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Hg^{2+} and Cd^{2+} induced inhibition of light induced proton efflux in the cyanobacterium *Anabaena flos-aquae*

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Light induced proton efflux in intact cells of *Anabaena flos-aquae* is inhibited by the heavy metals Hg^{2+} and Cd^{2+}. Furthermore, Hg^{2+} and Cd^{2+} reduced the $^{14}CO_2$ fixation, oxygen evolution and carbonic anhydrase activity responsible for H^+ efflux.

Keywords: *Anabaena flos-aquae*, carbon fixation, carbonic anhydrase, heavy metals, Hill activity, oxygen evolution, proton efflux

Introduction

A light induced acidification followed by an alkalinization of the medium has been reported for several cyanobacteria (Scholes *et al.* 1969, Scherer & Boger, 1984). However, the mechanism of the process has not yet been investigated in detail. Proton translocation due to the respiratory electron transport chain associated with the cytoplasmic membrane has been suggested to be responsible for light induced proton efflux in *Plectonema boryanum*. Hawkesford *et al.* (1983) and Lucas (1983) have suggested that light induced alkalinization is due to OH^- efflux caused by regulation of intracellular pH. Bicarbonate is taken up and converted to CO_2 plus OH^- inside the cell whereby OH^- is extruded and CO_2 is assimilated.

The interior side of cyanobacterial thylakoid is found to be more electropositive than the outer surface (Padan & Shimon 1978). It has been observed that if there is accumulation of excess cationic charge on the outer surface of the membrane, it tends to arrest the inside out proton (H^+) pump and result in the shrinkage of the membrane (Dilley & Rothstein 1967, Heber 1969, Kalosaka *et al.* 1985).

The present work deals with the effect of the heavy metals Hg^{2+} and Cd^{2+} on proton efflux in the cyanobacterium *Anabaena flos-aquae* in relation to oxygen evolution, $^{14}CO_2$ fixation and carbonic anhydrase activity.

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Materials and methods

Organism and growth conditions

A. flos-aquae was grown in batch culture in modified Chu-10 medium (Gerlof *et al.* 1950) under illumination with cool white fluorescent light for 14 h per day with trace elements (Allen & Arnon 1955) at 24 ± 1 °C. Light intensity of 10 W m^{-2} was provided with white fluorescent tubes on the surface of the culture vessel.

Measurement of growth

Growth was recorded by measuring the protein concentration as described by Lowry *et al.* (1951). The growth rate u (h^{-1}) was calculated according to the Kratz & Myers (1955). A plot of V_0/V of 2 was used to calculate the 50% inhibitory (I_{50}) concentration of metals as mentioned by Samuel & Bose (1987), where V_0 is the control value (without addition) and V is the value at different metal concentrations. The inhibitory concentration corresponding to V_0/V of 2 was taken as the actual concentration required to cause 50% inhibition.

Measurement of proton uptake

The homogeneous cell suspension in the growth medium was taken and the pH of the medium was adjusted to 7.0 with 1N HCl. Then, 0.3 mM $NaHCO_3$ solution was added

to the cell suspension and kept on a magnetic stirrer. The red light was turned on the surface of the vessel. The measurement of proton uptake or OH^- efflux was recorded by a pH meter fitted with an automatic recorder. Alkalinization of the medium in the presence and absence of the metal was recorded in terms of proton uptake mg protein^{-1} min^{-1}.

Measurement of oxygen evolution

Oxygen was measured with a Clark-type oxygen electrode (Hansatech, UK) fitted with a circulating water jacket. The temperature was adjusted to 25 °C. The light intensity on the surface of the vessel was 10 W m^{-2}. Heavy metals were added to the cell suspension and then incubated under illumination for 15 min prior to the measurement of oxygen evolution. The assay mixture contained phosphate buffer (pH 7.5, 100 mM). The cell suspension was light incubated for 15 min prior to the measurement of oxygen uptake.

Measurement of carbonic anhydrase activity

Exponentially growing cells were broken using a French Press (SLM, Urbana, IL, USA) at 15 000 p.s.i. Samples were suspended in Vernol buffer (pH 8, 0.1 M) containing 0.5 M NaCl at 30 °C. The reaction was started by adding CO_2 saturated solution in 0.5 M NaCl kept at 0 °C. The time required for the pH to change from 8 to 7 was recorded. Specific enzyme activity was calculated by employing the following formula

$$V = (T_0/T) - 1$$

where T_0 is the time required for pH change without cells and T is the time required for pH change with cells.

$^{14}CO_2$ incorporation

Carbon fixation was assayed in terms of $^{14}CO_2$ uptake from $NaH^{14}CO_3$ (0.05 μCi ml^{-1}) obtained from the Isotope Division of BARC (India). Exponentially growing cells preincubated in the dark for 24 h were transferred to glass scintillation vials (Beckman, USA). The reaction vessel contained 10 ml of cyanobacterial suspension (500 μg protein ml^{-1}) supplemented with metals and 0.05 μCi ml^{-1} of the $NaH^{14}CO_3$. Furthermore, incorporation of $^{14}CO_2$ was stopped by adding 0.1 ml of 2N HCl followed by the addition of 5 ml scintillation cocktail prepared by mixing four parts of 0.8% PPO (2,5-diphenyloxazole; Sigma, USA) plus 0.01% dimethyl POPOP (1,4-bis(4-methyl-5-phenyl-2-oxazole)benzene; Sigma, USA) in toluene (Merck, India) and three parts of ethanol (BCPL, India). Such a reaction mixture was surface blown for 2 min to remove the $^{14}CO_2$ and the clear solution was subjected to counting the emission of β-particles from incorporated $^{14}CO_2$ in a liquid scintillation counter (Beckman model LS 7000, USA). Mean values of counts were corrected for counting efficiency of the equipment.

Results

Effect of heavy metals on growth

Results indicate that all the concentrations (0.1–5.0 μM) of Hg^{2+} and Cd^{2+} were inhibitory to growth. A plot of V_0/V of 2 indicates that the Hg^{2+} and Cd^{2+} concentrations required for 50% inhibition of growth rate were 0.2 and 0.4 μM, respectively (Figure 1A & B).

Effect of heavy metals on proton uptake

A change in the pH of the medium was recorded as a function of graded concentrations of Hg^{2+} and Cd^{2+} (10–100 μM). Addition of $NaHCO_3$ saturated solution without metal resulted into alkalinization of the medium which was recorded in terms of H^+ uptake (nmol mg protein^{-1} min^{-1}). Addition of graded concentrations of mercury and cadmium resulted in a concentration dependent inhibition of alkalinization. A 50% inhibition of H^+ uptake activity in *A. flos-aquae* was observed at 22 μM Hg^{2+} and 45 μM Cd^{2+}. Experiments were conducted to optimize the concentration of $NaHCO_3$ and we found that the proton efflux was stimulated at 100–200 μM $NaHCO_3$ for $HCO_3 + CO_2$ (Figure 2A & B).

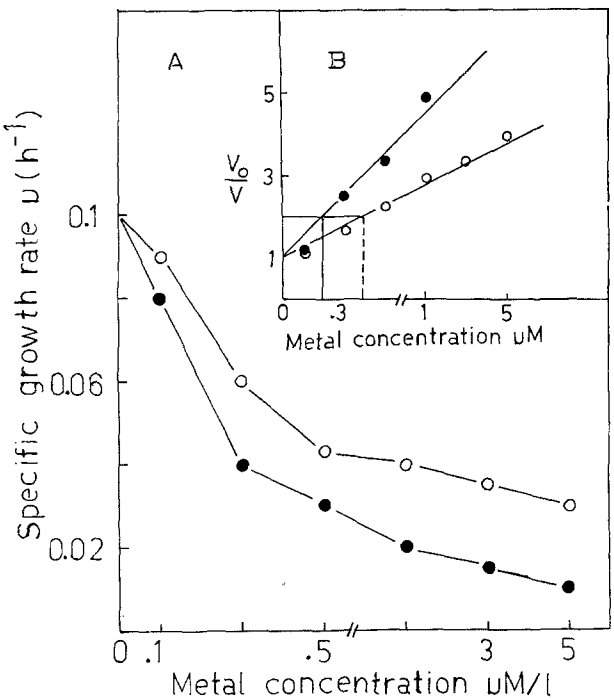

Figure 1. (**A**) Effect of a graded concentration (0.1–5 μM) of Hg^{2+} (●) and Cd^{2+} (○) on the specific growth rate $u(h^{-1})$ of *A. flos-aquae*. A plot of V_0/V of 2 was used to calculate the I_{50} as described in Materials and methods (**B**).

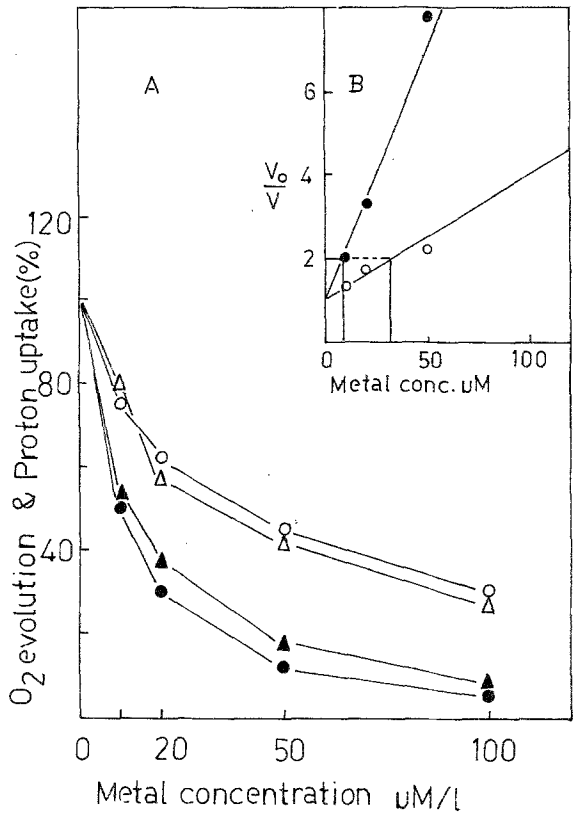

Figure 2. (**A**) Photosystem II activity was measured in terms of oxygen evolution. A range of concentrations of Hg^{2+} (●) and Cd^{2+} (○) was added to the permeaplast of *A. flos-aquae* suspended in phosphate buffer (pH 7.5) and light incubated for 15 min prior to the measurement of oxygen evolution. The inhibition pattern shows the effect of different concentrations (10–100 $\mu M\ l^{-1}$) of Hg^{2+} (▲) and Cd^{2+} (△) with the addition of 5 $\mu M\ l^{-1}$ of $NaHCO_3$ on the rate of proton uptake in intact cells of *A. flos-aquae*. The rates of oxygen evolution and proton uptake are given in terms of percentages. A plot of V_0/V of 2 was used to calculate the I_{50} (**B**).

Effect of heavy metals on oxygen evolution

Experiments were carried out to determine the effect of graded concentrations of Hg^{2+} and Cd^{2+} on oxygen evolution. The result suggested that oxygen evolution in the permeabilized cells of *A. flos-aquae* was inhibited with an increase in metal concentration. A plot of V_0/V of 2 indicates a 50% inhibition of oxygen evolution at 9 μM Hg^{2+} and 30 μM Cd^{2+} (Figure 2A). The result thus indicates that oxygen evolution was more sensitive to Hg^{2+}.

Effect of heavy metals on carbonic anhydrase activity

The carbonic anhydrase activity was inhibited by Hg^{2+} and Cd^{2+} in a concentration dependent manner (Figure 3A). A 50% inhibition of the activity was observed at 25 μM Hg^{2+} and 64 μM Cd^{2+} (Figure 3B).

Effect of heavy metals on CO_2 incorporation

The rate of CO_2 fixation was inhibited by Hg^{2+} and Cd^{2+} in a concentration dependent manner. However, Hg^{2+} was found to be more inhibitory to carbon fixation than Cd^{2+}. A 50% inhibition of the rate of carbon fixation was observed at 22 μM Hg^{2+} and 38 μM Cd^{2+}. The metal concentrations required for inhibition of carbon fixation are comparable with the concentrations required for the inhibition of carbonic anhydrase activity.

Discussion

The light induced acidification in *A. flos-aquae* was followed by alkalinization of the medium (Figure 2A) exhibiting a linear relationship with the inhibition of oxygen evolution at varying concentrations of the heavy metals Hg^{2+} and Cd^{2+}. Scherer & Boger (1984) have also reported the linear correlation between light induced proton efflux and oxygen

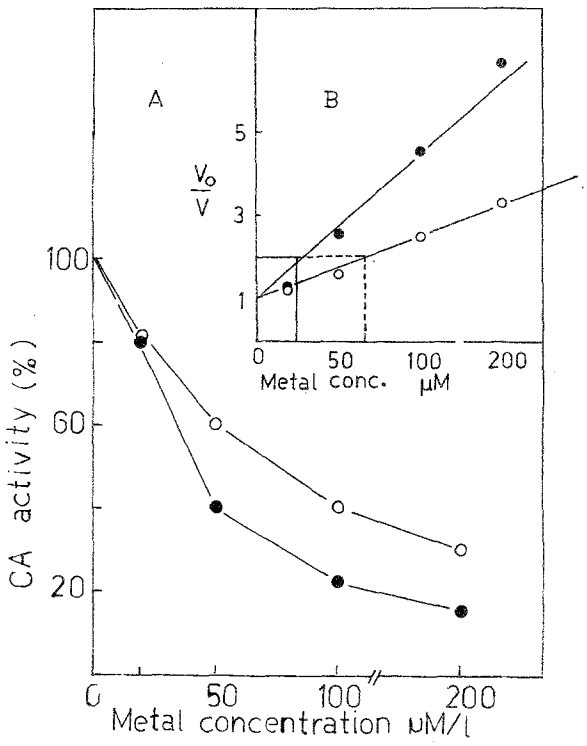

Figure 3. (**A**) Effect of different concentrations of Hg^{2+} (●) and Cd^{2+} (○) on carbonic anhydrase activity. A plot of V_0/V was used to calculate I_{50} (**B**).

evolution in intact cells of *Anabaena*, *Nostoc*, *Anacystis* and *Aphanocapsa* sp. The stoichiometry between oxygen evolution and proton efflux/alkalinization of the medium (O_2:OH) in *Anabaena* sp. is reported to be in a 1:1 ratio at equilibrium at alkaline pH (Miller & Colman 1980, Scherer & Boger 1984).

As evident from Figure 2(A), photosynthetic oxygen evolution in the cyanobacterium *A. flos-aquae* was extremely sensitive to different levels of heavy metals. The inhibition curve clearly suggests that cations affect the process of photosynthetic oxygen evolution. However, heavy metals might cause the inhibition of photoautotropic growth of *A. flos-aquae*; an inhibition of photoautotropic growth by heavy metals might result from their inhibitory effect on the in *vivo* process of photosynthetic oxygen evolution (Kallqvist & Meadows 1978).

The heavy metals appear to have inhibited both oxygen evolution and carbon assimilation in a parallel fashion. The significant inhibition of photosynthetic oxygen evolution did not seem to greatly influence the utilization of CO_2 (Singh & Singh 1987), thus the simplest explanation could be that heavy metals appear to inhibit carbon fixation mainly as a result of their inhibitory effect on the photosynthetic process, as suggested for heavy metals in *A. inaequalis* (Stratton *et al.* 1979). The uptake of CO_2 is due to the presence of carbonic anhydrase. CO_2 binds to the subunit and is converted to HCO_3. The nascent HCO_3 generated in this process is immediately available at or very near the site of its formation, to a HCO_3 porter which transfer it to the inner membrane surface (Volokita *et al.* 1984). The charged HCO_3 ion encounters resistance in diffusing through the cell wall and within the membrane, whereas CO_2 meets with less resistance in the wall and may be very efficiently transferred within the membrane by a carbonic anhydrase like moiety. It seems that the metal induced inhibition of alkalinity of the medium might be from $NaHCO_3$ dependent carbonic anhydrase activity. Thus, it can reasonably be inferred that the inhibition of CO_2 fixation is preceded by the inhibition of carbonic anhydrase activity.

Kalosaka *et al.* (1985) demonstrated that the surface charge potential of the thylakoid membrane is the key regulatory factor during the charge separation across the membrane. Hawkesford *et al.* (1983) demonstrated that light induced proton efflux by *P. boryanum* is thought to be due to redox-coupled H^+ pumping by a cytoplasmic membrane bound respiratory chain.

The heavy metal induced general increase in the light scattering property of the chloroplast membrane is found to be associated with the high energy state (H gradient) of the membrane (Heber 1969). A high energy state of the membrane is known to exert a back pressure on the electron transport (McCarty 1980). Thus, metal induced inhibition of Hill activity was due to the collapse of the high energy state of the membrane which is evident for the inhibition of light induced acidification followed by alkalinization of the medium. Dilly & Rothstein (1967) have also demonstrated metal induced modulation of H^+ movement which accounts for the shrinkage of the chloroplast membrane. Finally, it is inferred that metal induced modulation of charge separation (H^+ movement) resulting in a high energy state of the membrane accounts for the simultaneous inhibition of transport of CO_2 across the membrane and Hill activity. The metal induced inhibition of the Hill activity may reduce the CO_2 consumption in photosynthesis, which ultimately affects the carbonic anhydrase activity.

It can, therefore, be said that light induced proton efflux may be due to the transport of CO_2 across the cytoplasmic membrane which must be converted to HCO_3 for transport across the membrane by the carbonic anhydrase like activity of the carbonate transporter, as suggested by Volokita *et al.* (1984).

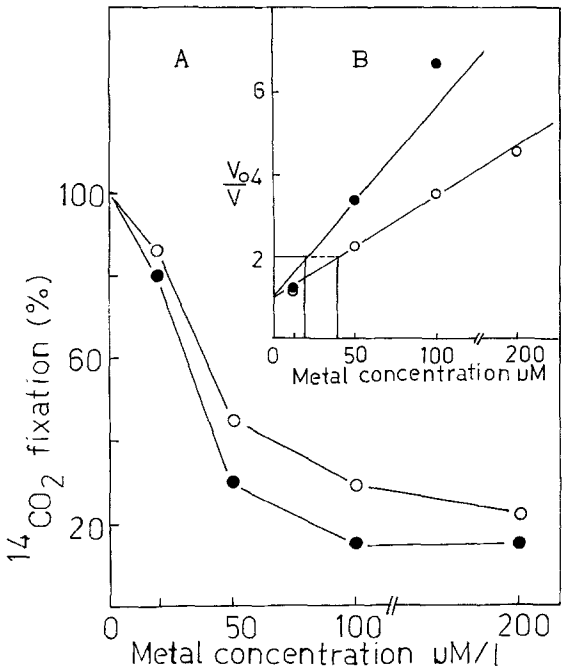

Figure 4. Effect of graded concentrations of Hg^{2+} (●) and Cd^{2+} (○) on $^{14}CO_2$ incorporation in *A. flos-aquae*.

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