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Hg^{2+}-induced leakage of electrolytes and inhibition of NO_3^- utilization in *Nostoc calcicola*

Role of interacting cations

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Summary. The effect of mercury (Hg^{2+}) in the absence and presence of methylmercury (CH_3Hg^+), cadmium (Cd^{2+}), copper (Cu^{2+}), nickel (Ni^{2+}) and calcium (Ca^{2+}) on *Nostoc calcicola* Bréb. has been studied in terms of electrolyte leakage, NO_3^- uptake and in vivo nitrate reductase (NR) activity to discover any possible correlation among such parameters under Hg^{2+} stress. Leakage of electrolytes from Hg^{2+}-treated cyanobacterial cells was directly proportional to Hg^{2+} concentrations and exposure time. In comparison to NO_3^- uptake, an about 60-fold slower rate of NR activity was observed in the untreated cultures, the former being five times more Hg^{2+}-sensitive. A non-competitive synergistic interaction of Hg^{2+} with CH_3Hg^+ or Cd^{2+} and antagonistic with that of Ni^{2+} or Ca^{2+} has been observed for both the processes of NO_3^- utilization. The antagonistic interaction of Cu^{2+} with Hg^{2+} in terms of NO_3^- uptake and synergistic with respect to NR activity, has been attributed to the dual bonding preference of Cu^{2+} for cellular ligands. These findings suggest that (a) a statistically significant correlation exists among such parameters; (b) Hg^{2+} predominantly attacks the cyanobacterial cell membrane; (c) Hg^{2+} inhibits NO_3^- utilization; (d) the presence of other cations increases or decreases the inhibitory actions of Hg^{2+}.

Key words: Hg^{2+} toxicity - *Nostoc calcicola* - Electrolyte leakage - NO_3^- uptake - Nitrate reductase - Metal interactions

Introduction

The extremely toxic metal cation of mercury (Hg^{2+}) has no known biological requirement. In natural habitats, the heavy metal may originate from domestic sewage, agricultural run-off and industrial or pharmaceutical wastes and ultimately enters biological systems as the active cation to interact with intracellular targets (Blinn et al. 1977; Sigmon et al. 1977; Jeffries 1982).

Hg^{2+} increases cell membrane permeability and induces the leakage of intracellular ions in eukaryotic algae (Shieh and Barber 1973; Rai et al. 1981); however, there are no similar reports on prokaryotic cyanobacteria. The transmembrane uptake of nitrate (NO_3^-) is largely an energy-dependent process and has been reported to be quite sensitive to Cd^{2+} in *Anacystis nidulans* (Singh and Yadava 1983) and to Cr^{2+} or Sn^{2+} in *Anabaena doliolum* (Rai and Dubey 1989). Nitrate reductase (NR), the key enzyme for NO_3^- reduction, operates at the expense of electrons derived through photosynthesis (Guerrero and Lara 1987), a process which is extremely sensitive to Hg^{2+} (Stratton et al. 1979; Singh and Singh 1987; Singh et al. 1989). Recently, in vivo NR activity was found to be quite sensitive to Cr^{2+} and Sn^{2+} (Rai and Dubey 1989). However, the reports so far available provide no information on the biology of Hg^{2+} toxicity to cyanobacteria in terms of electrolyte leakage, concurrently with NO_3^- uptake and in vivo NR activity.

Apart from various biotic factors, Hg^{2+} toxicity is greatly influenced by the presence of other interacting cations (Stratton and Corke 1979; Singh and Singh 1987; Singh et al. 1987). Since the natural habitats are generally characterized by the co-existence of a large number of toxic and non-toxic cations (Whitton 1970; Henriksen and Wright 1978; Strarodub et al. 1987) and since algal growth media also contain a large number of cations, it is necessary to study Hg^{2+} toxicity in the presence of other interacting cations.

The present study, therefore, was aimed at investigating such aspects in order to discover a possible correlation in electrolyte leakage, NO_3^- uptake and in vivo NR activity in the diazotrophic cyanobacterium *Nostoc calcicola* Bréb. exposed to either Hg^{2+} alone or to its combinations with CH_3Hg^+, Cd^{2+}, Cu^{2+}, Ni^{2+} or Ca^{2+}.

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Materials and methods

Experimental organism and growth conditions. *Nostoc calcicola* Bréb. was axenically grown in liquid growth medium (Allen and Arnon 1955) free from any combined nitrogen sources. The cultures were routinely maintained in 200 ml medium, contained in 500-ml conical flasks and illuminated with cool fluorescent light (50 μmol photon $\cdot m^{-2} \cdot s^{-1}$) in a culture room at 24±1° C with an 18-h/6-h light/dark regime. Protein was quantified by the method of Lowry et al. (1951), modified by Herbert et al. (1971), using lysozyme (Sigma, USA) as standard.

Electrolyte leakage. Exponentially growing *N. calcicola* cells were harvested by centrifugation and, after washing three times with sterile distilled water, inoculated into fresh sterile liquid medium (500 μg protein/ml culture), containing graded concentrations of Hg^{2+} (0.5–20.0 μM as $HgCl_2$; BDH, UK). Such sets were phototrophically incubated for 16 h and the samples (25.0 ml), withdrawn at 2-h intervals, were centrifuged and the pellets resuspended in sterile distilled water for 30 min to ensure the leakage of electrolytes. Such solutions were further centrifuged and the ion content of the resultant cell-free supernatant was monitored by recording the specific conductance in a conductivity meter (sensitivity range 0.02–200 mS/mg protein; Systronics, India). The values, expressed as μS/mg protein, were obtained after deducting the background specific conductance of sterile distilled water.

Nitrate uptake. The N_2-grown log-phase *N. calcicola* cells were inoculated in a sterile basal medium (500 μg protein/ml culture), containing graded concentrations of NO_3^- (1.0–25.0 mM; BDH, UK), to obtain the saturating NO_3^- concentrations for optimum uptake. To assess the 50% inhibitory Hg^{2+} concentration, the cyanobacterial cells were exposed to graded concentrations of Hg^{2+} (1.25–20.0 μM) at saturating NO_3^- concentration (20.0 mM) in the basal medium. This inhibitory Hg^{2+} concentration (2.5 μM) was used in subsequent experiments on metal interactions, involving equimolar concentrations (2.5 μM each) of either CH_3Hg^+ (as CH_3HgCl; Wilson Lab., India), Cd^{2+}, Cu^{2+}, Ni^{2+} or Ca^{2+} (as $CdCl_2$, $CuCl_2$, $NiCl_2$ or $CaCl_2$, respectively; BDH, UK) and graded NO_3^- concentrations (1.25–20.0 mM). After treatments, the sets were incubated phototrophically for 6 h and the amount of NO_3^- taken up by the cyanobacterial cells was determined by estimating the disappearance of NO_3^- from the external medium. The aliquots were withdrawn at regular 1-h intervals and NO_3^- in the supernatant fluid analyzed using the Brucine/H_2SO_4 method (Nicholas and Nason 1957).

Nitrate reductase activity. The in vivo NR activity in Hg^{2+}-treated (2.5–20.0 mM) *N. calcicola* cells was monitored under identical experimental conditions to NO_3^- uptake (see above) by estimating the amount of NO_2^- produced in the NO_3^--supplemented (20.0 mM) basal medium. Metal-interaction experiments were also performed in the same way as for NO_3^- uptake, except that 12.5 μM concentration (the 50% inhibitory Hg^{2+} level) of each interacting cation was used as separate bimetallic combinations with Hg^{2+}. The samples (5.0 ml), withdrawn at regular 30-min intervals, were permeabilized with 0.5 ml toluene (Merck, India), followed by rigorous shaking and incubation at 4° C for 10 min. Such samples were centrifuged to remove the toluene layer and the cell extract subjected to colorimetric estimations by using the azo-coupling method of Snell and Snell (1949).

Results

Electrolyte leakage under Hg^{2+} stress

The two-way analysis of variance (ANOVA) of the data represented in Table 1 shows that the leakage of electrolytes from Hg^{2+}-treated *N. calcicola* cells varied significantly ($P<0.005$) with respect to increasing metal concentrations and exposure time. Apart from cell death at Hg^{2+} concentrations exceeding 10.0 μM, the leakage of electrolytes was also found operative for the untreated cyanobacterial cells at a much slower pace (0.75 μS $\cdot$ mg protein$^{-1} \cdot h^{-1}$). A progressive increase in the extent of electrolyte leakage was observed for cells treated with 0.5–5.0 μM Hg^{2+}. However, on exposure of more than 16 h to 10.0 μM Hg^{2+} showed maximum rate of electrolyte efflux (i.e. 16.75 μS $\cdot$ protein$^{-1} \cdot h^{-1}$, as determined between 4–8-h exposures). Even higher Hg^{2+} concentrations (10.0–20.0 μM) neither stimulated the rate of electrolyte leakage nor caused a proportional increase in the specific conductance; instead, severe cell lysis was observed microscopically.

Table 1. Leakage of electrolytes from *Nostoc calcicola* cells exposed to Hg^{2+}

Hg^{2+} (μM)	Leakage (μS/mg protein) after incubation for				
	2 h	4 h	8 h	12 h	16 h
(control)	4	7	10	13	18
0.5	16	26	36	44	52
1.25	20	44	82	102	121
2.5	30	64	118	156	182
5.0	52	90	142	182	208
10.0	64	105	172	208	216
15.0	66	108	178	210	218
20.0	72	118	182	214	222

The data for electrolyte leakage (Table 1) and in vivo NR activity in relation various metal treatments (Fig. 4) and exposure time, were verified by analysis of variance (ANOVA) to test their significance at a particular probability level, and the variance ratio (F) calculated by the equation F=(treatment mean square)/(residual mean square). The value for incubations was $F_{4,28}=28.864$, $P<0.005$; for Hg^+ concentrations $F_{7,28}=26.480$, $P<0.005$

NO_3^- uptake and reduction in untreated cultures

Compared to reduction of NO_3^- at cell interior (16.0 nmol $NO_2^- \cdot$ μg protein$^{-1} \cdot h^{-1}$), its uptake from external medium proceeded >60 times faster (1.0 μmol $NO_3^- \cdot$ μg protein$^{-1} \cdot h^{-1}$) with linearity over 6 h (Fig. 1a). The respective rates have been calculated from the linear portion of the curves (i.e. between 2–4 h of incubation). A distinct lag of 1 h was observed for the in vivo NR activity in untreated cyanobacterial cells. Statistical analyses of the data established a significant correlation ($r=0.988$, $df\,4$, $P<0.01$; $\hat{y}=12.4x-11.52$) between the two processes in terms of NO_3^- utilization (Fig. 1b).

Hg^{2+} sensitivity of NO_3^- uptake and in vivo NR activity

A comparison of the slopes in Fig. 2a and the corresponding 50% inhibitory Hg^{2+} concentrations (2.5 μM

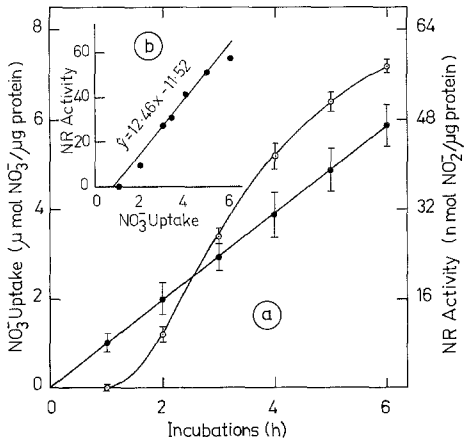


Fig. 1. Pattern of NO_3^- uptake and in vivo nitrate reductase activity in *N. calcicola* at 20.0 mM KNO_3. (**a**) Direct plot of NO_3^- uptake (●) versus NR activity (○). Values are mean ±3 SE; $r=0.988$, $df\,4$, $P<0.01$. (**b**) Regression analysis. $x=NO_3^-$ uptake and $\hat{y}=$ in vivo nitrate reductase activity

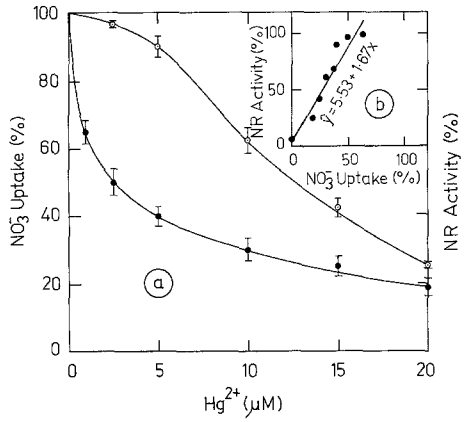
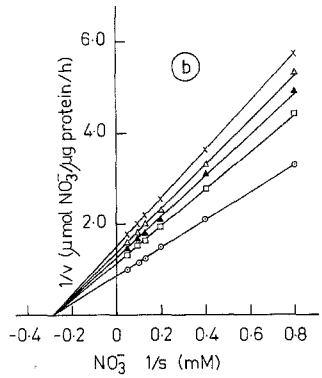


Fig. 2. A comparison of NO_3^- uptake and in vivo nitrate reductase activity at 20.0 mM KNO_3 in *N. calcicola* after 6-h exposure to graded Hg^{2+} concentrations. (**a**) Direct plot of NO_3^- uptake (●) versus NR activity (○). Values are mean ±3 SE; $r=0.916$, $df\,4$, $P<0.05$. (**b**) Regression analysis. $x=NO_3^-$ uptake and $\hat{y}=$ in vivo nitrate reductase activity

and 12.5 μM, respectively; as determined after 6-h exposures) suggested that the heavy metal inhibited NO_3^- uptake more severely than its subsequent reduction at cell interior (about fivefold difference). While even low Hg^{2+} concentrations (<5.0 μM) were most effective against the former, acute inhibition of the latter could be achieved only at more than 5.0 μM Hg^{2+}. The data in Fig. 2b further substantiate a positive, significant correlation ($r=0.916$, $df\,4$, $P<0.05$) and regression ($\hat{y}=5.53+1.67x$) between these metabolic events in terms of Hg^{2+} inhibition.

Interaction of CH_3Hg^+, Cd^{2+}, Cu^{2+}, Ni^{2+} or Ca^{2+} with Hg^{2+}

NO_3^- uptake kinetics. NO_3^- uptake at graded KNO_3 concentrations (1.25–20.0 mM), followed Michaelis-



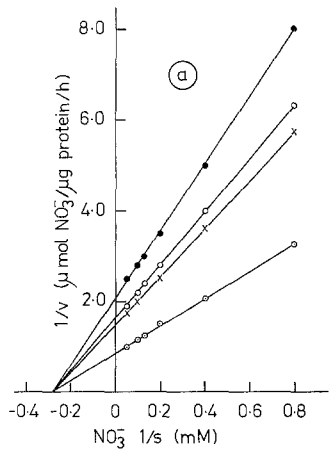


Fig. 3. Lineweaver-Burk plots for NO_3^- uptake in *N. calcicola*. (**a**) At graded KNO_3 concentrations and 2.5 μM Hg (50% inhibitory concentration) with other metal cations (equimolar); metal-less control (⊙—⊙), Hg^{2+} alone (×—×), $Hg^{2+}+Cd^{2+}$ (○—○) and $Hg^{2+}+CH_3Hg$ (●—●). (**b**) Showing interaction of Hg^{2+} (2.5 μM) with other metal cations (equimolar): metal-less control (⊙—⊙), Hg^{2+} alone (×—×), $Hg^{2+}+Ni^{2+}$ (△—△), $Hg^{2+}+Cu^{2+}$ (▲—▲) and $Hg^{2+}+Ca^{2+}$ (□—□)

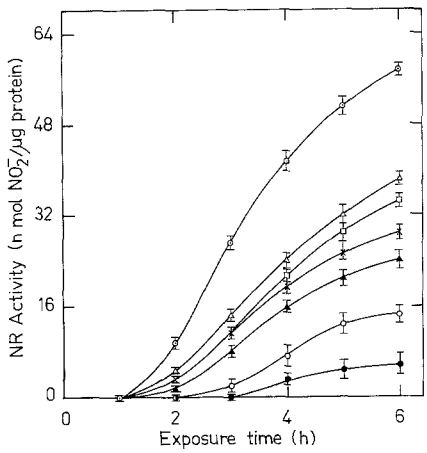


Fig. 4. In vivo nitrate reductase activity in *N. calcicola* at 20.0 mM KNO_3 showing interaction of Hg^{2+} (50% inhibitory concentration, 12.5 μM) with other metal cations (equimolar): metal-less control (⊙—⊙), Hg^{2+} alone (×—×), $Hg^{2+}+Cd^{2+}$ (○—○), $Hg^{2+}+CH_3Hg$ (●—●), $Hg^{2+}+Ni^{2+}$ (△—△), $Hg^{2+}+Cu^{2+}$ (▲—▲) and $Hg^{2+}+Ca^{2+}$ (□—□); values are mean ±3 SE; $F_{\text{hours }4,24}=19.267$ and $F_{\text{metal combinations }6,24}=20.437$, $P<0.005$

Menten kinetics with K_m and V_{max} values of 3.57 mM and 1.18 μmol $NO_3^-\cdot$μg protein$^{-1}\cdot h^{-1}$, respectively (Fig. 3a, b). The simultaneous addition of 2.5 μM Hg^{2+} (the 50% inhibitory level) did not alter the K_m; nevertheless, V_{max} was reduced to 0.67 μmol $NO_3^-\cdot$μg protein$^{-1}\cdot h^{-1}$). The bimetallic combinations of Hg^{2+} with either Cd^{2+} or CH_3Hg^+, further reduced the V_{max}

to 0.61 and 0.49 μmol NO_3^- · μg protein^{-1} · h^{-1}, respectively (Fig. 3a). However, Hg^{2+} in combination with either Ni^{2+}, Cu^{2+} or Ca^{2+} enhanced the corresponding V_{max} values (0.74, 0.80 and 0.91 μmol NO_3^- · · μg protein^{-1} · h^{-1}) over the cyanobacterial cells exposed to Hg^{2+} alone (Fig. 3b).

In vivo nitrate reductase activity. Interaction experiments on similar lines showed that the reduction of NO_3^- was reduced to half (8.0 nmol NO_2^- · μg protein^{-1} · h^{-1}) in presence of 12.5 μM Hg^{2+}, but the simultaneous addition of equimolar concentrations (12.5 μM each) of either Ca^{2+} or Ni^{2+} with Hg^{2+}, resulted in the recovery of enzyme activity (9.2 and 9.6 nmol NO_2^- · μg protein^{-1} · h^{-1} respectively; Fig. 4). In contrast, Hg^{2+} in combination with either Cu^{2+}, Cd^{2+} or CH_3Hg^+ reduced the in vivo activity rates of nitrate reductase to 7.2, 3.2 and 1.6 nmol NO_2^- · μg protein^{-1} · h^{-1}, respectively. Such variations in the NR activity rates in response to different Hg^{2+} combinations and exposure time were found to be statistically significant at less than 0.5% probability level.

Discussion

The negligible efflux of electrolytes from untreated *N. calcicola* cells (0.75 μS · mg protein^{-1} · h^{-1}; Table 1) suggests that the cyanobacterial cell membrane actively regulates the transmembrane movement of electrolytes. The proportional increase in electrolyte leakage corresponding to the exposure to less than 5.0 μM Hg^{2+} suggests it arises as a consequence of the irreversible binding of Hg^{2+} with the adsorption sites on the cell surface, as reported for *Anabaena inaequalis* (Stratton et al. 1979). While the irregular enhancement in the electrolyte loss at higher Hg^{2+} concentrations (5.0–20.0 μM) may be attributed to the disruption of the cell membrane, as reported for ion loss in other cyanobacteria induced by Cu, Cd, Cr or Pb (Mierle and Stokes 1976; Singh and Yadava 1986; Rai and Raizada 1987, 1988). A corollary of these findings is that the cell membrane represents the primary site of Hg^{2+} action in *N. calcicola* and even very low Hg^{2+} concentrations may disrupt its integrity, leading to the uncontrolled leakage of intracellular electrolytes. The cell lysis at Hg^{2+} concentrations exceeding 10.0 μM possibly represents the ultimate fate of the cell membrane. Thus, it is suggested that the electrolyte leakage may be taken as a reliable criterion to assess cell membrane integrity in cyanobacteria.

In comparison to NO_3^- uptake, the NR activity not only showed a lag of 1 h but also a slow activity rate in untreated *N. calcicola* cells (about 60-fold difference; Fig. 1). Such data are in conformity with those of other cyanobacteria in that NO_3^- enters the cell via a transport system and the anion itself acts as the inducer of nitrate reductase (Flores et al. 1983). The overall comparison of the inhibition events in general, and in particular of the corresponding 50% inhibitory Hg^{2+} concentrations for NO_3^- uptake (2.5 μM) and NR activity (12.5 μM), suggests a five-fold enhanced Hg^{2+} sensitivity of the former (Fig. 2). The severe inhibition of enzyme activity exclusively at more than 5.0 μM Hg^{2+}, and NO_3^- uptake at less than that, suggests that suboptimal availability of NO_3^- is needed to induce the nitrate reductase in the cell interior. The unaltered K_m compared to V_{max} indicates a condition of non-competitive interaction between NO_3^- and Hg^{2+} and rules out the possibility of a common site of entry for both ions or a competitive interaction (Fig. 3). However, such observations are in contrast to that of competitive Cd^{2+} inhibition of NO_3^- uptake in a non-N_2-fixing cyanobacterium (Singh and Yadava 1983), suggesting that the nature of NO_3^- – heavy-metal interaction may differ, not only from metal to metal, but also from organism to organism.

The apparent reduction of V_{max} for NO_3^- uptake (Fig. 3a) and NR activity rate (Fig. 4) with $Hg^{2+} + CH_3Hg^+$ combinations over that of Hg^{2+}-treated *N. calcicola* cells, suggests that the inorganic and organic mercury species interact synergistically with each other. The comparatively lesser extent of reduction in V_{max} and activity rate with $Hg^{2+} + Cd^{2+}$ combinations, suggests a lesser degree of synergism between these cations. Such synergistic interactions suggest that Hg^{2+} either becomes co-adsorbed on the common cell surface with CH_3Hg^+ and Cd^{2+} or it facilitates the influx of such toxic cations, which result in the enhancement of inhibitory actions of Hg^{2+}, as reported in terms of photosynthetic O_2 evolution and $^{14}CO_2$ incorporation in *N. calcicola* (Singh and Singh 1987). On the other hand, the enhancement of V_{max} and enzyme activity rates with $Hg^{2+} + Ca^{2+}$ or Ni^{2+} combinations over that of sets containing only Hg^{2+}, indicates antagonism between such cation combination (Figs. 3b, 4). The reduced degree of antagonism for $Hg^{2+} + Ni^{2+}$ combinations over that of $Hg^{2+} + Ca^{2+}$ may result either from direct competition for common uptake/binding site(s), or saturation of such site(s) preferentially by Ni^{2+} in the presence of Hg^{2+}, as reported for *Anabaena inaequalis* (Stratton and Corke 1979). Cu^{2+} ions showed a dual nature of interaction with Hg^{2+} depending on the parameters, i.e. antagonism for NO_3^- uptake and synergism for NR activity, which may be the result of its dual bonding preference for cellular ligands (Jones 1984).

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