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Ultrastructural changes in *Synechococcus leopoliensis* produced by cadmium as influenced by pH

Tang, Ming, Ph.D.

City University of New York, 1993

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A

ULTRASTRUCTURAL CHANGES IN *SYNECHOCOCCUS LEOPOLIENSIS* PRODUCED BY CADMIUM AS INFLUENCED BY pH

by

Ming Tang

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A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirement for the degree of Doctor of Philosophy, The City University of New York

1993

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This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

ULTRASTRUCTURAL CHANGES IN *SYNECHOCOCCUS LEOPOLIENSIS* PRODUCED BY CADMIUM AS INFLUENCED BY pH

by

Ming Tang

Advisor: Professor Thomas E. Jensen

Synechococcus leopoliensis was used as a representative of fresh-water cyanobacteria to study the toxic effect of the heavy metal, cadmium, on their growth and survival. The Cd^{2+} concentration that limited the *S. leopoliensis* population growth at pH 7.2 by 50%, EC_{50}, in 96 hours was determined to be 0.08 μM. Cell cultures were subsequently exposed to 0.08 and 0.8 μM Cd^{2+} at pH 5.5, 7.2 and 9, then compared with control cells for ultrastructural changes shown in micrographs produced with the transmission electron microscope (TEM). Statistical analyses of the morphometric data showed that more cellular inclusions were changed in their relative volumes in Cd^{2+} exposed cells at pH 9 than 5.5, both than at 7.2. The nucleoplasm relative volumes were 5.71, 7.68 and 8 in the control cells grown at pH 5.5, 7.2 and 9; however, they decreased to 1.11, 5.91 and 2.45 respectively in the cells exposed to 0.8 μM Cd^{2+}. When the organism was grown at pH 9, the average diameter of polyphosphate bodies (PPB's) increased from 0.14 nm in the control to 0.21 nm in the 0.8 μM Cd^{2+} treated cells. The relative volume of polyhedral bodies increased from 0.78 to 2.19 and the outer membrane of the Cd^{2+} exposed cells formed blebs. In one-third of the cells exposed to pH 5.5 the cytoplasm and the cytoplasmic membrane were separated from the cell wall.

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In cells exposed to 0.8 μM Cd^{2+} at pH5.5, electron dense and spherical inclusions in polyhedral bodies occurred at a frequency of 43% in contrast to 0 in the control cells. Elemental analysis of these inclusions using the scanning transmission mode of the TEM in conjunction with a PGT energy dispersive X-ray spectrometer (EDX) demonstrated that the inclusions inside polyhedral bodies contained P and Ca and were in fact PPB's.

Cd was detected mainly in the cytoplasmic and cell wall sectors with the EDX analysis, when *S. leopoliensis* cells were exposed to Cd^{2+} at 80 μM for 4 hours. As the exposure Cd^{2+} concentration increased to a higher concentration, such as 600 μM, most Cd was accumulated into the PPB's. Although the cell surface might be able to control the penetration of the Cd^{2+} at low concentrations, Cd was taken up intracellularly as the exposure concentration increased. The phosphorus rich PPB's in the cytoplasm attracted most of the Cd. The same pattern of uptake observed in the cells exposed to 600 μM Cd^{2+} at 4° C in the dark suggested that the Cd uptake in the first 4 hrs is mostly by passive diffusion.

Gel electrophoresis of cell proteins revealed differences in the protein band patterns of exposed cells from those of control cells. Nine polypeptides were enhanced in relative quantity in the exposed cells. When cells were exposed to Cd^{2+} at 25, 50 and 100 μM, the 20 K polypeptide band in the membrane and cell envelope fractions, also 15 K band in the latter appeared significantly dose-dependent. On the other hand polypeptides, 41, 33 and 70 K, in the cytoplasmic fraction were reduced in amount in Cd^{2+} exposed cells; however, the 70 K was enhanced in the cell envelope fraction. The significant enhancement of proteins in the sediment fractions (cell envelope and membrane) might suggest that the Cd^{2+} effect could be one of the protein denaturation or disassembly of the proteinaceous structures in cells.

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To my mother and father

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First, I wish to acknowledge my gratitude to my advisor, Professor Thomas Jensen, for his time, trust and guidance through my graduate experience. I thank Professor William Corpe for helpful suggestions, enthusiasm and support. I also thank Professor Jack Valdovinos for his support and help. Sincerest thanks are also extended to Professor Dwight Kincaid and Professor Eleanore Wurtzel for offering many helpful suggestions in this work. I would also like to thank Professor Sabine Huhndorf for reading the dissertation and serving on my committee.

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INTRODUCTION

Review of pertinent literatures

Metals can be either essential or toxic to living organisms. Iron is a component of hemoglobin and other important enzymes; its deficiency could lead to disease. Other metals stimulatory in trace amounts when supplied in excess could be toxic and lead to disease. Copper and zinc are nutrients required in very small amounts in the metabolism of living organisms, but are toxic at concentrations one or two orders of magnitude higher (Stokes, 1983; Vallee, 1991). Cadmium and mercury, on the other hand, have no known biological functions and are considered consistently toxic at all concentrations (Stokes, 1983; Reed and Gadd, 1989). Metals such as Cd, Hg, Pb, Cu, Zn, and Al are commonly encountered as environmental pollutants and are often referred to as heavy metals.

Distributed everywhere in the earth's crust, Cd is one of the most toxic metals (Reed and Gadd, 1989). It has become a pollutant due to increased excavation and industrial consumption since 1950. Sources of Cd emission are mining, industrial electroplating, and battery factories. Since it is commonly present in water, food, and tobacco, as well as in soil and the atmosphere, Cd is often exposed to living systems and seem to be readily absorbed by the body (Merian, 1990) and has been shown to be very harmful to human beings; the target tissues include the kidney and the liver.

Cd occurs mainly in the form of water soluble bivalent ions in nature (Bruland, 1989). It was estimated rain water may contain 0.05-0.8 μg Cd/L, fog water 0.3-7 μg/L, much more than river water with 0.01-0.15 μg/L and lake water containing even lower concentrations (Merian, 1990). In highly polluted lakes,

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such as Lake Erie in the Great Lakes, cadmium concentration ranges up to 1 μg/L (Doyle *et al.*, 1975). In the New York and New Jersey Harbor Estuary area, a high concentration of Cd, along with Cu, Pb, Ni, Ag and Zn in water and sediment categorized them as toxins with respect to their effect on marine life (Squibb *et al.*, 1991).

In aquatic systems, algal diversity and productivity have decreased as a general result of pollution by metals (Rai *et al.*, 1981). The latter have reported that a very toxic metal such as Cd generally inhibits cell growth at very low concentrations. Cd was earlier reported to cause significant growth inhibition of the cyanobacterium, *Anabaena inaequalis,* at concentrations greater than 0.02 ppm and complete growth inhibition occurs at 0.06 ppm (Stratton and Corke, 1979). Another aquatic organism, *Amoeba proteus,* was sensitive to Cd^{2+} at concentrations as low as 10^{-8} M, as the division and survival rates of the cells decreased to 95% and 80% for a 3 day exposure to Cd^{2+} at 10^{-8} M. (Ord and Al-Atia, 1979).

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The intracellular effects of heavy metals to some aquatic organisms as determined by electron microscopy and evaluated by morphometric analysis

Amoeba proteus, was studied as a model of the unicellular aquatic organism for the cadmium toxicity on the cellular structures (Ord and Al-Atia, 1979). Membrane fragility or rupture with cytolysis was commonly induced within 1-2 hours exposure to Cd^{2+} concentrations exceeding $5x10^{-5}$ M. Under the transmission electron microscope, the morphological changes were found to occur in many membrane bound organelles in the exposed cells. Mitochondria showed distorted shapes, vacuolation and centrally reduced cristae, and rupture of their membranes with Cd treatment.

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A number of researches have used morphometry to quantify the effects of heavy metals on cellular ultrastructure, to study the intracellular effects of heavy metals (Sicko-Goad, 1982; Rachlin *et al.*, 1982a; Rai *et al.*, 1990). Morphometry, also called stereology by some authors, is a method for obtaining quantitative three-dimensional information from essentially two-dimensional microscopical sections. Before biological morphologists began to quantitate their results, geologists and metallurgists had developed and were using these quantitative methods. Morphometry is now widely applied to histology, histopathology and electron microscopy of cells (Aherne and Dunnill, 1987).

For volumetric analysis, the geologist, Delesse, developed the fundamental relations of stereology called the Delesse principle in 1846 (cited in Weibel *et al.*, 1966). The principle says that the volume fraction, Vvi, of a component, i, in the tissue can be estimated by measuring the area fraction, AAi, of a random section occupied by transections of i:

$$Vvi=AAi$$

AAi was determined by planimetry which is rather cumbersome and tedious. Glagoleff (1933) found that planimetry could be done by superimposing a regular point lattice on the section and counting the points which lie on transections of the structures. This simplification made morphometry much easier to use. Similar procedures were proposed for application in biology by Chalkey (1943) and Attardi in 1953 (cited in Weibel *et al.*, 1966).

$$Vvi=PPi$$

The fraction PPi of points lying on transection of i would thus be an estimate of Vvi.

The surface of any structure will appear in sections as a contour of the transection and the length of these contours on the unit test area of the section will be proportional to the surface density, Svi. If a test line of fixed length (LT),

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for example, 1 cm, is randomly placed in the tissue it will pierce through the surface of these structures, whereby the number of interactions, Ni, between test line and surface will be proportional to Svi and to Lt. Tomkeieff and Hennig in 1956 and 1957 (cited in Weibel *et al.*, 1966) have independently derived the relation:

$$Svi = \frac{2\,Ni}{LT} = 2\,N_{Li}$$

Weibel and Bolander (1973) described the application of this technique for electron microscopic morphometry of cells. It has been used at the ultrastructural level to study the cellular differentiation in plants (Verbeke, 1989; Valdovinos *et al.*, 1985), the appearance of cyanobacterial cells on different nutrient media (Jensen and Rachlin, 1984) and the evaluation of the ultrastructure of cell parts of various methylotrophic bacteria (Jensen and Corpe, 1988). Using this technique, the effects of toxic metal ions on cell ultrastructure when compared to control cells were also evaluated (Sicko-Goad and Stoermer, 1979; Rachlin *et al.*, 1984; Rai *et al.*, 1990).

Morphometric analysis has been widely used to quantify the toxic effects of metals on cellular structures. Morphometric analysis of cells of *Diatoma tenue* var. *elongatum* revealed that lead at 0.05 µg/l caused a significant decrease in the number of mitochondria with a concomitant increase in their volume compared to control cells. Exposure of cultures to 0.08 µg/l copper resulted in reduction of the number of polyphosphate bodies (Sicko-Goad and Stoermer, 1979). Using the morphometric technique, Sicko-Goad (1982) studied the response of three algae, *Melosira granulata*, *Fragilaria capucina*, and *Anacystis cyanea* after exposure to 10 ppb lead or 5 ppb copper for 2, 3, and 24 hours. According to the quantitative ultrastructural changes caused by the metals (Sicko-Goad, 1982), it was suggested that all were more sensitive to copper than

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to lead. *Fragilaria* was more sensitive to both metals than the other two species. Mitochondria and photosynthetic membranes appeared to be more sensitive to both metals. Rachlin *et al.* (1982a) studied the morphometric response of *Plectonema boryanum* cells to eight metals all at 10 ppm and found Mn^{2+}, Zn^{2+}, Hg^{2+} and Cd^{2+} produced no changes in overall cell volumes while Pb^{2+} and Cu^{2+} produced significant increase in cell volumes and Co^{2+} and Ni^{2+} caused significant decrease in cell volumes. Metals Mn^{2+}, Pb^{2+}, Co^{2+}, Ni^{2+}, and Cd^{2+} caused significant increases in the surface area of the cell's thylakoids and Ni^{2+}, Co^{2+}, Zn^{2+}, and Hg^{2+} caused a significant reduction in the volume of the intrathylakoid spaces. The increase of the surface area of the thylakoids was also observed in *Anabaena variabilis* cells exposed to 52 and 114.7 μM Zn and *Anabaena flos-aquae* cells to 114.7 μM Zn when both organisms were treated with Zn at 20.6, 52 and 114.7 μM (Rachlin *et al.*, 1985). Rachlin *et al.* (1984), again using electron microscopic morphometry in studies of *A. flos-aquae*, established that Cd^{2+} caused a reduction in the surface area of the thylakoids, a decrease in cell volume, a reduction in the volume of polyhedral bodies, and in the number and volume of polyphosphate bodies. In the same organism, these reductions were more pronounced in Cd^{2+} exposed cells grown at acidic and alkaline pH than at pH 7.2 (Rai *et al.*, 1990). Lysis of the cell wall was more evident in acidic than in alkaline conditions, and in both higher and lower pH than at pH 7.2. It would seem that pH alters the sensitivity of cells to Cd^{2+}, but the reason for this has not been explained.

Metal uptake and accumulation by cells

The cell surface is the first defense against heavy metal ions and is reported to be also important in metal uptake (Beveridge, 1989; Remacle, 1990).

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In certain cases, metal crystallization may occur on algal surfaces by forming metal-sulfide microcrystals (Wood and Wang, 1983) or bacterial cell surfaces as reported by Mullen *et al.* (1989) that the heavy metal La (1 mM) accumulated at the surface of *Pseudomonas aeruginosa* cells as needlelike, crystalline precipitates. Silver precipitated as discrete colloidal aggregate at the surface and occasionally in the cytoplasm of *Bacillus cereus* cells. Macaskie *et al.* (1987) suggested an envelope-bound phosphatase generates enough HPO^- to surface-precipitate large quantities of Cd^{2+} as $CdHPO_4$ in *Citrobacter* sp.. The surface binding of metals may be especially important in slime-producing organisms or those organisms that grow in an extracellular matrix, the extracellular material acting as an "impermeable barrier" (Reed and Gadd, 1989). Surface bound Cd was reported to be loosely bound and easily removed (Mushrifuh and Peterson, 1991). Mushrifah and Peterson (1991) studied the uptake of Cd on the surface, insoluble fraction and soluble fraction of *A. flos-aquae.* They found that at low Cd^{2+} concentration the cell surface seemed able to control the penetration of Cd^{2+} by adsorbing the ions; but high absorption of Cd to the thylakoid membrane and cell wall components seemed to correspond with an increase in metal concentration.

There are other reports that metals are transported internally and localized within the protoplast. The most characterized internal metal deposit is Fe_3O_4 in the form of magnetite, found in the cytoplasm of certain bacteria as small membrane-bound magnetosomes (Blakemore, 1982). Other metals such as copper are preferentially accumulated within vacuoles and the nucleus, and lead is deposited in the nucleus of *Scenedesmus acutiformis* (Silverberg, *et al.*, 1976). In cyanobacteria and some eukaryotic microalgae, metals are sequestered within polyphosphate bodies (Jensen *et al.*, 1982a; 1982b; 1986). The metal-binding proteins, metallothioneins or phytochelatins, induced by heavy metals

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in animals, plants and microorganisms are located in the cytoplasm (Vallee, 1991; Rauser, 1990).

The metal uptake was suggested to be a two phase system: by a fast, temperature-independent step of passive adsorption, and by a slow, temperature- and metabolism-dependent process (Gadd and Griffths, 1978; Reed and Gadd, 1989). In the case of cadmium uptake by *Chlorella pyrenoidosa*, the fast phase was reported to be 8 min and the slow phase was 24 hours (Gipps and Coller, 1980). The slow phase depended not only on temperature, but also on pH whereas the fast phase depended on neither. Surface uptake of metal by the cell was considered as passive-uptake whereas some intracellular uptake was metabolism-dependent (Gadd and Griffths, 1978; Reed and Gadd, 1989; Beveridge, 1989). One example of the metabolism-dependent uptake is the metal-binding proteins as mentioned above. A more detailed discussion about the metal-binding proteins is followed. The amount of heavy metals taken up by passive mechanisms is often quite low, when compared to those transported by metabolism dependent processes (Gadd and Griffths, 1978). Skowronski (1984) studied the uptake of cadmium by both heat-killed and living cells of the filamentous green alga, *Stichococcus bacillaris*. The result showed that Cd was mostly taken up by the passive process; it was found that the dead cells adsorbed 80% of the amount of cadmium taken up by the living cells, which took up 20% more, apparently by an energy-dependent process.

In the present study, we were interested in learning the cellular location of Cd taken up by cells. Previous data from energy dispersive x-ray (EDX) studies indicated that most of the Cd was sequestered in polyphosphate bodies in microorganisms (Jensen, 1990; Jensen *et al.*, 1982a) while a small amount was concentrated in the cytoplasm and cell wall.

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