newton WE eds.) **pp 577-583, Junk Publications, Dordrecht, Nijhoff.**

Muallem A, Bruce D and Hall DO (1983): Photoproduction of H_2 and $NADPH_2$ by polyurethane immobilized cyanobacteria. **Biotech. Letts. 5; 365-368.**

Mullineauex PM, Gallon JR and Chaplin AE (1981): Acetylene reduction (nitrogen fixation) by cyanobacteria grown under alternating light dark cycles. **FEMS Microbiol. Letts. 10; 245-247.**

Musgrave SC, Kerby NW, Codd GA, Rowell P and Stewart WDP (1983b): Reactor types for the utilization of immobilized photosynthetic microorganisms. In: Advances in fermentation: **Process Biochem. pp 184-190, Rickmansworth, Wheatland J. Ltd.**

Musgrave SC, Kerby NW, Codd GA and Stewart WDP (1982): Sustained ammonia production by immobilized filaments of nitrogen fixing cyanobacterium *Anabaena* 27893. **Biotechnol. Letts. 4; 647-652.**

Musgrave SC, Kerby NW, Codd GA and Stewart WDP (1983a): Structural features of calcium alginate entrapped cyanobacteria modified for ammonia production. **Eur. J. Microbiol. Biotechnol. 17; 133-136.**

Muzzucco CE and Benson DR (1984): [^{14}C]-methylammonium transport by *Frankia* sp. strain CB II. **J. Bacteriol. 160; 636-641.**

Neilson AH and Larson T (1980): The utilization of organic nitrogen for growth of algae: Physiological aspects. **Physiol. Plant. 48; 542-553.**

Neilson AH and Nordlund S (1975): Regulation of nitrogenase synthesis in intact cells of *Rhodospirillum rubrum* inactivation of nitrogen fixation by ammonia, L-glutamine and L-asparagine: **J. Gen. Microbiol. 91; 53-62.**

Nichols JM and Admas DG (1982): Akinetes. In: The biology of cyanobacteria (Carr NG & Whitton BA eds.) **pp 387-412, Blackwell Scientific publications, Oxford.**

Nierzwicki-Bauer SA and Haselkorn R (1986): Differences in mRNA levels in *Anabaena* living freely or in symbiotic association with *Azolla*. **EMBO J. 5; 29-35.**

Ninfa AJ, Ueno-Nishio S, Hunt TP, Robustell B and Magasanik B (1986): Purification of nitrogen regulator II, the product of the *gln* L (*ntr* B) gene of *Escherichia coli*. **J. Bacteriol. 168; 1002-1004.**

Ohmori M (1981): Effect of ammonia on dark CO_2 fixation by *Anabaena* cells treated with methionine sulfoximine. **Plant & Cell Physiol. 22; 709-716.**

Ohmori M and Hattori A (1970): Induction of nitrate & nitrite reductase in *Anabaena cylindrica*. **Plant & Cell Physiol. II; 873-878.**

Ohmori M, Saino T and Ohmori K (1985): Occurrence of Glutamine-2-oxoacid transaminase activity in the blue-green alga *Anabaena cylindrica*. **J. Gen. Appl. Microbiol 31; 171-176.**

Orme-Johnson WH (1985): Molecular basis of biological nitrogen fixation. **A Rev. Biophys. Chem. 14; 419-459.**

Orr J and Haselkorn R (1982): Regulation of glutamine synthetase activity and synthesis in free living and symbiotic *Anabaena* spp. **J. Bacteriol. 152; 626-635.**

Paparao J and Singh HN (1983): Methylamine regulation of heterocyst and nitrogenase in a methylamine resistant blue-green alga *Anabaena doliolum*. **Z. Pflanzenphysiol. Bd. 109; 69-79.**

Pargent W and Kleiner D (1985): Characteristics and regulation of ammonium (methylammonium) transport in *Rhizobium meliloti*. **FEMS Microbiol. Letts. 30; 257-259.**

Pope MR, Murrell SA and Ludden PW (1985): Covalent modification of the iron protein of nitrogenase from *Rhodospirillum rubrum* by adenosine diphosphoribosylation of a specific arginine residue. **Proc. Natl. Acad. Sci. USA 82; 3173-3177.**

Postgate JR (1982): The fundamentals of nitrogen fixation. **Cambridge Univ. Press, London.**

Rai AK, Kashyap AK and Gupta SL (1981a): ATP dependent uptake of nitrate in *Nostoc muscorum* and inhibition by ammonium ions. **Biochim. Biophys. Acta 674; 78-86.**

Rai AN (1988): Nitrogen metabolism. In: Handbook of lichenology, vol.1 (Galun M ed.) **pp 201-237, CRC Press, Boca Raton, Florida (USA).**

Rai AN (1990): Handbook of symbiotic cyanobacteria. **pp 1-253, CRC Press, Boca Raton, Florida (USA).**

Rai AN and Bergman B (1986): Modification of NO_3^- metabolism in heterocysts of the N_2-fixing cyanobacterium *Anabaena* 7120 (ATCC 27893). **FEMS Microbiol. Letts. 36; 133-137.**

Rai AN, Borthakur M, Singh S and Bergman B (1989): *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. **J. Gen. Microbiol. 135; 385-395.**

Rai AN, Lindblad P and Bergman B (1986a): Absence of glutamine-synthetase linked methylammonium (ammonium) transport system in the cyanobiont of cycas-cyanobacterial symbiosis. **Planta 169; 379-381.**

Rai AN and Prakasham R (1989): Characteristics of the methyl-ammonium/ammonium transport systems of the nitrogen-fixing cyanobacterium *Anabaena* 7120 (ATCC 27893). **Ind. J. Biochem. Biophys. 26; 219-226.**

Rai AN, Rao VV and Singh HN (1985): The Biology of cyanobacterial akinetes (spores). **J. Pl. Sci. 1; 1-20.**

Rai AN, Rao VV and Singh HN (1988): Metabolic changes associated with akinete germination in the cyanobacterium *Anabaena doliolum*. **New Phytol. 109; 133-138.**

Rai AN, Rowell P and Stewart WDP (1980): NH_4^+ assimilation and nitrogenase regulation in the lichen *Peltigera aphthosa* Wild. **New Phytol. 55; 545-555.**

Rai AN, Rowell P and Stewart WDP (1981b): Glutamine synthetase activity in symbiotic cyanobacteria. **J. Gen. Microbiol. 126; 515-518.**

Rai AN, Rowell P and Stewart WDP (1982): Glutamate synthase activity of heterocysts and vegetative cells of the cyanobacterium *Anabaena variabilis* Kutz. **J. Gen. Microbiol. 128; 2203-2205.**

Rai AN, Rowell P and Stewart WDP (1984): Evidence for an ammonium transport system in free-living and symbiotic cyanobacteria. **Arch. Microbiol. 137; 241-246.**

Rai AN, Singh DT and Singh HN (1986b): Regulation of ammonium/methylammonium transport by ammonium in the cyanobacterium *Anabaena variabilis*. **Physiol. Plant. 68; 320-322.**

Rao VV, Rai AN and Singh HN (1984): Metabolic activities of akinetes of the cyanobacterium *Anabaena doliolum*: oxygen exchange, photosynthetic pigments and enzymes of nitrogen metabolism. **J. Gen. Microbiol. 130; 1299-1302.**

Rawson DM (1985): The effects of exogenous amino acids on growth and nitrogenase activity in the cyanobacterium *Anabaena cylindrica* PCC 7122. **J. Gen. Micriobiol. 131; 2549-2554.**

Reed RH, Warr SRC, Kerby NW and Stewart WDP (1986): Osmotic shock-induced release of low molecular weight metabolites from free-living and immobilized cyanobacteria. **Enz. Microb. Technol. 8; 101-104.**

Reglinski A, Rowell P, Kerby NW and Stewart WDP (1989): Characterization of methylammonium/ammonium transport in mutant strains of *Anabaena variabilis* resistant to ammonium analogues. **J. Gen. Microbiol. 135; 1441-1451.**

Reich S, Almon H and Boger P (1986): Short term effect of ammonia on nitrogenase activity of *Anabaena variabilis* ATCC 29413. **FEMS Microbiol. Letts. 34; 53-56.**

Reich S, Almon H and Boger P (1987): Comparing short term effects of ammonia and methylamine on nitrogenase activity in *Anabaena variabilis* ATCC 29413. **Z. Naturforsch. 42C; 902-906.**

Reinman S and Thornber JP (1979): The electrophoretic isolation and partial characterisation of three chlorophyll protein complexes from blue-green algae. **Biochim. Biophys. Acta 547; 188-197.**

Reitzer LJ and Magasanik B (1986): Transcription of *gln* A in *E. coli* is stimulated by activator bound to sites far from the promoters. **Cell 45; 785-792.**

Reynaud A and Roger PA (1978): Nitrogen fixing algal biomass in senegal rice fields. In: Environmental role of nitrogen fixing blue-green algae & asymbiotic bacteria (Granhall V ed.) **pp 148-157, Ecol Bull. (Stockholm) NFR 26.**

Rippka R, Deruelles J, Waterbury JB, Hardman M and Stanier RY (1979): Genetic assignments, strain histories and properties of pure cultures of cyanobacteria. **J. Gen. Microbiol. 111; 1-61.**

Rippka R and Stanier RY (1978): The effects of anaerobiosis on nitrogenase synthesis and heterocyst development by *Nostocacean* cyanobacteria. **J. Gen. Microbiol. 105; 83-94.**

Ris H and Singh RN (1961): Electron microscope studies on blue-green algae. **J. Biophys. Biochem. Cytol. 9; 63-80.**

Roberts GP and Brill WJ (1981): Genetics and regulation of nitrogen fixation. **Ann. Rev. Microbiol. 35; 207-235.**

Robson RL, Eady RR, Richardson TH, Miller RW, Hawkins M and Postgate JR (1986a): The alternative nitrogenase of *Azotobacter chroococcum* is a vanadium enzyme. **Nature 322; 388-390.**

Robson R, Woodley P and Jones R (1986b): Second gene (*nif* H) coding for a nitrogenase iron-protein in *Azotobacter chroococcum* is adjacent to a gene coding for a ferredoxin-like protein. **EMBO J. 5; 1159-1163.**

Rosen BP (1971): Basic amino acid transport in *Escherichia coli*. **J. Biol. Chem. 246; 3653-3662.**

Rosen BP and Kashket ER (1978): Energitics of active transport. In: Bacterial transport (Rosen BP ed.) **pp 559-620, Marcel Dekker, New York.**

Rowell P, Enticott and Stewart WDP (1977): Glutamine synthetase and nitrogenase activity in the blue-green alga *Anabaena cylindrica*. **New Phytol. 79; 41-54.**

Rowell P, Kerby NW, Darling AJ and Stewart WDP (1985a): Physiological and biochemical aspects of N_2-fixing cyanobacteria. In: Nitrogen fixation research progress (Evans HJ, Bottomley PJ & Newton WE eds.) **pp 387-393, Martinus Nijhoff,** Dordrecht.

Rowell P, Rai AN and Stewart WDP (1985b): Studies on the nitrogen metabolism of the lichen *Peltigera aphthosa* and *Peltigera canina*. In: Lichen physiology and cell biology (Brown DH ed.) **pp 145-160, Plenum press, New York.**

Rowell P, Sampaio MJAM, Ladha JK and Stewart WDP (1979): Alteration of glutamine synthetase activity *in vivo* in response to light and NH_4^+. **Arch. Microbiol. 120; 195-200.**

Salminen SO (1981): Effect of NH_4^+ on nitrogenase activity in nodule breis and bacteriods from *Pisum sativum* L. **Biochim. Biophys. Acta 658; 1-9.**

Sampaio MJAM, Rowell P and Stewart WDP (1979): Purification and some properties of glutamine synthetase from the nitrogen-fixing cyanobacteria *Anabaena cylindrica* and a *Nostoc* sp. **J. Gen. Microbiol. 111; 181-191.**

Schaeffer F and Stainer RY (1978): Glucose-6-phosphate dehydrogenase of *Anabaena* sp. kinetics and molecular properties. **Arch. Microbiol. 116; 9-19.**

Schrautemeier B and Bohme H (1984): Different functions assigned to NAD(H) & NADP(H) in light dependent nitrogen fixation by heterocysts of *Anabaena variabilis*. **FEMS Microbiol. Letts. 25; 215-218.**

Schrautemeier B and Bohme H (1985): A distinct ferredoxin for nitrogen-fixation isolated from heterocysts of the cyanobacterium *Anabaena variabilis*. **FEBS Letts. 184; 304-308.**

Scott ID and Nicholls DG (1980): Energy transduction in intact synaptosomes. Influence of plasma membrane depolarization on the respiration & membrane potential of internal mitochandria determined *in situ*. **Biochem. J. 186; 21-23.**

Shah VK, Imporial J, Ugalde RA, Ludden PW and Brill WJ (1986): *In vitro* synthesis of the iron-molybdenum cofactor of nitrogenase. **Proc. Natln. Acad. Sci. USA. 83; 1636-1640.**

Silver S (1978): In: Bacterial transport (Rosen BP ed.) **pp 221-324, Marcel Dekker, New York.**

Simson FB and Burris RH (1984): A nitrogen pressure of 50 atmosphere doesnot prevent evolution of hydrogen by nitrogenase. **Science 224; 1095-1097.**

Singh RN (1961): Role of blue-green algae in nitrogen economy of Indian agriculture. **ICAR, New Delhi.**

Singh HN, Bagchi SN and Singh RK (1983a): L-Methionine-DL-sulfoximine resistant Het^+ Nif^+ and Het^- Nif^- strains of *Nostoc muscorum* assimilating methylammonium as ammonium nitrogen source. **FEMS Microbiol. Letts. 20; 31-34.**

Singh DT, Ghosh R and Singh HN (1987): Physiological characterization of the ammonium transport system in the free-living diazotrophic cyanobacterium *Anabaena cycadeae*. **J. Pl. Physiol. 127; 231-239.**

Singh DT, Modi DR and Singh HN (1986): Evidence for glutamine synthetase and methylammonium (ammonium) transport system as two distinct primary targets of methionine sulfoximine inhibitory action in the cyanobacterium *Anabaena doliolum*. **FEMS Microbiol. Letts. 37; 95-98.**

Singh HN, Rai AN and Bagchi SN (1985a): Evidence for a common genetic regulation of glutamine synthetase and nitrate uptake and reductase in the cyanobacterium *Anabaena cycadeae*. **Mol. Gen. Genet. 198; 367-368.**

Singh HN, Rai UN, Rao VV and Bagchi SN (1983b): Evidence for ammonia as an inhibitor of heterocyst and nitrogenase formation in the cyanobacterium *Anabaena cycadeae*. **Biochem. Biophys. Res. Commun. 11; 180-187.**

Singh DT, Rai AN and Singh HN (1985b): Methylammonium (ammonium) uptake in a glutamine auxotroph of the cyanobacterium *Anabaena cycadeae*. **FEBS Letts. 186; 51-53.**

Smillie RM (1965): Isolation of two proteins with chloroplasts ferredoxin activity from a blue green alga. **Biochem. Biophys. Res. Commun. 20; 621-629.**

Smith AJ (1982): Modes of cyanobacterial carbon metabolism. In: The biology of cyanobacteria (Carr NG & Whitton BA eds.) **pp 47-86, Blackwell Scientific Publications, Oxford.**

Smith RL, Baalen CA and Tabita FR (1987b): Alteration of Fe protein of nitrogenase by oxygen in the cyanobacterium *Anabaena* sp. strain CA. **J. Bacteriol. 169; 2537-2542.**

Smith BE, Campbell F, Eady RR, Eldridge M, Ford CM, Hill S, Kavanagh EP, Lowe DJ, Miller RW, Richardson TH, Robson RL, Thorneley RNF and Yates MG (1987a): Biochemistry of nitrogenase and the physiology of related metabolism. **Phill. Trans. R. Soc. Lond. B 317; 131-146.**

Smith DC and Douglas AE (1987): The Biology of Symbiosis. (Smith DC & Douglas AE eds.) **pp 1-287, Edward Arnold Ltd, London, Great Britain.**

Speelmans G, Vrij W-DE and Konings WN (1989): Characterization of amino acid transport systems in membrane vesicles from the thermophilic fermentative bacterium *Clostridium fervidus*. **J. Bacteriol. 171; 3788-3795.**

Spiller H, Labarre C, Hassan ME and Shanmugam KT (1986): Isolation and characterisation of nitrogenase derepressed mutant strains of cyanobacterium *Anabaena variabilis*. **J. Bacteriol. 165; 412-419.**

Sprent JI (1979): In: Biology of Nitrogen fixing organisms. p 53, **Maidenhead, McGraw Hill, UK.**

Sprent JI, Sutherland JM and DeFaria SM (1987): Some aspects of the biology of nitrogen fixing organisms. **Phill. Trans. R. Soc. Lond. B 317; 111-129.**

Stanier RY (1977): The position of cyanobacteria in the world of phototrophs. **Carlberg Res. Commun. 42; 77-78.**

Stanier RY and Cohen-Bazire G (1977): Phototrophic prokaryotes: The cyanobacteria. **Ann. Rev. Microbiol. 31; 225-274.**

Stanier RY, Sistrom WR, Hansen TA, Whitton BA, Castenholz RW, Pfennig N, Gorlenko VN, Kondratieva EN, Eimhjellen KE, Whittenbury R, Gherna RL and Truper HG (1978): Proposal to place the nomenclature of cyanobacteria (Blue-green algae) under the rules of the International code of nomenclature of bacteria. **Int. J. Syst. Bacteriol. 28; 335-336.**

Stewart WDP (1977): A botanical ramble among the blue-green algae. **Br. Phycol. J. 12; 89-115.**

Stewart WDP (1980); Some aspects of structure and function in Nitrogen fixing cyanobacteria. **Ann. Rev. Microbiol. 34; 497-536.**

Stewart WDP (1982): Nitrogen fixation - its current relevance and future potential. **Israel J. Bot. 31; 5-44.**

Stewart WDP, Fitzgerald GP and Burris RH (1967): *In situ* studies on N_2-fixation using the acetylene reduction technique. **Proc. Natl. Acad. Sci. USA 58; 2071-2078.**

Stewart WDP and Lex M (1970): Nitrogenase activity in the blue-green alga *Plectonema boryanum* strain S94. **Arch. Mikrobiol. 73; 250-260.**

Stewart WDP, Pemble M and Al-Ugily (1978): Nitrogen and phosphorus storage and utilization in blue green algae. **Mitt. Int. Verein. Limnol. 21; 224-247.**

Stewart WDP and Rodgers GA (1977): The cyanophyte • heptic symbiosis. II. Nitrogen fixation and the interchange of nitrogen and carbon. **New Phytol. 78; 459-471.**

Stewart WDP and Rowell P (1975): Effects of L-Methionine DL-sulfoximine on the assimilation of newly fixed ammonia, acetylene reduction & heterocyst production in *Anabaena cylindrica*. **Biochem. Biophys. Res. Commun. 65; 846-856.**

Stewart WDP and Rowell P (1977): Modification of nitrogen fixing algae in lichen symbiosis. **Nature 265; 371-372.**

Stewart WDP, Rowell P, Cossar JD and Kerby NW (1985): Physiological studies on N_2-fixing cyanobacteria. In: Nitrogen fixation and CO_2 metabolism (Ludden PW & Burris JE eds.) **pp 269-279, Elsevier, New York.**

Stewart WDP, Rowell P, Hawkesford M, Sampaio MJAM and Ernst A (1982): Nitrogenase and aspects of its regulation in cyanobacteria. **Isrl. J. Bot. 31; 168-189.**

Stewart WDP, Rowell P, Kerby NW, Reed RH and Machray GC (1987): N_2-fixing cyanobacteria and their potential applications. **Phill. Trans. R. Soc. Lond. B 317; 245-258.**

Stewart WDP, Rowell P, Ladha JK and Sampaio MJAM (1979): Blue-green algae (cyanobacteria)-some aspects related to their role as sources of fixed nitrogen in paddy soils. In: Nitrogen & Rice. **pp 263-284, LosBanos, IRRI, Philippines.**

Stewart WDP, Rowell P and Rai AN (1980): Symbiotic nitrogen-fixing cyanobacteria. In: Nitrogen fixation (Stewart WDP & Gallon JR eds.) **pp 239-277, Academic Press, London, New York.**

Stewart WDP, Rowell P and Rai AN (1983): Cyanobacteria eukaryotic plant symbiosis. **Ann. Microbiol. (Inst. Pasteur) 134 B; 205-228.**

Strasser P and Falkner G (1986): Characterisation of the glutamate/aspartate transport system in a symbiotic *Nostoc* sp. **Planta 168; 381-385.**

Streicher Sl, Shanmugam KT, Ausubel F, Morandic and Goldberg R (1974): Regulation of nitrogen fixation in *Klebsiella pneumoniae* evidence for a role glutamine synthetase as a regulator of nitrogenase synthesis. **J. Bacteriol. 120; 815-821.**

Thomas J, Meeks JC, Wolk CP, Shaffer PW, Austin SM and Chin WS (1977): Formation of glutamine form [^{13}N] ammonia, [^{13}N] dinitrogen & [^{14}C] glutamate in heterocysts isolated from *Anabaena cylindrica*. **J. Bacteriol. 129; 1545-1555.**

Thomas JH, Mullineaux, Cronshaw AD, Chaplin AE and Gallon JR (1982): The effects of structural analogues of amino acids on ammonium assimilation and acetylene reduction (nitrogen fixation) in *Gloeocapsa (Gloeothece)* sp. CCAP 1403/3. **J. Gen. Microbiol. 128; 885-893.**

Tien HT (1974): Bilayer lipid membranes. **pp 117-188, Marcel Dekker, New York.**

Tumer NE, Robinson SJ and Haselkorn R (1983): Different promotors for the *Anabaena* glutamine synthetase gene during growth with molecular or fixed nitrogen. **Nature 306; 337-342.**

Turpin HD, Edie SA and Canvin DT (1984): *In vivo* nitrogenase regulation by ammonium and methylammonium and the effect of MSX on ammonium transport in *Anabaena flosaquae*. **Pl. Physiol. 74; 701-704.**

Vaishampayan A (1982): Amino acid nutrition in the blue-green alga *Nostoc muscorum*. **New Phytol. 90; 545-549.**

Venkataraman GS (1980): Key not address on algal biofertilizers. Potential & Problems. **Proc. Natln. Workshop Algal system, IIT, New Delhi, Indian Society of Biotechnology.**

Wall JD and Gest H (1979): Derepression of nitrogenase activity in glutamine auxotrophs of *Rhodopseudomonas capsulata*. **J. Bacteriol. 137; 1459-1463**

Walker NA, Smith FA and Beilby MJ (1979): Amine uniport at the plasmalemma of Charophyte cells. 2. Ratio of matter of change transported and permeability of free bases. **J. Membr. Biol. 49; 283-296.**

Walsby AE (1982): The permeability of gas vesicles, vegetative cells and heterocysts of *Anabaena flosaquae* to oxygen and nitrogen. **Abstract C-33, IV international Symposium on Photosynthetic Prokaryotes, Bombannes, France.**

Watt GD, Wang ZC and Knotts R (1986): Redox reactions of the nucleotide binding to the iron-protein of *Azotobacter vinelandii*. **Biochem. 25; 8156-8162.**

Whitton BA and Carr NG (1982): Cyanobacteria: Current Perspectives. In: The biology of cyanobacteria (Carr NG & Whitton BA eds.) **pp 1-8, Blackwell Scientific Publications, Great Britain.**

Winkenbach F and Wolk CP (1973): Activities of enzymes of the oxidative and the reductive pentose phosphate pathway in heterocysts of blue-green alga. **Pl. Physiol. 52; 480-483.**

Wolk CP (1968): Movement of carbon from vegetative cells to heterocysts in *Anabaena cylindrica*. **J. Bacteriol. 96; 2138-2143.**

Wolk CP (1982): Heterocysts. In: The biology of cyanobacteria (Carr NG & Whitton BA eds.) **pp 359-386, Blackwell Scientific Publications, Great Britain.**

Wolk CP, Thomas J, Shaffer PW, Austin SM and Galonsky A (1976): Pathway of nitrogen metabolism after fixation of ^{13}N-labeled nitrogen gas by the cyanobacterium *Anabaena cylindrica*. **J. Biol. Chem. 251; 5027-5034.**

Yoch DC, Zhang ZM and Claybrook DL (1983): Methylamine metabolism and its role in nitrogenase "switch-off" in *Rhodopseudomonas capsulata*. **Arch. Microbiol. 134; 45-48.**

Zumft WG (1985): Regulation of nitrogenase activity in the anoxygenic photosynthetic bacteria. In: Nitrogen Fixation research progress (Evans JH, Bottomley PJ & Newton NW eds.) **pp 551-557, Dordrecht, Nijhoff.**

Zumft WG and Castillo F (1978): Regulatory properties of the nitrogenase from *Rhodopseudomonas palustris*. **Arch. Microbiol. 117; 53-60.**

PUBLICATIONS

1. A.N. Rai and **R. Prakasham** (1989): Characteristics of the methylammonium/ammonium transport systems of the nitrogen-fixing cyanobacterium *Anabaena* 7120 (ATCC 27893). **Ind. J. Biochem. Biophys. 26; 219-226.**

2. **R. Prakasham** and A.N. Rai (1990): Methylammonium/ammonium transport and metabolism in the heterocystous cyanobacteria: *Anabaena* 7120 and *Nostoc* ANTH. **Phycos (Spl. Issue) (in press).**

3. A.N. Rai and **R. Prakasham** (1990): Transport of inorganic nitrogen in cyanobacteria and its relevance in use of cyanobacteria as biofertilizers. In: International Symposium on Nitrogen-fixation Associated with Rice Production and Rice Culture, **Oxford & IBP Publ. Co., New Delhi, India (in press).**