GENERAL INTRODUCTION
The cyanobacteria* (blue-green algae) are prokaryotic microorganisms capable of both photoautotrophy and diazotrophy ($N_2$-supported growth). In the presence of simple inorganic salts and using solar energy and water they convert atmospheric $CO_2$ and $N_2$ into cell protein. Under certain conditions they evolve $H_2$ also. Cyanobacteria were amongst earth's early inhabitants and have been recorded from Early Precambrian (nearly $3 \times 10^9$ years ago). These microbes were probably responsible for the initial oxygenation of the earth's atmosphere and it is generally believed that present day chloroplasts of eukaryotes might have evolved from them.

Cyanobacteria are ubiquitous in nature occupying a range of habitats which are rivalled only by the bacteria. They predominate in the flora of arid deserts, are abundant on rocky surfaces exposed to high temperatures and also show massive growth in temperate fresh water lakes. They are dominant life forms in the frigid lakes of the Antarctic (Fogg et al., 1973) and on the other hand also grow in the hot springs at temperatures above $70^\circ$C (Castenholz, 1969).

* The term cyanobacteria is currently in use following the realisation in the recent years that it is scientifically more accurate. The term has therefore been used, in preference to blue-green algae, in this thesis.
However, it is in the tropics that they are especially abundant and grow luxuriously, outdoing other free-living diazotrophs. The cyanobacteria of tropical paddy fields are now generally considered to be major contributors of fixed nitrogen in agricultural productivity.

The ability for photoautotrophic diazotrophy is central to the importance of cyanobacteria; their ability to fix $N_2$ and the resulting agronomic benefits have attracted research workers to cyanobacteria. In the last few decades these microbes have been the subject of careful observation both in the field as well as in the laboratory. These studies have led to a greater understanding of the physiology, biochemistry and ecology of these organisms. In view of the profound importance of biological $N_2$ fixation and also in view of the emphasis in the present thesis on the physiological and biochemical factors affecting $N_2$ fixation the following account is mainly aimed at a general review of only such aspects of cyanobacteria as are relevant to the process of $N_2$ fixation and assimilation. More than 125 strains of cyanobacteria are now known to possess $N_2$-fixing capacity and these include all major types viz., unicellular/filamentous, heterocystous/non-heterocystous, free-living/symbiotic etc. (see Stewart, 1980).

The heterocystous cyanobacteria fix $N_2$ aerobically while few non-heterocystous types can fix $N_2$, but only anaerobically or
micro-aerobically. The unicellular cyanobacterium *Gloeothece* is an exception and fixes $N_2$ aerobically (Rippka et al., 1979) as also does *Trichodesmium*, as a result of its bundled habit (Carpenter and Price, 1976). Vegetative cells of some heterocystous forms have also been shown to have nitrogenase under anaerobic conditions (Fleming and Haselkorn, 1974).

Although initially cyanobacteria appeared to differ from most other diazotrophs in their capacity to fix $N_2$ aerobically it has gradually become clear that cyanobacterial nitrogenase is little different from that of other $N_2$-fixing prokaryotes. What is different is the cellular background of structure, function and molecular organisation in which nitrogenase functions in cyanobacteria. Thus cyanobacterial nitrogenase like that of other diazotrophs, catalyses the reduction of $N_2$, protons (Haystead and Stewart, 1972; Benemann and Weare, 1974a), cyanide (Haystead and Stewart, 1972) and $C_2H_2$ (Stewart et al., 1967). The reductions of methyl isocyanide, azide, nitrous oxide and cyclopropene by cyanobacterial nitrogenases have not yet been demonstrated. All cyanobacterial nitrogenases, studied so far, from *Anabaena cylindrica* (Tsai and Mortenson, 1978; Hallenbeck et al., 1979), *Anabaena 7120* (Reynaud, 1977; 1978) and *Plectonema boryanum* (Hallenbeck et al., 1979) require ATP, reductant, $Mg^{2+}$ and two oxygen sensitive proteins which reduce $N_2$ only when together (Smith et al., 1971).
Recently partial purification of cyanobacterial nitrogenases has been achieved. The MoFe-protein of *A. cylindrica* is an acidic tetramer (mol.wt. 216000) of two non-identical subunits (mol.wt. 52600 and 55000) and resembles, the MoFe-protein of *Rhodospirillum rubrum* most and that of *Klebsiella pneumoniae* least (Hallenbeck et al., 1979), in amino acid composition. The Fe-protein is a highly labile dimer (mol. wt. 60000) of two identical subunits. There are, as yet, no reports of a Fe-Mo-cofactor as is known in heterotrophic bacteria (Shah and Brill, 1977; Eady et al., 1980) or of two forms of nitrogenase as in *R. rubrum* and *Azospirillum lipoferum* (Ludden and Burris, 1976; Carithers et al., 1979; Burris et al., 1980). The MoFe-and Fe-proteins of *A. cylindrica* and *P. boryanum* cross react at 65 - 90% efficiency of the homologous crosses (Hallenbeck et al., 1979). The MoFe-protein of *A. cylindrica* complements with the Fe-protein of *Azotobacter* (Hallenbeck et al., 1979) and *Clostridium* (Tsai and Mortenson, 1978) at 75% and 50% efficiency respectively, thus demonstrating the similarity of various nitrogenases. The Fe-protein from *A. cylindrica* or *P. boryanum* shows little cross reactivity with MoFe-protein from *A. vinelandii* and *R. rubrum* (Hallenbeck et al., 1979) or *C. pasteurianum* (Tsai and Mortenson, 1978).

Cyanobacterial nitrogenase is highly oxygen sensitive. Inhibition of nitrogenase activity by O₂ can be reversed in the
short term (Gotto et al., 1979) but prolonged oxygenation irreversibly inhibits the nitrogenase activity (Stewart and Lex, 1970; Rippka and Stanier, 1978) and represses the synthesis of nitrogenase proteins (Fleming and Haselkorn, 1974) as is the case with other diazotrophs too (see Robson and Postgate, 1980). Heterocysts provide an anaerobic milieu for nitrogenase (Stewart et al., 1969; Thomas, 1970; Thomas and David, 1972; Fleming and Haselkorn, 1973) and the heterocystous forms can therefore afford to fix N₂ aerobically. Recent data showing higher rates of nitrogenase activity by isolated heterocysts (Peterson and Burris, 1976), incorporation of ¹³N₂ into heterocysts (Thomas et al., 1977), much higher proportion of ⁵⁵Fe-labelled nitrogenase proteins in heterocysts relative to vegetative cells (Peterson and Wolk, 1978 a) and transport of nitrogen from heterocysts (Thomas et al., 1977) have established heterocysts, beyond doubt, to be the sole sites of aerobic N₂ fixation in heterocystous cyanobacteria. Oxygen protection mechanisms in the heterocysts include a thick, multi-layered envelope acting as a barrier to gaseous diffusion of O₂ (Haury and Wolk, 1978), the general reducing atmosphere (Thomas and David, 1972), the uptake hydrogenase which provides respiratory protection (Peterson and Wolk, 1978 b; Bothe et al., 1980), absence of photosystem II (Thomas, 1970; Van Gorkom and Donze, 1971; Donze et al., 1972) and lack of photoevolution of O₂ (Tel-Or and Stewart, 1975), high activities of superoxide
dismutase (Henry et al., 1978), and $\text{H}_2$ production which may protect against $\text{O}_2$ inactivation through uptake hydrogenase (Peterson and Wolk, 1978 b).

Several possible routes of supply of reductant to nitrogenase have been identified in cyanobacteria. These include photolysis of $\text{H}_2\text{O}$ and electron transfer through photosystems II and I (Tel-Or and Stewart, 1977; Gallon, 1980), organic compounds feeding into photosystem I dependent light reactions (Murai and Katoh, 1975), $\text{H}_2$ via an uptake hydrogenase and photosystem I (Benesmann and Weare, 1974 b; Bothe et al., 1977), oxidative pentose phosphate pathway linked with ferredoxin-NADP$^+$-oxidoreductase (Apte et al., 1978; Lockau et al., 1978; Gallon, 1980) and glycolysis and/or Kreb's cycle (Gallon et al., 1973; Lockau et al., 1978; Bothe et al., 1980). It is generally accepted that NADPH and/or ferredoxin is the terminal electron donor to nitrogenase, but the relative importance of these routes in light and dark is yet to be resolved. It is also not clear whether more than one route can operate simultaneously and, if so, whether the effect is additive. More recently evidence has been obtained that a reversed electron flow driven by a proton motive force regulates electron flow from NADPH to ferredoxin in cyanobacteria (Hawkesford et al., 1981), as is the case in the aerobic diazotroph Azotobacter also (Haaker et al., 1980). Source of ATP to cyanobacterial nitrogenase is
generally considered to be cyclic photophosphorylation or total photophosphorylation in light and oxidative phosphorylation and substrate level phosphorylation in dark (Bottomley and Stewart, 1976).

Glutamine synthetase (GS) coupled with glutamate synthase (GOGAT) forms the main route of primary NH$_4^+$ assimilation in most cyanobacteria (Dharmawardene et al., 1973; Thomas et al., 1975; Wolk et al., 1976; Meeks et al., 1977). It is established that NH$_4^+$ per se does not inhibit nitrogenase synthesis in cyanobacteria (Stewart and Rowell, 1975). In heterotrophic bacteria, GS had earlier been shown to regulate nitrogenase synthesis at transcriptional level (Streicher et al., 1974). But in cyanobacteria adenylylation of GS does not occur (Rowell et al., 1977; Stacey et al., 1979; Tuli and Thomas, 1981), and the effect of NH$_4^+$ on repression of GS and nitrogenase does not correlate (Rowell et al., 1977; Stewart and Rowell, 1977; Tuli and Thomas, 1980). Also a cloned segment of *Anabaena* chromosome carrying *gln A* gene (structural gene for GS) when introduced into a Gln^-Ntr^- mutant of *Klebsiella pneumoniae* restores GS activity but does not derepress nitrogenase or other ntr functions (Tuli et al., 1982). The possibility of regulation of nitrogenase synthesis by GS or *gln A* is therefore unlikely in cyanobacteria. Instead the products of GS activity viz., glutamine (Ganby, 1977), or a derived product like carbamoyl phosphate (Lawrie, 1979) are considered more likely to do so.
Thus, there have been remarkable advances in our knowledge of cyanobacteria in the last two decades, and several aspects have now been resolved satisfactorily. Prominent among these are: the prokaryotic nature and similarity to Gram-negative bacteria, nature and role of various cell inclusions, identification of heterocysts as the sites of $N_2$ fixation, demonstration of nitrogenase activity in non-heterocystous forms under microaerophilic conditions, realization of the similarity of cyanobacterial nitrogenase with those from other diazotrophs, GS-GOGAT as the central pathway of primary $NH_4^+$ assimilation, non-involvement of GS itself in regulation of nitrogenase synthesis, ability of cyanobacteria for both oxygenic and non-oxygenic photosynthesis, and oxidative pentose phosphate pathway as the main route of carbon dissimilation. The controversial question of transcriptional control of enzyme synthesis (or its absence) in cyanobacteria (Hood et al., 1969) is also becoming clear now and evidence in favour of its occurrence is being obtained especially for $N_2$ metabolism (Lau et al., 1977). Apart from these some estimates of $N_2$ fixation by cyanobacteria in natural and agricultural eco-systems are available now and have helped to assess the contribution of these microbes in the nitrogen economy (Singh, 1961; Venkatraman, 1972; Watanabe et al., 1977; Stewart et al., 1979; Venkatraman, 1979; Reddy, 1982).
Inspite of all this, our knowledge of \( N_2 \)-fixing cyanobacteria is by no means complete. In fact several questions which intrigued the early workers persist largely unanswered even today. The obligate photoautotrophy of most cyanobacteria is a typical example. Very little is known about cyanobacterial genetics and our knowledge in this field lags at least a decade behind that of the genetics of heterotrophic diazotrophs. Cyanobacterial nitrogenase has not yet been purified to homogeneity or characterised in detail. The exact source of reductant to cyanobacterial nitrogenase is yet to be resolved and the importance of membrane potential in the regulation of electron flow to nitrogenase needs to be assessed further. The possible role of nitrogenase and/or its products in the regulation of heterocyst pattern is still controversial. Intercellular transport processes in cyanobacteria are a neglected topic. Although it has been realised long back that the vegetative cells and heterocysts exchange carbon and nitrogen metabolites (and possibly others), so far, there have been no attempts to study permeases or other translocases which may possibly be involved. Ion transport has similarly received only scant attention in cyanobacteria. Mainly \( K^+ \) transport has been studied in some detail in the unicellular non-diazotroph \textit{Anacystis} (Dewar and Barber, 1973) and recently in \textit{Anabaena} (Reed et al., 1981). Nothing is known of the transport of ions involved in a vital
process like \( \text{N}_2 \) fixation viz., molybdenum or iron. Apart from the early work by Allen and Arnon (1955 a) and Kratz and Myers (1955) and later by Stanier et al. (1971), there has been little systematic effort to study the nutritional requirements of cyanobacteria or to optimise the concentrations of required minerals in the medium. In some of the cases where a requirement has been found the metabolic basis of such a requirement has not been investigated e.g., requirement of sodium (\( \text{Na}^+ \)). Studies of the metal ions involved in cyanobacterial \( \text{N}_2 \) fixation are rare except for molybdenum, although a role in \( \text{N}_2 \) fixation has been suspected for \( \text{Ca}^{2+} \) (Allen, 1956) and \( \text{Na}^+ \) (Allen and Arnon, 1955 b; Brownell and Nicholas, 1967). Possible regulation of cyanobacterial nitrogenase by \( \text{Mn}^{2+} \), as is known in some diazotrophic bacteria (Burris et al., 1980) has not been looked into.

A requirement of \( \text{Na}^+ \) for normal growth has been known in cyanobacteria for some time. Several unicellular non-\( \text{N}_2 \)-fixing cyanobacteria such as *Chroococcus* (Emerson and Lewis, 1942), *Microcystis* (McLachlan and Gorham, 1961), *Anacystis* (Kratz and Myers, 1955) and also the \( \text{N}_2 \)-fixing filamentous forms *Anabaena cylindrica* (Allen and Arnon, 1955 b), *A. variabilis* and *Nostoc muscorum* (Kratz and Myers, 1955) and *A. flos-aquae* (Bostwick et al., 1968) were all found to require \( \text{Na}^+ \) for logarithmic growth in the presence of nitrate. Allen and Arnon (1955 b) and Brownell
and Nicholas (1967) reported a requirement for $\text{Na}^+$ also under $\text{N}_2$-fixing conditions. However, in spite of this awareness for last three decades there have been no attempts to investigate the biochemical nature of such a requirement in cyanobacteria. $\text{Na}^+$ is the predominant cation in most agricultural habitats especially in the brackish and saline/alkali soils where cyanobacterial populations are abundant, but nothing is known about the physiological basis of salt tolerance in cyanobacteria. Knowledge of the transport of $\text{Na}^+$ in cyanobacteria would appear to be an essential prerequisite for understanding their salt tolerance but it has not been studied at all, except in $A. \text{ nidulans}$ (Paschinger, 1977).

The present study is aimed at obtaining information on the physiological and biochemical basis of $\text{Na}^+$ requirement in $\text{N}_2$-fixing cyanobacteria. The effects of $\text{Na}^+$ deficiency on nitrogen fixation and growth and some aspects of the interrelated consequences on photosynthesis and general metabolism have been examined. $\text{Na}^+$ transport has been studied in a fresh water and a saline, diazotrophic cyanobacterium and its relationship to their salt tolerance has been investigated. In addition, attempts have been made to grow cyanobacteria on saline 'Kharland' soils from coastal areas of Maharashtra State and the possibility of reclamation of such soils using cyanobacteria has been explored.
The subject matter of these investigations have been described in three chapters which are delineated as follows:

Chapter I: Requirement of sodium for growth and nitrogenase activity.

Chapter II: Effect of sodium on photosynthesis and general metabolism related to N$_2$ fixation and assimilation.

Chapter III: Sodium transport and its relationship to salt tolerance.
CHAPTER I

REQUIREMENT OF SODIUM FOR GROWTH AND NITROGENASE ACTIVITY
INTRODUCTION

Over the last four decades there have been several reports indicating a requirement of sodium (Na\(^+\)) for cyanobacterial growth. In particular, the nitrate (\([\text{NO}_3^-]_2\))-grown cultures of many cyanobacteria have been reported to exhibit a dependence of growth on the presence of Na\(^+\). Emerson and Lewis (1942) found that a species of unicellular, adiazotroph Chroococcus grew poorly in the absence of Na\(^+\). Requirement of Na\(^+\) was also reported in (\([\text{NO}_3^-]_2\))-supplemented cultures of two other unicellular adiazotrophic cyanobacteria, *Microcystis aeruginosa* (McLachlan and Gorham, 1961) and *Anacystis nidulans* (Kratz and Myers, 1955). The necessity for Na\(^+\) was clearly demonstrated by Kratz and Myers (1955) for (\([\text{NO}_3^-]_2\))-grown cultures of the diazotrophic cyanobacteria *Anabaena cylindrica* and *Nostoc muscorum* which showed cessation of logarithmic growth in the absence of Na\(^+\). Very little is known about the requirement of Na\(^+\) for \(N_2\)-fixing cultures of cyanobacteria except in the case of *A. cylindrica* which has been found to show a specific requirement for Na\(^+\) in the absence of combined nitrogen (Allen and Arnon, 1955 b; Brownell and Nicholas, 1967). Whether Na\(^+\) is needed in the presence of other forms of combined nitrogen, such as \(\text{NH}_4^+\), has not been looked into.

The requirement for Na\(^+\) in cyanobacteria has sometimes been confused with that for K\(^+\). For example Allen (1952) found
23 strains of cyanobacteria which could grow in media where $K^+$ salts were replaced by $Na^+$ salts. The validity of such data is questionable since it is possible that the apparently complete replacement of $K^+$ by $Na^+$ might have been only partial. Indeed, more careful studies later showed independent requirement for both $K^+$ and $Na^+$ in cyanobacteria (Allen and Arnon, 1955 b; Kratz and Myers, 1955; Bostwick et al., 1968).

The reported concentrations of $Na^+$ required by cyanobacteria are quite low. Batterton and Van Baalen (1971) showed that $Na^+$ requirement was saturated at 1 p.p.m. ($\approx 40 \mu M$) NaCl in fresh water cyanobacteria and at 100 p.p.m. (4 mM) in marine forms. Allen and Arnon (1955 b) reported $Na^+$ requirement in the range of 1 - 5 p.p.m. (40 - 220 $\mu M$) while Kratz and Myers (1955) found a much higher requirement of 4 - 40 p.p.m. ($\approx 75 - 750 \mu M$). These concentrations are at least 10 - 20 times higher than those reported for some of the known essential micronutrients of cyanobacteria, like manganese (0.01 - 0.5 p.p.m.), molybdenum (0.1 - 0.5 p.p.m.), boron (0.1 p.p.m.), cobalt (0.2 - 0.4 p.p.m.), copper (0.02 p.p.m.) and zinc (0.05 p.p.m.) (see Wolk, 1973).

There has been very little effort to examine the nature of $Na^+$ nutrition in cyanobacteria. Brownell and Nicholas (1967) noticed a much higher requirement of $Na^+$ during growth on ($NO_3^-$)$^2$ than during growth on dinitrogen ($N_2$) for $A. cylindrica$ and
observed that in the presence of $(\text{NO}_3)^{2-}$, $\text{Na}^+$ deficiency enhanced nitrate reductase activity. $\text{Na}^+$-deficient cultures of *A. cylindrica* accumulated toxic levels of nitrite which caused chlorosis. The same authors also found that $\text{Na}^+$ deficiency decreased the incorporation of $^{15}\text{N}_2$ but increased the incorporation of $^{15}\text{NH}_3$ or $^{14}\text{C}$-glutamate into protein and attributed decreased growth to decreased $\text{N}_2$ reduction. More recently Apte and Thomas (1980) clearly showed a requirement of $\text{Na}^+$ for nitrogenase activity in cyanobacteria, but the precise role of $\text{Na}^+$ in the synthesis or for the activity of nitrogenase was not ascertained. Apart from these two studies there have been no major attempts to identify the role of $\text{Na}^+$ in cyanobacterial growth and metabolism.

The only other metal cation known, in addition to $\text{Na}^+$, to be required during growth of cyanobacteria on $\text{N}_2$ is molybdenum*. Such a requirement for molybdenum in cyanobacteria, first shown by Bortels (1940), has since been studied by several workers (Wolfe, 1954 *a,b, Taha and Elrefai, 1962; Fay and de Vasconcelos, 1974).

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* Molybdenum, a transition metal, displays variable valency states (II, III, IV, V and VI) in its complexes with other elements, with $+\text{VI}$ as the most stable oxidation state particularly in solutions. Throughout this text, therefore, use of the symbolic form(s) have been avoided and the element is referred to as molybdenum. Same also applies to other transition elements, tungsten and vanadium.
Nagatani and Haselkorn, 1978). Molybdenum forms part of the iron molybdenum cofactor (FeMoCo) (Shah and Brill, 1977; Eady et al., 1980), a constituent of the MoFe protein or component I of nitrogenase (Eady and Postgate, 1974). There is indirect evidence that molybdenum is the substrate-binding active site in the functional nitrogenase (Nagatani and Brill, 1974; Smith, 1977; Thorneley et al., 1980). Molybdenum has also been implicated in the regulation of nitrogenase synthesis although its precise role has been a matter of controversy (Brill et al., 1974; Kennedy and Postgate, 1977). Thus molybdenum is reported to be essential for the synthesis of both component I and II in Clostridium (Cardenas and Mortenson, 1975) while in Azotobacter (Nagatani and Brill, 1974; Pienkos et al., 1981), Klebsiella (Kahn et al., 1982), A. cylindrica (Hallenback and Benemann, 1980) and Plectonema boryanum (Nagatani and Haselkorn, 1978) nitrogenase synthesis is independent of molybdenum.

The present study examined the requirement of Na⁺ in two heterocystous, aerobic diazotrophs Anabaena torulosa and Anabaena L-31 and a non-heterocystous, micro-aerophilic diazotroph Plectonema boryanum. In order to gain information on the mode of action of Na⁺, the results were compared with the molybdenum requirement in these organisms. The results show that in cyanobacteria Na⁺ is essential for the catalytic activity of nitrogenase though not for the synthesis of its components. As a result, growth on N₂ is dependent on the presence of Na⁺.
Cyanobacteria

Two filamentous, heterocystous cyanobacteria Anabaena torulosa (Lagerh.) and Anabaena L-31 were isolated in this laboratory and used in axenic condition. A. torulosa, a known inhabitant of brakish waters (see Desikachary, 1969) was isolated from a saline soil and has been shown to sporulate under certain conditions (Fernandes and Thomas, 1982). Anabaena L-31, a non-sporulating form of fresh waters, was isolated from a paddy field soil (Thomas, 1970). Plectonema boryanum-594, a non-heterocystous, filamentous cyanobacterium was a gift from Dr. R. Haselkorn, University of Chicago, Illinois, U.S.A..

Chemicals, radioisotopes and gases

All the inorganic salts were from Sarabhai M. Chemicals, Baroda or British Drug Houses, Bombay, except Ca(NO$_3$)$_2$·4H$_2$O which was from E. Merck AG, Darmstadt, Germany. ($^{22}$Na) sodium chloride was acquired from Amersham International plc, Amersham, England, U.K. and ($^{99}$Mo) ammonium molybdate and ($^{59}$Fe) ferric chloride from Isotope Division, Bhabha Atomic Research Centre, Trombay, Bombay. Tetraphenylphosphonium bromide (−phenyl−$^{14}$C−) was from New England Nuclear, Boston, U.S.A.. Molecular weight standards (for electrophoresis) were obtained from Pharmacia Fine
Chemicals, Uppsala, Sweden. All other chemicals were supplied by Sigma Chemical Co., St. Louis, U.S.A. Argon, acetylene and gases for gas chromatographic analysis were purchased from Indian Oxygen Ltd., Bombay and standard ethylene from Matheson Gas Products, New Jersey, U.S.A.

Culture conditions and growth

Cyanophycean medium (David and Thomas, 1979) diluted five-fold (CM/5) was modified to avoid molybdenum or Na⁺ or both and enriched with combined nitrogen and was used for the maintenance of all cyanobacteria. For Anabaena spp. the medium was supplemented with 3 mM ammonium chloride (NH₄Cl) and buffered with 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) to pH 7.0. For P. boryanum 10 mM Ca(NO₃)₂·4H₂O was added to the medium at pH 7.0. The pH was maintained with LiOH or HCl. The cyanobacteria were grown photoautotrophically at 25°C under continuous illumination (5000 lx) and aeration (2 l.min⁻¹). Chlorophyll a content determined after Mackinney (1941) served as the index of growth.

Induction of nitrogenase and differentiation of heterocysts

For removal of Na⁺ the medium was passed through a column of hydrated antimony pentoxide (HAP) followed by elution with 12 N HCl, evaporation of acid, resolubilisation in distilled
water and restoration of pH to 7.0. Such treated medium when examined by atomic absorption spectrometry showed the presence of less than 15 μM Na⁺ as contaminant. Cultures from logarithmic phase of growth in media containing NH₄⁺ or (NO₃)₂⁻ were harvested on Whatman GF/C filter paper circles (Whatman Ltd., England, U.K.), aseptically washed with H₃P-treated sterile glass distilled water (Na⁺ content < 6 μM) and reinoculated in CM/5, free of combined nitrogen, for induction of nitrogenase and differentiation of heterocysts (in Anabaena spp.). CM/5, which was suitably modified to avoid molybdenum or Na⁺ or both, served as the basal medium for all the experiments. In Anabaena spp. nitrogenase was induced under aerobic conditions and in P. boryanum-594 under microaerophilic conditions ensured by continuous sparging with argon. At suitable time aliquots were removed from cultures of Anabaena spp. and were microscopically examined in a haemocytometer for differentiation of heterocysts. Percent total heterocysts (proheterocysts + mature heterocysts) were computed from a count of at least 2000 cells per sample.

Acetylene reduction assay for nitrogenase activity

Nitrogenase activity was measured as reduction of acetylene (C₂H₂) to ethylene (C₂H₄) in five ml capacity stoppered glass vials. Two ml cyanobacterial suspension was incubated with 0.1 atm C₂H₂ in air on a rotary wheel (11 r.p.m.) at 5000 lx and
22°C for 30 min. In the case of *P. boryanum* all the operations including assays were performed under a gas phase of argon. Assays were terminated by the injection of ammoniacal silver nitrate (10 mg ml⁻¹, final concentration) as described by David *et al.* (1980). Aliquots of the gas phase from the vials were withdrawn and analysed with the help of a gas chromatograph (type RIO4, Toshniwal Instruments, Bombay). Operating conditions for the gas chromatograph were as follows: column - Porapak T, 183 by 0.3 cm; nitrogen as carrier gas at a flow rate of 30 ml min⁻¹; temperature - column = 90°C, injection port = 110°C, flame ionisation detector = 120°C. Under these conditions the retention time for C₂H₂ and C₂H₄ was 30 sec and 46 sec respectively. C₂H₄ formed was estimated from a standard curve prepared using pure C₂H₄.

Measurement of molybdate and sodium uptake

Uptake of molybdate $\left(\text{MoO}_4^{2-}\right)$ and Na⁺ was examined by employing their radiotracers $^{99}$Mo and $^{22}$Na respectively. Logarithmic phase cultures of *A. torulosa* were harvested, washed and inoculated in CM/5, without $\left(\text{MoO}_4^{2-}\right)$ and Na⁺, and were grown either in the presence of 10 mM Ca(NO₃)₂.4H₂O or 3 mM NH₄⁺ or in the absence of combined nitrogen for 24 h. These $\left(\text{MoO}_4^{2-}\right)$-deficient and Na⁺-deficient cultures were harvested, washed, resuspended in the fresh medium and used in uptake experiments.
Assay mixture for Na\(^+\) uptake contained \(^{22}\text{Na}\) sodium chloride (0.25 µCi.ml\(^{-1}\)) mixed with stable NaCl to give 0.5 mM Na\(^+\). Molybdate uptake was measured in a mixture of \(^{99}\text{Mo}\) ammonium molybdate (4 µCi.ml\(^{-1}\)) and stable molybdic acid (MoO\(_3\)) to give 50 µM (MoO\(_4\))\(^{2-}\). Final volume of assays was 2.5 ml. After the desired period, assays were terminated by filtering 2 ml suspensions on Whatman GF/C circles. Residues were washed three times with stable NaCl or MoO\(_3\) (at equimolar concentrations of Na\(^+\) and (MoO\(_4\))\(^{2-}\) in the assay mixture) and then three times with distilled water. The entire washing procedure took less than one minute. Filter paper circles were dried and counted in 10 ml 2-5-di-(5-tert-butyl-2-benzoxazolyl)-thiophene (BBOT) (0.4%, w/v in 1:1 toluene : methanol) using a Beckman LS-100 C liquid scintillation counter (Beckman Instruments Inc., California, U.S.A.).

Determination of membrane potential

Cells were suspended in CM/5 containing 20 mM HEPES/LiOH at pH 7.0 and were incubated with 5 µM phenyl-\(^{14}\)C-TPMP\(^+\) (0.1 µCi/ml assay) and 20 µM tetraphenylboron which facilitated complete equilibration in 10 min. Assay was terminated after 15 min by rapidly centrifuging 0.2 ml suspension at 12500 x g for 1 min in an Eppendorf microcentrifuge. Pellet was subsequently washed with 5 ml experimental medium without radiotracer followed by washing with 5 ml glass distilled water. Radioactivity in the
cells was determined by liquid scintillation spectrometry as described earlier. Intracellular radioactivity was corrected for cell volume and membrane potential was calculated using the Nernst equation (Harold, 1972).

**Determination of packed cell volume**

Cyanobacterial suspensions were centrifuged at 12500 x g for 30 min in pre-calibrated plastic tubes for estimation of packed volume of cells. Centrifugation at higher 'g' values collapsed the cells and was therefore not done. Internal cell water was assumed to be 50% of packed cell volume (for comparison see Paschinger, 1977).

**Labelling of cultures with $^{59}$Fe and polyacrylamide gel electrophoresis (PAGE) of native proteins**

A. torulosa was grown in 100 ml of suitable culture media supplemented with $^{59}$FeCl$_3$ (100 μCi, 5.9 μCi/mg Fe$^{-1}$) for 30 h to allow incorporation of the radiotracer. Cultures were harvested and concentrated cell suspensions were evacuated and regassed with argon to 1 atm, three times. Pellet was suspended in 0.025 M Tris (hydroxymethyl)methylamine-HCl at pH 8.7, containing 20% (w/v) sucrose and 5 mM Na$_2$S$_2$O$_4$ and frozen at -60°C overnight. The pellet was then thawed at 4°C, centrifuged at 12500 x g for 30 min and the supernatant was used in PAGE.
Two mm thick slab gels were cast as described by Takács (1979). The running gel contained 10% (w/v) acrylamide and 0.13% (w/v) bis-acrylamide in 0.375 M Tris-HCl, pH 8.7. The stacking gel contained 3% (w/v) acrylamide and 0.1% (w/v) bis-acrylamide in 0.125 M Tris-HCl, pH 6.8. For anaerobiosis the buffer in the upper tank was flushed with argon, 50 mg Na$_2$S$_2$O$_4$ was added and current was passed through the gel for 15 min before introduction of the samples as described by Peterson and Wolk (1978a). Electrophoresis was carried out using 0.025 M Tris-glycine, pH 8.7 for 5-6 h at 40 mA. Slabs were fixed, stained with Coomassie Brilliant Blue-R and photographed.

Detection of $^{59}$Fe in polyacrylamide slab gels

Slab gel was cut into individual slots each of which was cut from top to bottom in 1 mm slices with a gel slicer (Arthur H. Thomas Co., Philadelphia, U.S.A.). Each slice was placed in 1 ml glass distilled water in a test tube and counted in a NaI crystal scintillation $\gamma$-spectrometer (Model NI-2T23, B.A.R.C., Bombay). The instrument settings were as follows: EHT, 1.25 kV; ampere gain, 1; window width, 10 volts; baseline, 3.35. The counting efficiency for $^{59}$Fe was $\approx$ 13%.
Preparation of sodium dodecyl sulphate extracts and SDS poly­
acrylamide gel electrophoresis

Cyanobacterial suspensions were concentrated to 50 μg chlo­
rophyll a.ml⁻¹, sonicated (2 min.ml⁻¹) in a MSE-Soniprobe
(Measuring & Scientific Equipment Ltd., London, U.K.) and
centrifuged at 1000 x g for 10 min. Supernatant was precipitated
with cold trichloroacetic acid (TCA) (final concentration: 10%,
w/v) and the pellet was washed twice with ethanol (70%, v/v) and
once with ether. After drying the pellet was solubilised in
0.025 M 2-amino-2(hydroxymethyl)propane-1,3-diol (Tris)-HCl buffer,
pH 6.8, containing 2% (w/v) sodium dodecylsulphate (SDS) and 5% 
(w/v) β-mercaptoethanol, by incubating in a boiling water bath
for 5 min.

A 22% to 15% gradient slab gel was prepared with 25 ml of
each of the following two solutions: (a) 14.55% (w/v) acrylamide,
0.45% (w/v) bis-acrylamide and 0.8 mg.ml⁻¹ ammonium persulphate
(APS) (b) 21.34% (w/v) acrylamide, 0.66% (w/v) bis-acrylamide
and 0.4 mg.ml⁻¹ APS. Both solutions were prepared in 0.1 M Tris-
HCl, pH 8.3 containing 0.1% (w/v) SDS and poured into a vertical
slab through a gradient mixer. The running gel was over-layered
with a stacking gel containing 2.5% (w/v) acrylamide, 0.625% (w/v)
bis-acrylamide and 0.1% (w/v) SDS in 0.1 M Tris-HCl, pH 6.8. The
solubilised preparations were centrifuged and loaded on to the
stacking gel and electrophoressed for 8 h at 20 mA.gel⁻¹ using
0.025 M Tris-glycine, pH 8.3 (containing 0.1%, w/v SDS) as the running buffer. Slabs were fixed, stained with Coomassie Brilliant Blue-R and photographed.

**Estimation of proteins**

Total proteins were estimated as described by Lowry et al. (1951) using bovine serum albumin (BSA) as standard.

**RESULTS**

**Sodium requirement for growth**

*Anabaena torulosa* (Fig. I.1) and *Anabaena I-31* (Fig. I.2) showed complete dependence of growth on Na\(^+\) under N\(_2\)-fixing conditions. Such a requirement was not seen when the medium was supplemented with combined nitrogen either in the form of (NO\(_3\))\(^{-}\) or NH\(_4^+\). The requirement was very specific for Na\(^+\) which could not be replaced by other cations, e.g. K\(^+\), Li\(^+\), Ca\(^{2+}\) or Mg\(^{2+}\) (Table I.1). All the salts of Na\(^+\) tested supported growth of cyanobacteria irrespective of the accompanying anion (Table I.1). The requirement was also found to be very low; concentrations of externally added Na\(^+\) above 5/μM in the case of *Anabaena I-31* and 8/μM in the case of *A. torulosa* supported good growth of cyanobacteria (Table I.2).
Fig. I.1. Sodium dependence of growth of *Anabaena torulosa* on various nitrogen sources. Cyanobacterium was inoculated in the media, with or without 1 mM NaCl and, containing no added nitrogen or 10 mM Ca(NO₃)₂·4H₂O or 3 mM NH₄Cl.
Fig. I.2. Sodium dependence of growth of *Anabaena* L-31 on various nitrogen sources. Details as in legend to Fig. I.1.
Table 1.1. Specificity of sodium requirement for the growth of N\textsubscript{2}-fixing Anabaena spp..

<table>
<thead>
<tr>
<th>Additions to the Na\textsuperscript{+}-deficient medium</th>
<th>Growth ((\mu g) chlorophyll (a). ml\textsuperscript{-1})</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anabaena torulosa</td>
<td>Anabaena L-31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>2.13</td>
<td>3.59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>18.12</td>
<td>20.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na\textsubscript{2}SO\textsubscript{4}</td>
<td>16.25</td>
<td>20.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na\textsubscript{2}HPO\textsubscript{4}.2H\textsubscript{2}O</td>
<td>17.90</td>
<td>19.45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>1.45</td>
<td>5.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K\textsubscript{2}HPO\textsubscript{4}.2H\textsubscript{2}O</td>
<td>2.15</td>
<td>3.59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LiOH.H\textsubscript{2}O*</td>
<td>1.46</td>
<td>2.96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgSO\textsubscript{4}.7H\textsubscript{2}O</td>
<td>2.00</td>
<td>3.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaCl\textsubscript{2}.2H\textsubscript{2}O</td>
<td>2.31</td>
<td>3.02</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Growth was measured 5 days after inoculation, into combined nitrogen-free medium. The chlorophyll \(a\) content at the time of inoculation was 3.43 \(\mu g\).ml\textsuperscript{-1} for A. torulosa and 3.92 \(\mu g\).ml\textsuperscript{-1} for Anabaena L-31. All the salts were added to yield 1 mM final concentration of the cation.

* pH was adjusted to 7.00 with 0.1 N HCl.
Table I.2. Requirement of sodium for the growth of N₂-fixing Anabaena spp..

<table>
<thead>
<tr>
<th>Na⁺ (mM)</th>
<th>Growth (μg chlorophyll a. ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anabaena torulosa</td>
</tr>
<tr>
<td>0.000</td>
<td>2.18</td>
</tr>
<tr>
<td>0.005</td>
<td>3.54</td>
</tr>
<tr>
<td>0.008</td>
<td>8.05</td>
</tr>
<tr>
<td>0.010</td>
<td>11.96</td>
</tr>
<tr>
<td>0.020</td>
<td>10.31</td>
</tr>
<tr>
<td>0.050</td>
<td>14.51</td>
</tr>
<tr>
<td>0.100</td>
<td>15.12</td>
</tr>
<tr>
<td>0.250</td>
<td>18.18</td>
</tr>
<tr>
<td>0.500</td>
<td>18.51</td>
</tr>
<tr>
<td>1.000</td>
<td>18.83</td>
</tr>
<tr>
<td>5.000</td>
<td>20.87</td>
</tr>
<tr>
<td>10.000</td>
<td>24.48</td>
</tr>
<tr>
<td>20.000</td>
<td>26.31</td>
</tr>
</tbody>
</table>

Growth was measured 5 days after inoculation into combined nitrogen-free medium. Na⁺ was added as NaCl. The chlorophyll a content at the time of inoculation was 3.43 μg.ml⁻¹ for Anabaena torulosa and 3.92 μg.ml⁻¹ for Anabaena L-31.
Effect of sodium on N₂ fixation by *Anabaena* spp.

Table 1.3 shows the effect of Na⁺ on heterocyst differentiation and nitrogenase activity of *Anabaena* spp.. In both species differentiation of heterocysts was not affected by the absence of Na⁺ in the medium and no gross alteration in their morphology could be noticed under a light microscope. In contrast, the nitrogenase activity was reduced under Na⁺ deficiency. The sensitivity of nitrogenase to Na⁺ deficiency was different in the two cyanobacteria; while in *A. torulosa* there was little detectable activity in the absence of Na⁺, *Anabaena* L-31 showed about 26% activity compared to its Na⁺-supplemented controls.

In the presence of Na⁺ in both *Anabaena* spp. nitrogenase activity appeared 20 - 21 h after inoculation into the combined nitrogen-free medium (Fig. I.3, I.4). Na⁺-starved *A. torulosa* cultures showed negligible nitrogenase activity (Fig. I.3) while corresponding *Anabaena* L-31 cultures showed about one-third of the activity of Na⁺-supplemented cultures (Fig. I.4). There was a remarkable enhancement in nitrogenase activity of both cyanobacteria when Na⁺ was added to Na⁺-deficient cultures around 19.5 h - 20.5 h. Detectable nitrogenase activity appeared in *A. torulosa* within 2.5 h after addition of Na⁺. Moreover, subsequently the activity increased very rapidly and within 6 h it fully attained the level in control cultures (Fig. I.3).
Table I.3. Effect of sodium on heterocyst differentiation and nitrogenase activity of *Anabaena* spp..

<table>
<thead>
<tr>
<th></th>
<th><em>Anabaena</em> torulosa</th>
<th><em>Anabaena</em> L-31</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)</td>
<td>(+)</td>
<td>(-)</td>
</tr>
<tr>
<td>Heterocysts (% cells)</td>
<td>9.70</td>
<td>9.90</td>
</tr>
<tr>
<td>Nitrogenase activity</td>
<td>2.98</td>
<td>52.63</td>
</tr>
</tbody>
</table>

(NMmol C\(_2\)H\(_2\) reduced \(\mu\)g chlorophyll \(a\)\(^{-1}\)h\(^{-1}\))

Thirty hours after inoculation into combined nitrogen-free medium, sampling was done. Number of heterocysts was calculated as % of total cells from a count of at least \(2 \times 10^3\) cells per sample. \(C_2H_2\) reduction was assayed with 2 ml culture aliquots incubated in 0.1 atm \(C_2H_2\) (in air) at 5000 lx intensity and 25°C on a rotary wheel (11 r.p.m.) for 30 min. (+) and (-) denote presence and absence of Na\(^+\) respectively.
Fig. 1.3. Sodium requirement for in vivo nitrogenase activity in *Anabaena torulosa*. Content of chlorophyll a was maintained at 2-3 µg.ml⁻¹ of cultures. Enzyme activity in the absence of Na⁺ was compared with cultures to which 1 mM Na⁺ was added either at 0 h or at the time indicated by arrow. Other details are described in the legend to Table 1.3.
Fig. 1.4. Sodium requirement for \textit{in vivo} nitrogenase activity in 
\textit{Anabaena} L-31. Details as in legend to Fig. 1.3.
Anabaena L-31 also exhibited a similar effect upon addition of Na\(^+\) to Na\(^-\) deficient cultures (Fig. I.4).

The amount of Na\(^+\) required for expression of nitrogenase activity in A. torulosa was found to be very low (Table I.4) and comparable to that required for growth (Table I.2). Nitrogenase activity in this cyanobacterium was seen at 10 \(\mu M\) of added Na\(^+\) (in addition to \(\approx 15 \mu M\) Na\(^+\) present as contaminant) and increased with increasing Na\(^+\) concentration. The threshold Na\(^+\) level for appearance of nitrogenase activity in A. torulosa was thus calculated to be approximately 25 \(\mu M\). In the fresh water cyanobacterium, Anabaena L-31, since Na\(^+\)-starved cultures retained substantial nitrogenase activity the threshold Na\(^+\) concentration for nitrogenase activity could not be determined.

Comparison of sodium requirement with that for molybdenum in
Anabaena spp.

The effect of Na\(^+\) on growth, heterocyst differentiation and nitrogenase activity of A. torulosa was compared with that of molybdenum - a known constituent of MoFe protein of nitrogenase, and tungsten - known to substitute molybdenum in nitrogenase from several microorganisms (Benemann et al., 1973; Brill et al., 1974; Cardenas and Mortenson, 1975; Nagatani and Haselkorn, 1978). Whereas response of the cyanobacterium to Na\(^+\) starvation was very rapid, molybdenum-starved
Table I.4. Effect of sodium on in vivo nitrogenase activity in *Anabaena torulosa*.

<table>
<thead>
<tr>
<th>Na⁺ (mM)</th>
<th>Amol C₃H₂ reduced</th>
<th>mg chlorophyll a⁻¹.h⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.000</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>0.005</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>0.008</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>0.010</td>
<td>2.30</td>
<td>6.02</td>
</tr>
<tr>
<td>0.020</td>
<td></td>
<td>10.20</td>
</tr>
<tr>
<td>0.050</td>
<td>21.60</td>
<td>25.90</td>
</tr>
<tr>
<td>0.100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.250</td>
<td></td>
<td>26.35</td>
</tr>
<tr>
<td>1.000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Twenty two hours after inoculation into combined nitrogen-free medium cyanobacterium was harvested. 15 ml aliquots of culture were incubated with NaCl in plastic containers under aeration for 5 h at 5000 lx light intensity and 25°C, before being assayed for nitrogenase activity. Other details were as described in the Materials and Methods.
cultures could be obtained only after several passages in molybdenum-free medium. Under both molybdenum as well as Na⁺ deficiency, growth and nitrogenase activity were inhibited (Fig. I.5: A, B, C). Tungsten, whenever present, inhibited growth and nitrogenase activity (Fig. I.5: E - H). Na⁺ and tungsten had no effect on heterocyst differentiation (Fig. I.5: B, D, H) but molybdenum deficiency resulted in enhancement of the heterocyst frequency (Fig. I.5: A, C, E, G). This enhancement was neither prevented nor stimulated further by tungsten (Fig. I.5: E, G).

Good growth and nitrogenase activity were observed only in the presence of both molybdenum and Na⁺ and in the absence of tungsten (Fig. I.5: D). Similar results were also observed with Anabaena L-31 (Fig. I.5).

In both Anabaena spp. molybdenum deficiency nearly doubled the number of heterocysts while the absence or presence of Na⁺ had no effect (Table I.5). Also in the absence of molybdenum the differentiation process seemed to extend over a much longer period since, even 30 h after transfer to nitrogen-free medium, a significant proportion of cells was still in the early stage (proheterocyst) of their differentiation.

When subcultured repeatedly in molybdenum-free medium, A. torulosa displayed a much reduced nitrogenase activity (5.2 \text{\mu mol C}_2\text{H}_2 \text{reduced mg chlorophyll} s^{-1} h^{-1}) compared to
Fig. 1.5. Effect of sodium (Na) molybdenum (Mo) and tungsten (W) on the growth, heterocyst differentiation ( ), and nitrogenase activity ( ) in Anabaena torulosa, grown under N₂-fixing conditions for 30 h. Additions to the basal medium were 50 μM (MoO₄)²⁻, 50 μM (WO₄)²⁻ or 0.5 mM NaCl in following combinations:

A: Mo(-)W(-)Na(-);  B: Mo(+)/W(-)Na(-);
C: Mo(-)W(-)Na(+);  D: Mo(+)/W(-)Na(+);
E: Mo(-)W(+)/Na(-);  F: Mo(+)/W(+)/Na(-);
G: Mo(-)W(+)/Na(+);  and H: Mo(+)/W(+)/Na(+).

(+) and (-) denote presence and absence of the element respectively.
Fig. 1.6. Effect of sodium (Na), molybdenum (Mo) and tungsten (W) on the growth (□), heterocyst differentiation (■■■■) and nitrogenase activity (□□□□□) in Anabaena L-31, grown under N₂-fixing conditions for 30 h. Other details as in legend to Fig. 1.5.
Table I.5. Comparison of the effect of sodium and molybdenum on heterocyst differentiation in *Anabaena* spp.:

<table>
<thead>
<tr>
<th>Cyanobacterium</th>
<th>Cation</th>
<th>% differentiated cells</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Proheterocysts</td>
<td>Heterocysts</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>1. <em>Anabaena</em></td>
<td>Sodium</td>
<td>(-)</td>
<td>0.80</td>
<td>10.08</td>
<td>10.88</td>
</tr>
<tr>
<td>torulosa</td>
<td>(+)</td>
<td>0.36</td>
<td>11.39</td>
<td>11.75</td>
<td></td>
</tr>
<tr>
<td>Molybdenum</td>
<td>(-)</td>
<td>4.22</td>
<td>14.64</td>
<td>18.85</td>
<td></td>
</tr>
<tr>
<td>(+)</td>
<td>0.47</td>
<td>10.76</td>
<td>11.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. <em>Anabaena</em></td>
<td>Sodium</td>
<td>(-)</td>
<td>0.96</td>
<td>9.04</td>
<td>10.00</td>
</tr>
<tr>
<td>L-31</td>
<td>(+)</td>
<td>0.24</td>
<td>9.99</td>
<td>10.23</td>
<td></td>
</tr>
<tr>
<td>Molybdenum</td>
<td>(-)</td>
<td>3.86</td>
<td>14.80</td>
<td>18.66</td>
<td></td>
</tr>
<tr>
<td>(+)</td>
<td>0.91</td>
<td>9.21</td>
<td>10.12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Cells were counted 30 h after transfer to combined nitrogen-free CM/5. (-) and (+) denote absence or presence of the cation respectively. Other details were as given in Table I.3.
molybdenum-supplemented controls (40 - 50 μmoles). Addition of molybdenum to such cultures enhanced their nitrogenase activity (Fig. 1.7) after a lag of nearly 4 h (compared to 2 h for Na⁺). The time course of reappearance of nitrogenase activity following addition of molybdenum (Fig. 1.7) closely resembled that observed with Na⁺ (Fig. 1.3) but for the difference in the duration of initial lag. The activity was fully restored by 5-6 h following addition of both cations.

The duration of initial lag (2-4 h) was not reduced even if the metal ions were added at 30 h, after transfer to combined nitrogen-free medium, or later. It could however be reduced if the cyanobacterial suspensions were concentrated to higher cell densities. A five-fold concentration (compared to that in Fig. 1.3) reduced the lag to only 90 min for Na⁺ and 2.5 h for molybdenum. However even at still higher concentrations of A. torulosa cultures the lag could not be eliminated altogether.

Uptake of molybdate and sodium and their interrelationship

To determine if the absorbed lag was a result of a similar lag in the transport of these ions and whether or not Na⁺ regulated molybdenum transport, uptake of Na⁺ and (MoO₄)²⁻ by whole filaments of A. torulosa was examined. Na⁺ uptake was a very rapid process and saturated within minutes while (MoO₄)²⁻ uptake continued at a slow rate for a long duration (Fig. 1.8).
Fig. I.7. Molybdenum requirement for in vivo nitrogenase activity in *Anabaena torulosa*. Cyanobacterium was grown in combined nitrogen-free medium under molybdenum deficiency and was subcultured in identical medium 5 times before experimentation. To one part of such molybdenum-deficient culture (○) 50 μM (MoO₄)²⁻ was added (△) and C₂H₂ reduction was measured at 1 h intervals. Other details as in legend to Table I.3.
Fig. 1.8. Initial kinetics of sodium and molybdate uptake in Anabaena torulosa. Uptake of (MoO₄)²⁻ was studied both in the absence (〇) and in the presence (●) of 1 mM Na⁺. Uptake of Na⁺ was similarly examined in the absence (△) as well as in the presence (▲) of 50 μM (MoO₄)²⁻. Details of the radioactivity assays are described in the Materials and Methods.
Uptake of both the ions was independent of the presence or absence of the other ion.

Nature of the nitrogen source during growth influenced both the requirement as well as the uptake of \( \text{Na}^+ \) and \( (\text{MoO}_4)^{2-} \).

A reduced requirement of molybdenum (0.1 p.p.m.) for maximum growth on \( (\text{NO}_3)^{2-} \) as compared to the requirement of molybdenum for maximum growth on \( \text{N}_2 \) (0.2 p.p.m.) has been shown earlier for \( \text{A. cylindrica} \) (Wolfe, 1954 a,b). \( \text{N}_2 \)-fixing \( \text{A. torulosa} \) absorbed much more \( \text{Na}^+ \) than when grown in the presence of \( (\text{NO}_3)^{2-} \) or \( \text{NH}_4^+ \) (Table 1.6). In the \( \text{NH}_4^+ \)-grown cultures uptake of both the ions was curtailed by over 50 per cent but \( (\text{NO}_3)^{2-} \)-grown cultures showed substantial uptake of \( (\text{MoO}_4)^{2-} \). \( \text{Na}^+ \), even at concentrations 50 times higher than those of molybdenum did not affect \( (\text{MoO}_4)^{2-} \) uptake, both in the presence or absence of \( \text{NH}_4^+ \) (Fig. I.9).

**Effect of inhibition of protein synthesis on nitrogenase activity**

Table 1.7 shows the effect of two inhibitors of protein synthesis on the enhancement of nitrogenase activity by \( \text{Na}^+ \) or molybdenum in cultures of \( \text{A. torulosa} \) deficient in one of these cations. No nitrogenase activity was observed when either cation was added along with either rifampicin - an inhibitor of transcription, or chloramphenicol - an inhibitor of translation, at 20.5 h (data not included) or even at 30 h. Addition of similar
Table I.6. Uptake of sodium and molybdate by *Anabaena torulosa* grown on various sources of nitrogen.

<table>
<thead>
<tr>
<th>Nitrogen source for growth</th>
<th>Uptake (cpm/μl packed cells⁻¹)</th>
<th>Na⁺</th>
<th>(MoO₄)²⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>N₂</td>
<td>260</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>(NO₃)²⁻</td>
<td>68</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>105</td>
<td>45</td>
<td></td>
</tr>
</tbody>
</table>

*A. torulosa* was grown either in combined nitrogen-free CM/b or the medium was supplemented with 10 mM Ca(NO₃)₂·4H₂O or 3 mM NH₄Cl + 10 mM HEPES/LiOH at pH 7.0. Uptake was measured during a 1 min (for Na⁺) or 10 min (for (MoO₄)²⁻) assay initiated by the addition of respective radiotracer. Other details were as described in the Materials and Methods.
Fig. 1.9. Effect of sodium on molybdate uptake by *Anabaena torulosa* grown on $N_2$ (○) or $NH_4^+$ (●) as the nitrogen source. Duration of the assay was 15 min when the $(MoO_4)_{2-}$ uptake was linear. Other details are described in the Materials and Methods.
Table I.7. Effect of protein synthesis inhibitors on the enhancement of nitrogenase activity in *Anabaena cornulosa* by addition of sodium or molybdenum.

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Additions</th>
<th>Time after addition (h)</th>
<th>Nitrogenase activity A</th>
<th>Nitrogenase activity B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium-deficient</td>
<td>-</td>
<td>2.02</td>
<td>2.02</td>
<td></td>
</tr>
<tr>
<td>Sodium</td>
<td>3</td>
<td>11.08</td>
<td>11.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>25.03</td>
<td>24.51</td>
<td></td>
</tr>
<tr>
<td>Sodium + Inhibitor</td>
<td>3</td>
<td>2.02</td>
<td>2.80</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2.17</td>
<td>3.04</td>
<td></td>
</tr>
<tr>
<td>Molybdenum-deficient</td>
<td>-</td>
<td>4.06</td>
<td>3.67</td>
<td></td>
</tr>
<tr>
<td>Molybdenum</td>
<td>3</td>
<td>9.57</td>
<td>9.86</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>25.76</td>
<td>25.93</td>
<td></td>
</tr>
<tr>
<td>Molybdenum + Inhibitor</td>
<td>3</td>
<td>4.91</td>
<td>5.62</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>4.56</td>
<td>5.02</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>50.15</td>
<td>49.26</td>
<td></td>
</tr>
<tr>
<td>Inhibitor</td>
<td>3</td>
<td>3.02</td>
<td>5.98</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2.75</td>
<td>4.96</td>
<td></td>
</tr>
</tbody>
</table>

Thirty hours after transfer to combined nitrogen-free CM/5, cultures deficient in either Na⁺ or molybdenum were concentrated to contain ≥ 10 μg chlorophyll a.ml⁻¹ and treated as shown in the table. Sodium was added as NaCl to give a final concentration of 1 mM and molybdenum was added as MoO₃ to give 50 μM. Concentrations of the inhibitors were: rifampicin, 2.5 μM; and chloramphenicol, 200 μg.ml⁻¹. Nitrogenase activity is expressed as μmol C₂H₂ reduced.mg chlorophyll a⁻¹.h⁻¹. A and B indicate separate experiments where rifampicin and chloramphenicol were added respectively, as indicated in the column "Additions."
concentration of these inhibitors to control (Na\(^+\) and molybdenum-supplemented) cultures decreased their existing nitrogenase activity by over 90 per cent in three hours. Rifampicin inhibited nitrogenase activity more rapidly than chloramphenicol.

**Effect of sodium on the synthesis of nitrogenase proteins**

Experiments were conducted to examine whether nitrogenase was synthesised in *A. torulosa* under Na\(^+\) deficiency. The approach used to visualise and identify nitrogenase proteins was the one described by Peterson and Wolk (1978 a) and consisted of labelling the iron proteins with radioactive \(^{59}\)Fe followed by electrophoresis on polyacrylamide gels under non-denaturing conditions and detection of radioactivity in gel slices. As shown in Fig. I.10 in extracts from diazotrophically grown *A. torulosa* (Fig. I.10 b,c) the radioactivity was distributed in six well resolved peaks. Of these, two peaks (i.e., band I centered at slice no. 6 and band II centered at slice no. 23 and 24) were conspicuous by their absence in extracts of NH\(_4\)^+-grown cultures (Fig. I.10 a) but were present in extracts of both Na\(^+\)-deficient as well as Na\(^+\)-supplemented cultures (Fig. I.10 d,e). Both peaks corresponded with distinct bands of proteins in electrophoretograms which were also absent in NH\(_4\)^+-grown culture extracts (data not shown). These proteins were identified as MoFe and Fe proteins of nitrogenase based on their
Fig. I.10. Distribution of radioactivity in non-denaturing electrophoregrams of soluble extracts from whole filaments of Anabaena torulosa. Cyanobacterium was grown in (a) CM/5 supplemented with 3 mM NH$_4$Cl, or (b) combined nitrogen-free CM/5 devoid of Na$^+$, or (c) combined nitrogen-free CM/5 containing 1 mM NaCl. Radioactive $^{59}$FeCl$_3$ was added to all the media (1/$\mu$Ci. ml$^{-1}$) and incorporation was allowed for 30 h. Amount of protein loaded on gels was (a) 190 $\mu$g, (b) 210 $\mu$g and (c) 230 $\mu$g. Counts per minute of $^{59}$Fe are expressed per slice without correcting for counting efficiency.
tentative molecular weights and rate of migration towards anode. Possible identities of the remaining peaks in Fig. I.10 include ferredoxin and haem proteins like cytochromes. These results are in agreement with those of Peterson and Wolk (1978 a) who used $^{55}$Fe labelling to identify nitrogenase proteins of A. variabilis.

**Effect of anaerobiosis on nitrogenase activity**

Experiments were conducted to ascertain that the observed effect of Na$^+$ deficiency on nitrogenase was not due to alteration(s) in the mechanisms which protect nitrogenase from oxygen. Protection of nitrogenase from oxygen in A. torulosa was tested in two ways and the results are shown in Fig. I.11. First, in a short term experiment (during the 30 min assay for nitrogenase), aerobically grown filaments were incubated in argon containing different volumes of oxygen (0 - 20%, v/v) and 0.1 atm C$_2$H$_2$, and C$_2$H$_4$ production was measured. Even this short term anaerobiosis caused a 50 per cent increase in nitrogenase activity of Na$^+$-supplemented cultures (Fig. I.11 a) indicating that in heterocysts oxygen protection of nitrogenase was not perfect. Secondly, when subjected to prolonged anaerobiosis by sparging the cultures for 90 min with argon prior to assay, Na$^+$-supplemented cultures showed a 3.3 fold increase in C$_2$H$_4$ production (Fig. I.11 b). The enhancement was probably because of further strengthening
Fig. I.11. Effect of anaerobiosis on the nitrogenase activity in *Anabaena* *tenuisosa*.

(a) aerobically grown cyanobacterial suspensions from Na⁺-deficient (△) and Na⁺-supplemented (▲) cultures, grown in combined nitrogen-free CM/5 for 30 h, were assayed for C₂H₂ reduction, under a gas phase of argon, containing various amounts (% gas phase, v/v) of oxygen.

(b) aerobically grown cultures were subjected to prolonged anaerobiosis, by continuous sparging with argon for 90 min, prior to assay. Na(−) and Na(+) represent nitrogenase activity of cultures grown in the absence or presence of 1 mM NaCl.
of protection of the enzyme due to oxygen exclusion as well as stimulation of nitrogenase derepression due to nitrogen starvation. Significantly, in both the experiments nitrogenase activity in Na⁺-deficient cultures remained unaffected.

Effect of preincubation under acetylene on nitrogenase activity

Preincubation under C₂H₂ is known to activate nitrogenase in several diazotrophs both in vivo (David and Fay, 1977; David et al., 1978; Apte et al., 1978) as well as in vitro (Thorneley and Eady, 1977) and attempts were therefore made to see if Na⁺ affected this property. As shown in Fig. 1.12 in Na⁺-supplemented cultures of A. torulosa this treatment resulted in a 2.2 fold enhancement of C₂H₂ reduction while Na⁺-deficient cultures showed no such effect.

In recent years the role of membrane potential in regulation of nitrogenase activity in aerobic diazotrophs has come into prominence (Haaker et al., 1980; Hawkesford et al., 1981). Attempts were therefore made to investigate whether Na⁺ influenced membrane potential and thereby nitrogenase activity. As shown in Table 1.8 Na⁺ deficiency resulted in hyperpolarisation of membrane potential in A. torulosa. This hyperpolarisation was partially reversed by addition of Na⁺ but was not neutralised even after six hours.
Fig. 1.12. Effect of preincubation under C₂H₂ on C₂H₂ reduction by Anabaena torulosa. Prior to assay for nitrogenase activity cyanobacterial suspensions from 30 h old, Na⁺-deficient (circles) and Na⁺-supplemented (triangles) cultures were alternately exposed to a gas phase of 0.1 atm C₂H₂ (in air) and to ambient atm, each period lasting for 30 min (○, △). Control samples (○, △) were incubated in air without C₂H₂ for the same period, prior to assay.
Table I.8. Effect of sodium on membrane potential of nitrogen-fixing *Anabaena torulosa*.

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Time after addition of Na&lt;sup&gt;+&lt;/sup&gt; (h)</th>
<th>Membrane potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na&lt;sup&gt;+&lt;/sup&gt;-supplemented</td>
<td>-</td>
<td>-65.2</td>
</tr>
<tr>
<td>Na&lt;sup&gt;+&lt;/sup&gt;-deficient</td>
<td>-</td>
<td>-94.3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>-85.7</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>-84.3</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>-80.1</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>-80.6</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>-68.2</td>
</tr>
</tbody>
</table>

Twenty four hours after transfer to combined nitrogen-free medium cyanobacterium was harvested and suspended in nitrogen-free GM/5 (with or without 1 mM NaCl) containing 20 mM HEPES/LiOH at pH 7.0. Membrane potential was determined using phenyl-<sup>14</sup>C-TPMP<sup>+</sup> in a 15 min assay followed by estimation of intracellular radioactivity and cell volume and application of Nernst equation.
Effect of sodium on *Plectonema borvanum* nitrogenase

The filamentous, non-heterocystous cyanobacterium *P. borvanum* also showed requirement of Na⁺ for nitrogenase activity (Fig. I.13). Nitrogenase activity in Na⁺-supplemented cultures was five times more than that in Na⁺-deficient cultures. The latter responded to addition of Na⁺ by enhancement of nitrogenase activity. The lag preceding this enhancement was short (60 min) compared to that (≈ two hours) observed with the heterocystous *A. torulosa*.

The electrophoretic pattern of denatured proteins from *P. borvanum* was examined for the presence or absence of nitrogenase proteins (Fig. I.14). Cultures repressed for nitrogenase synthesis, by addition of (NO₃)₂⁻ to the medium and by aeration, were used as controls (Fig. I.14 A) and were compared with Na⁺-deficient (Fig. I.14 B) and Na⁺-supplemented (Fig. I.14 C) cultures induced to synthesise nitrogenase under microaerophilic conditions. Purified *Klebsiella pneumoniae* nitrogenase proteins (Kp1 and Kp2) were also electrophoresed for comparison. Four proteins having molecular weights near 86 K, 63 K, 50 K and 33 K daltons were found to be uniquely synthesised under N₂-fixing conditions, irrespective of the presence or

*These were kindly provided by Dr. R. Eady from ARC Unit of N₂ fixation, Sussex, U.K.

**K = 10³**
Fig. 13. Requirement of sodium for in vivo nitrogenase activity in *Plectonema boryanum*-594. Nitrogenase was induced in combined nitrogen-free medium in the presence (□) or absence (▲) of 1 mM Na⁺, under micro-aerophilic conditions. The experiment was performed 18 h after transfer to combined nitrogen-free medium. NaCl (1 mM) was added to Na⁺-deficient cultures (▲) at the time indicated by arrow.
Fig. 1.14. Electrophoretic pattern of proteins from *Plectonema boryanum* 594. SDS-extracts of proteins were electrophoresed on a 22% to 15% polyacrylamide gradient slab gel with 0.1% (w/v) SDS. The culture conditions were: A: 10 mM Ca(NO\textsubscript{3})\textsubscript{2}·4H\textsubscript{2}O, aerobic; B: combined nitrogen-free, Na\textsuperscript{+}-deficient, argon; and C: combined nitrogen-free, Na\textsuperscript{+}-supplemented, argon. The *in vivo* specific activities of cultures A, B and C were 0, 12 and 59 \(\mu\text{mol C_2H_2 reduced mg chlorophyll a}^{-1} \text{h}^{-1}\) respectively. Amount of protein loaded was A: 230 \(\mu\text{g}\) and B and C: 200 \(\mu\text{g}\). Positions of bands belonging to molecular weight standards and Kp1 and Kp2 are indicated on the left side. Bands marked on the right side show proteins synthesised only under N\textsubscript{2}-fixing conditions of which 63K and 33K bands have been previously identified as *Plectonema* nitrogenase proteins (see text).
absence of Na\(^+\) (Fig. 1.14 B,C). Protein bands at 63 K and 33 K fall within the known range of molecular weights of nitrogenase proteins (see Yates, 1960) and corresponded with the known molecular weights of Plectonema nitrogenase proteins (see Nagatani and Haselkorn, 1978) and were assigned to component I and II of nitrogenase, respectively.

**DISCUSSION**

Two filamentous, heterocystous species of *Anabaena*, *A. torulosa* and *Anabaena* L-31 show a specific requirement of Na\(^+\) for growth (Fig. 1.1, 1.2; Table I.1) and nitrogenase activity (Fig. 1.3, 1.4). The amount of Na\(^+\) required is very low; 20 - 25 \(\mu\)M being the threshold level required by *A. torulosa* for growth and nitrogenase activity alike (Table I.2, I.4). The observed threshold level is much below the range of Na\(^+\) concentrations previously reported to be essential for cyanobacteria (Allen and Arnon, 1955 b; Kratz and Myers, 1955). The optimum Na\(^+\) concentrations for the growth of *Anabaena* L-31 and *A. torulosa* are 0.5 mM and 20 mM respectively (Table I.2).

Dependence of growth on Na\(^+\) is observed only under \(\mathrm{N}_2\)-fixing conditions (Fig. 1.1, 1.2) unlike *A. cylindrica*, which, when grown on \((\mathrm{NO}_3)^{-2}\), required much higher levels (400 \(\mu\)M) of Na\(^+\) than when grown without combined nitrogen (4 \(\mu\)M). \((\mathrm{NO}_3)^{-2}\)
grown, Na⁺-deficient *A. cylindrica* did not grow, as a result of chlorosis (Brownell and Nicholas, 1967). *Anabaena* spp. used in the present study show no such effect at least in the first subculture in (NO₃)₂-supplemented, Na⁺-deficient medium. They could also be successfully maintained in Na⁺-deficient media supplemented with NH₄⁺ for over two years. This aspect is further discussed in Chapter II.

A requirement of Na⁺ for cyanobacterial N₂ fixation has been suspected earlier (Allen and Arnon, 1955b). Based on the decreased ¹⁵N₂ incorporation and yet increased ¹⁵NH₃ or ¹⁴C-glutamate incorporation into protein, in Na⁺-deficient *A. cylindrica*, Brownell and Nicholas (1967) suggested that Na⁺ was likely to be "required for conversion of N₂ gas into ammonia". However, a direct requirement for nitrogenase was not shown. Present results clearly demonstrate a requirement of Na⁺ for nitrogenase activity. Although the conversion of ¹⁵N₂ to ¹⁵NH₃ has not been measured, the lack of C₂H₂ reduction (Fig. I.3, I.4) under Na⁺ deficiency together with the lack of growth (Fig. I.1, I.2) under these conditions is clearly indicative of a lack of N₂ reduction to NH₃.

The differentiation of heterocysts is unaffected by Na⁺ deficiency (Table I.3, I.5) nor are the heterocysts "leaky" (structural or biochemical lesions) since even under anaerobiosis Na⁺-deficient *A. torulosa* does not express nitrogenase activity (Fig. I.11). The requirement is also observed in the non-heterocystous
Plectonema (Fig. 1.13) and thus appears to be a general requirement for $N_2$-fixing cyanobacteria.

The response of both Anabaena spp. to Na deficiency is similar to that observed during molybdenum deficiency (Fig. 1.5, 1.6) except that in the latter case heterocyst differentiation is remarkably enhanced (Table I.5). Such stimulation of heterocyst frequency was earlier observed in A. cylindrica (Fay and de Vasconcelos, 1974). In cyanobacteria the heterocyst pattern is thought to be determined by a specific nitrogenous inhibitor, produced by already existing heterocysts, which diffuses through the vegetative cells establishing a decreasing concentration gradient away from heterocysts. New heterocysts arise from those vegetative cells where the concentration of this inhibitor falls below a critical level. This hypothesis originally proposed by Fogg (1949), has been supported and slightly modified by subsequent work (Wolk, 1967; Wilcox et al., 1973; Reddy and Talpasayi, 1974; Wolk and Quine, 1975). Molybdenum starvation leads to nitrogen starvation and both are presumed to result in a fall in the level of nitrogenous inhibitor thereby increasing the number of heterocysts.

The present data (Fig. I.5, I.6 and Table I.3, I.5) show that while molybdenum-deficient cultures probably do not possess sufficient amount of the postulated inhibitor, it is
present in the Na+-deficient cultures in quantities adequate to regulate the heterocyst pattern. Since both molybdenum and Na+
deficiency eventually result in nitrogen starvation, due to lack of nitrogenase activity, it appears that nitrogen starvation may not be the sole cause for the absence of such inhibitor. Further, this inhibitor (or its precursor) is not a direct product of N₂ fixation and can be synthesised irrespective of an active nitrogenase. These conclusions are in agreement with the following findings of other workers: (i) commitment to heterocyst differentiation occurs very early and the pattern is established long before the commencement of N₂ fixation (Wilcox, 1970; Kulassoorya et al., 1972; Bradley and Carr, 1976, 1977), (ii) the pattern is observed even in an atmosphere of A/O₂ (Rippka and Stanier, 1978), (iii) some Nif⁻ mutants exhibit a normal pattern of heterocysts (Wilcox et al., 1975; Currier et al., 1977).

Tungsten inhibits growth and N₂ fixation (Fig. I.5, I.6) suggesting that its substitution for molybdenum in the nitrogenase of Anabaena spp. renders the enzyme inactive. Similar results have been reported for tungsten and vanadium substituted nitrogenases from Azotobacter (Benemann et al., 1973; Nagatani and Brill, 1974), Klebsiella (Brill et al., 1974), A. cylindrica (Fay and de Vasconcelos, 1974) and P. boryanum (Nagatani and Haselkorn, 1978). Tungsten somewhat amplifies the inhibitory
effect of molybdenum deficiency on nitrogenase but not that of Na⁺ deficiency (Fig. 1.5, I.6) suggesting difference in the molecular basis of molybdenum and Na⁺ requirement.

Nitrogenase activity in Na⁺ or molybdenum-deficient cultures of A. torulosa appears after a lag of two or four hours following addition of Na⁺ or molybdenum respectively (Fig. 1.3, I.7). This lag is not due to a corresponding delay in the transport of these ions (Fig. 1.8). The kinetic difference in the initial rates of Na⁺ and (MoO₄)²⁻-transport possibly accounts for the difference in the duration of lag following their addition. The observed lag may perhaps be due to barrier(s) to the transport of these ions at the step of their entry into the heterocysts. This conclusion gains support from the observation that in the non-heterocystous P. boryanum the lag is much shorter (Fig. I.13).

Transport of molybdate is independent of the presence or absence of Na⁺ (Fig. 1.9) ruling out a possible symport or antiport of molybdate with Na⁺ as has been observed for certain cations (Dewar and Barber, 1973), amino acids (Lanyi et al., 1976) and sugars (Stock and Roseman, 1971) in bacteria. Clearly, therefore, the Na⁺ requirement of nitrogenase is not consequent to a possible role of Na⁺ in molybdenum transport.

Restoration of nitrogenase activity following addition of molybdenum and Na⁺ to respective deficient cultures affords
two interpretations, namely that these cations are essential either for the synthesis or activity of the enzyme. In heterocystous cyanobacteria it is difficult to ascertain the exact time of induction of nitrogenase synthesis, independently of heterocyst differentiation. Although there is evidence that nitrogenase proteins are synthesised even in the proheterocysts (Fleming and Haselkorn, 1974), the appearance of fully differentiated heterocysts and detectable nitrogenase activity is usually concomitant (Thomas and David, 1972; Bradley and Carr, 1976). Even in the anaerobically grown A. variabilis, where the requirement of heterocysts was uncoupled from nitrogenase expression, detectable nitrogenase activity was observed only after 20 h (Rippka and Stanier, 1978). A similar time lag is observed in Anabaena spp. (Fig. 1.3, 1.4) while in anaerobically grown P. boryanum induction of nitrogenase is observed after 8-9 h under present experimental conditions. As against these enhancement of activity, following addition of Na⁺ or molybdenum to respective deficient cultures of cyanobacteria, occurs much faster (Fig. 1.3, 1.4, 1.13) and activity is fully restored in Anabaena spp. in a short time. These observations favour a role of Na⁺ and molybdenum in activation of the enzyme, presynthesised in their absence rather than in its de novo synthesis. Both A. torulosa as well as P. boryanum induced to synthesize nitrogenase do indeed show protein bands corresponding to presumptive component I (MoFe protein).
and II (Fe protein) of nitrogenase, both under Na⁺ deficiency and in its presence (Fig. I.10, I.14). Thus the nitrogenase proteins are synthesised under Na⁺ deficiency but remain catalytically inactive.

The experiments to demonstrate activation of existing nitrogenase by Na⁺ while fresh synthesis was blocked by chloramphenicol/rifampicin gave negative results in A. torulosa (Table I.7). This is however misleading since these inhibitors rapidly inhibit nitrogenase activity (> 90% in 3 h) even in control cultures and since the effect of added Na⁺ or molybdenum is observed only two to four hours later, these experiments proved to be inconclusive. Attempts were also made to examine the presence of nitrogenase proteins in Na⁺-deficient A. torulosa and P. boryanum culture extracts using antibodies raised against purified Klebsiella pneumoniae nitrogenase proteins (since neither purified nitrogenase nor their antibodies are easily available from cyanobacteria). Anti-Kp1 and anti-Kp2 were provided by Dr. R. Eady from ARC Unit of N₂ Fixation, Sussex, U.K.. No immunological cross reactivity was observed between these antibodies and concentrated nitrogenase antigens from both Na⁺-deficient as well as Na⁺-supplemented cultures of these cyanobacteria (data not shown).
The close similarity in the requirement of Na⁺ and molybdenum for cyanobacterial \(N_2\) fixation and growth is quite striking. Uptake of both ions is curtailed in the \(NH_4^+\)-grown cultures (Table I.6). This is expected for molybdenum which is a constituent of two nitrogen metabolising enzymes, nitrogenase and nitrate reductase, both of which are absent in the presence of \(NH_4^+\). For Na⁺, it is a new and interesting finding, which is explained by its requirement for nitrogenase activity. The similarity in kinetics of reappearance of nitrogenase activity by the addition of Na⁺ (Fig. I.3) or molybdenum (Fig. I.7) suggests that their roles are complementary to \(N_2\) fixation. Non-rerequirement of molybdenum per se for the synthesis of nitrogenase proteins is common to almost all the diazotrophs, except *Clostridium* (Cardenas and Mortenson, 1975) where a reexamination is necessary, and the inactive component I has been shown to be rapidly activated by the addition of \((MoO_4)^{2-}\) or Fe-Mo cofactor (Nagatani and Brill, 1974; Pienkos et al., 1981; Kahn et al., 1982). Molybdenum independence of nitrogenase synthesis is also established in cyanobacteria (Nagatani and Haselkorn, 1978; Hallenbeck and Benemann, 1980) and results show that it is true for Na⁺ also (Fig. I.10, I.14). The contrasting effects of these cations on heterocyst differentiation and the difference in their interaction with tungsten (Fig. I.5, I.6) indicate that the actual mechanism of action of these cations is probably different.
The reason(s) for the catalytic inactivity of nitrogenase under Na⁺ deficiency is not yet clear. Subjecting Na⁺-deficient *A. torulosa* to anaerobiosis does not promote nitrogenase activity (Fig. I.11) and in the anaerobically induced *P. boryanum* also nitrogenase remains inactive under Na⁺ deficiency. This rules out the possibility of a lack of protection from oxygen to nitrogenase during Na⁺ deficiency being the cause of its inactive nature.

The enhancement of C₂H₂ reduction upon preincubation under C₂H₂ **in vivo** has been shown to be due to conformational change(s) in the nitrogenase leading to increase in its affinity towards C₂H₂ (Apte *et al.*, 1978). With purified enzyme preparations from *K. pneumoniae* such treatment has been shown to result in increased electron flow through nitrogenase thereby enhancing its activities, i.e. the proportion of forms capable of reducing C₂H₂ and C₂H₂ + N₂ increases over those capable of reducing only protons (Thorneley and Eady, 1977). However the inactive nitrogenase of Na⁺-deficient cultures does not respond to this treatment (Fig. I.12).

The nature of "activation" of nitrogenase by Na⁺ – whether due to a direct effect on enzyme molecule or an indirect effect – remains unresolved. Data provided in this chapter show that cyanobacterial N₂ fixation and growth are dependent on Na⁺.
The requirement is for nitrogenase activity and nitrogenase proteins are synthesized in the absence of Na\(^+\) but function only in its presence. Na\(^+\) is not involved in promoting nitrogenase activity, indirectly, by influencing heterocyst differentiation, molybdenum transport or protection of nitrogenase from oxygen. Na\(^+\) deficiency does influence membrane potential which in turn is known to regulate nitrogenase activity at least in aerobic diazotrophs like Azotobacter (Haaker et al., 1980) and Anabaena variabilis (Hawkesford et al., 1981). In both these organisms high nitrogenase activity is correlated with hyperpolarisation of membrane potential while depolarisation results in inhibition of nitrogenase. In A. torulosa also, hyperpolarisation (-81.2 mV) induced by nigericin (1 \(\mu\)g.ml\(^{-1}\)) results in \(\sim 45\%\) enhancement of nitrogenase activity (data not included). This however distinctly contrasts with the situation observed under Na\(^+\) deficiency where hyperpolarisation is accompanied by loss of nitrogenase activity (Table I.8). Clearly therefore the effects of Na\(^+\) on membrane potential and nitrogenase activity are independent. It is possible though that Na\(^+\) may be involved in maintenance of a requisite membrane potential conducive to general metabolism during diazotrophic growth.

The requirement of Na\(^+\) for manifestation of nitrogenase activity in cyanobacteria may, in fact, be a general requirement for nitrogen fixation in several or all diazotrophs. Na\(^+\) is a
constituent of culture media of all the important N$_2$-fixing microbes (see Dalton, 1980) viz., *Rhizobium*, *Azotobacter*, *Klebsiella*, *Bacillus*, *Clostridium*, *Azospirillum* and even the hydrogen bacterium *Xanthobacter*, the methane-oxidising bacterium *Methylococcus* and the sulfur bacterium *Thiobacillus*. The reasons for inclusion of Na$^+$ in media are however not known. It is also the most common accompanying cation or a contaminant in many organic and inorganic compounds employed in the preparation of media and buffers. *In vitro* assays of nitrogenase are most often performed using sodium dithionite (Na$_2$S$_2$O$_4$) and sometimes Na$^+$-ATP. It is therefore quite likely that a requirement of Na$^+$ for diazotrophy has been overlooked. This needs to be examined critically.