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Influence of chromium on some physiological variables of *Anabaena doliolum*: interaction with metabolic inhibitors

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The impact of 2,4-dinitrophenol and chlorophenyl dimethylurea on ATP content, carbon fixation, O_2 evolution, nitrogenase activity and Cr uptake of *Anabaena doliolum* has been studied. 2,4-Dinitrophenol has been found to be more toxic than chlorophenyldimethylurea for all these processes. However, when Cr toxicity to above variables was assessed in their presence the interaction was less than additive. An initial (10–15 min) concentration-dependent rapid Cr uptake, followed by a slow one, indicates a biphasic uptake. A significant inhibition of Cr uptake in the presence of both these metabolic inhibitors suggests the involvement of metabolic processes in Cr uptake.

Keywords: *Anabaena doliolum*, Cr uptake, chlorophenyl dimethylurea, 2,4-dinitrophenol, ATP

Introduction

Chromium, a non-essential toxic metal, finds its way into the environment through its use in steel production, wood preservation, leather tanning, paints, pigments, metal plating and various other applications (Papp 1985). However, a threat to the very existence of aquatic organisms not only depends on the presence of toxic metals in the surrounding medium, but also on its uptake and accumulation by the organisms in question. Though extensive studies have been made on the uptake and accumulation of heavy metals by different groups of plants including algae (Cain *et al.* 1980, Khumongkol *et al.* 1982, Singh *et al.* 1989), reports dealing with Cr are mainly confined to bacteria, bryophytes and yeasts (Lull *et al.* 1983, Mouvet 1984, Baldi *et al.* 1990).

The two processes known to be involved in metal uptake by microbes are: (i) rapid binding of cations to the negatively charged groups on the cell surfaces and (ii) a subsequent metabolism-dependent intracellular uptake (Norris & Kelly 1979, Gipps & Coller 1980, Khumongkol *et al.* 1982, Stary & Kratzer 1984). However, it is worth stating that controversy still exists on the mode of uptake and accumulation of heavy metals by microorganisms. One of the reasons for such controversy is the accumulation of metals by dead algae (Sakaguchi *et al.* 1979) which suggests an energy-independent uptake of metals. In order to resolve this controversy with regard to Cr uptake in the cyanobacterium *Anabaena doliolum*, attempts have been made to study the effect of two metabolic inhibitors, viz. 2,4-dinitrophenol (inhibitor of oxidative phosphorylation) and chlorophenyl dimethylurea (inhibitor of photosynthetic electron transport chain) on Cr uptake by the test cyanobacterium. The other objective was to explore the interactive effects of these metabolic inhibitors and Cr on cellular ATP content and some energy-dependent metabolic processes of *A. doliolum*.

Materials and methods

A. doliolum Bharadwaja was grown axenically in the modified medium of Allen & Arnon (1955) buffered with 4 mM Tris/HCl pH 7.5. The cultures were incubated in a 14-h/10-h light/dark cycle at 24 ± 2 °C under 72 μmol photon $m^{-2} s^{-1}$ light intensity. Stock solutions of chromium trioxide, 2,4-dinitrophenol (DNP) and chlorophenyl dimethylurea (CMU) were prepared and filter-sterilized

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by passing through Millipore membrane filters (0.45 μm) before supplementing to the culture medium. The concentration of DNP and CMU used was 1 μM. Biochemicals used were obtained from Sigma, St Louis, MO, USA. $NaH^{14}CO_3$ was supplied by BARC, Bombay, India; other chemicals from BDH, India.

Estimation of protein

Protein was estimated following the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Measurement of ATP content

Total ATP content was estimated by the luciferin/luciferase assay using an LKB-1250 luminometer. The ATP was extracted in 4% trichloroacetic acid supplemented with 2 mM EDTA (Larsson & Olsson 1979).

Measurement of carbon fixation and O_2 evolution

Carbon fixation was measured by recording the uptake of ^{14}C from $NaH^{14}CO_3$ (specific activity 185 MBq) as described by Rai & Raizada (1986). Photosynthetic oxygen evolution was measured by an oxygen electrode. Approximately 15 ml algal suspension (absorbance at 663 nm = 0.2) was taken in a reaction vessel, which was connected with a thermocirculator to regulate its temperature. Oxygen evolved was recorded (as ppm/min) with an oxygen analyzer (Biochem 76T model) and converted to μmol O_2 evolved mg protein^{-1} h^{-1}.

Nitrogenase assay

Nitrogenase activity was measured by the acetylene reduction technique (Stewart *et al.* 1968). Activity was measured in triplicate in serum vials of about 7.5 ml capacity. The acetylene concentration was kept at 10% (by volume) and 2 ml algal suspension was injected into each vial. Reactions were terminated following injection of 0.8 ml 15% (mass/vol) trichloroacetic acid. The acetylene formed was measured in a CIC gas chromatograph (Baroda, India) equipped with a Porapak-R column and a hydrogen flame ionization detector. Activity was expressed in terms of nmol C_2H_4 formed μg Chl a^- h^{-1}.

Chromium uptake

For analyzing Cr content of algal cells (as by the method of Singh & Yadav 1985), 10 ml cyanobacterial suspension (around 500 μg protein ml^{-1}) was withdrawn at desired intervals and centrifuged. The washed pellet was dried and treated with 1 ml $HNO_3/HClO_4$ (10:1, by volume) and kept in a boiling water bath for 30 min to ensure digestion and release of metal. Samples, when cool, were diluted to a total volume of 5 ml with triple glass-distilled water and centrifuged. The Cr content in the supernatant was analyzed in Perkin-Elmer 2380 atomic absorption spectrophotometer.

Results

Effect of Cr, CMU and DNP on cellular ATP content

A reduction of approximately 37, 54 and 71% in ATP content of the test cyanobacterium was noticed with Cr, CMU and DNP, respectively, which indicates a greater toxic potential of DNP than CMU (Table 1). When both the metabolic inhibitors were supplemented in combination with Cr, an increased toxicity was noticed. However, the observed percentage inhibition was less than the expected values (i.e. the combined inhibition was less than their additive values).

Table 1. Impact of chromium on ATP content of *A. doliolum*: interaction with CMU and DNP

Supplementation	ATP content (μg μg protein^{-1}) at		
	0.5 h	1.0 h	2.0 h
Control	0.93 ± 0.005	0 .91 ± 0.007	1.05 ± 0.005
LC_{50} Cr	0.63 ± 0.003 (32.3)	0.53 ± 0.001 (41.8)	0.66 ± 0.001 (37.2)
CMU	0.58 ± 0.002 (37.7)	0.52 ± 0.003 (42.9)	0.48 ± 0.002 (54.3)
DNP	0.54 ± 0.002 (42.0)	0.42 ± 0.003 (55.9)	0.31 ± 0.002 (70.5)
LC_{50} Cr + CMU	0.42 ± 0.003 (55.9)	0.37 ± 0.002 (59.4)	0.34 ± 0.001 (67.7)
LC_{50} CR + DNP	0.41 ± 0.001 (56.0)	0.34 ± 0.002 (62.7)	0.26 ± 0.002 (75.3)

Data in parentheses show percentage inhibition. Values are significant at $P < 0.001$. The lethal concentration of Cr for 50% of algal cells (LC_{50}) was 40 μg ml^{-1}.

O_2 evolution and CO_2 fixation

Approximately 40% inhibition of O_2 evolution was observed at sublethal concentrations of Cr; whereas inhibition of this process was only 25% and 32% for CMU and DNP, respectively (Table 2). The inhibition however, became more pronounced when DNP was supplemented simultaneously with Cr (54%) as compared to CMU with Cr (49%).

Maximum inhibition of carbon fixation was found with DNP (43%) followed by Cr (41%) and CMU (39%, Table 2). Like O_2 evolution, an increase in the toxicity occurred when CMU and DNP were added with Cr. However, the interaction was less than additive. A significant positive correlation ($P < 0.05$) for ATP content versus O_2 evolution/carbon fixation was observed.

Nitrogenase activity

Inhibition of nitrogenase by CMU, DNP, Cr, Cr + CMU and Cr + DNP followed the trend of O_2 evolution and carbon fixation (Table 2). When inhibitors were spiked with Cr, an inhibition of 86% for Cr + DNP and 72% for Cr + CMU was noticed as compared to 67, 61 and 33% for DNP, CMU and Cr alone, respectively. The correlation coefficient (r) between nitrogenase versus ATP content/carbon fixation also showed a highly significant value ($P < 0.01$).

Cr uptake

The data incorporated in Figure 1 clearly demonstrate a concentration-dependent uptake of Cr. An initial (first 10–15 min) rapid uptake was common to all the five Cr concentrations used, after which the rate decreased gradually. Considerable inhibition of Cr uptake was noticed following supplementation of CMU and DNP. However, DNP was found to be more efficient (58%) in reducing the uptake than CMU (20%).

Discussion

A low ATP content in cultures treated with DNP and CMU (Table 1) reflects the inhibitory effect of these metabolic inhibitors towards ATP. Hence the inhibition of O_2 evolution, carbon fixation and nitrogenase following supplementation of CMU and DNP (Table 2) is attributed to the low-energy status of the cell. A greater inhibition of nitrogenase as compared to other parameters can be explained in the light of the observations of Almon & Böhme (1982) who vividly demonstrated a high requirement of ATP (i.e. 16 molecules of ATP) have to be provided for the reduction of one molecule of nitrogen) for nitrogenase activity.

When Cr toxicity was assayed in the presence of DNP and CMU an increase in inhibition was noticed. The values were, however, less than additive. This might be due to the reduced uptake of Cr in the presence of DNP and CMU (Figure 1b). Reduction in Cr uptake by *A. doliolum* in the presence of CMU and DNP suggests its dependence on cellular energy level (see Gadd & Griffiths 1978). DNP was found to be more efficient than CMU in reducing the uptake of Cr. Since CMU is a known inhibitor of photosynthetic electron transport, it indirectly affects the energy production, whereas DNP directly inhibits the oxidative phosphorylation, thus causing immediate depletion of ATP, *vis-a-vis*

Table 2. Impact of Cr on photosynthetic oxygen evolution, carbon fixation and nitrogenase activity of *A. doliolum*: interaction with CMU and DNP

Supplementation	Oxygen evolution (μmol O_2 mg protein^{-1} h^{-1})	Carbon fixation (cpm × 10^3)	Nitrogenase activity (nmol C_2H_4 μg protein^{-1} h^{-1})
Control	41.9 ± 0.11	22.738 ± 0.08	8.4 ± 0.005
LC_{50} Cr	25.3 ± 0.03	13.418 ± 0.05	5.6 ± 0.005
	(39.7)	(41.0)	(33.4)
CMU	31.6 ± 0.05	13.984 ± 0.11	3.3 ± 0.001
	(24.6)	(38.5)	(60.8)
DNP	28.5 ± 0.03	12.919 ± 0.04	2.8 ± 0.002
	(32.0)	(43.2)	(66.7)
LC_{50} Cr + CMU	21.5 ± 0.03	9.845 ± 0.05	2.3 ± 0.002
	(48.7)	(56.8)	(17.7)
LC_{50} Cr + DNP	19.3 ± 0.05	7.788 ± 0.09	1.2 ± 0.005
	(54.0)	(65.8)	(85.8)

Data in parentheses denote percentage inhibition. Values are significant at $P < 0.001$. For LC_{50} of Cr see Table 1.

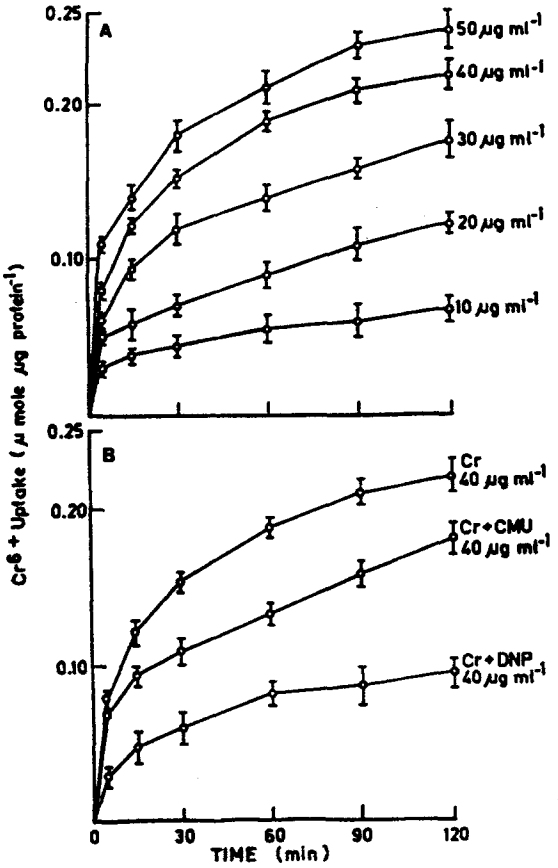

Figure 1. (**A**) Uptake of chromium by *A. doliolum* at different concentrations and (**B**) influence of CMU and DNP on Cr uptake of *A. doliolum*.

reducing chromium uptake. The initial rapid uptake followed by a slow one also suggests the existence of a biphasic uptake pattern in the test cyanobacterium. This study therefore suggests that Cr uptake in cyanobacteria is directly related to ATP-generating processes.

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