Ammonium (NH$_4^+$) uptake in the cyanobacterium Nostoc muscorum ISU (Anabaena ATCC 27893) and interaction of copper (Cu$^{2+}$) and sulfhydryl agents was studied. N$_2$-grown cells scavenged extracellular NH$_4^+$ via two energy-dependent transport systems: the 'high-' ($K_m=11\ \mu M$, $V_{max}=0.22\ \text{nmol/min/mg protein}$) and 'low-affinity' ($K_m=66\ \mu M$, $V_{max}=1.25\ \text{nmol/min/mg protein}$). Both transport systems were competitively inhibited by methylamine (high-affinity $K_i=20\ \mu M$; low affinity $K_i=80\ \mu M$), and showed distinct pH profiles. Addition of Cu$^{2+}$ (0.1 $\mu M$) stimulated NH$_4^+$ uptake by the high-affinity system ($K_m=8\ \mu M$, $V_{max}=0.42\ \text{nmol/min/mg protein}$). Similar effect was not observed with other bivalent cations (Hg$^{2+}$, Ni$^{2+}$, Zn$^{2+}$, Mn$^{2+}$) applied at equimolar concentrations. The sulfhydryl reducing agents, cysteine and dithiothreitol, inhibited the high-affinity system noncompetitively and caused efflux of accumulated NH$_4^+$. Cu$^{2+}$ eliminated the inhibitory effect of sulfhydryl reducing agents on NH$_4^+$ uptake. Inhibition of NH$_4^+$ uptake by sulfhydryl blocking agents (N-ethylmaleimide or p-chloromercuribenzoate) which was not reversible by Cu$^{2+}$ suggested that oxidation of available sulfhydryl residues of membrane proteins (carriers) is an important factor in NH$_4^+$ translocation in Nostoc muscorum.

The free-living N$_2$-fixing microorganisms are known to respond to the fixed form of nitrogen in the immediate environment by repressing nitrogenase synthesis. The nature of the regulatory mechanism is not well understood. However, a possibility exists, though unproved, that NH$_4^+$ uptake is one of the regulatory mechanisms which may be similar to catabolite repression resulting from transport of sugar across the inner membrane ($I$, $2$). The existence of ammonia as NH$_4^+$...
ion at physiological pH (dissociation constant $10^{-9.25}$) and rather negligible permeability of biomembranes towards NH$_4^+$ suggest the requirement of specific carrier(s) for NH$_4^+$ transport (3). Strong evidence for the existence of NH$_4^+$ carriers has been provided for several bacterial species (4-8). Further, the dithiol-disulfide interconversion mediated methylamine transport has been reported in methylothroph <i>Arthrobacter</i> PI (9).

While the pathway involved in assimilation of various forms of nitrogen has been studied in cyanobacteria (10), less is known about the mechanism of uptake of NH$_4^+$ (11). The dearth of information on the cyanobacterial NH$_4^+$ transport system prompted us to study the properties of the NH$_4^+$ transport system in N$_2$-fixing cyanobacterium <i>Nostoc muscorum</i>. Further, in an attempt to define the state of carrier responsible for NH$_4^+$ influx, interaction of the NH$_4^+$ uptake system with sulfhydryl agents was investigated. The results presented here indicate (a) the existence of two energy-dependent (carrier mediated) uptake systems, and (b) the involvement of oxidation-reduction of high-affinity carrier(s) system in the transport.

**MATERIALS AND METHODS**

*Organism and growth conditions.* <i>Nostoc muscorum</i> ISU (<i>Anabaena</i> ATCC 27893), obtained through the courtesy of Prof. R. Haselkorn, University of Chicago (USA), was cultivated in modified Chu No. 10 medium (12) with A-6 trace elements. Nitrogen free medium (N$_2$-medium) was obtained by replacing calcium nitrate with calcium chloride (0.07 mM). The cultures were grown in 250 ml, cotton stoppered, Erlenmyer flasks at 24±1°C (2,400 lux, fluorescent illumination) with 18/6 hr light/dark cycle.

*Ammonium uptake experiments.* Exponentially growing culture (8–10 days) was harvested by filtration through sintered glass filter (4.0 µm), washed with water, and resuspended in the assay medium (medium as above, without added nitrogen source and pH adjusted to desired level either by citrate or phosphate buffer) to a final density of 450 µg protein/ml and incubated for 30 min. NH$_4^+$ in the form of NH$_4$Cl (British Drug Houses, India) was added to the assay medium ranging from 1.0–350 µM to account for saturating levels of NH$_4^+$. The samples were taken out at regular intervals of 10 min, centrifuged (4,000× g, 2 min), and cell-free supernatant was analysed for NH$_4^+$ levels (in terms of nmol/mg protein) by Nessler’s reagent (13), phenyl hypochlorite method (14), or by ammonia electrode (Orion Ion Analyzer, model 901). Protein was estimated by the method of Lowry et al. (15). Kinetic constants ($K_m$ and $V_{max}$) were determined from LINEWEAVER and BURK (16) plots.

For inhibition studies, the cells were pretreated with an inhibitor prior to addition of NH$_4^+$. In case of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), the chemical was dissolved in ethanol before diluting 100 fold in the assay medium.
In such experiments, all the treatments including control were run with 0.1% ethanol. In certain experiments involving competition studies, unless otherwise mentioned, an inhibitor was added simultaneously with NH$_4^+$ into the assay medium.

Ammonium gradient formation was estimated by monitoring changes in intracellular and extracellular (in the assay medium) NH$_4^+$ concentrations following the uptake experiments. Procedure for determination of NH$_4^+$ under the latter condition was similar to that described above. For determination of intracellular concentration of NH$_4^+$, cyanobacterial samples were rapidly washed with phosphate buffer (0.04 M, pH 7.0) by centrifugation and the pellet was immediately treated with 0.5 N H$_2$SO$_4$ (30 min, 25°C). The suspension was centrifuged, supernatant neutralized with 1.0 M NaOH, and assayed NH$_4^+$ concentration using ion specific electrode. Intracellular concentrations were subsequently determined by dividing the difference by five times the dry weight of the cyanobacterial sample, assuming that aquatic prokaryotes contain about 80% water.

**Chemicals.** Methylamine hydrochloride, cysteine, dithiothreitol (DTT), N-ethylmaleimide (NEM), p-chloromercuribenzoate (pCMB) and DCMU were obtained from Sigma Chemicals. Copper (Cu$^{2+}$) was used as CuSO$_4$·5H$_2$O (British Drug Houses, India).

### RESULTS AND DISCUSSION

**Kinetics of NH$_4^+$ uptake**

Addition of NH$_4^+$ (26 µM) in the assay medium revealed a lag of about 30 min in NH$_4^+$ uptake, after which it was linear with respect to time for 1.5 hr. The lag in NH$_4^+$ could be reduced if the N$_2$-fixing cells were preincubated for 30 min with a low level of NH$_4^+$ (5-10 µM) prior to the uptake experiments. The rate of uptake under the two conditions was similar, being 0.21 nmol/min/mg protein. The results thus suggested that the cells have to be induced for NH$_4^+$ uptake.

When NH$_4^+$ uptake was followed in different concentrations of NH$_4^+$ (1.0–350 µM) using the induced cells, it followed the Michaelis-Menten type of saturation kinetics (Fig. 1A), although two inflections were observed. This biphasic nature of the curve suggested the possible involvement of two transport systems: a ‘high-affinity’ system with an apparent $K_m$ of 11 µM and a ‘low-affinity’ system with an apparent $K_m$ of 66 µM. The $V_{\text{max}}$ values were 0.22 nmol/min/mg protein and 1.25 nmol/min/mg protein for the two transport systems, respectively. The existence of multiple transport systems has been documented for bacterial species (4-7) and in yeast (17). Measurement of NH$_4^+$ gradient at the saturating levels of the two transport systems (26.6 and 266.0 µM, respectively) revealed that 10 and 50 fold gradients were formed following 90 min of NH$_4^+$ uptake.

The transport systems seem to be specific for NH$_4^+$ since simultaneous addition of methylamine competitively inhibited NH$_4^+$ uptake either by high- or low-
Fig. 1. (A) Concentration dependent NH$_4^+$ uptake by *N. muscorum* cells grown on elemental nitrogen showing dual isotherm Michaelis-Menten kinetics. Changes in concentration of ammonium were monitored using the phenyl hypochlorite method. (B) Lineweaver-Burk plot for NH$_4^+$ uptake by high-affinity system (NH$_4^+$ concentration range 1-35 µM). NH$_4^+$ uptake was followed in absence (○) or presence (●) of 20 µM methylamine. (C) Lineweaver-Burk plot for NH$_4^+$ uptake by low-affinity system NH$_4^+$ concentration range (35-350 µM). NH$_4^+$ uptake was followed in absence (○) or presence (●) of 80 µM methylamine.

Table 1. Effect of DCMU (10$^{-5}$ M) on NH$_4^+$ uptake by high-and low-affinity systems on *N. muscorum*.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>NH$_4^+$ uptake (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High-affinity system</td>
</tr>
<tr>
<td>Light (control)</td>
<td>100</td>
</tr>
<tr>
<td>Light + DCMU (10$^{-5}$ M)</td>
<td>25</td>
</tr>
<tr>
<td>Dark</td>
<td>0.0</td>
</tr>
</tbody>
</table>

The N$_2$-grown cyanobacterium was exposed to light or dark (48 hr), washed and suspended in the assay-medium containing NH$_4^+$ (26 or 266 µM for high-and low-affinity systems, respectively), and uptake was monitored using Nessler's reagent. The photosystem II inhibitor, DCMU, was added prior to addition of NH$_4^+$. 
affinity systems with $K_1$ values of 20 and 80 µM, respectively (Figs. 1© and 1©). The transport systems were dependent on external pH. The experiments showed the high- and low-affinity systems had optimum pH at 6.5 and 7.5, respectively. The transport of NH$_4^+$ was inhibited if the cells were either preincubated in dark or treated in light with DCMU (10⁻⁴ M), the photosystem II inhibitor (18, 19) (Table 1). Since the cyanobacteria are obligate photoautotrophs, it is anticipated that the major source of energy is derived from the light reactions (20). Light dependent NH$_4^+$ uptake has also been observed in the green alga Chlorella (21).

On the basis of the above observations, it may be concluded that Nostoc muscorum cells scavenge traces of NH$_4^+$ from the medium via two energy dependent transport systems. The evidence is also in line with the hypothesis of ALEF and KLEINER (6) that NH$_4^+$ transport systems are widespread among the free-living bacteria including the photoautotrophic ones. Considering the multiplicity of transport systems in N. muscorum, further investigations were restricted to the high-affinity system only.

Effect of Cu$^{2+}$ on ammonium uptake

Effect of graded concentrations of Cu$^{2+}$ (0.05–2.0 µM) on ammonium uptake (assayed at 26.6 µM NH$_4^+$) revealed that 0.1 µM Cu$^{2+}$ stimulated NH$_4^+$ uptake which was about 30% higher than the rate observed in the absence of Cu$^{2+}$. Higher concentrations of Cu$^{2+}$ ions (0.5–2.0 µM) were found to competitively inhibit the NH$_4^+$ uptake ($V_{\text{max}}$=0.22 nmol/min/mg protein, $K_i$=0.5 µM). Thus the stimulation of NH$_4^+$ transport by 0.1 µM Cu$^{2+}$ was observed at high NH$_4^+$ concentration (26.6 µM) with negligible effect of competitive inhibition by Cu$^{2+}$. The extent of stimulation by Cu$^{2+}$ in dark incubated cells was similar to that of light incubated cells (Fig. 2). The results thus indicate that the stimulatory role of Cu$^{2+}$ is not affected by the presence or absence of light. Further, the stimulation seems to be specific for Cu$^{2+}$ as a similar effect was not observed if other metal ions, such as Hg$^{2+}$, Ni$^{2+}$, Zn$^{2+}$ and Mn$^{2+}$, were incorporated at equimolar concentrations (0.1 µM). A Lineweaver-Burk plot of the data on Cu$^{2+}$ (0.1 µM) interaction revealed that the $K_m$ value decreased to 8.0 µM with concomitant increase in $V_{\text{max}}$ (0.42 nmol/min/mg protein). Thus the $V_{\text{max}}$ value in the presence of Cu$^{2+}$ was approximately two fold higher (Fig. 3©) than the control (0.22 nmol/min/mg protein). Stimulation of amino acid (proline, glutamic acid) uptake by Cu$^{2+}$ in membrane vesicle preparations of Mycobacterium phlei has been demonstrated (22). It is also known that Cu$^{2+}$ plays an important role in various hydroxylases (23) and causes oxidation of sulfhydryl groups of proteins (24). Thus the results obtained for Cu$^{2+}$ stimulated NH$_4^+$ uptake in N. muscorum suggest that it may result from specific interaction of Cu$^{2+}$ ions with the carrier(s) system.

Effect of sulfhydryl reducing agents and interaction with Cu$^{2+}$

Since NH$_4^+$ uptake may be associated with the oxidation of sulfhydryl groups
of carrier(s) and is stimulated by Cu²⁺ (24), it was of interest to determine the effect of sulfhydryl reducing agents, cysteine and dithiothreitol. Addition of dithiothreitol to the assay medium inhibited the NH₄⁺ uptake in a concentration dependent manner (Fig. 3). Experiments employing 50% inhibitory concentration of DTT (0.1 µM) revealed that inhibition was of non-competitive type (V_max = 0.15 nmol/min/mg protein). The rate of NH₄⁺ uptake was similar to that of control if the addition of Cu²⁺ was followed by that of DTT. On the other hand, if the cells were preincubated with DTT and Cu²⁺ was added subsequently, the inhibitory action of DTT could be partially reversed (V_max = 0.20 nmol/min/mg protein) (Fig. 3). Similar observations were made when cysteine (0.2 µM) was used as a sulfhydryl reducing agent (data not shown).

Further experiments were conducted to demonstrate the effect of sulfhydryl reducing agents on cells which have accumulated NH₄⁺ following uptake. Addition of DTT (0.5 µM) to such cells (steady state condition) caused efflux of NH₄⁺.
in the medium which remained negligible in the absence of DTT (Fig. 4). Similar observations were made when cysteine (0.5 µM) was used instead of DTT, although the extent of efflux was lower.

Taken together, the data suggest that sulphydryl reducing agents inhibited
NH₄⁺ uptake which was reversible by Cu²⁺. These observations indicate that the oxidation/reduction state of probable carrier(s) is an important factor in NH₄⁺ transport. Involvement of such an oxidation-reduction state of carrier(s) in transport of methylamine by facultative methylotroph Arthrobacter P1 (9) and amino acids transport in Mycobacterium phlei (22) has also been suggested.

Effect of sulfhydryl inhibitors and interaction with Cu²⁺

The possibility that oxidation of sulfhydryl groups of membrane proteins/carer(s) was associated with the stimulated NH₄⁺ uptake was ascertained by using NEM, an irreversible inhibitor (25), as well as pCMB, a reversible inhibitor (26). In such experiments, NEM (1.5 µM) was added prior to the addition of Cu²⁺ in order to block the available sulfhydryl groups. As shown in Fig. 5, NEM inhibited the Cu²⁺ stimulated uptake. Similar observations were made with pCMB (5.0 µM). The results, therefore, suggest the involvement of the oxidation-reduction state of sulfhydryl groups of carrier(s) responsible for NH₄⁺ transport.

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