Order Number 9405591

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

Tang, Ming, Ph.D.

City University of New York, 1993

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

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

by

Ming Tang

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

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.

8/11/93
Date

Chairman of Examining Committee
Dr. T. Jensen, Lehman College

8/23/93
Date

Executive Officer
Dr. Richard L. Chappell

Dr. D. Kincaid, Lehman College

Dr. J. Valdovinos, Lehman College

Dr. W. Corpe, Columbia University

Dr. S. Huhndorf, New York Botanical Garden

Supervising Committee

The City University of New York

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.

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.

To my mother and father

ACKNOWLEDGMENTS

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.

Special thanks are extended to Mr. Michael Baxter for his assistance and suggestions, and to my friend and colleague, Edythe Boyer Jones, for her friendship and help.

I wish to thank the Department of Biological Sciences at Lehman for financial support.

My thanks also go to my husband and my son, my parents and parents-in-law for their love and encouragement.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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,

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).

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.

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),

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

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).

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

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.

polyphosphate bodies (PPB's) and their possible role in metal concentrations in cells

PPB's are found to be common cytoplasmic inclusions in microorganisms, such as bacteria, algae and fungi. Jensen (1968; 1969) modified a lead sulfide-staining procedure, which was reported to be specific for PPB's (Ebel *et al.* 1958) and demonstrated which cellular inclusion is the PPB under the transmission electron microscope. Under the electron beam, PPB's are identified as electron dense, spherical and sometimes porous granules (Jensen, 1990). They, because of their dense nature, often fall out or get chipped out in sectioning (Jensen, 1968). The bodies can also be seen in living cells by phase-contrast microscopy.

PPB's used to be referred as volutin, Babes-Ernst granules, or metachromatic granules (Harold, 1966) since they were stained metachromatically with certain basic dyes. Wiame (1947a, 1947b; 1949) and Schmidt *et al.* (1946) were the first to identify volutin granules as deposits of inorganic polyphosphate. Inorganic polyphosphates, found in almost all living organisms, are linear polymers of orthophosphate in anhydrous linkage, and their phosphoanhydride bond is high-energy. PPB's in cells are also visualized by electron microscopy; combined with EDX analysis, it is possible to detect the phosphorus (Doonan *et al.*, 1979; Baxter and Jensen, 1980a).

PPB's from *Micrococcus lysodeikticus* were isolated and analyzed and found to contain 27% phosphate, 24% protein, 30% lipid, and the remainder of the components were RNA, carbohydrate and metals (Friedberg and Avigad, 1968). It is possible some of these constituents were contaminants adsorbed by PPB's during isolation. Coleman *et al.* (1972) studied PPB's of *Tetrahymena pyriformis* by electron microprobe analysis and found that the granules often contained P, Mg, Ca, K, Na, Cl, and C (Coleman *et al.*, 1972). Recent studies on

the cyanobacterium, *Plectonema boryanum*, using EDX analysis have shown that PPB's of air dried cells have four common elemental components, Mg, P, K, and Ca (Baxter and Jensen, 1980a).

PPB's are usually located in the cytoplasm of the bacterial cell. In eukaryotic cells they are found mainly in the vacuoles. However they were observed to be in association with a number of subcellular entities, for example, lipid deposits and glycogen inclusions (Harold, 1966). Jensen and Sicko (1973) recognized minute, spherical, electron dense structures, which appeared to be PPB's, within some polyhedral bodies of the cyanophyte *Chlorogloea fritschii*. They were subsequently seen in other cyanobacterial species (Lawry and Jensen, 1979; Jensen *et al.*, 1977; Jensen and Rachlin, 1984) and referred to as polyphosphate minibodies (Lawry and Jensen, 1979) or minipolyphosphate bodies (Jensen *et al.*, 1982a; Jensen and Rachlin, 1984).

Polyhedral bodies are conspicuous inclusions with polygonal profiles found in cyanobacteria (Jensen, 1990) and are also found in other autotrophic bacteria (Codd and Marsden, 1984). These inclusions, first described by Jensen and Bowen in 1961, are limited by a membrane monolayer. Recently polyhedral bodies have been isolated from the cyanobacteria and shown to have as their main component ribulose 1,5-biphosphate carboxylase/oxygenase (RuBisCO) (Lanaras and Codd, 1981; Shively, 1988). Polyhedral bodies are therefore called carboxysomes by some workers and suggested to be active in photosynthesis (Lanaras and Codd, 1981; Shively, 1988; Shively and English, 1991). The presence of polyphosphate body-like inclusions in some polyhedral bodies certainly raises questions about their functions in polyhedral bodies and in cells. An analytical proof that these inclusions are in fact PPB's must be developed.

All phosphates, including polyphosphates, are highly charged anions and thus adhere strongly to cations (Peverly *et al.*, 1978). Using the EDX analysis,

Jensen and his group (Jensen *et al.*, 1982a; 1982b; 1986; Baxter and Jensen, 1980b) studied the uptake of various heavy metals by cells of *Anabaena variabilis*, and *Plectonema boryanum*. They found that PPB's in these cells concentrated large amounts of exposed metals, such as Ba, Cd, Co, Cu, Hg, Mg, Ni, Zn, and Pb; smaller amounts of the metals were concentrated in the cytoplasm and cell walls. Similar experiments (Jensen *et al.*, 1982b) on other microorganisms, Chlorophyta and Bacillariophyta, suggested that PPB's can accumulate metals and might be involved in metal detoxication. Using the same analytical method, Pettersson *et al.* (1985) also demonstrated that aluminium was mostly taken up in PPB's of *Anabaena cylindrica*. *Klebsiella aerogenes* cells were found to be more sensitive to cadmium when they grew in phosphate limited medium (Aiking *et al.*, 1984). Therefore phosphorus nutrition in relation to heavy metal uptake or concentration of intracellular metals has been a matter of researches interested (Jensen *et al.*, 1986; Watanabe *et al.*, 1989).

"Stress" responses of cells exposed to heavy metals and possible relationship to other "stress" conditions

Living organisms respond at the cellular level to unfavorable conditions or stressful situations, such as exposure to heat shock, heavy metal ions, ethanol, and amino acid analogues by the rapid, vigorous, and transient acceleration in the rate of expression of a small number of specific genes (Ashburner, 1982; Morimoto *et al.*, 1990; 1992; Neidhardt *et al.*, 1984; Lindquist and Craig, 1988; Schlesinger, 1990). This phenomenon is called the heat shock response because it was discovered in heat shocked *Drosophila* larvae. It is also called the stress response.

In the early sixties, Ritossa, a geneticist, reported that when *Drosophila* larvae raised at 25° C, were heat shocked by being exposed at 30-32° C for about 30 minutes, several new puffs appeared on the giant salivary gland chromosomes (Ashburner, 1982). In 1974, Tissieres *et al.* reported the striking results revealed on protein gel electrophoresis that "heat shock" led to the appearance of approximately seven new protein species, which was reminiscent of the appearance of seven heat shock puffs on the polytene chromosomes. These protein species were the products directed from mRNAs made at heat shock puff sites from the induced genes. This phenomenon of inducible gene expression in *Drosophila* is called the heat shock response and the products of these gene are called heat shock proteins (hsps) (Ashburner, 1982; Morimoto *et al.*, 1990; Neidhardt *et al.*, 1984). Although it was first reported for *Drosophila* cells, the universality of the heat shock response from bacteria to human was recognized shortly thereafter. In *E. coli*, there are 17 identified hsps; ten of them are the products of known genes (Lindquist and Craig, 1988). In all organisms, the heat shock response is induced remarkably rapidly, for example, as early as 15 seconds after a temperature elevation in *E. coli* (Neidhardt *et al.*, 1984). It is also characterized as a vigorous and transient response. Besides the activation of heat shock genes, the expression of most other genes is inhibited as a result of stress (Morimoto *et al.*, 1990). The hsps also proved to be very highly conserved; the major heat shock protein, hsp 70, has about 50% of its sequence conserved between *E. Coli* and human (Schlesinger, 1990).

Heavy metal ions, such as As or Cd, were later found to induce proteins similar to those induced by heat in various biological systems (Li *et al*, 1982). In a study in chick embryo cells and in human foreskin cells (Levinson *et al.*, 1980), the transition series metals, Co, Cd, Zn, and Hg induced the synthesis of four

proteins, which comigrated on acrylamide gel with proteins induced by heat shock. They hypothesized that a sulfhydryl-containing molecule was the target of the induction since all four metals bind to sulfhydryl groups.

Although metals, such as As, Cd, and Hg, induced the hsps in *Drosophila* cells, heat shock still elicited the strongest response (Bournias-Vardiabasis *et al.*, 1990) and was also a faster inducer in terms of levels of all hsps induced and of the exposure time needed for the induction (Courgeon *et al.*, 1984). VanBogelen *et al.* (1987) studied the ability of heat, $CdCl_2$ and various inhibitory chemicals to induce proteins of the heat shock, the oxidation stress, and the SOS regulons in *E. coli.* These regulons appear to be related in the sense that they respond to the same environmental stimuli, share member genes, or have protein products which interact. Heat was found to initiate solely a heat shock response while $CdCl_2$ strongly induced the heat shock, oxidation stress and SOS regulons. However, Cd only induced half of the heat shock proteins and, on the other hand, induced uniquely 17 unidentified proteins.

The toxic effect of heat shock and related "stresses" was reported to denature and inactivate enzymes (Bensaude *et al.*, 1990; Nguyen *et al.*, 1989). It was suggested that the production of abnormal proteins and /or an overloading of the ubiquitin-dependent protein degradation pathway might be the inducer of the heat shock or stress response (Munro and Pelham, 1985). A number of studies have supported this suggestion. For example, the addition of amino acid analogs (Hightower, 1980) and the mutation in genes (Hiromi and Hotta, 1985), both resulting in the production of abnormal proteins, induced the expression of hsps. The heat shock genes were also induced in E. coli when a foreign protein which fails to fold correctly is expressed in the cells (Goff and Goldberg, 1985).

It is believed that hsps protect cells from the toxic effects of "stresses" (Lindquist and Craig, 1988; Hahn and Li, 1990). Studies have shown that organisms subjected to a pre-shock treatment that induce the hsps can be more tolerant to a more extreme temperature (Schlesinger, 1990; Lindquist and Craig, 1988) and this protective effect is transient. When other stresses, such as heavy metal ions, induce hsps, they also induce thermotolerance.

Tolerant and resistant responses to toxic metals

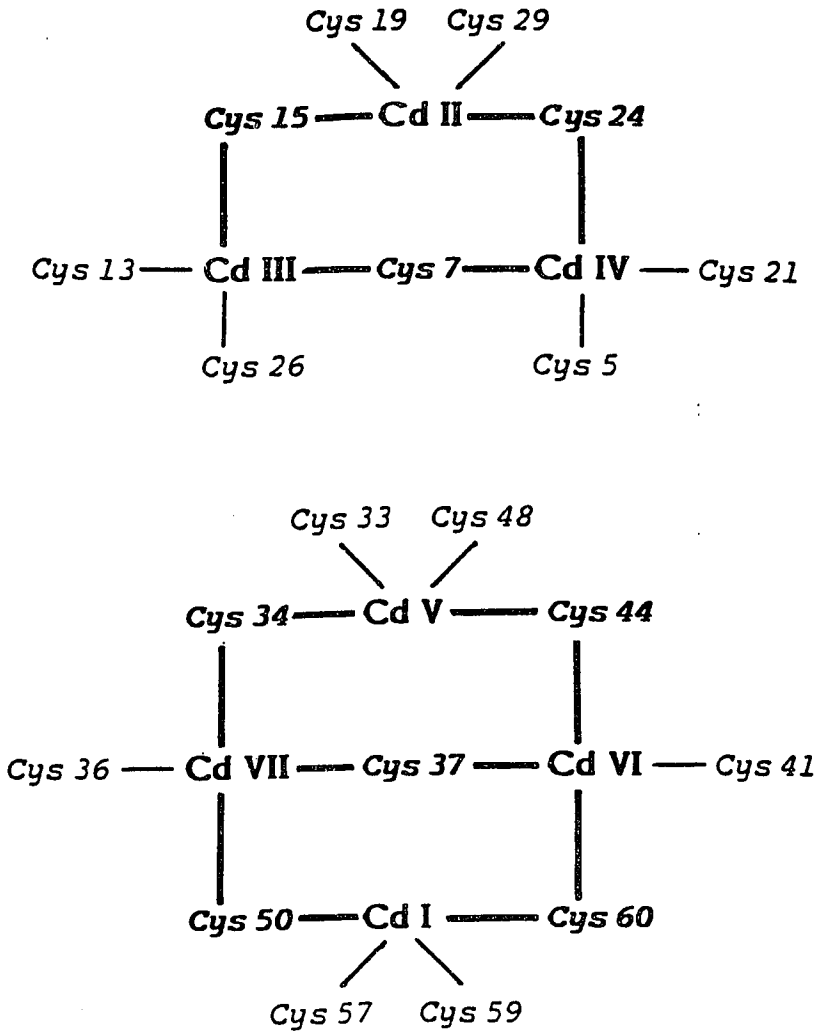
Metal tolerance or resistance, often used interchangeably, has been reported in various organisms, such as fungi, bacteria, and algae (Bischoff, 1982; Reed and Gadd, 1989). Some cyanobacteria such as *Oscillatoria, Phormidium, Plectonema,* and *Schizothrix* isolated from zinc enriched water are reported to be often resistant to zinc (Shehata and Whitton, 1981). The fungi, *Rhizopus stolonifer* and *Cunninghamella blakesleeana,* were trained to be copper-tolerant after growing in elevated concentrations of copper. They acquired a tolerance to elevated levels of cadmium, cobalt, nickel, and lead simultaneously (Toledo *et al.*, 1985). The tolerance was the result of physiological adaptation rather than of the selection of resistant mutant cells. In these cases, metals seem to act as agents of directional selection leading to the establishment of metal-tolerant ecotypes.

Metal tolerance or resistance may be made possible by [1] exclusion of the toxic metal by binding metals with extracellular materials such as polysaccharides and hydroxamates (Wang and Tischer, 1973); [2] internal detoxification including metal binding by metallothioneins or phytochelatins (Vallee, 1991), polyphosphate bodies (Jensen, 1990), and the formation of precipitates such as inclusion bodies or mineral concretions (Reed and Gadd,

1989); [3] metal transformation to lesser toxic forms by oxidation, reduction, and methylation (Reed and Gadd, 1989); and [4] genetic determinants (Silver and Misra, 1988). Metallothioneins and genetic determinants are briefly discussed here, since both explain the metal resistance phenomenon at the biological molecular level; therefore their mechanisms may be helpful to find out how the heavy metals exert toxicity at the molecular level in organisms.

metallothioneins

Unlike zinc, which is involved in virtually all aspects of metabolism and has been found to be an integral component of nearly 300 enzymes in different species of all phyla (Vallee, 1991), cadmium is a metal with no known biological functions. However, a low molecular weight protein containing 20-25 mg of cadmium per gram dry weight measured by spectrography and by colorimetric analysis was isolated from equine kidney cortex in 1957 (Margoshes and Vallee, 1957). Analysis by ultraviolet absorption of the protein revealed that it had low content of aromatic groups. That was the first report of metallothionein. Now metallothionein has been found throughout the animal kingdom (Hamer, 1986). The metallothionein has a highly unusual amino acid composition - no aromatic or heterocyclic amino acids while one third of its residues are cysteines. The other unusual feature was its extraordinary metal content -7 g-atoms of metal per mole (Kojima *et al.*, 1976). Moreover, it was a small protein of just 60 or so amino acids and was inducible in response to heavy metals *in vivo* and to a great variety of metabolites (Vallee, 1991). The amino acid sequence was Cys-X-Cys where X is an amino acid other than Cys (Rauser,1990). The figure on page 15 shows the arrangements of the 20 cysteines coordinated to the 7 metal ions in ^{113}Cd-substituted human metallothionein-2, which is established by 2-dimensional heteronuclear $^{1}H^{113}Cd$ NMR correlation spectroscopy (Kägi, 1991).



Cd-Cys coordination in human liver Cd_2-metallothionein-2 (Kägi, 1991). *Top*: Three-metal thiolate cluster. *Bottom*: Four-metal thiolate cluster. The roman numerals specifying the metal sites refer to the corresponding ^{113}Cd NMR resonance positions. The arabic numerals refer to the residue position in the sequence. The thick - and thin-lettered residues denote the bridging and terminal cysteines, respectively.

Grill *et al.* (1985a, 1985b) discovered that higher plant (*Rauvolfia serpentina*) cell suspension cultures, upon exposure to cadmium, synthesized cysteine-rich peptides, called phytochelatins. It was also noticed (Grill *et al.*, 1985a) that the synthesis of phytochelatins is also induced by other heavy metal ions such as Cu^{2+}, Hg^{2+}, Pb^{2+}, and Zn^{2+} . Cell extracts not exposed to metals did not yield any phytochelatins within the detection limit. Cell cultures of various higher plants, including both dicotyledons and monocotyledons, synthesized large amounts of the peptides when exposed to Cd^{2+}. The Cd binding peptides were also isolated from the algae *Chlorella fusca* (Gekeler *et al.*, 1988). The phytochelatins had a structure of (r-glu-cys)ngly (n=3-7), (n=2-5 in *C. fusca*), which is quite different from metallothioneins reported from animals. The ratio of Cd^{2+}: cysteine was about 1:2. Because of the r-glutamic acid bonds, the phytochelatins cannot be regarded as primary gene products (Grill *et al.*, 1985a). Their biosynthesis was connected to glutathione metabolism rather than to direct ribosomal synthesis (Rauser, 1990). Metallothioneins were reported to have been isolated from cyanobacteria such as *Synechococcus* sp. (Olafson, 1991). The work in cyanobacteria, however, was preliminary.

Metallothioneins or phytochelatins found in animals, plants and microorganisms exposed to heavy metals are considered as a major detoxicification mechanism because of their high content of metals. The standard method to isolate metallothioneins is gel filtration (Vasák, 1991). Gel electrophoresis is also an efficient way to quantify and identify the low-molecular weight metallothioneins extracted from chicks (McCormick and Lin, 1991).

Genetic Mechanisms

The genetic determinants of resistances to heavy metals are often found on plasmids and transposons in many bacterial strains (Silver and Misra, 1984).

Some heavy metal resistances, such as arsenic and antimony resistances, are governed by plasmids that also code for antibiotic resistances (Silver and Misra, 1988). The same is true for copper resistance, which is on an antibiotic resistance plasmid. These co-resistant plasmids are often found in strains isolated from hospitals. However, heavy metal resistance plasmids without antibiotic resistance determinants were found in *E. coli* from an industrially polluted environment (Nakahara and Kozukue, 1982). Bacteria with these plasmids can tolerant the metal at higher concentration; for example, *E. coli* strains without the plasmid grew in the presence of 0.06 mM $CuSO_4$ while strains harboring the plasmid grew in the presence of 10 mM $CuSO_4$ (Trevors *et al.*, 1985; Trevors and Cotter, 1990).

The plasmid R100 mercury resistance system is the most studied (Silver and Misra, 1988; Schiering *et al.*, 1991). The regulatory gene of the system *merR* produces a trans-acting inducer-repressor, followed by a promoter/operator region and structural genes, *merT* (for a Hg^{2+} transport system that brings extracellular Hg^{2+} into the cell), *merP*, *merA* (for the subunits of mercuric reductase, which catalyzes the reduction of Hg^{+} to nontoxic elemental mercury Hg^{0}), and *merD*. The *merT* was discovered in hypersensitive mutants. Deletion of the *merT* and *merP* region eliminates that hypersensitivity. The *merP* encodes a periplasmic mercury-binding protein. The function of *merD* is uncertain.

There are several systems for bacterial cadmium resistance. The mechanisms for *cadA* system is Cd^{2+} efflux (Yoon and Silver, 1991), while that for *cadB* is increased binding of Cd^{2+}. One system found with a chromosomal mutation is in *Bacillus subtilis* that resulted in a change in the membrane manganese transport system so that Cd^{2+} is no longer accumulated.

Cyanobacteria are important producers in ecosystems. They evolve oxygen through photosynthesis and some species can fix nitrogen. The impact of Cd^{2+} on aquatic cyanobacteria is influential on the environment since they have a determinant effect on the productivity of the aquatic environment. Therefore, it is important to know how cyanobacteria respond to metals and how metals affect cells. *Synechococcus* is an excellent research organism. It is widely distributed in nature (Johnson and Sieburth, 1979). This rod-shaped unicellular organism is easily grown in the laboratory. It has been studied for some time in our laboratory (Lawry, 1979).

In the present research, *Synechococcus leopoliensis* is used as a representative of freshwater cyanobacteria to study its cultural and growth responses to Cd^{2+} and to explore the effect of Cd^{2+} on the cell structure to lay a ground-work for understanding the mechanism of action of Cd as a toxic element.

MATERIALS AND METHODS

A culture of the freshwater cyanobacterium, *Synechococcus leopoliensis* (UTEX 2434), was obtained from the Starr Culture Collection (Starr and Zeikus, 1987). Cells of both stock and experimental cultures were cultivated in modified Fitzgerald's medium (Fitzgerald *et al.*, 1952; Zender and Gorham, 1960) at pH 7.2 (see Appendix A). The cells were kept in a Sherer Controlled Environment Chamber (Model Cel B) at 25° C and illuminated with 500 ft candles of cool white fluorescent light, supplemented by two 25W incandescent bulbs. The chamber was set to a 12 hour light/12-hour dark cycle. All cultures were agitated by shaking the flasks several times each day. Cell growth was determined by optical density (turbidity technique) with a Bausch & Lomb's Spectronic 20 spectrophotometer at a wavelength of 440 nm, which was determined by dilution curves (Sorokin, 1975). Cell count (cell number per ml) was determined with a hemocytometer under a light microscope. Cadmium chloride from Sigma (Sigma Chemical Company, P. O. Box 14508, St. Louis, MO 63178) was used in the study.

Sublethal Cd^{2+} exposure:

The cell count of a stock culture was determined, and a fixed volume of the culture was transferred with a sterile pipette to 80 ml fresh sterile medium to achieve a cell count of $5x10^6$ cells/ml. This stock was then distributed in 5 ml aliquotes into fifteen sterile disposable 50 ml Falcon flasks (Becton Dickinson and Company, 2 Bridgewater Lane, Lincoln Park, New Jersey 07035). Cadmium chloride ($CdCl_2$), in 10 mM stock solution, was added to the flasks and the cultures were exposed to $CdCl_2$ at final concentrations of 0.01, 0.10, 0.15, and 0.20 μM in triplicate (Rachlin *et al.*, 1984). The minute volume change due to the

addition of $CdCl_2$ to the cultures was considered negligible. The effective concentration of Cd which reduces the population growth by 50%, EC_{50}, was expected to fall within the range of these four concentrations, which was determined by preliminary runs. The flasks were placed in the growth chamber and cells were agitated daily. After a 96-hour exposure, the optical density of each experimental culture in the flasks was measured. The optical densities of replicates were averaged and calculated for the EC_{50} by the method of probit analysis (Finney, 1964).

Cd uptake by whole cells:

S. leopoliensis cells were collected from rapidly growing cultures by centrifugation in a clinical centrifuge and washed once with deionized and distilled water (ddH_2O). The cells were then exposed to 8 µM, 80 µM, 200 µM and 600 µM $CdCl_2$ respectively, in ddH_2O for 4 hours at room temperature. Some cells were also exposed to 600 µM $CdCl_2$ at 4°C with no illumination in a refrigerator for the same period of time. After the 4 hour exposure, the cells were prepared by the air-dry method (Baxter and Jensen, 1980a). The air dried cells (see below) were then examined with the TEM. Analysis with EDX (see below) was carried out in the STEM mode by placing the probe on the PPB's, and cytoplasm of the cell for a period of 100 seconds as has been described above. The analysis of the cell wall was done by placing the probe on the margin of the cell. Counts were also taken on the formvar film near the cell and these were subtracted (Jensen *et al.*, 1982a) before obtaining the spectra (For convenience the result of the EDX analysis, displayed as a series of peaks, will be referred to as a "spectrum".) of the cytoplasm and cell wall. The spectra of PPB's were obtained after subtracting the spectra of the cytoplasm.

Electron microcopy:

Cells were fixed in 1% osmium tetraoxide (see appendix B) as described by Pankratz and Bowen (1963). After fixation, cells were dehydrated in ethanol followed by propylene oxide then embedded in Epon (Luft, 1961) (see appendix C). Sections cut with a diamond knife were collected with copper grids and post-stained using uranyl acetate (Stempak and Ward, 1964) (see appendix D). The cells were then examined with a Hitachi H-7000 transmission electron microscope (TEM) at 75 kV.

Preparation of air-dried cells:

As demonstrated by Baxter and Jensen (1980a), the air-dry method is simple and efficient; most importantly, it is a method that can prevent serious elemental changes in the preparation process. It is therefore frequently used to prepare cells for EDX. Cells were collected from growing cultures by centrifugation, washed 3 times in distilled H_2O, then placed on formvar-coated (0.25%) grids and air dried at room temperature. The cells after being totally dried were ready for study.

Elemental analysis with energy dispersive X-ray spectrophotometer:

With the TEM in the Scanning transmission (STEM) mode and coupled to a Princeton Gamma Tech System (PGT) 4 Plus energy dispersive X-ray spectrophotometer (EDX), elemental analyses were carried out on cellular items of interest. These items were first located under the TEM; the instrument was then switched to the STEM mode for use in analysis by placing the probe (spot mode) on the appropriate cellular items (Baxter and Jensen, 1980a), each for a period of 100 seconds. The probe diameter at 100,000 x is about 20 nm. Count rate varied from 800-1200 counts per second.

Cd^{2+} exposure at selected pH values:

The media for the experimental cultures were buffered to pH 5.5, pH 7.2 and pH 9 with 10 mM HEPES buffer (Wehr *et al.*, 1986). The pH values of the media were adjusted by addition of either 1 N NaOH or 1 N HCl and was confirmed after autoclaving of the media. Cells from the stock culture were washed once with the medium and then inoculated into 10 ml of the medium to provide a cell count of $5x10^6$ cells/ml. The cells were then exposed to Cd^{2+} at the concentrations of the EC50 value determined in the last experiment and one log above the EC_{50} at the various pH's (Rai *et al.*, 1990). The cultures were then incubated in the growth chamber for 96 hrs with daily agitation. The pH and increase in cell number of each culture were determined at the end of the 96-hr period.

Morphometric analysis:

The cells from the above experiment were centrifuged, then fixed, embedded, sectioned and stained as described in the section of electron microscopy. Although several sections were collected onto one grid, only 1 section on a grid was used for the analysis to avoid repeated sampling of the same organism on the adjacent sections for each treatment (Sicko-Goad and Stoermer, 1979). Images of 30 randomly chosen sections of cells including small grazing tips were taken at a magnification of 20,000 x. Photomicrographs were obtained at a magnification of 3 times and therefore, a final magnification of 60,000 x; these were then overlayed with a transparent sampling lattice, containing 1.0 cm square spacings for quantitative measurements (Fig. 1). Using the procedures for morphometric analysis (Weibel and Bolender, 1973; Sicko-Goad *et al.*, 1977; Sicko-Goad and Stoermer, 1979; Mori and Christensen, 1980;

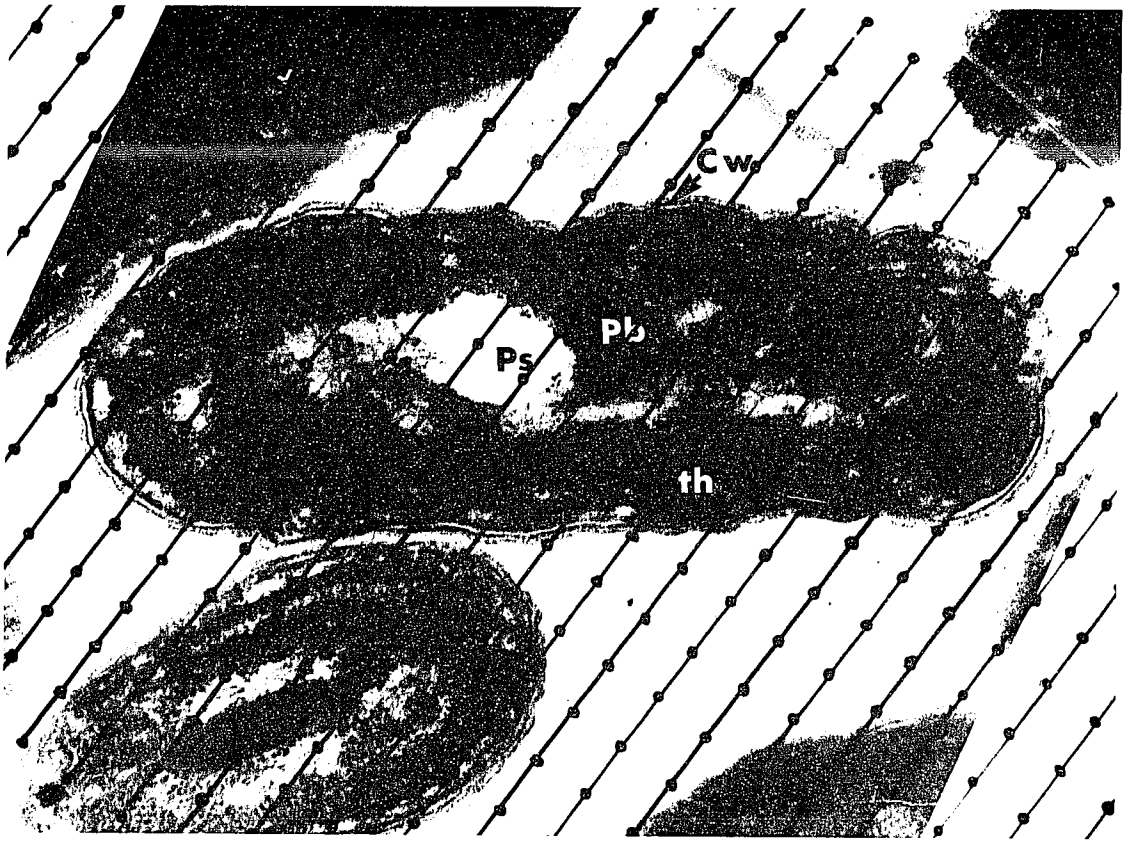


Figure 1: Micrograph of the section of a cell overlaid with a counting grid used throughout the morphometric analysis, which was carried out on the cell wall (Cw), polyhedral bodies (Pb), thylakoid (th), nucleoplasm (D), and polyphosphate bodies shown in the micrograph as the space left after the loss of the body (Ps). The section of the cell was magnified at 65,000 X.

Jensen and Corpe, 1988), the cells were analyzed for surface area of the thylakoids, and relative volume of nucleoplasm, cell wall, PPB's, polyhedral bodies and intrathylakoid spaces. The data were stored in SAS program on CUNY/VM mainframe and analyzed for descriptive statistics and variances (ANOVA) according to Sokal and Rohlf (1981). The frequency of occurrence of PPB's, polyhedral bodies and inclusions in polyphosphate bodies in cells incubated under different pH's and cadmium concentrations were determined by counts from the electron micrographs.

Elemental analysis of cell inclusions:

Using the fixed and embedded sample from the morphometric experiment, sections for the elemental analysis were cut at a thickness of about 150 nm and stained with uranyl acetate. Elemental analysis of PPB's, polyhedral bodies and the inclusions within polyhedral bodies in sections of cells was accomplished with EDX. Counts were also taken on the epoxy background near the cell sections and these were subtracted (Jensen *et al.*, 1982a) before obtaining the spectra.

Preparation of cell extracts:

Rapidly growing cultures of *S. leopoliensis* (1,500 ml) were exposed to 25 μM, 50 μM and 100 μM Cd^{2+} by the addition of 10 mM stock $CdCl_2$ solution, which was sterilized by filtering through a Nalgene disposable sterile filter (0.2 μm) (Nalge Company, Rochester, NY 14602). Then the cultures were kept in the growth chamber at 25° C for 2-day exposure.

The exposed cells were collected by centrifugation at 3,750 rpm with a Beckman GPR centrifuge (Beckman Instruments Inc., Palo Alto, California 94304) at 20° C. Then they were washed with 20 mM Tris-HCl buffer at pH 8.0

(Weckesser and Jurgens, 1988) and stored at 4° C. The cells were then suspended in 5 ml of the same buffer (ice-cold) and sonicated with a MSE Ultrasonic Power Unit (Instrument Associate Inc., 17 West 60th Street, New York 23, New York 212 C150840) for 2 min at 2 min intervals for 4 rounds. The temperature of the suspension was kept low throughout the process by keeping the cuvette in an ice bath containing NaCl to lower the thawing point. Extent of cell breakage was assessed by examination with a phase contrast light microscope. When broken cells were over 90%, the suspension was centrifuged at 5,000x g twice in a Sorvall Superspeed RC2-B centrifuge. The cell free extract and the rest were subsequently centrifuged (Fig. 2) in a Beckman L7-55 Ultracentrifuge at 4° C. The sediments of 10,000 g and 100,000 g were washed once with the Tris-HCl buffer, centrifuged again at 10,000 g for 30 min and 100,000 g for 1 hr respectively, and the sediments were suspended in protein extraction buffer (2% SDS, 10% glycerol, and 0.01% mercaptoethanol in 10 mM Tris-HCl, pH 8.0). The suspension was incubated at 70° C for 20 min for solubilization (Weckesser and Jurgens, 1988; Inouye and Guthrie, 1969). After centrifugation to discard insoluble items, i. e. peptidoglycan, proteins were precipitated by the addition of the same volume of ice-cold acetone (Pohl, 1990). After another wash with 50% acetone, the sediment after centrifugation was air dried to remove solvent and water. The protein precipitate was dissolved in sample buffer (see appendix O). The final supernatant fluid presumably containing cytoplasmic proteins was precipitated by acetone as mentioned above, the sediment then air dried to remove solvent residues and dissolved in the sample buffer (as above). The cell free extract was similarly treated. The protein content of each fraction was determined by the Bio-Rad colorimetric method (Bio-Rad Laboratories, 1414 Harbour Way South, Richmond, CA 94804), using procedures provided by the manufacturer. Bovin

serum albumin, which came with the protein colorimetric kit, was used to prepare a standard curve.

Polyacrylamide gel electrophoresis (PAGE) of proteins:

The sodium dodecyl sulfate (SDS)-polyacrylamide gels (see appendix P) at 15% was used (Sambrook *et al.*, 1989). A vertical slab gel apparatus (Model #SE6119SM, Hoefer Scientific Instruments, San Francisco, CA 994107) was employed. The separating gel was 1 mm thick X 15 cm wide X approximately 12 cm high. The protein samples (40 μg each) were boiled for 5 minutes in buffer, cooled and loaded onto gels. The electrophoresis was performed at 50 mA for about 10 hours. After electrophoresis, the gels were stained in Comassie R-250 (see appendix Q) and dried on a gel drier (Model No. SE1160, Hoefer Scientific Instruments) after destaining. The low range SDS-PAGE molecular weight standards from Bio-Rad was used with each of the gels.

Electron microscopy of broken cell fractions:

Broken cell fractions were washed 3 times with ddH_2O and then air dried on formvar coated copper grids. The fractions of unbroken cells, cell envelope fragments and membrane fragments were fixed. The cell free fraction was centrifuged in an Eppendorf centrifuge (Micro Centrifuge 5415 C, Brinkmann Instruments, Inc., Cantiague Road, Westbury, NY 11590) at 16,000 g for 30 minutes and the sediment was fixed. All fixed fractions were then embedded, sectioned and stained as described above. Both air dried and thin sectioned samples were then studied under the TEM and analyzed with EDX system.

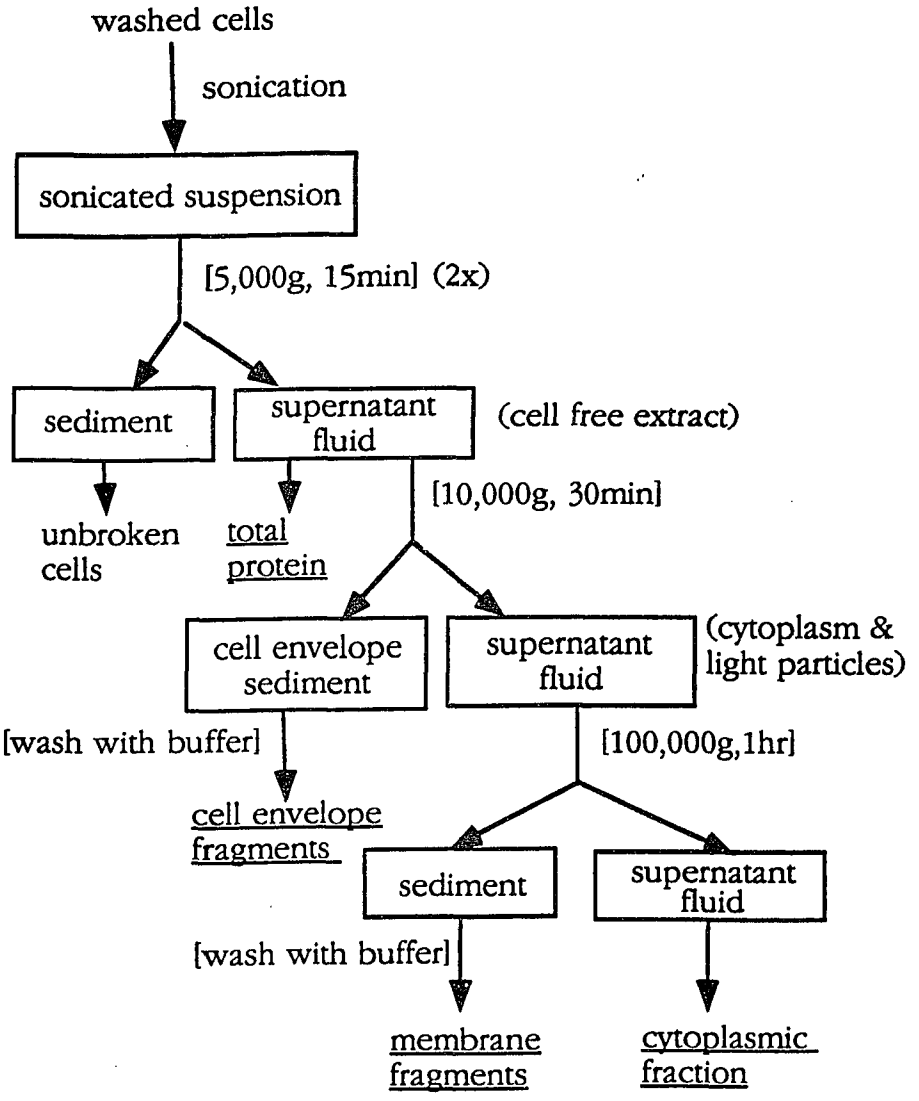


Figure 2. Fraction scheme for separation of the cell envelope and membrane fractions from the cytoplasmic fraction.

RESULTS

Determination of EC_{50} of Cd^{2+} to *S. leopoliensis*

The *S. leopoliensis* culture while grown under normal growth conditions began the rapid growing phase in 1-2 days after inoculation and reached a maximum yield after 20 days of growth (data not shown). The growth of cultures after the 96-hr exposure to Cd^{2+} was estimated and shown in table 1. The Cd exposed cultures were inhibited in growth as their optical densities (O.D.) after the 96-hr exposure were 0.358-0.091 while the control culture reached 0.4 (O.D.) in 96 hrs (Table 1). When the control cell growth was considered as 100%, the cell growth of Cd^{2+} exposed cultures varied from 89.5% to 22.75%. Table 1 also gives the calculated regression equation representing the response of *S. leopoliensis* to cadmium during a 96-hr exposure and the EC_{50} concentration of Cd^{2+}, 0.08 μM, to *S. leopoliensis* under the experimental conditions.

Effect of Cd^{2+} on ultrastructure of cells grown at different pH values

S. leopoliensis has a four layered cell wall (Fig. 12). Layer one is electron transparent, external to the plasma membrane and about 8 nm thick. The electron dense mucopolymer (peptidoglycan) is layer two and about 5 nm in thickness. Layer three is periplasm, a transparent layer of 8 nm thick outside of the mucopolymer layer. The outer membrane is layer four and is about 10 nm thick. When cells were subjected to pH 9 with 0.08 and 0.8 μM Cd^{2+} (Fig. 13), the cell wall seemed expanding and protruding; part of the wall layer one enlarged to an average thickness of 16 nm and it could reach up to 50 nm, and the outer membrane formed blebs. At pH 5.5, the wall layer one of some cells reached 70 nm in thickness with an average of 25 nm for control cells, 31 nm for cells exposed to 0.08 μM Cd^{2+} and 23 nm for cells exposed to 0.8 μM Cd^{2+} (Fig.

14); the cytoplasmic membrane appeared partially lysed in cells with an enlarged wall layer one. Cells grown at pH 5.5 (Fig. 4-6) and also cells at pH 9 with 0.8 μM Cd^{2+} (Fig. 11) had electron dense material deposited on the cell walls.

Table 1. The EC_{50} value of cadmium calculated from the percent response of *Synechococcus leopoliensis* after 96 hr. exposure, with representative regression equation (Data based on triplicate runs).

Conc. (μM)	Log Conc.	Initial O. D.	Final O. D.	% control growth	Empirical Probit
control	-----	0.100	0.400	100	-----
0.010	-2.0000	0.100	0.358	89.50	6.2536
0.100	-1.0000	0.100	0.200	50.00	5.0000
0.150	-0.8239	0.100	0.143	35.75	4.6362
0.200	-0.6990	0.100	0.091	22.75	4.2546

$Y=-1.446X + 3.401$; $r^2=0.977$; $\log EC(50)=-1.1057$; $EC_{50}=0.08$ μM

Polyhedral bodies were found in every medially sectioned cell irrelevant to the treatment. They (Fig. 3) were polygonal shaped and membrane bound and had fairly constant dimensions with an average diameter of 0.18 μm. The occurrence of the polyhedral bodies in the Cd^{2+} exposed cells increased as compared to that in the control cells; however, there was a slight drop of the occurrence in the cells exposed to 0.8 μM Cd^{2+} at pH 9 (Table 2).

Table 2: The occurrence of polyhedral bodies and PPB's (diameters in parenthesis) in 30 sections of cells exposed to selected concentrations of Cd^{2+} at different pH's.

	pH5.5			pH7.2			pH9		
$[Cd^{2+}]$ (uM)	0	0.08	0.8	0	0.08	0.8	0	0.08	0.8
No. Polyhedral body	10	19	22	21	25	14	15	25	29
No. PPB's	12	9	11	8	4	17	9	8	8
(diameter in nm)	(0.13)	(0.15)	(0.16)	(0.10)	(0.12)	(0.12)	(0.14)	(0.12)	(0.21)

Figure 3. Micrograph of a section of a control cell grown at pH 7.2 showing the four layered cell wall (Cw). Note the porous nature of the polyphosphate body (Pp). A polyhedral body (Pb), thylakoid (th) and DNA fibrils (D) are also visible. X 70,000.

Figure 4. Micrograph of a section of a control cell grown at pH 5.5. A space (Ps) was left after the loss of a polyphosphate body. The outer membrane of the cell wall is associated with electron dense material. X 46,000.

Figure 5. Micrograph of a section of a cell grown at pH 5.5 with 0.08 μM Cd. Note an electron dense inclusion (I) in the polyhedral body (Pb). X 56,500.

Figure 6. Micrograph of a section of a cell grown at pH 5.5 with 0.8 μM Cd. Note the space left by an inclusion (Is) in the polyhedral body (Pb). The outer membrane of the cell wall is associated with electron dense material. X 56,000. The inset micrograph shows two inclusions found in one polyhedral body. X 60,000.


4
Ps

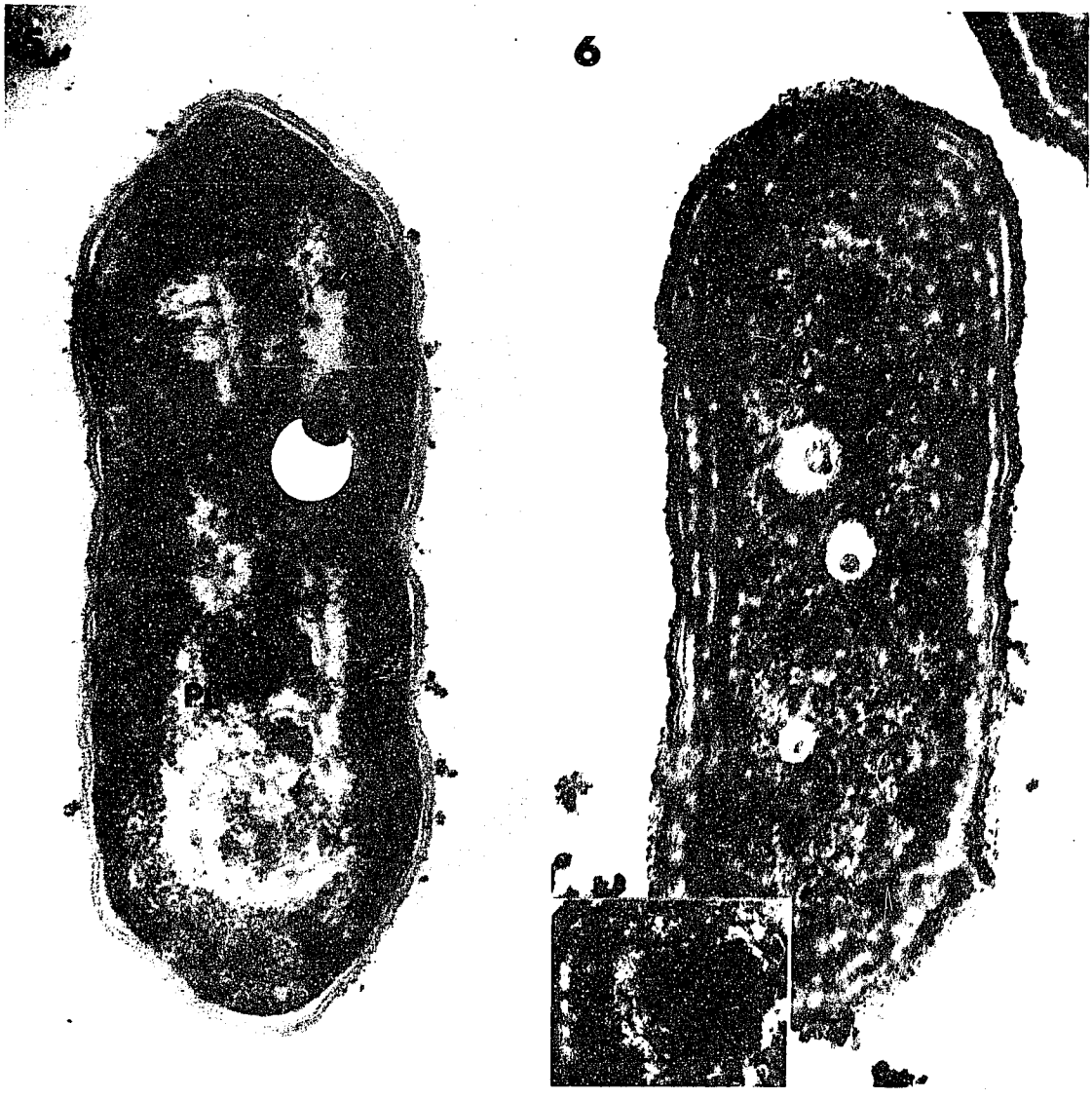

6

Figure 7. Micrograph of a section of a cell grown at pH 7.2 with 0.08 μM Cd^{2+}. X 52,000.

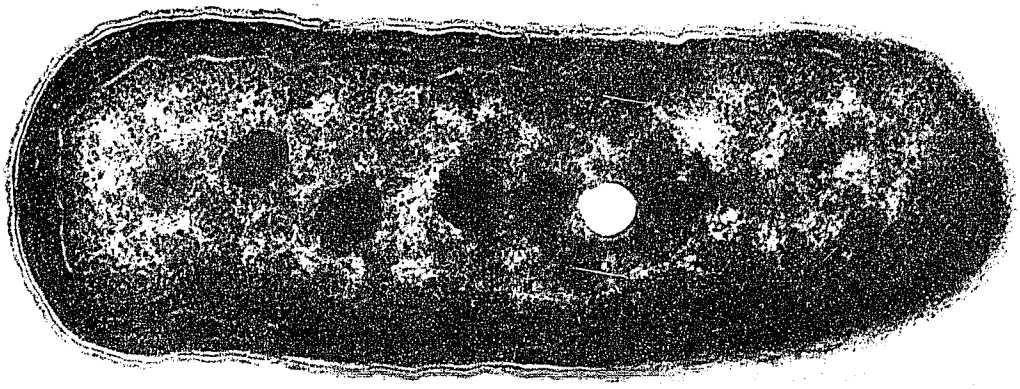
Figure 8. Micrograph of a section of a cell grown at pH 7.2 with 0.8 μM Cd^{2+}. X 52,000.

Figure 9. Micrograph of a section of a control cell grown at pH 9.0. Note the intrathylakoid space (arrow). X 68,000.

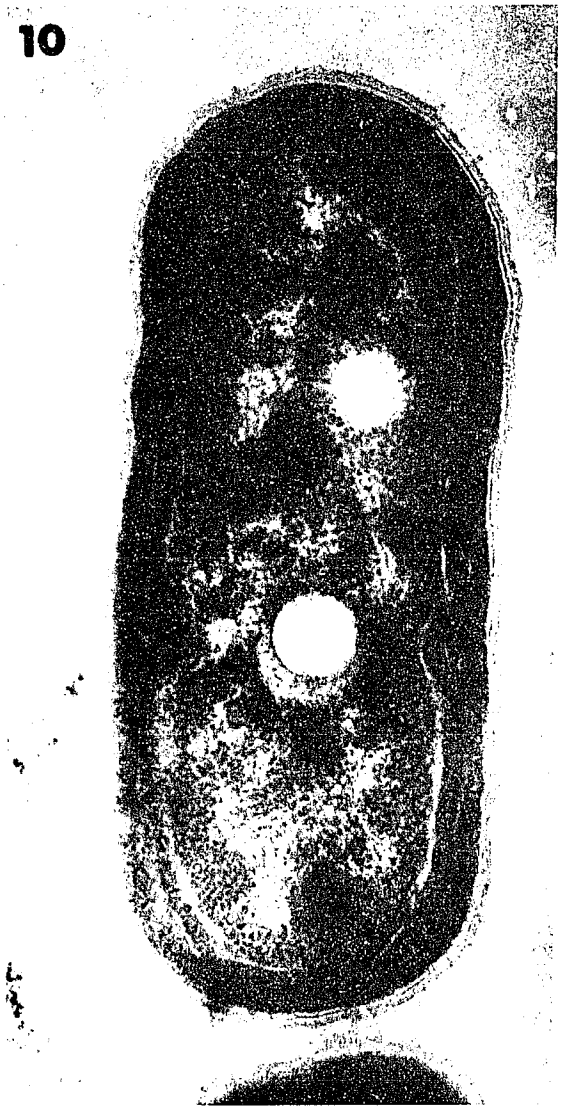
Figure 10. Micrograph of a section of a cell grown at pH 9.0 with 0.08 μM Cd^{2+}. Note the intrathylakoid space. X 53,000.

Figure 11. Micrograph of a section of a cell grown at pH 9.0 with 0.8 μM Cd^{2+}. Note the blebs (arrow) on the outer membrane. X 54,000.

9



10



11

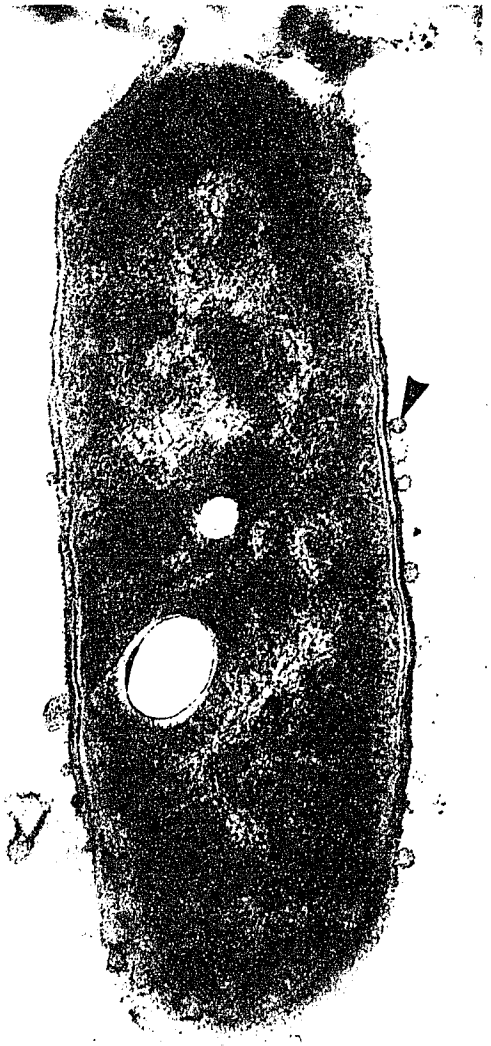
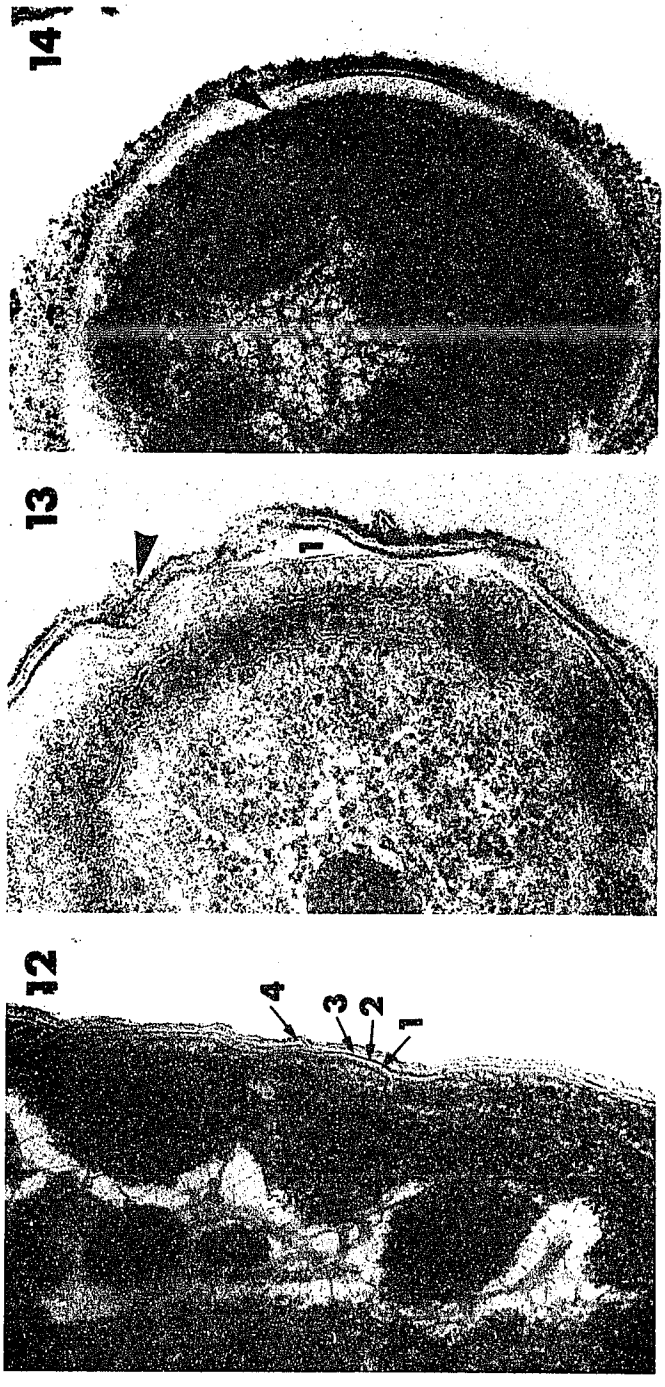


Figure 12. Micrograph of a section of a portion of a control cell showing the detail of the cell wall (layers 1-4). X 85,000.

Figure 13. Micrograph of a section of a portion of a cell exposed to 0.8 μM Cd^{2+} at pH 9.0 showing the enlargement of wall layer 1 at certain areas. Note the blebs (arrow) formed on the outer membrane (layer 4), which is also associated with electron dense material. X 85,000.

Figure 14. Micrograph of a section of a portion of a cell exposed to 0.8 μM Cd^{2+} at pH 5.5 showing the enlargement of wall layer 1. Note the lysis of the plasma membrane (arrow). The outer membrane (layer 4) with electron dense material associated with it is also visible. X 85,000.


14
13
1
12
4
3
2
1

Electron dense, cytoplasmic inclusions, sometimes porous were recognized as PPB's in thin sectioned cells (Fig. 3). They measured an average 0.16 μm in diameter ranging from 0.03 to 0.35 μm. They tended to fall out of the sections and left holes (Fig. 4, 5). A general increase was observed in the averaged diameters of PPB's in cells exposed to Cd^{2+} at any pH's (Table 2); the increase was more prominent in cells exposed to 0.8 μM Cd^{2+} at pH 9. Electron dense, spherical, sometimes porous inclusions (Fig. 5, 6, 23) were also observed in polyhedral bodies but they measured an average of only 0.03 μm in diameter, ranging from 0.017 to 0.06 μm. They, like PPB's, fell out of sections occasionally (Fig. 6).

Table 3. Frequency (%) of occurrence of inclusions within polyhedral bodies in thin sections of cells grown at different pH's and Cd^{2+} concentrations.

Cd^{2+}(μM)	pH 5.5	pH 7.2	pH 9
0	20	0	0
0.08	16	4	4
0.8	43	21	3

Data collected from the 30 thin sectioned cells from each growth condition showed that when cells were grown with 0.8 μM Cd at pH 5.5, about half of polyhedral bodies contained at least one detectable inclusion (Table 3) and 25% of them contained more than one (Fig. 6). Under this condition of growth, the inclusions inside polyhedral bodies were larger with an average diameter of 0.035 μm.

Thylakoids were often seen in the peripheral area of the cytoplasm running parallel to the plasma membrane. The thylakoid vesicles appeared tripartite, having a typical "unit membrane" structure. The membranes are usually closely opposed to each other in the flattened vesicles, without an internal lumen (Fig. 6). When cells were subjected to pH 9, however, intrathylakoid spaces were observed in 37% control cells and 57% cells exposed to 0.08 μM Cd^{2+} (Fig. 9, 10). The thylakoid membranes in cells exposed to 0.8 μM Cd^{2+} at pH 5.5 or 9 appeared damaged (Fig. 6, 11, 13). The nucleoplasm was more electron transparent than the rest of the cytoplasm, with fibrils of nucleic acids as shown in Fig. 3. In cells exposed to Cd, the nucleoplasm diminshed. The deterioration of cellular structures was observed in the cells exposed to Cd^{2+} especially at pH 5.5 (Fig. 6) and pH 9 (Fig. 11).

A summary of the morphometric data of the cells exposed to selected Cd^{2+} concentrations at different pH's is given in Table 4. The thylakoid Sv reported in the table was the thylakoid surface area / cell volume ratio. The cell wall Vv, polyphosphate body Vv, polyhedral body Vv and nucleoplasm Vv were reported as % cell volume.

Effect of pH on the cell ultrastructures:

Single classification ANOVA was applied to study the effect of pH on the cellular structures. The morphometric data were obtained from 90 sections of cells as 30 from each of the three pH's. The result showed pH caused a variation in cells for only polyhedral Vv ($P<0.05$) (Table 5). Further analysis by Fisher's LSD pairwise tests showed that the polyhedral body Vv decreased significantly ($P<0.05$) when the cells grew at pH 9 or pH 5.5 compared to the cells subjected to pH 7.2.

Effects of Cd on cell ultrastructures at different pH values:

The morphometric data obtained from the cells exposed to selected Cd^{2+} concentrations at pH 7.2 were analyzed by single classification ANOVA (Table 7). The analysis showed Cd^{2+} caused an overall significant change in the PPB's Vv ($P<0.05$). The Fisher's LSD tests demonstrated that the PPB's Vv increased ($P<0.05$) in cells grown at 0.8 μM Cd^{2+} compared to those of control cells and cells exposed to 0.08 μM Cd^{2+} at this pH (Fig. 18).

At alkaline pH, Cd^{2+} was responsible for a significant difference in nucleoplasm Vv ($P<0.001$), cell wall Vv ($P<0.01$) and polyhedral body Vv ($P<0.05$) analyzed by single classification ANOVA (Table 8). The Fisher's LSD tests gave the following information. The nucleoplasm Vv of cells exposed to 0.8 μM Cd^{2+} decreased ($P<0.05$) compared to cells exposed to 0.08 μM Cd^{2+}; and both were significantly smaller than that of control cells ($P<0.05$) (Fig. 16). At 0.8 μM Cd^{2+} caused a significant increase ($P<0.05$) in polyhedral body Vv compared to that of control cells or cells exposed to 0.08 μM Cd^{2+} (Fig. 19). At 0.8 μM Cd^{2+} also caused an significant increase of cell wall Vv (Fig. 15) compared to control cells ($P<0.05$).

When the pH was 5.5, Cd^{2+} accounted for significant changes in the nucleoplasm Vv ($P<0.01$) and cell wall Vv ($P<0.05$) (Table 6). The Fisher's LSD tests showed that the nucleoplasm Vv decreased significantly when cells were exposed to 0.8 μM Cd compared to control cells (Fig. 16). Cell wall Vv of cells exposed to 0.08 μM Cd increased significantly compared to control cells (Fig. 15).

Evaluation of Cd and pH interactions on cellular ultrastructures:

Subsequent two-way ANOVA's were applied to assess the source of variation (Table 9). The data were pooled from 270 cells, as 30 cells from each of

Table 4: Summary of the morphometric data of *synechococcus leopoliensis* cells exposed to selected concentrations of Cd^{2+} at different pH's. Values reported are means (standard error in parenthesis) of a sample size of 30 sections of cells.

	pH 5.5			pH 7.2			pH 9		
$[Cd^{2+}]$ µM	0	0.08	0.8	0	0.08	0.8	0	0.08	0.8
cell volume	29.60 (2.34)	35.60 (2.66)	32.37 (2.75)	30.50 (2.09)	36.30 (2.60)	36.70 (3.16)	36.17 (2.60)	40.40 (2.87)	41.87 (4.74)
cell wall Vv	8.121 (1.04)	12.83 (1.44)	9.06 (1.12)	10.27 (1.19)	7.90 (0.77)	10.32 (0.98)	9.34 (0.84)	6.54 (0.60)	11.09 (1.21)
nucleoplasm Vv	5.71 (1.19)	4.03 (1.25)	1.11 (0.57)	7.68 (0.92)	7.14 (0.72)	5.91 (0.68)	8.00 (0.95)	6.21 (1.03)	2.45 (0.56)
PPB's body Vv	1.18 (0.47)	0.51 (0.19)	0.87 (0.35)	0.22 (0.13)	0.13 (0.13)	1.02 (0.42)	0.55 (0.19)	0.56 (0.32)	1.44 (0.59)
polyhedral body Vv	1.01 (0.34)	1.39 (0.36)	1.60 (0.32)	1.98 (0.44)	2.46 (0.60)	1.01 (0.34)	0.78 (0.24)	2.06 (0.48)	2.19 (0.47)
intrathylakoid Vv	—	—	—	—	—	—	1.48 (0.45)	1.93 (0.58)	—
thylakoid Sv	21.17 (3.25)	29.13 (2.26)	28.87 (2.87)	27.22 (2.61)	25.24 (2.23)	20.22 (2.21)	18.99 (2.54)	17.75 (2.26)	16.91 (2.29)

Table 5: Comparison of cell volume, thylakoid surface area and relative volumes (Vv) of other cellular components among cells of *S. leopoliensis* subjected to different pH's, by single classification ANOVA. Values reported are the means from 30 cells.

	pH5.5	pH 7.2	pH 9	ANOVA df	F	P
cell volume	29.60	30.50	36.17	2	2.29	0.11
polyhedral body Vv	1.00	1.98	0.78	2	3.39	0.038*
PPB's Vv	1.18	0.22	0.55	2	2.57	0.082
thylakoid Sv	21.17	27.22	18.99	2	2.29	0.11
cell wall Vv	8.12	10.26	9.34	2	1.09	0.34
nucleoplasm Vv	5.71	7.68	8.00	2	1.46	0.24

* = $0.01 < P \leq 0.05$

Table 6: Comparison of cell volume, thylakoid surface area and relative volumes (Vv) of other cellular components among the cells *S. leopoliensis* exposed to selected Cd^{2+} concentration at pH 5.5, by single classification ANOVA. Values reported are the means obtained from 30 cells.

	Cd^{2+} (μM)			ANOVA		
	0	0.08	0.8	df	F	P
cell volume	29.60	35.60	32.37	2	1.34	0.027
polyhedral body Vv	1.00	1.39	1.60	2	0.80	0.45
PPB's Vv	1.18	0.51	0.86	2	0.88	0.42
thylakoid Sv	21.17	29.13	28.87	2	2.57	0.083
cell wall Vv	8.12	12.83	9.06	2	4.24	0.018*
nucleoplasm Vv	5.71	4.03	1.11	2	4.91	0.0095**

* = $0.01 < P \leq 0.05$
** = $0.001 < P \leq 0.01$

Table 7: Comparison of cell volume, thylakoid surface area and relative volumes (Vv) of other cellular components among the cells *S. leopoliensis* exposed to selected Cd^{2+} concentration at pH 7.2, by single classification ANOVA. Values reported are the means obtained from 30 cells.

	Cd^{2+} (μM)			ANOVA		
	0	0.08	0.8	df	F	P
cell volume	30.50	36.30	36.70	2	1.71	0.19
polyhedral body Vv	1.98	2.46	1.01	2	2.48	0.09
PPB's Vv	0.22	0.13	1.02	2	3.49	0.035*
thylakoid Sv	27.22	25.24	20.22	2	2.34	0.10
cell wall Vv	10.32	7.90	10.32	2	1.94	0.15
nucleoplasm Vv	7.68	7.14	5.91	2	1.35	0.26

* = $0.01 < P \leq 0.05$

Table 8: Comparison of cell volume, thylakoid surface area and relative volumes (Vv) of other cellular components among the cells *S. leopoliensis* exposed to selected Cd^{2+} concentrations at pH 9, by single classification ANOVA. Values reported are the means obtained from 30 cells.

	Cd^{2+} (μM)			ANOVA		
	0	0.08	0.8	df	F	P
cell volume	36.17	40.40	41.87	2	0.71	0.50
polyhedral body Vv	0.78	2.06	2.19	2	3.61	0.031*
PPB's Vv	0.55	0.56	1.43	2	1.59	0.21
thylakoid Sv	18.99	17.75	16.90	2	0.20	0.82
cell wall Vv	9.34	6.54	11.08	2	6.25	0.029*
nucleoplasm Vv	8.00	6.21	2.45	2	10.54	0.0001***

* = $0.01 < P \leq 0.05$

*** = $P \leq 0.001$

Table 9: Two way ANOVA results for *S. leopoliensis* cells subjected to selected Cd concentrations and 3 different pH's.

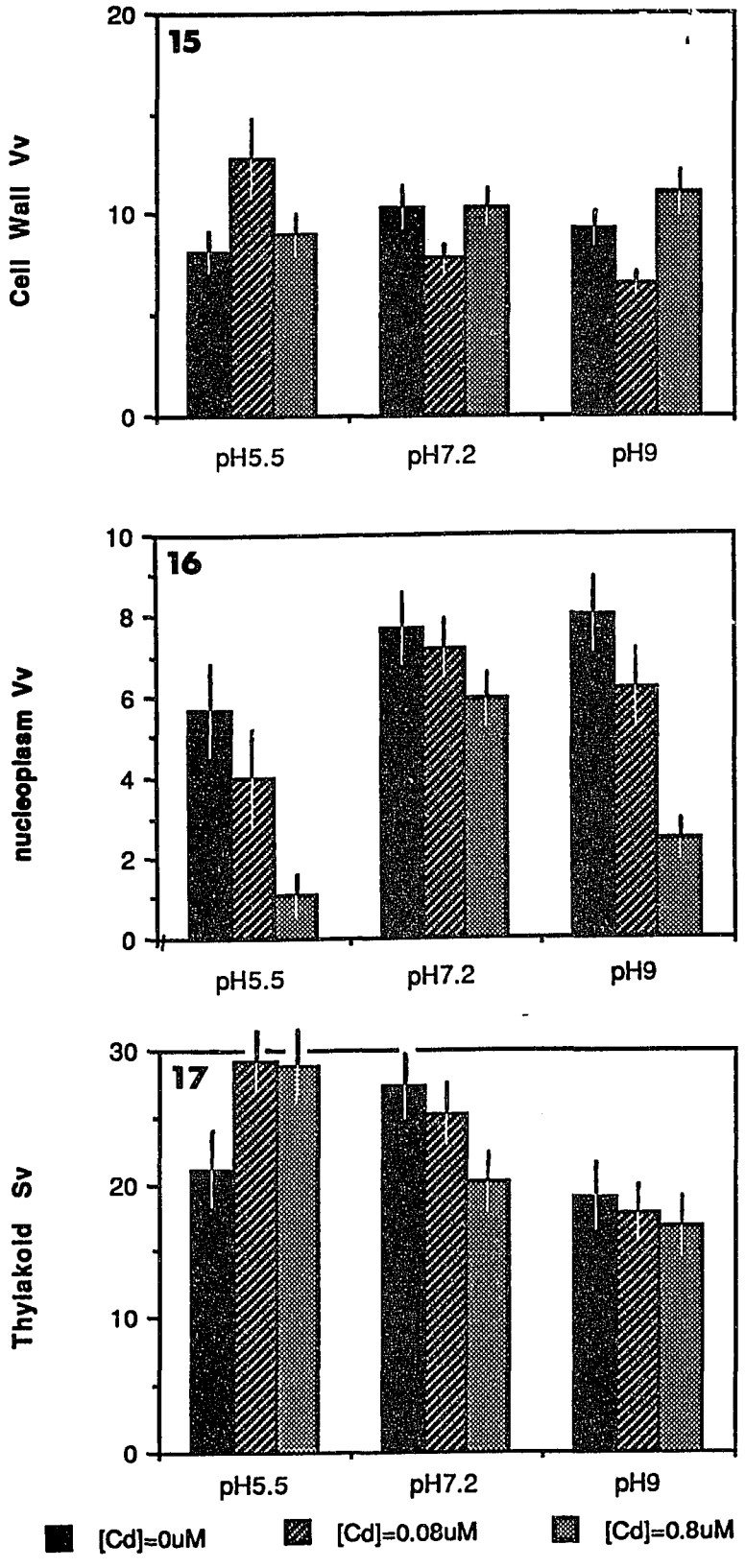
Variable	Source	df	SS	F	P
cell volume	$[Cd^{2+}]$	2	1580	3.01	0.051
	pH	2	2312	4.41	0.013*
	$[Cd^{2+}]$ x pH	4	209	0.20	0.939
polyhedral	$[Cd^{2+}]$	2	23	2.29	0.104
body Vv	pH	2	11	1.11	0.331
	$[Cd^{2+}]$ x pH	4	52	2.57	0.039*
PPB's Vv	$[Cd^{2+}]$	2	23	3.25	0.040*
	pH	2	9	1.28	0.279
	$[Cd^{2+}]$ x pH	4	14	0.94	0.441
thylakoid	$[Cd^{2+}]$	2	206	0.54	0.583
Sv	pH	2	3518	9.21	0.0001***
	$[Cd^{2+}]$ x pH	4	1866	2.44	0.047*
cell wall Vv	$[Cd^{2+}]$	2	60	0.91	0.403
	pH	2	46	0.70	0.496
	$[Cd^{2+}]$ x pH	4	742	5.63	0.0002***
nucleoplasm Vv	$[Cd^{2+}]$	2	737	14.9	0.0001***
	pH	2	494	9.96	0.0001***
	$[Cd^{2+}]$ x pH	4	120	1.21	0.307

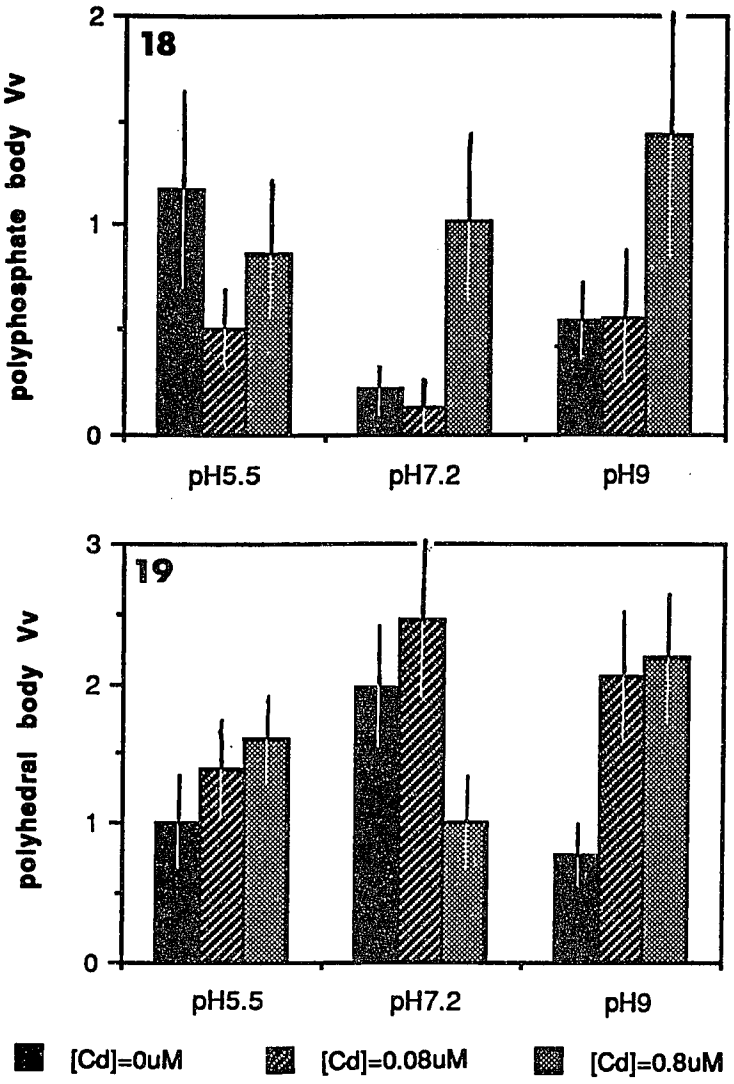
* = $0.01 < P \leq 0.05$

** = $0.001 < P \leq 0.01$

*** = $P \leq 0.001$

Figures 15-17. Bar graphs showing mean values of relative volumes of cell wall (Fig. 15), nucleoplasm (Fig. 16), and relative surface area of thylakoids (Fig. 17) of both the control and Cd^{2+} exposed cells at different pH's. Standard errors ($\pm$1 S.E.) are indicated by the bar markers.


15
Cell Wall Vv
20
10
0
pH5.5
pH7.2
pH9
16
nucleoplasm Vv
10
8
6
4
2
0
pH5.5
pH7.2
pH9
17
Thylakoid Sv
30
20
10
0
pH5.5
pH7.2
pH9
[Cd]=0uM
[Cd]=0.08uM
[Cd]=0.8uM

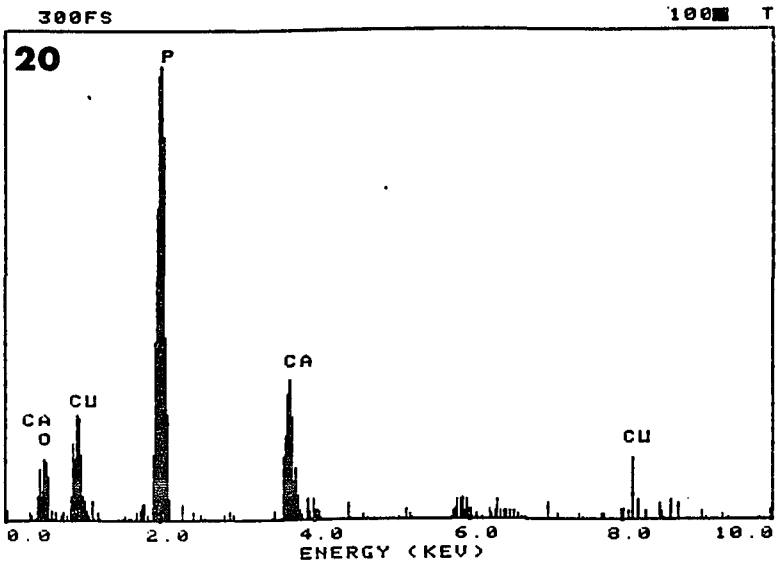


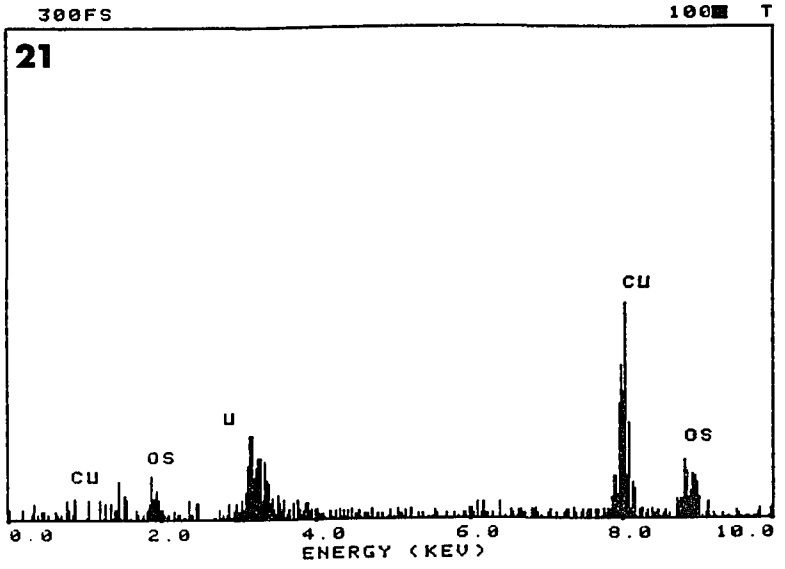
Figures 18-19. Bar graphs showing mean values of relative volumes of polyphosphate bodies (Fig. 18) and polyhedral bodies (Fig. 19) of both the control and Cd^{2+} exposed cells at different pH's. Standard errors ($\pm$1 S.E.) are indicated by the bar markers.

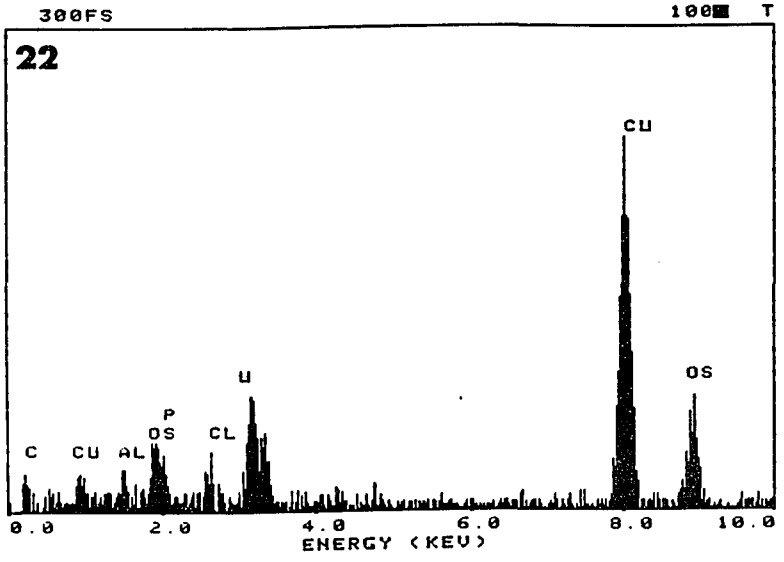
Figure 20. Spectrum of a cytoplasmic polyphosphate body of a sectioned cell exposed to 0.8 μM Cd^{2+} at pH 5.5. P and Ca are typical peaks. Other peaks, O and Cu, are shown also.

Figure 21. Spectrum of a polyhedral body of a sectioned cell exposed to 0.8 μM Cd^{2+} at pH 5.5. Identifiable peaks are Os, U, and Cu, elements from sample preparation.

Figure 22. Spectrum of the cytoplasm of a sectioned cell exposed to 0.8 μM Cd^{2+} at pH 5.5. Identifiable peaks are C, P, Al, U, Os and Cu.


300FS
100
T
20
P
CA
CU
CA
O
CA
CU
0.0
2.0
4.0
6.0
8.0
10.0
ENERGY (KEV)


300FS
100
T
21
CU
U
OS
CU
OS
0.0
2.0
4.0
6.0
8.0
10.0
ENERGY (KEV)


300FS
100
T
22
CU
U
OS
P
OS
CL
C
CU
AL
0.0
2.0
4.0
6.0
8.0
10.0
ENERGY (KEV)

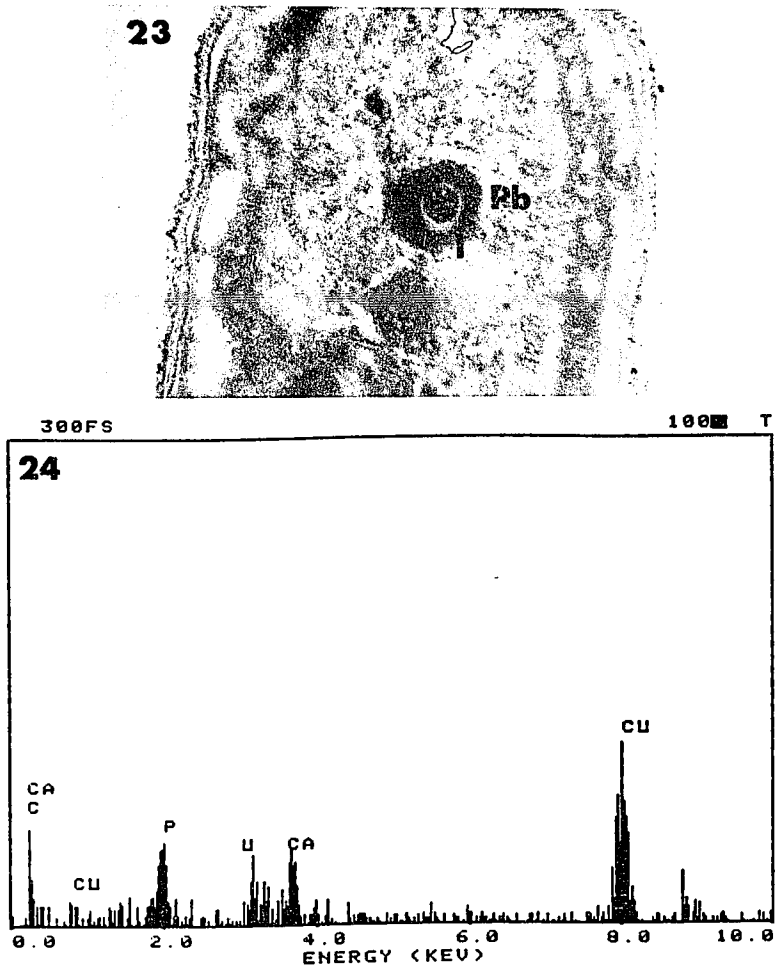


Figure 23. Micrograph of a section of a portion of a cell exposed to 0.8 μM Cd^{2+} at pH5.5. A porous inclusion (I) is shown inside a polyhedral body (Pb).
X 74,000

Figure 24. Spectrum of the inclusion in the polyhedral body shown in Fig. 23. Typical peaks of polyphosphate bodies, P and Ca, are shown. Other identifiable peaks are C, U, Os and Cu.

the 9 combination of pH and Cd^{2+} concentrations. Among these morphometric variables, the cell volume was influenced more by pH ($P<0.05$) than by Cd^{2+} concentrations. The nucleoplasm Vv was influenced by both pH and Cd^{2+} concentrations at the same significant level ($P<0.001$) without interaction between the two. The presence of Cd^{2+} resulted in a decrease of the nucleoplasm Vv at any pH (Fig. 16). Variation in the PPB's Vv was primarily attributed to Cd^{2+} ($P<0.05$). Interactions of pH and Cd^{2+} concentrations, however, were present in the PPB's Vv ($P<0.001$), the thylakoid Sv ($P<0.05$) and the cell wall Vv ($P<0.001$). For example, the presence of Cd^{2+} with pH 7.2 or pH 9 resulted in a decrease of thylakoid Sv rather than an increase as with pH 5.5 (Fig. 17).

Elemental study of cellular inclusions

Cells exposed to 0.8 μM Cd^{2+} at pH 5.5 were sectioned and observed in the STEM mode of the TEM. Both PPB's and polyhedral bodies were very evident. The inclusions inside polyhedral bodies (Fig. 23), however, were barely visible in the STEM mode. The diameters of the inclusions were about 0.035 μm and only slightly larger than a ribosome, which is about 20 nm in diameter. They were located under the TEM before switching to STEM mode. The PPB's, polyhedral bodies and inclusions inside polyhedral bodies were analyzed with EDX.

All spectra of the cytoplasmic PPB's (Fig. 20) and inclusions within polyhedral bodies (Fig. 24) had identifiable P and Ca peaks, as well as Cu, a spectral contaminant from copper grids. The bodies within polyhedral bodies also had Os and U in their spectra as did polyhedral bodies, the portion of them not occupied by the inclusions (Fig. 21). Both Os and U were spectral contaminants from fixation and post staining. When the probe was placed in

the cytoplasm, the spectra generated had small peaks of S, P, C, O, Al, Cu, Os, and U (Fig. 22). None of them generated identifiable Cd peaks. These observations show that these inclusions possess large amounts of the elements P and Ca which is consistent with those in PPB's.

Use of EDX to study Cd uptake

Figure 25 shows the image of an air dried *S. leopoliensis* cell under the TEM. The cells were about 3 μm long and 0.8 μm wide. Each cell could be seen to have about 5-10 electron dense PPB's, which ranged from 0.1-0.04 μm. Little internal organization of cells other than the PPB's could be observed.

The PPB's were also very evident when the cells were examined in the STEM mode. The EDX analysis showed that all PPB's in control cells had peaks of O, P, K, and Mg (Fig. 26). Half of the bodies gave a very small Ca peak and one out of ten had Fe or Na. Analysis of the cytoplasm revealed (Fig. 27) a prominent peak of C, smaller ones of P, S, K, Mg, O, and also a small peak of Ca sometimes. The spectra generated from the cell walls varied; some of them were similar to that of cytoplasm except that the peaks were smaller, and some only had the peak of C, and small peaks of K, or Ca (Fig. 28).

Analysis of the cells exposed to 8 μM Cd^{2+} showed no distinctive difference in the spectra of the PPB's, cytoplasm and cell walls from those of the control cells. There was no Cd detected in any of the analyzed area in the cells.

In the cells exposed to 80 μM Cd^{2+}, no Cd could be detected in their PPB's. However, the cytoplasm was found to give a small Cd peak (Fig. 29) and also generated a higher S peak so that its S/P count was greater than that of control cells or cells exposed to 8 μM Cd^{2+}. The cytoplasm also gave a Si peak. In about 4 out of 10 cells Cd was detected in the cell wall and the spectra had P and S peaks as in those of the cytoplasm (Fig. 30). In the spectra of the cell

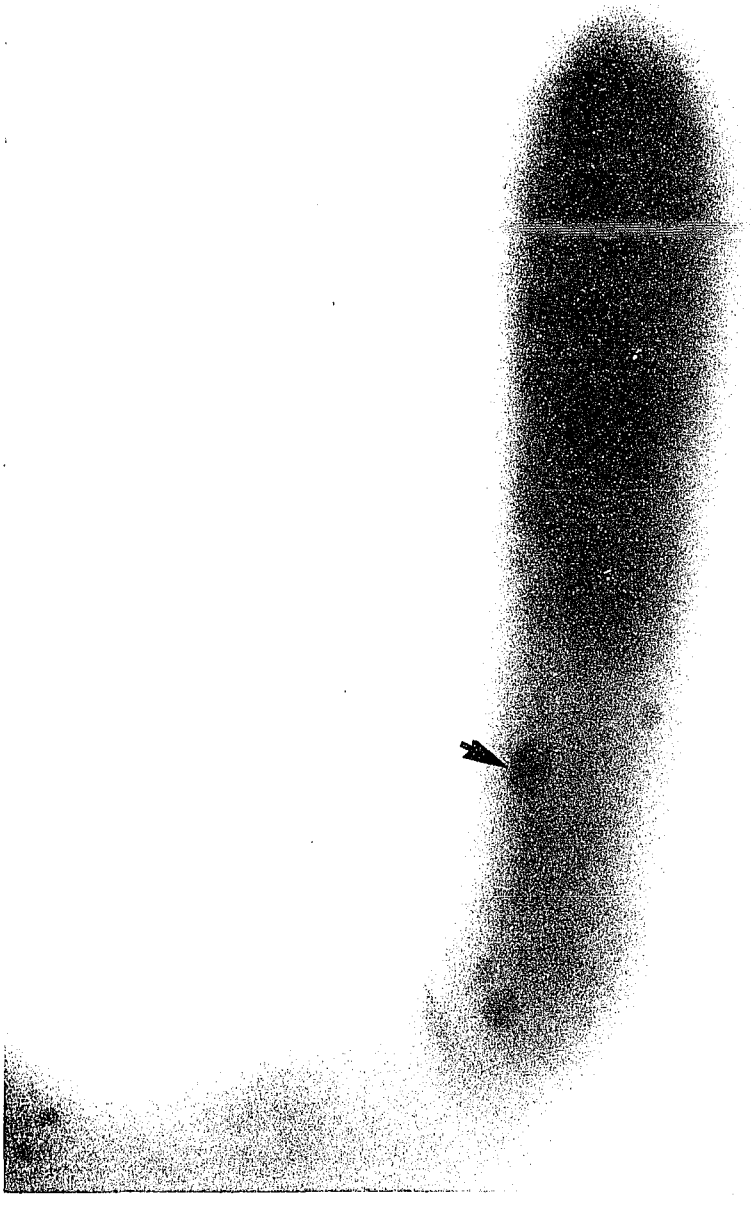
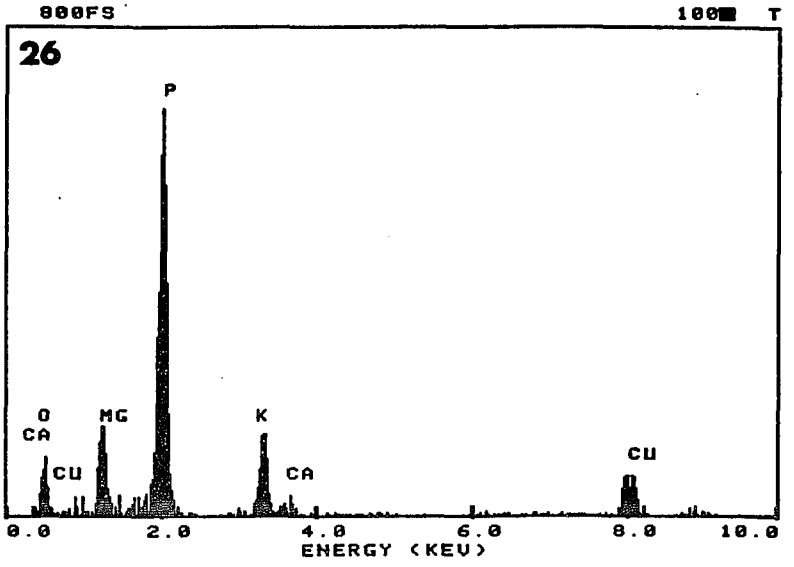


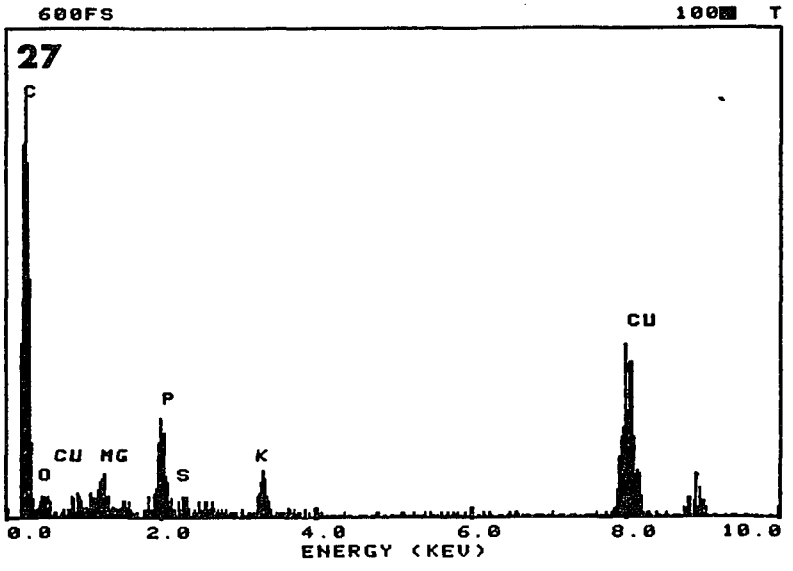
Figure 25. TEM image of an air dried *S. leopoliensis* cell. All the dense bodies in the cell are polyphosphate bodies (arrow). X 47,000.

Figure 26. Spectrum of a polyphosphate body of an air dried control cell. Identifiable peaks are O, Mg, P, K, Ca and Cu.

Figure 27. Spectrum of the cytoplasm of an air dried control cell. Identifiable peaks are C, O, Mg, P, K, S, and Cu.

Figure 28. Spectrum of the cell wall of an air dried control cell. Identifiable peaks are C, K and Cu.


800FS
100 T
26
P
O
CA
MG
K
CU
CA
CU
0.0
2.0
4.0
6.0
8.0
10.0
ENERGY (KEV)


600FS
100 T
27
C
CU
P
CU MG
K
O
S
0.0
2.0
4.0
6.0
8.0
10.0
ENERGY (KEV)

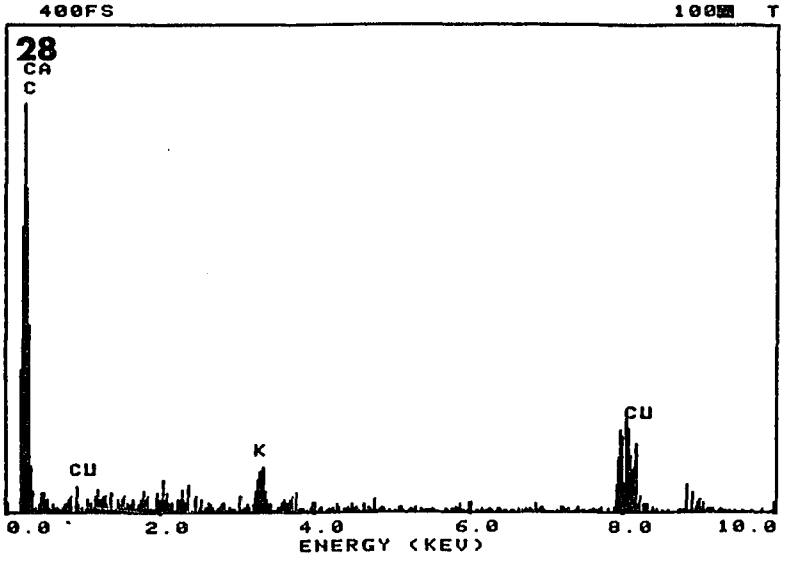
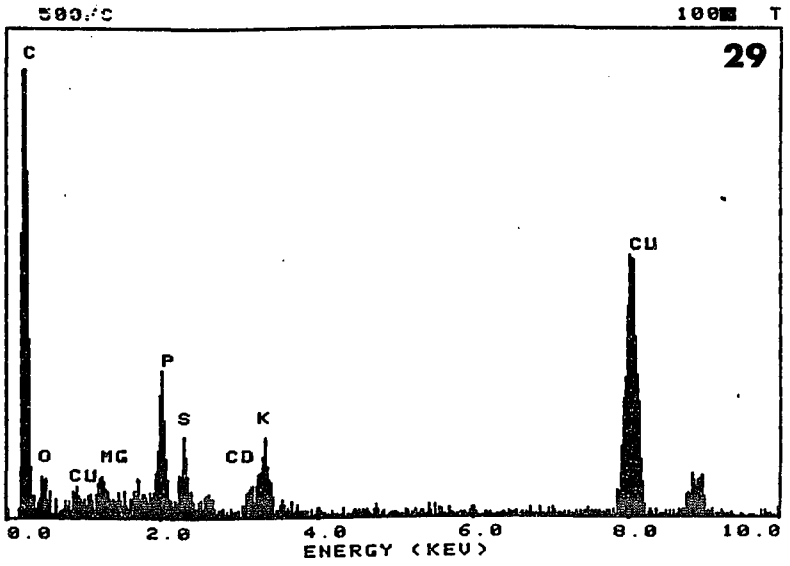
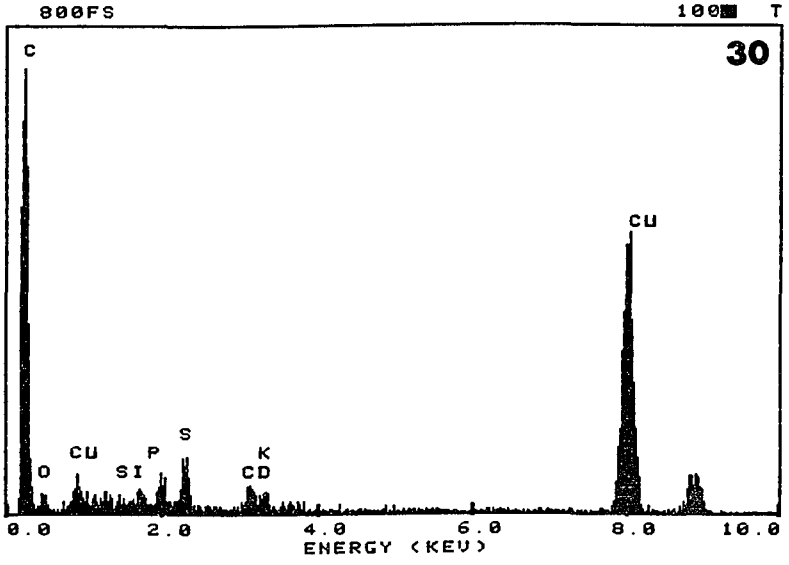

400FS
100 T
28
CA
C
CU
K
CU
0.0
2.0
4.0
6.0
8.0
10.0
ENERGY (KEV)

Figure 29. Spectrum of the cytoplasm of an air dried cell exposed to 80 μM Cd^{2+}. Note the Cd peak and a more prominent S peak in the cell compared to the control cell.

Figure 30. Spectrum of the cell wall of an air dried cell exposed to 80 μM Cd^{2+}. Note the Cd peak and Si peaks. Other identifiable peaks are C, O, P, S, K, and Cu.

Figure 31. Spectrum of the cell wall of an air dried cell exposed to 80 μM Cd^{2+}. Note no Cd peak but there is a prominent Si peak. Other identifiable peaks are C, O, K, Cu.


500FS
100 T
29
C
CU
P
S
K
O
MG
CU
CD
0.0
2.0
4.0
6.0
8.0
10.0
ENERGY (KEV)


800FS
100 T
30
C
CU
S
CU
P
K
O
SI
CD
0.0
2.0
4.0
6.0
8.0
10.0
ENERGY (KEV)

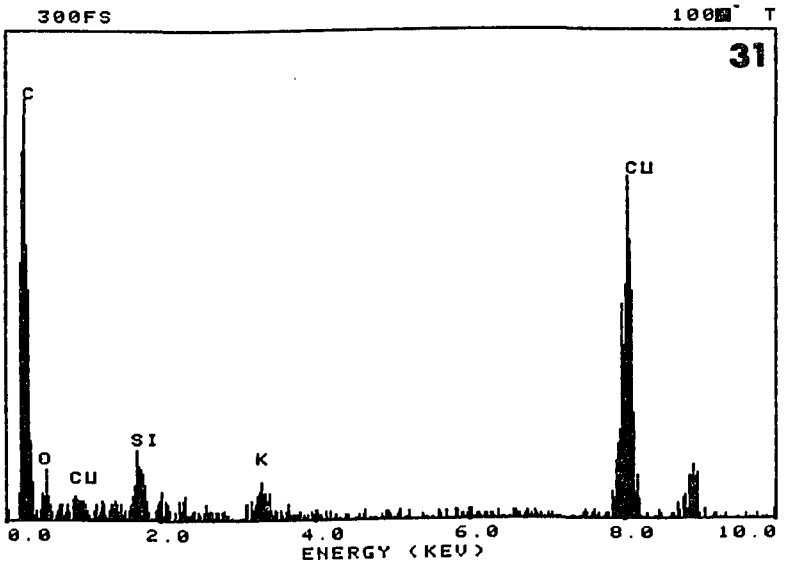
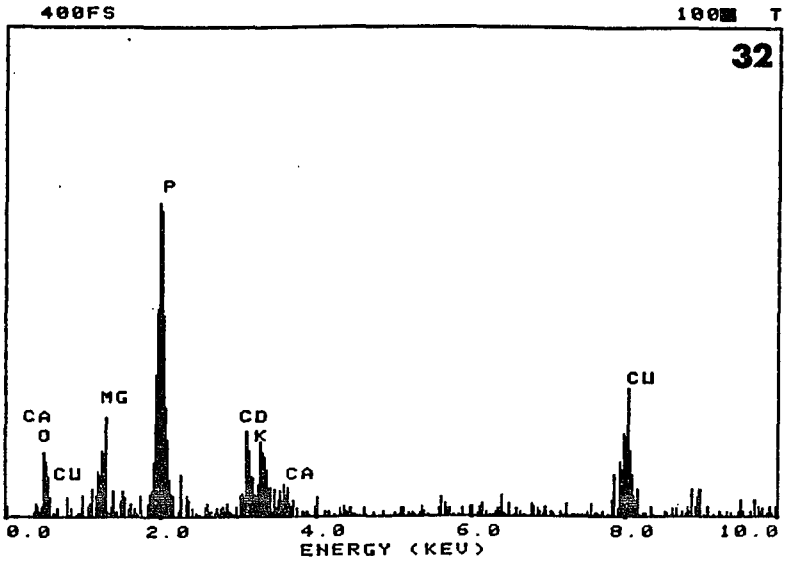
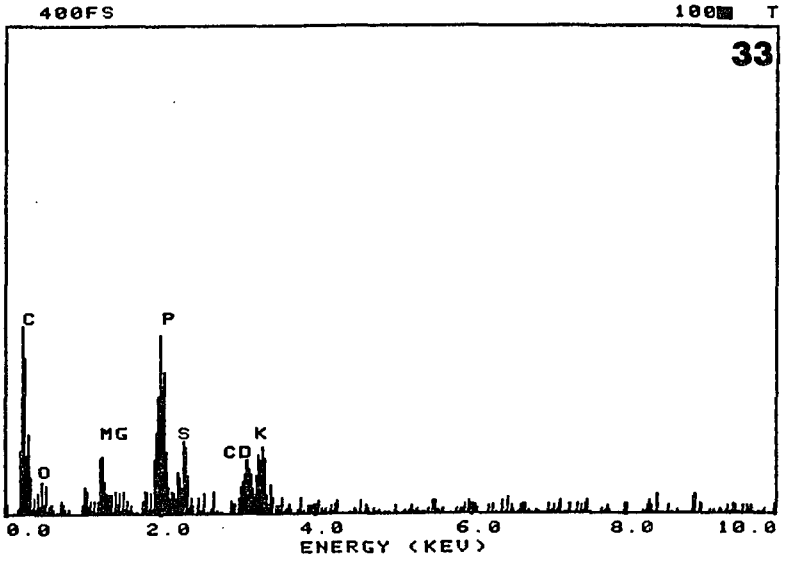

300FS
100 T
31
C
CU
SI
K
O
CU
0.0
2.0
4.0
6.0
8.0
10.0
ENERGY (KEV)

Figure 32. Spectrum of a polyphosphate body of an air dried cell exposed to 200 μM Cd^{2+}. Note the Cd peak.

Figure 33. Spectrum of the cytoplasm of an air dried cell exposed to 200 μM Cd^{2+}. Note the Cd peak.

Figure 34. Spectrum of the cell wall of an air dried cell exposed to 200 μM Cd^{2+}. Note the Cd and Si peaks


400FS
100
T
32
P
CA
O
CU
MG
CD
K
CA
CU
0.0
2.0
4.0
6.0
8.0
10.0
ENERGY (KEV)


400FS
100
T
33
C
P
O
MG
S
CD
K
0.0
2.0
4.0
6.0
8.0
10.0
ENERGY (KEV)

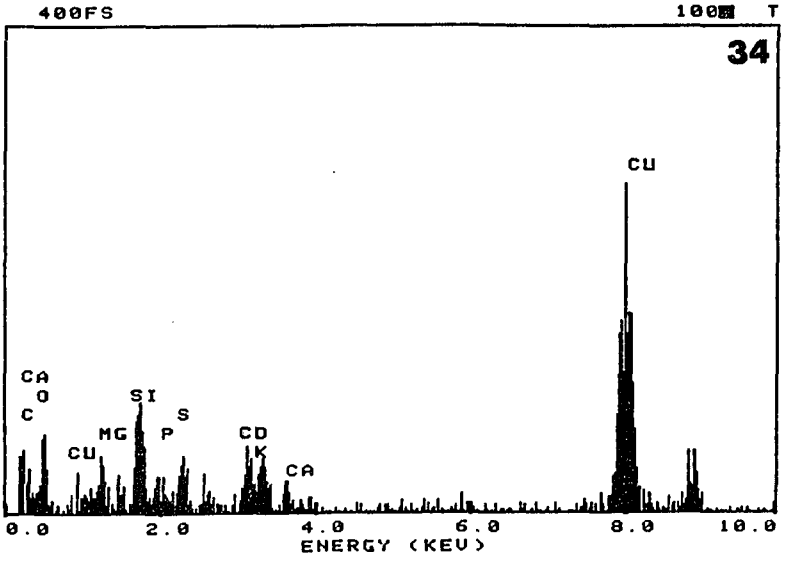
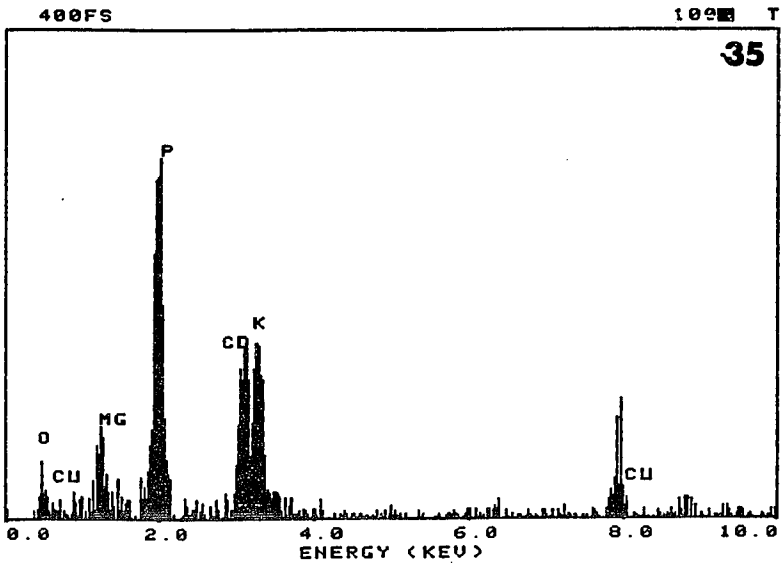
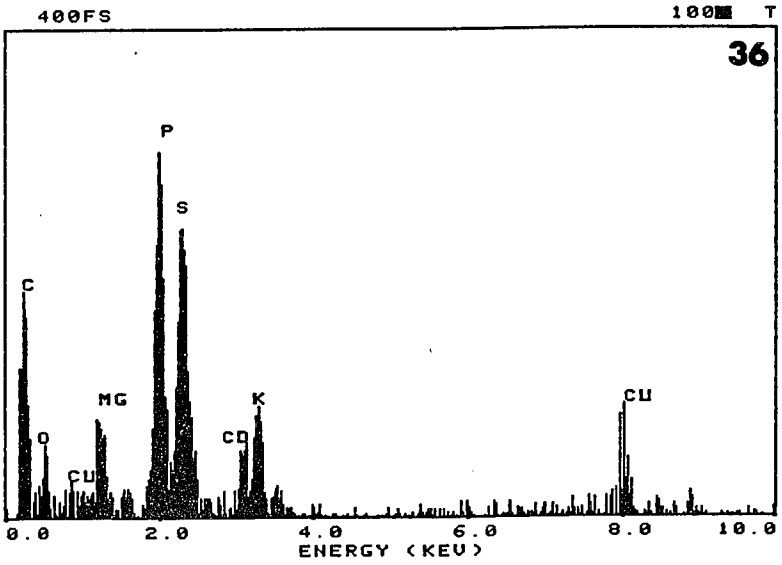

400FS
100
T
34
CU
CA
O
C
SI
MG
CU
P
S
CD
K
CA
0.0
2.0
4.0
6.0
8.0
10.0
ENERGY (KEV)

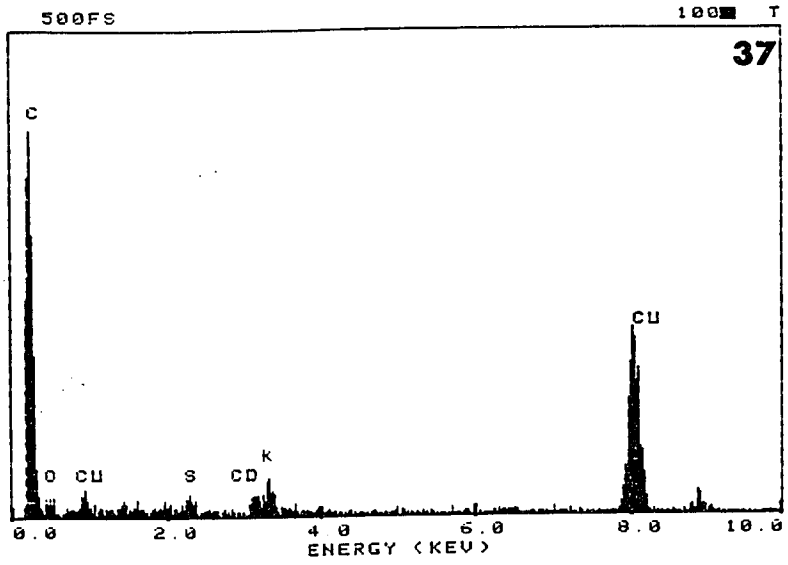
Figure 35. Spectrum of a polyphosphate body of an air dried cell exposed to 600 μM Cd^{2+}. Note the prominent Cd peak.

Figure 36. Spectrum of the cytoplasm of an air dried cell exposed to 600 μM Cd^{2+}. Note the Cd peak and prominent S peak.

Figure 37. Spectrum of the cell wall of an air dried cell exposed to 600 μM Cd^{2+}. Note the Cd peak.


400FS
T
35
P
K
CD
MG
O
CU
CU
0.0
2.0
4.0
6.0
8.0
10.0
ENERGY (KEV)


400FS
T
36
P
S
C
MG
K
CU
O
CD
CU
0.0
2.0
4.0
6.0
8.0
10.0
ENERGY (KEV)


500FS
T
37
C
CU
K
O
CU
S
CD
0.0
2.0
4.0
6.0
8.0
10.0
ENERGY (KEV)

walls, the S peaks were higher than the P peaks. In the other cells, the cell wall gave no Cd peak but gave the peaks of only C, O, K and Si (Fig. 31).

In about half of the cells exposed to 200 μM Cd^{2+}, Cd was detected in their PPB's (Fig. 32). In about 50% of the cells, Cd was also detected in the cytoplasm (Fig. 33), but not necessarily the same cells where Cd was detected in their PPB's. In about 20% of the cells, Cd was detected in their cell wall (Fig. 34). A large Si peak was generated by the cell wall also.

In every cell exposed to 600 μM Cd^{2+}, all of the PPB's gave prominent Cd peaks (Fig. 35). In 40% of the bodies, the height of Cd peaks exceeded the K peaks. The cytoplasm (Fig. 36) and cell wall (Fig. 37) gave smaller Cd peaks in contrast to those the PPB's generated. The cytoplasm also gave a prominent S peak.

When cells were exposed to 600 μM Cd^{2+} at 4° C in the dark, the PPB's, the cytoplasm and the cell wall gave similar spectra as those cells treated with the same concentration but at the room temperature.

Effect of Cd^{2+} on protein patterns

Upon visual inspection of the gel prepared from the total protein extracts of the cells exposed to 25 μM, 50 μM and 100 μM Cd^{2+}, approximately 4 polypeptides with apparent molecular masses of 44,000 dalton (44 K), 23 K, 20 K, and 15 K appeared to be enhanced in amount. Except the polypeptide 15 K, which was present in significant amount in the control cells, other polypeptides, 44 K, 23 K, and 20 K were minimal in the control. The enhancement of polypeptides 23 K, 20 K and 15 K appeared dose-dependent (Fig. 38C).

The gel prepared from the cytoplasmic fraction (Fig. 38B) showed an apparent enhancement of polypeptides 74 K, 44 K, 23 K and 20 K in the exposed cells. There were almost no polypeptides, 74 K and 44 K, shown in the

gel of the control. The polypeptides 23K and 20K were present in significant amount in the control cells, but enhanced slightly in the exposed cells; their enhancement was also dose-dependent. However, a polypeptide of 41 K found in the control cells was lost in the exposed cells. Another two polypeptides, 33 K and 70 K was present in lesser amount in the exposed cells when compared to that in the control cells. As shown in the gel prepared from the membrane fraction, the polypeptides 42 K and 20 K were also enhanced by Cd^{2+} exposure; the latter was also dose dependent (Fig. 38A). Both were minimal in the control cells.

The gel prepared from the cell envelope fraction showed that the polypeptides of 70 K, 60 K, 57 K, 20 K and 15 K were enhanced in amount because of Cd^{2+} exposure (Fig. 39). All were minimal in the control cells and increased in amount tremendously in the exposed cells, especially the polypeptides 70 K, 15 K, and 20 K.

A total of 9 polypeptides were enhanced due to Cd^{2+} exposure. Most of these polypeptides were present in the cell envelope fraction rather than in the other two fractions. Although the enhanced polypeptide 20 K was found in every fraction, its enhancement was more significant in the membrane and envelope fractions than in the cytoplasmic fraction.

Study of the broken cell fraction with EM

Under the TEM, the sonicated suspension after being air-dried was found to have a few unbroken cells and also cellular remains from broken cells, and clusters of PPB's (Fig. 42). When studied with the EDX, the PPB's gave spectra (Fig. 43), which had peaks O, Si, P, K, Ca, Fe, and Cu. Since the sample was obtained from cells exposed to Cd^{2+} for 2 days, the PPB's also gave a very prominent Cd peak. The P peak was still the largest. The Ca peak was

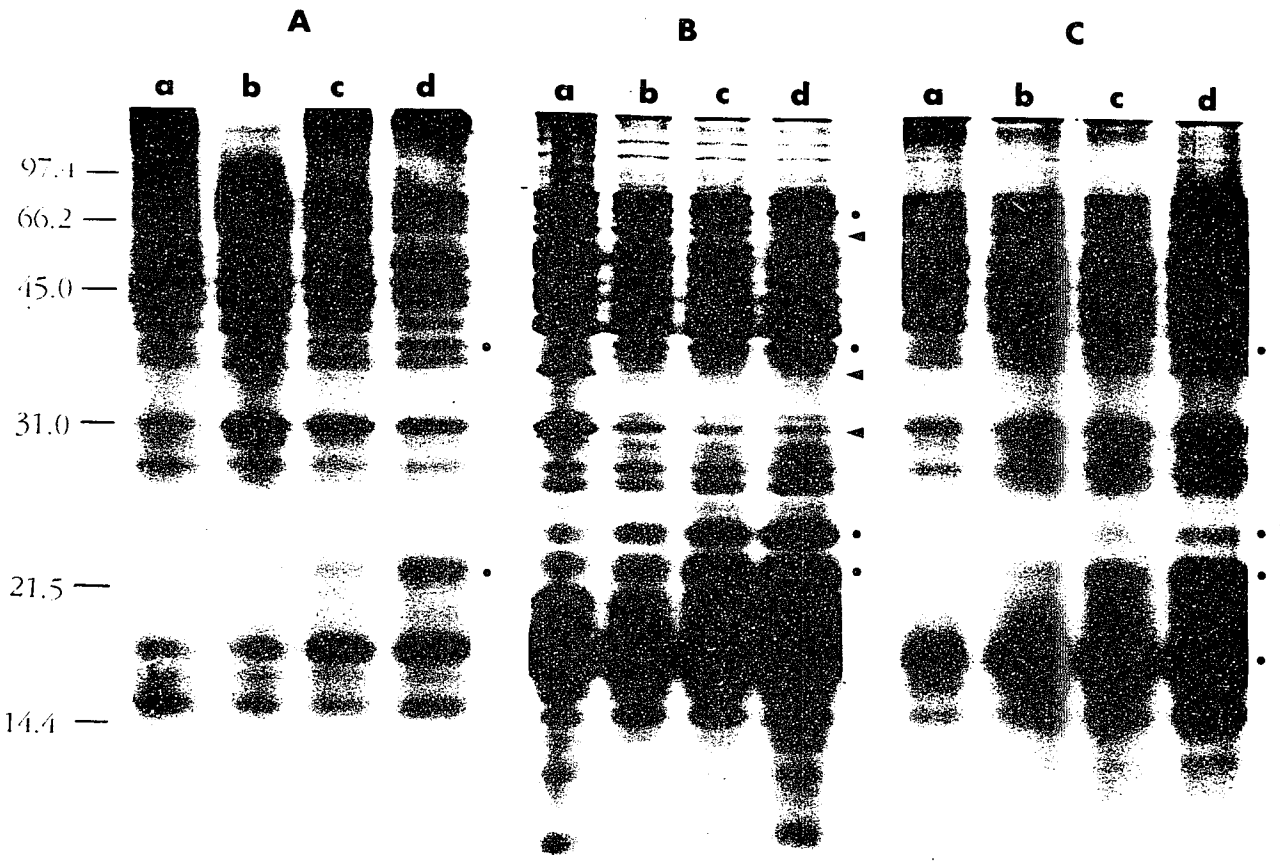
significantly enhanced in contrast to that of air-dried cells. The Fe and Si peaks, due to their presence in the Tris-HCl buffer used to wash the cells, were present in the spectrum of every PPB.

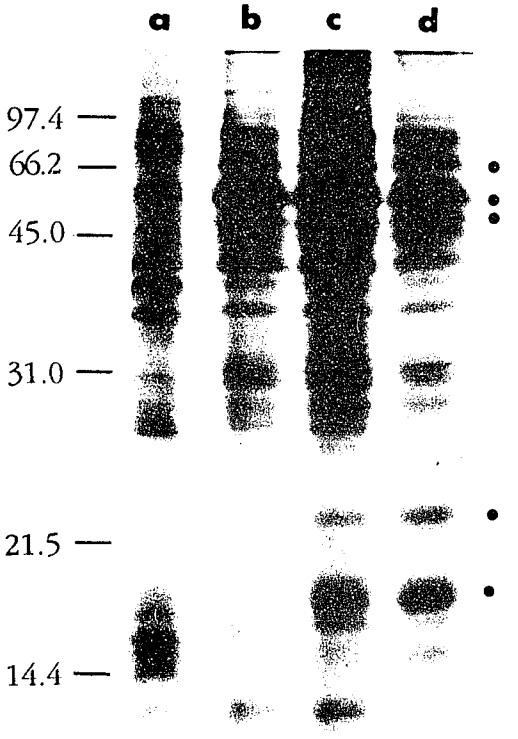
When studied under the TEM, the unbroken cell fraction (Fig. 2) was found to contain intact or partially broken cells. There were many PPB's released from broken cells in this fraction also. As shown in figure 40, an unbroken cell was surrounded by the free PPB's. The electron dense PPB's were also proved heavier than polyhedral bodies and other cellular inclusions since the PPB's were found to mix with unbroken cells in this fraction. When these PPB's were studied with the EDX system, they generated spectra having peaks of O, P, Ca, Fe as shown in figure 41. U and Cu were spectra contaminants from poststaining and the copper grid.

The cell free extract was studied under the TEM. There were fragments of the cell wall and membranes, and also other cellular remains (Fig. 44). Most of the membrane fragments, such as the fragments of thylakoid membranes, appeared circular. The cell wall fragment sometimes adhered to another wall fragment. Polyhedral bodies were found in this fraction also (Fig. 45).

Under the TEM, the cell envelope fraction was found to contain cell wall fragments (Fig. 46) and many aggregates of cellular debris. This fraction also had a few large pieces of membrane. The membrane fraction contained mainly membrane fragments (Fig. 47) and also a few tiny wall fragments.

Figures 38. The SDS-PAGE gels showing the protein patterns of the memnrane fraction (A), the cytoplasmic fraction (B), and the total protein fraction (C). The samples were from (a) control cells, and cells exposed to (b) 25 μM Cd^{2+}, (c) 50 μM Cd^{2+}, and (d) 100 μM Cd^{2+}. Dots indicate polypeptides that are enhanced in amount in the Cd^{2+} treated. Arrows indicate polypeptides that are reduced in amount in the Cd^{2+} treated. The position of molecular markers are indicated in kilodaltons.


A
B
C
a
b
c
d
97.4
66.2
45.0
31.0
21.5
14.4



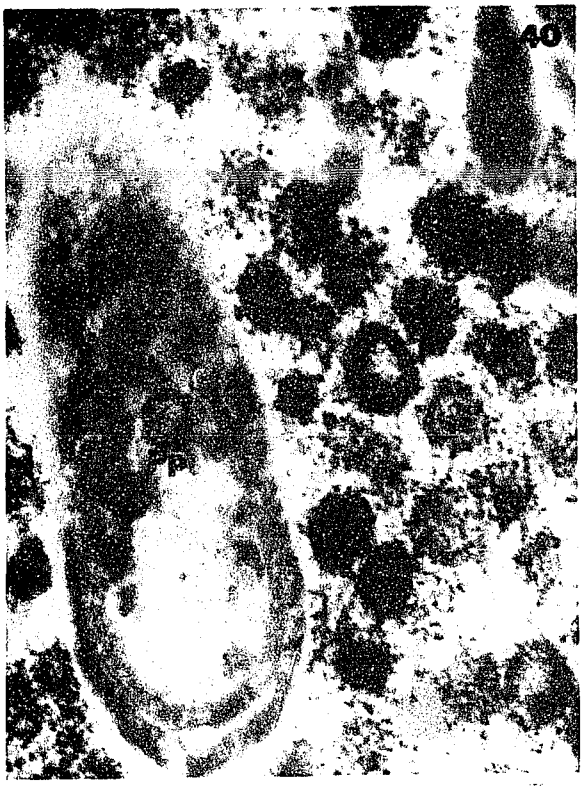
Figures 39. The SDS-polyacrylamide gel showing the protein patterns of the cell envelope fraction of (a) control cells, and cells exposed to (b) 25 μM Cd^{2+}, (c) 50 μM Cd^{2+}, and (d) 100 μM Cd^{2+}. Dots indicate polypeptides that are enhanced in amount in Cd^{2+} treated or heat shocked cells. The position of molecular markers are indicated in kilodaltons.

Figure 40. Micrograph of a thin section of the 5,000 g sediment showing an unbroken cell with a polyphosphate body (Pp) inside. Free polyphosphate bodies (Pp) surround the cell. X 80,000.

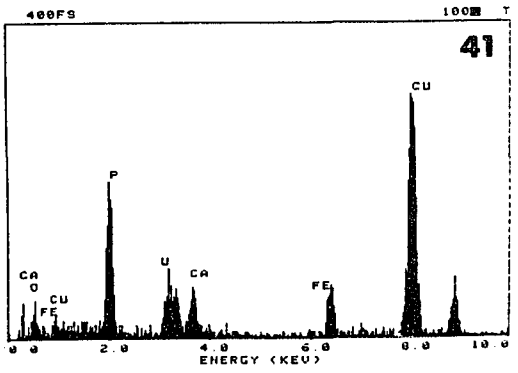
Figure 41. Spectrum of a free polyphosphate body in thin section (Fig. 40). Identifiable peaks are P, Ca, O, Fe, U and Cu.

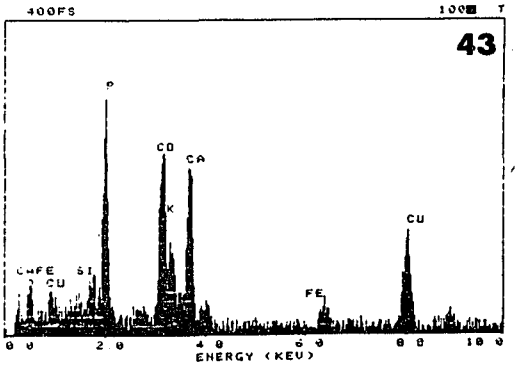
Figure 42. TEM image of the air dried cell extract showing isolated polyphosphate bodies (Pp) and other cellular fragments remaining. X 55,000.

Figure 43. Spectrum of a polyphosphate body in Fig. 44. Peaks, P, K, Ca, O, Fe, Si, and Cu are shown. Since the cells had been exposed to 100 μM Cd^{2+} for 2 days, a prominent Cd peak is also present.


40


42


400FS
100
T
41
CU
P
U
CA
CA
O
CU
FE
FE
0.0
2.0
4.0
6.0
8.0
10.0
ENERGY (KEV)

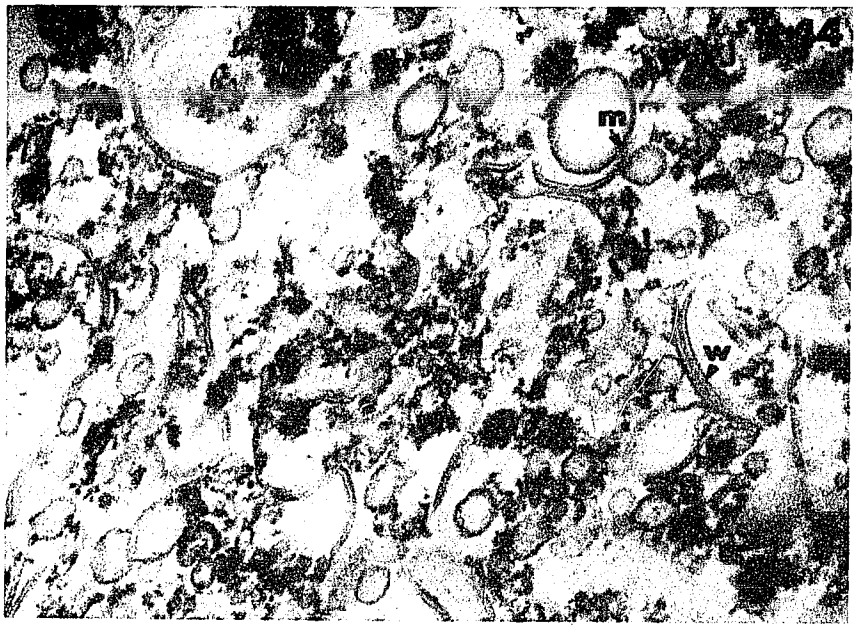

400FS
100
T
43
P
CO
CA
K
CU
FE
FE
0.0
2.0
4.0
6.0
8.0
10.0
ENERGY (KEV)

Figures 44. Micrograph of a thin sections of the total protein fraction showing fragments of membranes (m) and cell wall (w). X 53,000

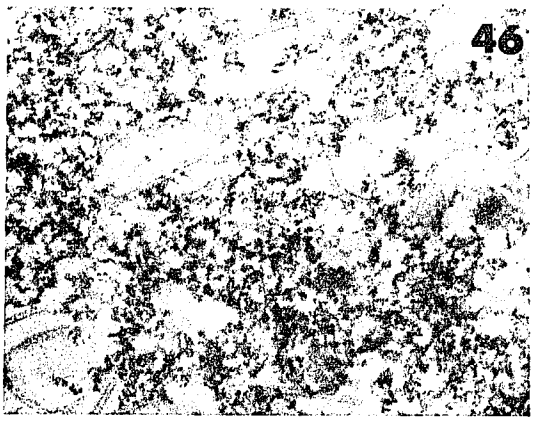
Figure 45. Micrograph of a thin section of the total protein fraction showing an isolated polyhedral body (arrow). X 53,000

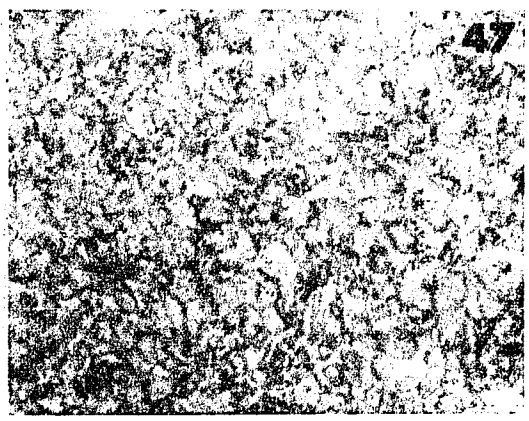
Figure 46. Micrograph of a thin section of the 10,000 g sediment. X 53,000

Figure 57. Micrograph of a thin section of the 100,000 g sediment. X 53,000


44
m
w


45


46


47

DISCUSSION

TOXICITY OF CADMIUM TO *SYNECHOCOCCUS LEOPOLIENSIS*

Effect of Cd on the growth of cell cultures

The toxic effect of cadmium on microorganisms can be obviously demonstrated by the fact that it inhibits the population growth in laboratory culture. This has been shown by the studies of the effective concentration of cadmium which reduces the population growth by 50% (EC_{50}). The EC_{50} of Cd^{2+} for the filamentous cyanobacteria *Anabaena variabilis* and *Anabaena flos-aquae* were 0.11 μM and 0.12 μM (Rachlin *et al.*, 1982a) respectively. The EC_{50} values of Cd^{2+} for eukaryotes are generally greater. For example, the EC_{50} values of Cd^{2+} for some eukaryotic microorganisms were 0.93 μM for *Chlorella saccharophila*, 0.34 μM for *Dunaliela minuta*, 4.2 μM for *Nitzschia closterium*, and 27 μM for *Navicula incerta* (Rachlin *et al.*, 1982a; Rachlin *et al.*, 1982b; Visviki and Rachlin, 1991). This indicates that cyanobacteria are more sensitive to Cd than eukaryotic algae in terms of the population growth. In this report, the EC_{50} of Cd^{2+} for *S. leopoliensis* was 0.08 μM, which is less than all the EC_{50} values mentioned above. It suggests that *S. leopoliensis* cells are very sensitive to Cd^{2+} and is therefore a good organism to use in studies of Cd toxicity.

Undoubtedly the pH has an important role to play in metal toxicity because metal availability to aquatic biota are influenced by pH (Starodub *et al.*, 1987; Luderitz and Nicklisch, 1989; Reed and Gadd, 1989; Peterson *et al.*, 1984). The H^+ concentration may exert its effect either directly by affecting uptake sites, or indirectly by determining the chemical speciation of the dissolved metal pool. For example, the uptake of the metal germanium by green algae, *Chlorella ellipsoideae*, and cyanobacteria, *Oscillatoria* sp., and *Spirulina*

platensis, was reported to be affected by pH (Yanagimoto *et al.*, 1983). However, there is no conclusive metal-pH effects that have been firmly established (Peterson *et al.*, 1984; Reed and Gadd, 1989). One hypothesis is that metals in acidic conditions tend to exist as more toxic free, hydrated ions, whereas in alkaline media they may precipitate as insoluble complexes; the other is H^+ may compete with free metal ions for uptake sites and so a decrease in pH may lead to a decrease in heavy metal toxicity.

By measuring the P uptake rate, Peterson *et al.* (1984) studied the inhibitory effect of Cd and of Cu on P uptake of the green alga, *Scenedesmus quadricauda*. It was found that Cd toxicity increased as pH increased from 5.5-8.5, and Cu toxicity increased from pH 5.0 to 6.5. Discordantly, the toxicity of Ni was potentiated as the pH was decreased to acidic level (Babich and Stotzky, 1982). Luderitz and Nicklisch (1989) examined (1) the effect of pH on the growth of the cyanobacteria, *Aphanizomenon gracile* and *Oscillatoria redekei*, without copper treatment, (2) copper toxicity in the organisms adapted to different pH values, and (3) the effects of simultaneous pH shock and copper treatment, with pH ranging from 5.1 to 8.2. A slow decrease in growth rate at a constant acidic pH was explained as the loss of the ability to regulate the cell-internal pH and an inhibiting influence of H^+ on the process of trace metal uptake by competing for uptake sites. A rapid increase in copper toxicity was observed when the pH dropped from 8.2 to 7.2 for *O. redekei* and when the pH dropped from 6.2 to 5.7 for *A. gracile*. The increased toxicity of Cd and of Cu was explained as both inorganic and organic complexation of metals generally increase with pH and decreases the free ion pool. The alkalinity has no obvious influence on copper toxicity to the organisms studied. They concluded that pH-metal interactions in modifying copper toxicity were a complex phenomenon. This might be also true for other metals.

The Cd inhibition on the population growth, e.g. the cell division, might be due to Cd interfering with normal cellular metabolism. The chemistry of Cd suggests (Vallee and Ulmer, 1972) that Cd binds to and affects the comformation of nucleic acids. Autoradiographs have shown that Cd (McLean and Williamson, 1977) blocked the G1, S or G2 stage of the cell cycle in exposed *Porphyra umbilicalis* cells. Cd inhibited the chloroplast ATPsynthase/ATPase (Teige *et al.*, 1990) and thereby the complete energy-metabolism in spinach plants. Many enzymes whose activities are enhanced or inhibited by Cd were discussed by Vallee and Ulmer (1972). Cd also substitutes for Zn in the Zn carboxypeptidase; the Cd carboxypeptidase is significantly active but does not hydrolyze peptides substrates in contrast to the Zn carboxypeptidase (Vallee and Ulmer, 1972). The Cd effects on cellular metabolism would result in alteration of the cellular ultrastructure and organization as shown in studies (Rai *et al.*, 1990; Rachlin *et al.*, 1984; Ord and Al-Atia, 1979).

Effect of Cd on cellular ultrastructures and organization as influenced by pH

Cell envelopes of the cells for most cases are the first target to be encountered by heavy metal ions. The outer membrane of the gram negative bacteria is a lipopolysaccharide-phospholipid-protein mosaic (Beveridge, 1989). Cd was found to cause the formation of membrane blebs from the outer membrane at alkaline pH in the present study. It might be explained as Cd interacts with phospholipids (Vallee and Ulmer, 1972) in the outer membrane so that the outer membranes become destabilized by Cd; the lipid packing order is disturbed and small membrane blebs off (Beverage, 1989). However, that the cytoplasmic membrane and cytoplasm of the cells exposed to Cd at pH 5.5 seemed to be pulled away from the cell wall and resulted in the enlarged cell wall layer 1 might be explained by the shrinkage of the cytoplasm as observed

by Rachlin *et al.* (1984) in *A. flos-aquae* cells exposed to Cd^{2+}. Since the enlarged layer 1 and lysis of plasma membrane were found in some of the cells subjected to pH 5.5 regardless of the Cd^{2+} concentrations, H^+ might play a role in this kind of destruction of cell envelopes. The cell wall of the cells subjected to the treatments mentioned above associated with the electron dense materials might also show that the cell walls underwent some chemical changes. In a study by Rai *et al.* (1990), it was found that the peptidoglycan layer of the cell wall of *A. flos-aquae* cells exposed to Cd was degraded.

The nucleoplasm, of course, contains the genetic material, DNA, which is important for the cell multiplications, the cellular metabolism and therefore the survival of the cells. The decrease of the nucleoplasm Vv in the Cd^{2+} treated cells observed in the present study might be a result of the cell lysis.

Polyhedral bodies are important cellular inclusions because they contain the enzyme ribulose 1,5-bisphosphate carboxylase (RuBisCO) (Codd and Marsden, 1984). The RuBisCO in the cytoplasm is active in photosynthesis, however, whether the RuBisCO in polyhedral bodies is active in photosynthesis is not very clear. The polyhedral bodies are suggested to function in CO_2 concentration (Badger and Price, 1992). The increase in the number of polyhedral bodies in cells exposed to Cd^{2+} at acidic or alkaline pH's (Table 2) and also a significant increase in the relative volume at alkaline pH in the present study might be explained as a compensation reaction to Cd^{2+}, which inhibits photosynthesis (Stratton and Corke, 1979), and the bodies could concentrate more CO_2 in order to keep up the photosynthetic rate.

Thylakoids are the photosynthetic units of the cell where almost all of the cellular chlorophyll and carotenoid pigments are localized (Fogg *et al.*, 1973). Although in the present study no significant changes in the thylakoid surface area were caused by Cd^{2+}, the loosely organized thylakoid membranes,

especially in cells exposed to Cd^{2+} at pH 9 (Fig. 13), in contrast to the thylakoid membranes in control cells (Fig. 11) might be due to the destruction by Cd. Ghoshroy and Nadakavukaren (1990) reported the destruction of photosynthetic structures, e.g. severe disruption of grana and irregularly spaced thylakoids, in soybean leaves of plants treated with 100 μM Cd^{2+} and explained that Cd^{2+} might interfere with the membrane synthesis and resulted in the absence of organized prolamellar bodies, which were reserves of membrane components and involved in the development of chloroplasts. The Cd^{2+} effect on photosynthetic structures might result in a decrease of the photosynthetic rate, which was reported in *A. inaequalis* (Stratton and Corke, 1979).

PPB's in microorganisms may take up and concentrate metals (Jensen *et al.*, 1982a; 1982b; 1986; Baxter and Jensen, 1980b). The present study found that Cd^{2+} is responsible for changing the volume of the cells occupied by PPB's and a general trend of increase in the dimensions of PPB's was observed due to Cd at any pH. van Groenestijn *et al.* (1988) reported that PPB's in *Acinetobacter* take up P and Mg^{2+} or Ca^{2+} simultaneously. When PPB's took up Cd, they might also simultaneously take up P to neutralize the cations.

The result of morphometric study in the present study demonstrated that the cell ultrastructures and organization were altered as a result of Cd toxicity. Based on the changes of their relative volumes, nucleoplasm and the cell wall were significantly affected by Cd at acidic pH and plus polyhedral bodies at alkaline pH, but PPB's seemed to be the target of Cd at neutral pH. It is concluded that Cd^{2+} accounted for more cellular changes at acidic or alkaline pH than at neutral pH, which is in good agreement with findings reported before (Rai *et al.*, 1990).

pH, which was able to affect the effect of heavy metal ions on the culture growth as discussed above, also affected the Cd effects on the cell ultrastructure

as shown in the present study and the previous study by Rai *et al.* (1990). The pH dependent effect of Cd were observed on the thylakoid surface area, and relative volumes of cell wall and polyhedtal bodies. Every cellular structures studied was affected by Cd or pH dependent Cd effect (Table 9), which was not limited to one structure.

Cd^{2+} INDUCED THE OCCURRENCE OF PPB'S IN POLYHEDRAL BODIES

The elemental analysis of the PPB's, polyhedral bodies and the inclusions inside the latter showed that either the PPB's or the inclusions generated P and Ca peaks and thus demonstrates that the electron dense inclusions found in the polyhedral bodies of *S. leopoliensis* cells are PPB's. Studies of the cyanobacterium, *P. boryanum*, using X-ray microanalysis of air dried cells showed that cytoplasmic PPB's had four common elemental components, P, Ca, Mg, and K, but fixation and embedding caused the loss of Mg and K, and the enhancement of Ca (Baxter and Jensen, 1980a). This accounts for only P and Ca being detected in those bodies.

One of the important environmental functions of cytoplasmic PPB's is their capacity to trap heavy metals present in high concentrations (Jensen, 1990; Jensen *et al.*, 1982a; 1982b). When heavy metals such as Pb, Hg, Co, Ni, Zn, Cd and Cu were added to cultures, they became concentrated in polyphosphate bodies (Jensen, 1990; Jensen *et al.*, 1982a; 1982b) but not always when supplied in trace amounts (Rachlin *et al.*, 1984; Rai *et al.*, 1990). Although the *S. leopoliensis* cells grown in 0.8 μM Cd^{2+} for 4 days did not take up detectable amount of the element in either PPB's, cellular growth however was inhibited, indicating that Cd was assimilated, producing biochemical changes, resulting in

polyphosphate accumulation in the polyhedral bodies, an event most prominently observed at low pH.

The polyhedral PPB's were first observed in *Chlorogloea fritschii* cells which were grown in sodium acetate enriched medium (Jensen and Sicko, 1973). Later they were observed in *Plectonema boryanum* cells which had been starved for phosphate for 5 days before being transferred to a phosphate rich medium for 4 hrs (Jensen *et al.*, 1977), and later in phosphate starved *Synechococcus leopoliensis* cells grown in a sulfur deficient medium (Jensen and Rachlin, 1984). Lawry and Jensen (1979) found that the diameter of polyhedral PPB's were doubled or tripled in *Synechococcus sp.* cells as the effects of sulfur starvation became manifest. In all of these examples cyanobacterial cells were grown in various "stress" conditions. In this present study, Cd^{2+} and a slightly acidic pH is probably a "stress" condition. When the control cells were grown at pH 7.2 or pH 9, the occurrence of polyhedral PPB's was so low that none were found in 30 randomly chosen cells which were analyzed (Table 3). The polyhedral PPB's were also found in naturally grown picoplanktonic cyanophytes, which might be under the natural "stress" of a low nutrient environment (Corpe and Jensen, 1992; Jensen and Corpe, 1993).

Polyhedral bodies are composed of 7 to 15 different polypeptides (Lanaras and Codd, 1981). Two of these are the RuBisCO subunits. Two others have been identified as part of the membrane monolayer, while the rest are unidentified (Codd and Marsden, 1984; Lanaras and Codd, 1981; Shively and English, 1991). The RuBisCO subunits are particulate and about 10 nm in diameter (Lanaras and Codd, 1981). Although RuBisCO is the only identified enzyme in polyhedral bodies at the present time, the presence of PPB's suggests that polyphosphate kinase and / or polyphosphate phosphotransferase may also be present. These enzymes have been reported

to be involved in polyphosphate formation in *E. coli* (Preiss, 1989; Wood and Clark, 1988).

The role of the particulate RuBisCO in polyhedral bodies is uncertain (Codd and Marsden, 1984). Polyhedral bodies have been suggested to be active in photosynthesis (Shively and English, 1991) or as a reservoir for CO_2 (Badger and Price, 1992). If they function in the Calvin cycle, a possible break in the cycle caused by stresses, e. g. Cd^{2+} (Stratton and Cork, 1979), could lead to a piling up of phosphorylated intermediates. Ribulose-1,5-bisphosphate molecules may then be oxidized in the process of photorespiration (Badger, 1985) giving rise to CO_2 and Pi. With the availability of Pi and polyphosphate phosphotransferase, ATP and polyphosphate kinase additional polyphosphate could be generated (Preiss, 1989; Wood and Clark, 1988). It would presumably be retained inside the polyhedral bodies whose membrane barrier would prevent the molecules from moving into the cytoplasm. The recognition of the function of PPB's in polyhedral bodies may help in determining the function of the latter inclusions in the cyanobacteria.

THE UPTAKE OF Cd BY PPB'S

The PPB's of the air dried *S. leopoliensis* control cells were composed of P, Mg, K, Ca and O, which is in good agreement with previous studies; the previous EDX studies demonstrated that elements P, K, Mg and Ca were found in the PPB's in cyanobacteria, green algae and fungi (Doonan *et al.*, 1979; Sicko-Goad *et al.*, 1975; Baxter and Jensen, 1980a). Since in improved EDX system was used, oxygen was also detected in the PPB's in the present study. Early works with EDX also demonstrated that PPB's in cells of cyanobacteria and green algae

were able to concentrate the metals when the cells were exposed (Jensen *et al.*, 1982a; 1982b; 1986; Baxter and Jensen, 1980b; Pettersson *et al.*, 1985).

As a continual study of the metal-uptake pattern, the Cd uptake by *S. leopoliensis* cells exposed to a series of Cd^{2+} concentrations was studied. Although no Cd was detected with EDX in any sectors of the cells exposed to 8 μM Cd^{2+}, Cd^{2+} must have entered the cells because the EC_{50} value of Cd^{2+} and the ultrastructural changes caused by Cd^{2+} in previous experiments demonstrated that Cd^{2+} exerted toxic effects to the cells at concentrations far below 8 μM. In a study (Jensen *et al.*, 1982b) of the metal uptake in *Chlorella saccharophila, Navicula incerta* and *Nitzschia closterium* cells exposed to Pb^{2+} and Zn^{2+} at various concentrations showed that the uptake of either metals by the cells were not detectable with EDX when the concentrations of Pb^{2+} or Zn^{2+} were low.

In a few of the cells exposed to 80 μM Cd^{2+}, Cd was only detected in the cytoplasmic and cell wall sectors. It seemed that Cd ions at a low Cd concentration were mostly adsorbed by the cell wall or cytoplasm in contrast to the PPB's as the Cd ions were taken up by the cells. A study by Mushrifah and Peterson (1991) showed that the cell surface of *A. flos-aquae* cells seemed able to control the penetration of Cd ions by binding to the ions when the Cd concentration was low. In the present study, the PPB's in the cells exposed to 200 μM Cd^{2+} could accumulate detectable Cd since PPB's in about half of the cells gave Cd peaks. As the Cd^{2+} concentration increased to 600 μM, the metal was mainly taken up into PPB's and also some in the cytoplasm and the cell wall, the pattern of the uptake of heavy metal ions as described by Jensen *et al.* (1982a; 1982b; 1986) and Pettersson *et al.* (1985). The result that Cd was mostly taken up in the PPB's demonstrated that the most of the Cd ions taken up in the

cells were attracted by the polyphosphates (Peverly *et al.*, 1978), the highly charged anions, in PPB's and concentrated in the PPB's (Jensen, 1990).

The prominent S peak generated by the cytoplasm sector in Cd^{2+} exposed cells could mean that the amount of S in the cytoplasm increased as the exposure Cd^{2+} concentration increased. When the Cd was mostly taken up in the PPB's with a small amount taken up in the cytoplasm, the S was accumulated in the cytoplasm (the chemical, $CdCl_2$, used in the study contained 0.05 % sulfide). It would be of interest to know whether the S was incorporated in the metal-binding proteins, whose syntheses were induced by heavy metals (Rauser, 1990; Vallee, 1991).

The Cd uptake by the cells for the first 4 hour exposure did not show much dependency on light and temperature. The close similarity of the Cd uptake patterns by the cells exposed to Cd^{2+} at room temperature and at 4°C in the dark suggests that the Cd uptake by the cell wall, the cytoplasm and PPB's of the cells during the first 4 hours might be due to passive diffusions. Pettersson *et al.* (1986) also showed that the rapid uptake of Al by *A. cylindrica* mainly occurs via passive diffusion because either the darkness or a chemical added to stop the phosphorylation did not stop the Al accumulation.

The EDX analysis on the isolated PPB's from the cells exposed to Cd^{2+} at 100 μM for 2 days again demonstrated that PPB's took up and sequestered elements. The isolated PPB's gave very significant Cd peaks similar to those given by PPB's in air dried cells exposed to 600 μM Cd^{2+} for 4 hours. However no Cd could be detected in PPB's of the air dried cells which had been exposed to 80 μM Cd^{2+} for 4 hours. This might be explained in that a slow or energy dependent process is involved in Cd uptake (Gadd and Griffths, 1978; Gipps and Coller, 1980).

It is therefore concluded that Cd was taken up intracellularly and the phosphorus rich PPB's in the cytoplasm attracted the largest amount of the positively charged metal ions. The Cd accumulated by PPB's increased with increasing external Cd concentration and exposure time.

CHANGES IN PROTEIN PATTERNS DURING Cd^{2+} EXPOSURE

When *S. leopoliensis* cells were exposed to Cd^{2+}, analysis of the protein patterns of the cellular fractions by SDS-PAGE indicated a total of nine polypeptides were elevated in amount and three polypeptides decreased in amount in cytoplasmic fraction. It shows that Cd^{2+} also exerts its effects on proteins in the exposed cells.

As discussed in many reviews, heavy metals are one of the stimuli inducing the so called "stress" response, and the synthesis of heat shock proteins (Neidhardt *et al.*, 1984; Schlesinger, 1990; Lindquist and Craig, 1988). Cd^{2+} is typically found to induce the "stress response" in various organisms or cells, such as chick embryo cells and human foreskin cells (Levinson *et al.*, 1980), *Drosophila* cells (Bournias-Vardiabasis *et al.*, 1990; Courgeon *et al.*, 1984), and *Agrobacterium tumefaciens* (Mantis and Winans, 1992). In a study with *Drosophila* embryonic cells, metals, such as Cd^{2+}, As^{2+} and Hg^{2+}, induced the entire set of heat shock proteins while other two metal ions, Ni^{2+} and Zn^{2+} were shown to induce only two of the stress proteins (Bournias-Vardiabasis *et al.*, 1990).

In the present study, some of the polypeptides elevated in amount in the Cd^{2+} exposed cells might be stress proteins induced by Cd^{2+}; however, future works will be needed to characterize the possible stress proteins.

Bensaude *et al.* (1990) reported that while a few differences between heat shocked and nonstressed mouse Ltk- fibroblasts were detected in the

electrophoretic pattern of proteins in the supernatant of 10,000 g, the major changes were increased amount of 85 and 70 kDa proteins in the heat shocked pellet because the two proteins became insoluble.

Cd^{2+}, in the present study, induced the enhancement in the amount of some polypeptides observed in the cell envelope fraction. The increased amount of the 15 K or 20 K polypeptide was very predominant in the cell envelope and membrane fractions; both polypeptides were barely detectable in the control cell envelope and membrane fractions. However, there was no obvious increase of the 15 K and only a slight increase of 20 K observed in the cytoplasmic fraction. Some polypeptides reduced in amount were found in the cytoplasmic fraction. One of the reduced polypeptides, 70 K, was enhanced in the cell envelope fraction. It is suggested that insolubilization and denaturation of some polypeptides might occur during Cd^{2+} exposure.

Excessive heat and other stresses often cause the denaturation of proteins and formation of incorrect aggregates (Ellis and van der Vies, 1991; Bensaude *et al.*, 1990). Cellular proteins were reported to become insolubilized during stress (Dubois *et al.*, 1987). Dubois and coworkers found that a cytoplasmic soluble enzyme, the p68 kinase, was in the supernatant fraction in control cell extracts while it is in the pellet from heat shocked cell extract.

Cadmium shows a strong affinity for cysteinyl and histidyl groups of protein (Vallee and Ulmer, 1972). Protein thiol groups can sequester cadmium and therefore can function in cadmium detoxification, for example, glutathione (Singhal *et al.*, 1987), metallothioneins and phytochelatins (Vallee, 1991; Steffens, 1990; Rauser, 1990). However, the protein thiol groups are also the targets for cadmium poisons (Fairlamb and Cerami, 1992). Cadmium inhibits a large number of enzymes having functional sulfhydryl groups (Vallee and Ulmer, 1972).

Protein thiol groups form disulfide bonds, which play an important role in protein tertiary structure, the functional structure of proteins. By interfering with the thiol groups, cadmium might cause proteins to become abnormal configuration and to inactivate some crucial enzymes, then to inhibit the cellular growth and disrupt the cellular structure or disassemble the proteinaceous structures.

SUPPLEMENT

THE STUDY OF EMBEDDING MEDIA

INTRODUCTION

The presence of Al in osmium tetraoxide, a fixative, and Nadic Methyl Anhydride (NMA), a component in many embedding media, lead to the PPB's in cell sections generating spectra with Al peaks when the elemental analysis was carried out with the EDX (Jensen *et al.*, unpublished). As an attempt to find an embedding medium which would not contaminate cell sections with Al and may be suitable for use in studies with EDX, experiments were carried out as the cells were fixed in glutaraldhyde, embedded in various media without NMA component.

MATERIALS AND METHODS

The *S. leopoliensis* cells were grown in the medium or in the medium without minor elements and then collected by centrifugation. Then the cells were fixed in 3% glutaraldhyde (in ddH_2O) for 1 hour and then washed in ddH_2O once. After that the cells were embedded in the media ordered from Electron Microscopy Sciences (P.O. Box 251, Ft. Washington, Pennsylvania 19034). All embedding media, except Epon 812 and Quetol 651, were chosen because they do not have the NMA component which was shown to contribute Al to PPB's (Jensen *et al.*, unpublished). Since it is conventionally used in our laboratory, Epon 812 is used as a comparison in this experiment. The embedding processes and media are listed below.

The fixed cells were:

1. dehydrated in ethanol followed by propylene oxide, then embedded in:

Epoxy 812; (see Appendix C)

Der 332-732; (see Appendix E)

Maraglas 732; (see Appendix F)

Araldite 502. (see Appendix G)

2. dehydrated in ethanol then embedded in:

Low viscosity Spurr's; (see Appendix H)

Araldite 6005. (see Appendix I)

3. dehydrated in acetone then embedded in:

Durcupan ACM. (see Appendix J)

4. embedded in water soluble media:

Durcupan water soluble; (see Appendix K)

Vestopal, then gelatin capsule; (see Appendix L)

LR white, then gelatin capsule; (see Appendix M)

Quetol 651. (see Appendix N).

After embedding the samples in the capsules (polyethylene capsules unless otherwise specified) they were placed in a 45°-60° C oven for 1-2 days till the media were fully polymerized. Sections cut with a diamond knife were collected with copper grids and cells were located under the TEM. PPB's were analyzed with the EDX in the STEM mode for a period of 100 seconds as described above. The spectra were compared with those of PPB's in air dried cells.

RESULTS AND CONCLUSION

Under the TEM, the cells fixed in glutaraldhyde showed little internal structure without post staining. The electron dense PPB's, however, were clearly seen (Fig. 48). The result of the EDX analysis showed that distinctive Al peaks were generated by almost every PPB's of the cell sections embedded in the embedding media of Maraglas, Spurr's, Araldite 812, Der-Ser, Vestopol, Quetol 651, LR White, and Epon 812, and by about half of the PPB's in the cell sections embedded Durcupan-ACM, Durcupan-water soluble and Araldite 6005 (Fig. 49, 50). An ideal embedding medium for EDX analysis was not found among the tested media.

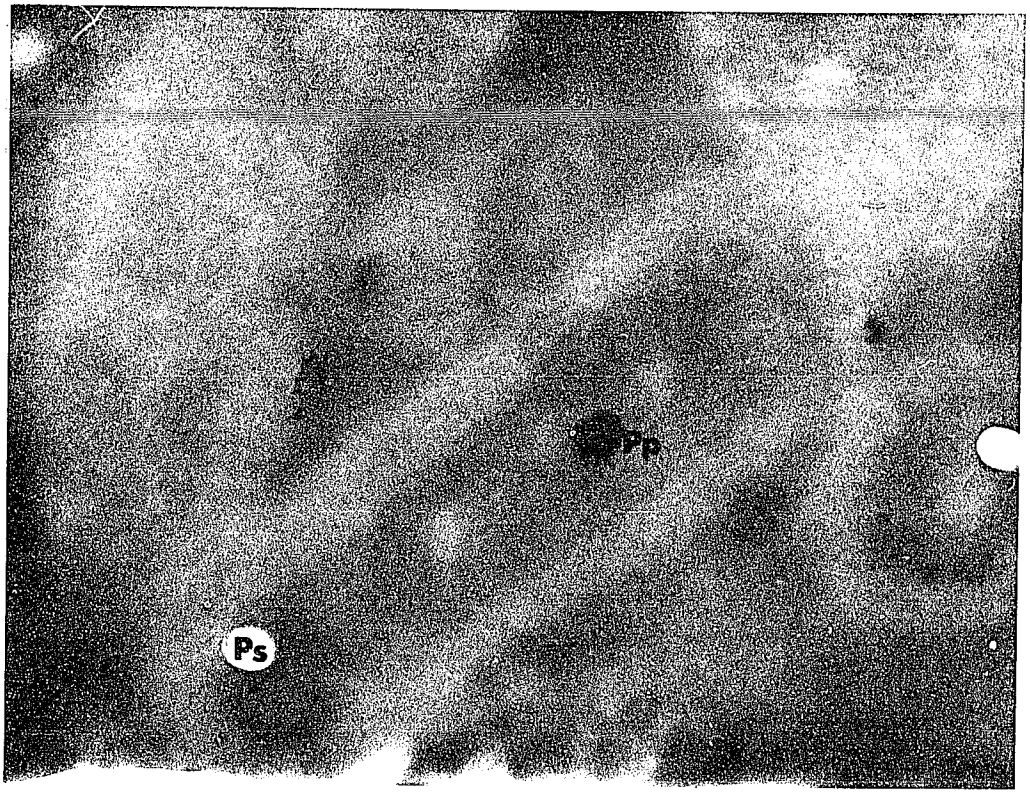
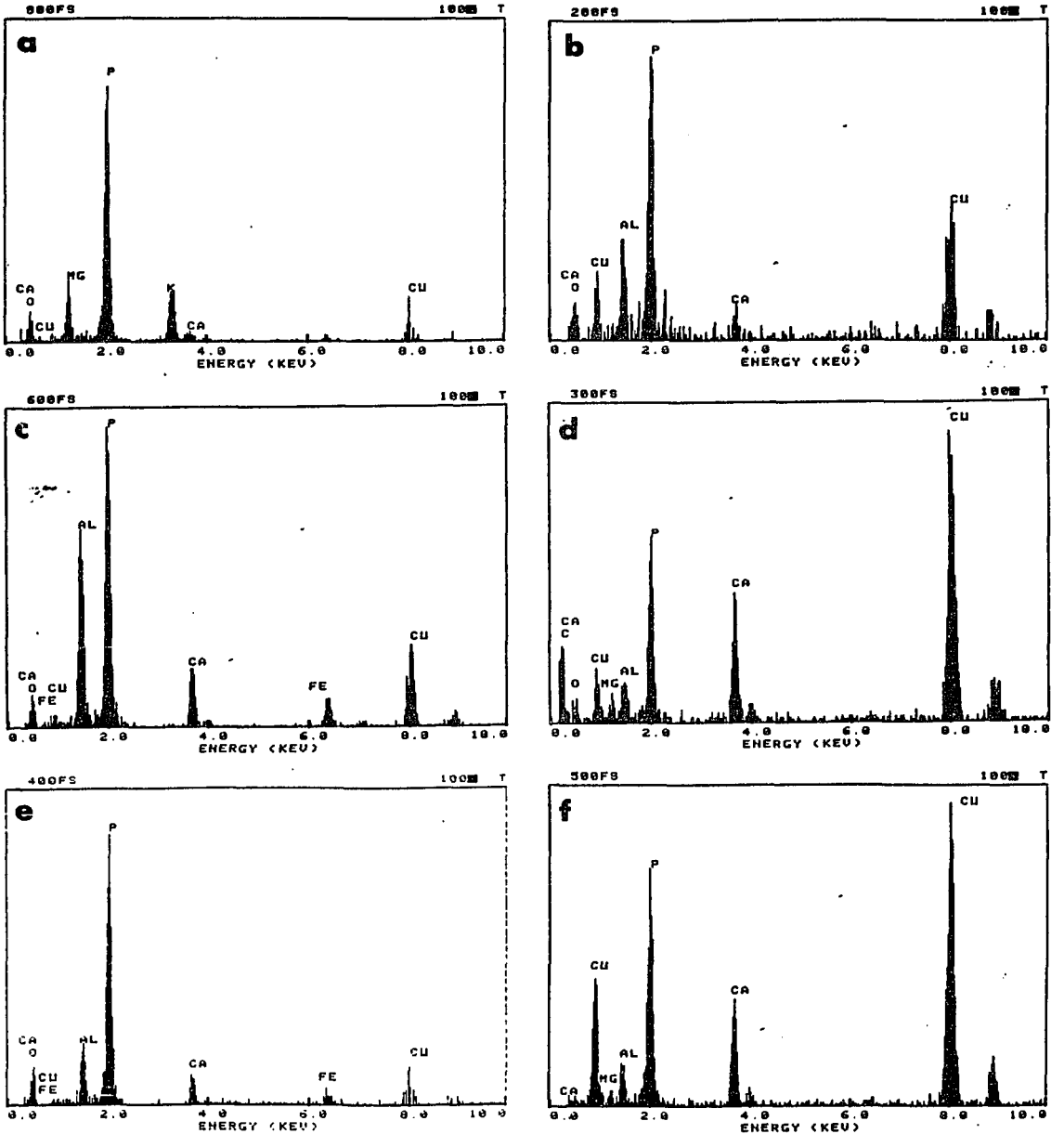


Figure 48. Micrograph of a section of a *S. leopoliensis* cell fixed in glutaraldehyde and embedded in araldite 13920 showing the electron dense and porous polyphosphate body (Pp) and a space left after loss of a body (Ps). X 62,000.



Figures 49. Spectra of polyphosphate body of (a) air dried cells and of sectioned cells embedded in (b) Der 332-732, (c) Maraglas, (d) Araldite 502, (e) Epon 816, and (f) Durcupan ACM.

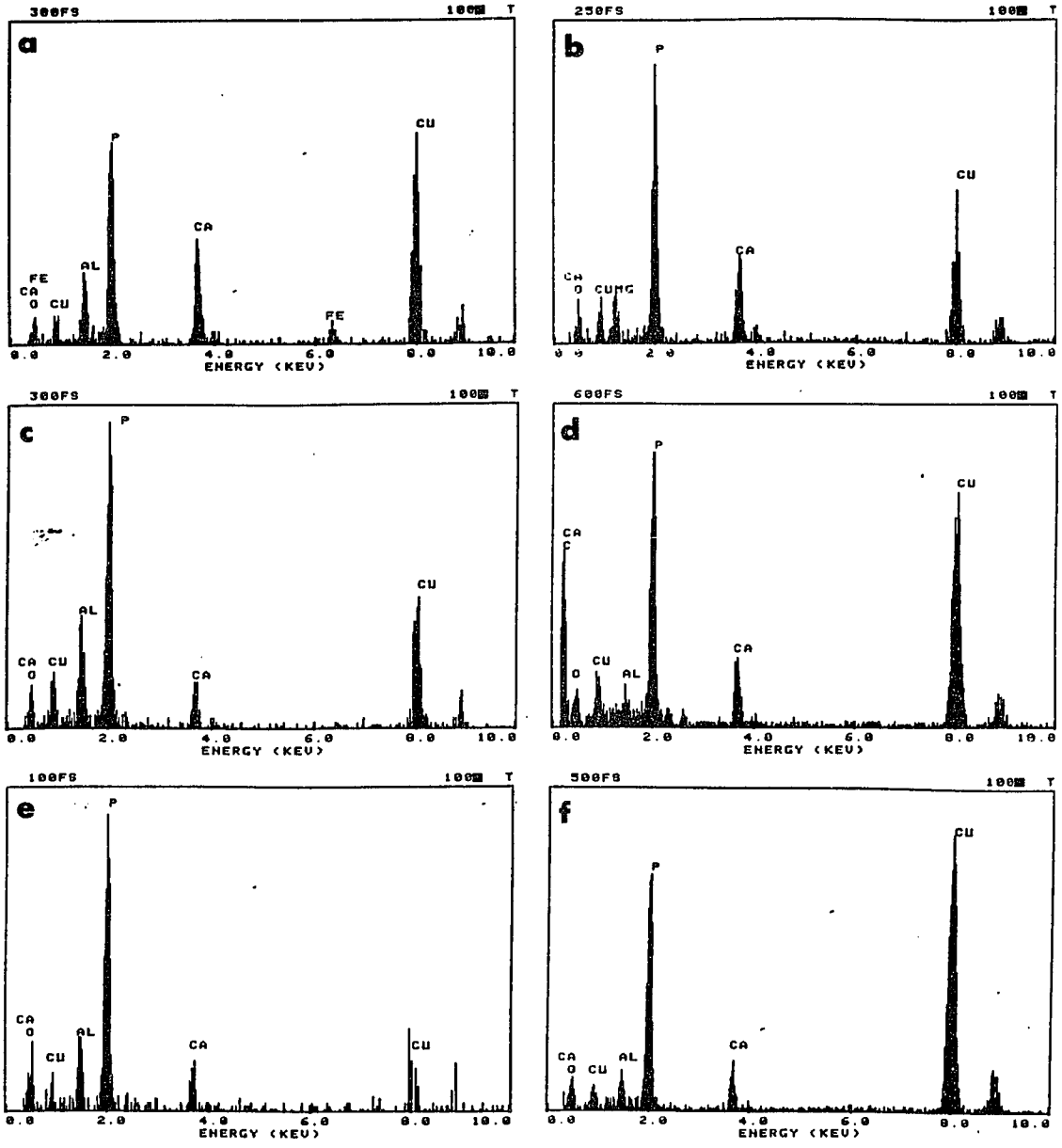


Figure 50. Spectra of polyphosphate bodies of sectioned cells embedded in (a) low viscosity Spurr's, (b) Araldite 6005, (c) Quetol 651, (d) Durcupan water soluble, (e) Vestopal, and (f) LR white.

APPENDICES

Appendix A

Composition of Culture Medium-Modified Fitzgerald (Fitzgerald *et al.*, 1952)

	mg/liter
$NaNO_3$	124
$K_2HPO_4 . 3H_2O$	13
$MgSO_4 . 7H_2O$	25
$CaCl_2 . 2H_2O$	36
Na_2CO_3	20
$Na_2SiO_3 . 9H_2O$	58
Ferric Citrate	3
Citric Acid	3
Gaffron's minor element solution -	0.04 ml.

pH is adjusted to 7.2 by adding 1 N HCl or 1 N NaOH.

Gaffron's solution:

	g/liter
H_3BO_3	3.10
$MnSO_4 . 4H_2O$	2.23
$ZnSO_4 . 7H_2O$	0.287
$(NH_4)_6Mo_7O_{24}. 4H_2O$	0.088
$CuSO_4 . 5H_2O$	0.125
$Co(NO_3)_2 . 6H_2O$	0.146
$Al_2(SO_4)_3KSO_4. 24H_2O$	0.474
$NiSO_4(NH_4)SO_4 . 6H_2O$	0.198
$Cd(NO_3)_2 . 4H_2O$	0.154

$Cr(NO_3)_3 \cdot 7H_2O$	0.037
$V_2O_4(SO_4)_3 \cdot 16H_2O$	0.035
$Na_2WO_4 \cdot 2H_2O$	0.033
KBr	0.119
KI	0.083

Appendix B

Fixation of experimental samples.

A: Preparation of solutions:

1. Stock Michaelis Buffer:

   a. Weight: 1.94 g sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$)
   2.94 g sodium barbital
   3.40 g sodium chloride

   b. Add water to (a) to reach 100 ml and dissolve the chemicals,then store the buffer in refrigerator.

2. Osmium Tetraoxide:

   A stock solution of 2% OsO_4 was made by dissolving 1 g of osmium tetraoxide in 50 ml of distilled water. The solution was stored in a Pyrex glass stoppered bottle, which was placed in a tin can, and stored in refrigerator.

3. 1% Bacto-tryptone:

   1 g of Difco bacto-tryptone and 0.5 g NaCl were dissolved in 100 ml distilled water. The solution was placed in small closed vials and stored frozen. The contents of the vials were thawed and mixed thoroughly before use.

B. Fixing Procedure of Modified Kellenberger (Kellenberger *et al.*, 1958; Pankratz and Bowen, 1963):

1. Preparation of solutions:

    a. 5 ml Stock Michaelis Buffer,

    7 ml 0.1 N HCl,

    13 ml distilled water,

    0.25 ml 1.0 M $CaCl_2$;

    then adjust the solution to pH 6.1 or 6.2.

    b. Dilute solution (a) with an equal amount (1:1) of 2% OsO_4

    then add 0.1ml 1% bacto-tryptone per ml.

2. Fix samples in solution (b) for 3 hours at room temperature.

Appendix C

Embedding with Epon 812 (Luft, 1961)

A: preparation of Epon:

1. Mixture A:

    62 ml Epon 812,

    100 ml DDSA (Dodenyl Succinic Anhydride).

2. Mixture B:

    100 ml Epon 812,

    89 ml NMA (Nadic Methyl Anhydride).

    Mixture A and mixture B were refrigerated, and brought to room temperature before opening to avoid water condensation.

3. Epon mixture for embeddiment: 1 part mixture A, and 2 parts mixture B were mixed in a disposable plastic beaker, and 0.2 ml of DMP-30 was added per 10 ml of the Epon mixture. It is important that all components are thoroughly mixed so that no "streaks " are visible. Epon mixture should be freshly made before embedding.

B: Dehydration and embedding of fixed samples:

50% ethanol	5 - 10 min
75% ethanol	5 - 10 min
95% ethanol	5 - 10 min
100% ethanol	5 - 10 min
100% ethanol	5 - 10 min
100% ethanol	5 - 10 min
propylene oxide	5 - 10 min
propylene oxide	5 - 10 min
propylene oxide	5 - 10 min
3 propylene oxide : 1 Epon	15 min
1 propylene oxide : 1 Epon	30 min
1 propylene oxide : 3 Epon	60 min

Transfer the sample into capsules then filled with pure Epon. Polymerize the Epon by leaving capsules at:

room temperature	overnight
35° C oven	8-24 hours
45° C oven	8-24 hours
60° C oven	3-5 days

Appendix D

Uranyl acetate-methanol stain (Stempak and Ward, 1964).

A: Preparation of the solution: 15 g of hydrated uranyl acetate ($UO_2(CH_3COO)_2 \cdot 2H_2O$) were dissolved in 50ml of acetone-free absolute methanol with a magnetic stirrer. The solution was then stored in a Pyrex glass-stoppered bottle at 4°C.

B: Staining: Grids were immersed in wells of a porcelain staining dish, section side up, for 10 minutes at room temperature. The wells were covered during this period to prevent evaporation of the methanol.

C: Washing: The grids were then rinsed with absolute methanol, ethanol, and water as listed below:

1. Absolute methanol	2-3 dips
2. Absolute methanol	10-20 dips
3. 100% ethanol	25 dips
4. 80% ethanol	50 dips
5. 50% ethanol	50 dips
6. distilled H_2O	50 dips

Appendix E

Embedding with DER 332-732 (Electron Microscopy Sciences)

A: preparation of the embedding mixture:

DER 332	70 g
DER 732	20 g
DDSA	50 g
DMP 30	2.8 g

Mix thoroughly at one or half hour before use. DER 332 should be slowly heated to about 60° C before use to reduce viscosity.

B: dehydrate the fixed samples as described in Appendix C-B until in the second change of propylene oxide (P.O.), then

P.O.: embedding mixture=1:1	1 hr at 45° C in capped containers,
embedding mixture	1 hr at 37° C in open containers.

Finally, transfer specimens to polyethylene capsules filled with embedding mixture, and heat at 37° C, 45° C and 60° C, for 24 hrs at each temperature.

Appendix F

Embedding with Maraglas 732 (Electron Microscopy Sciences)

A: preparation of embedding mixture:

Maraglas 655	36.0 ml
DER 732	8.0 ml
Dibutyl phthalate	5.0 ml
Benzyl dimethylamine	1.0 ml

B: dehydrate the fixed specimens as described in Appendix C-B until in the second change of propylene oxide, and transfer the specimens in embedding mixture in polyethylene capsules. Leave them overnight at room temperature. Then polymerize in an oven at 52° C for 17 hrs.

Appendix G

Embedding with Araldite 502 - EMBED 812 (Electron Microscopy Sciences)

A: preparation of embedding mixture:

EMBED 812	25 ml
Araldite 502	15 ml
DDSA	55 ml
DMP-30	1.4 ml (added just prior to use)

B: dehydrate the fixed specimens as described in Appendix C-B. Then transfer the specimens in

P.O.: embedding mixture=2:1	1 hr at room temperature
P.O.: embedding mixture=1:2	1 hr at room temperature

Finally transfer the specimens in polyethylene capsules filled with 100% embedding mixture. Leave them for 2-4 hrs at room temperature, then in an oven at 40°-80° C for about 12 hrs.

Appendix H

Embedding with Araldite 6005 (Electron Microscopy Sciences)

A: Preparation of solutions for infiltration and embedding:

Mixture I: 10 ml Araldite 6005 plus 9 ml DDSA and 0.5 ml BDMA (benzyldimethylamine)

Mixture II: 10 ml Araldite 6005 plus 9 ml DDSA;

Mixture III: 20 ml Araldite 6005 plus 18 ml DDSA, 0.5 ml BDMA, and 1-3 ml Dibutyl phthalate plasticizer.

B: Fixed samples were dehydrated in ethanol solutions as described in Appendix C-B until in the third change of 100% ethanol, and transferred in

100% ethanol / mixture 1(1:1)	overnight at room temperature
mixture II	overnight at 48° C
mixture III (preheated to 48° C)	20 hours at room temperature

Embedding is done using freshly prepared mixture III, and placing the cells into capsules with small amount of the resin and then filled. Leave them in an oven at 48° C for at least 16 hours.

Appendix I

Embedding with Low Viscosity Embedding Media (Spurr's Kit) (Electron Microscopy Sciences)

A: Preparation of the embedding medium:

ERL 4206 (vinyl cyclohexene dioxide (VCD))	10 g
DER 736 (diglycidyl ether)	6.0 g
NSA (nonenyl succinic anhydride)	26.0 g
DMAE (dimethylaminoethanol)	0.4 g

B: Fixed cells were dehydrated as described in Appendix C-B until the 3rd change of 100% ethanol. The infiltration is done by adding the embedding

media to an equal quantity (1:1) of the 100% ethanol left in the vial with the tissue. Swirl the mixture and let sit for 30-60 min.. Add the same amount of embedding media to the existing infiltration media. Swirl and let sit for another 30 min.. Pour and drain the mixture and add more embedding media. A second change can be made before the final embedding. Polymerization takes 8 hours at 70° C.

Appendix J

Embedding with Vestopal W (Electron Microscopy Sciences)

A: Preparation of Vestopal mixture:

Mixture I	Vestopal / parts acetone (1:3);
Mixture II	Vestopal / part acetone (1:1);
Mixture III	Vestopal / acetone (3:1);
Mixture IV	99% Vestopal, 0.5% tertiary butyl perbenzoate, and 0.5% of a 3% solution of cobalt naphthenate in styrene.

B: Dehydrate and embed the fixed samples in:

25% acetone	20 min
50% acetone	20 min
75% acetone	20 min
90% acetone	20 min
100% acetone	30 min
100% acetone	30 min
Mixture I	30 min
Mixture II	30 min
Mixture III	30 min
Mixture IV	30 min

Mixture IV	overnight

The samples were then placed in gelatin capsules filled with mixture IV and the capsules were kept at 60° C for 12-24 hours for polymerization.

Appendix K

Embedding with Durcupan ACM (Electron Microscopy Sciences)

A: Preparation of embedding mixtures:

a. No.1 Durcupan ACM mixture:

Epoxy resin	10 ml
964 hardener	10 ml
Dibutyl phthalate Fluka	0.1-0.2 ml

b. No. 2 Durcupan ACM mixture:

Epoxy resin	10 ml
964 hardener	10 ml
964 accelerator	0.3-0.4 ml
Dibutyl phthalate Fluka	0.1-0.2 ml

Both mixtures are preferably prepared at least 15 minutes before use, and kept at 50°C for this period so that they are thoroughly mixed.

B: The fixed specimens were dehydrated and embedded in

30% acetone	15 min
50% acetone	30 min
70% acetone	30 min
90% acetone	30 min
Dry acetone	30 min
Dry acetone	30 min
Dry acetone : No. 1 mixture=3:1	1 hr
Dry acetone : No. 1 mixture=2:2	1 hr

Dry acetone : No. 1 mixture=1:3	1 hr
No.1 mixture	1-2 hrs at 50°C
No.1 mixture	1-2 hrs at 50°C
No.2 mixture	1-2 hrs at 50°C

The specimens were placed in polyethylene capsules, which were then filled with No. 2 mixture. Polymerization takes place at 50°-80° C for at least 48 hrs.

Appendix L

Embedding with Durcupan water soluble embedding medium (Electron Microscopy Sciences)

A: preparation of the embedding mixture:

component A	5 ml
component B	11.7 ml
component C	1.0-1.2 ml
component D	0.2-0.4 ml

B: The fixed specimens were transferred in

50% component A with 50% water	30 min
70% component A with 30% water	45 min
90% component A with 10% water	45 min
100% component A	90 min
100% component A	90 min

Then, transfer the specimens into gelatin capsules, which were then filled with freshly made embedding mixture. Polymerization takes place at 37°-45° C for 3-4 days.

Appendix M

Embedding with LR White (Electron Microscopy Sciences).

Fixed specimens were transferred in:

70% ethanol	30-60 min
70% ethanol	30-60 min
L.R. White/70% ethanol (2:1)	30 min
L.R. White	1 hr
L.R. White	overnight

Then, the specimens were transferred to gelatin capsules filled with fresh L.R. White. Polymerization takes place at 50° C for 24 hrs.

Appendix N

Embedding with Quetol 651 (Electron Microscopy Sciences).

A: Preparation of embedding medium:

Quetol 651	35 ml
NSA (nonenyl succinic anhydride)	54 ml
NMA (nadic methyl anhydride)	11 ml
DMP-30	1.5-2.0 ml

B: The fixed specimens were transferred in

50% Quetol 651	20 min
70% Quetol 651	20 min
90% Quetol 651	20 min
100% Quetol 651	20 min
100% Quetol 651	20 min
Quetol 651/n-BGE (n-butyl glycidyl ether) (1:1)	30 min
n-BGE	30 min
n-BGE/embedding mixture (1:1)	1-2 hrs
embedding mixture	2-3 hrs

embedding mixture	2-3 hrs

Then, transfer the specimens into polyethylene capsules filled with the mixture. Polymerization takes place at 60° C for about 24 hrs.

Appendix O

Protein sample buffer (Bio Rad):

Distilled water	3.2 ml
0.5 M Tris-HCl pH 6.8	1.0 ml
Glycerol	1.6 ml
10% (W/V) SDS	1.6 ml
B-mercaptoethanol	0.4 ml

Appendix P

Solutions for preparing 20 ml 15% resolving gels for Tris-glycine SDS-polyacrylamide gel electrophoresis:

H_2O	4.6 ml
30% acrylamide mix	10.0 ml
1.5 M Tris (pH8.8)	5.0 ml
10% SDS	0.2 ml
10% ammonium persulfate	0.2 ml
TEMED	0.008 ml

Solution for preparing 10 ml 5% stacking gels for Tris-glycine SDS-polyacrylamide gel electrophoresis:

H_2O	6.8 ml
30% acrylamide mix	1.7 ml
1.0 M Tris (pH6.8)	1.25 ml

10% SDS	0.1 ml
10% ammonium persulfate	0.1 ml
TEMED	0.01 ml

Appendix Q

Staining SDS-polyacrylamide gels with coomassie brilliant blue (CBB):

A: preparation of staining solutions:

solution I:	Isopropanol	250 ml
	Acetic acid	100 ml
	10 mg/ml CBB	3 ml
	H_2O	650 ml
solution II:	Isopropanol	100 ml
	Acetic acid	100 ml
	10 mg/ml CBB	3 ml
	H_2O	800 ml
solution III:	acetic acid	100 ml
	10 mg/ml CBB	3 ml
	H_2O	900 ml

Stain the gel in solution I for 30 min, and stain in solution II overnight, then in solution III for 2 hours. After that, destain the gel in 10% acetic acid. Wash the gel in running water and then dry it.

REFERENCES

Aherne, W. A., and M. S. Dunnill. 1987. Morphometry. Edward Arnold, Great Britain.

Aiking, H., A. Stijnman, C. van Garderen, H. van Heerikhuizen, and J. van't Riet. 1984. Inorganic phosphate accumulation and cadmium detoxification in *Klebisiella aerogenes* NCTC 418 growing in continuous culture. Appl. Environ. Microbiol. 47:374-377.

Ashburner, M. 1982. The effects of heat shock and other stress on gene activity: an introduction, p.1-10. *In* M. J. Schlesinger, M. Ashburner, and A. Tissieres (eds.), Heat Shock from Bacteria to Man. Cold Spring Harbor Laboratory.

Babich, H., and G. Stotzky. 1982. Nickel toxicity to microbes: effect of pH and implication for acid rain. Environ. Res. 29:335-350.

Badger, M. R. 1985. Photosynthetic oxygen exchange. Ann. Rev. Plant Physiol. 36:27-53.

Badger, M. R., and G. D. Price. 1992. The CO_2 concentrating mechanism in cyanobacteria and microalgae. Physiol. Plant 84:606-615.

Baxter, M., and T. E. Jensen. 1980a. A study of methods for in situ X-ray energy dispersive analysis of polyphosphate bodies in *Plectonema boryanum.* Arch. Microbiol. 126: 213-215.

Baxter, M., and T. E. Jensen. 1980b. Uptake of Magnesium, Strontium, Barium, and Manganese by *Plectonema boryanum* (Cyanophyceae) with special reference to polyphosphate bodies. Protoplasma 104:81-89.

Bensaude, O., M. Pinto, M. Dubois, N. V. Trung, and M. Morange. 1990. Protein denaturation during heat shock and related stress, p. 89-99. *In* M. J. Schesinger, M. G. Santoro, and E. Garaci (eds.), Stress proteins. Springer-Verlag.

Beveridge, T. J. 1989. Role of cellular design in bacterial metal accumulation and mineralization. Annu. Rev. Microbiol. 43:147-171.

Bischoff, B. 1982. Effects of cadmium on microorganisms. Ecotoxicology and Environmental Safety. 6:157-165.

Blakemore, R. P. 1982. Magnetotactic bacteria. Annu. Rev. Microbiol. 36:217-238.

Bournias-Vardiabasis, N., C. Buzin, and J. Flores. 1990. Differential expression of heat shock proteins in Drosophila embryonic cells following metal ion exposure. Exp. Cell Res. 189:177-182.

Bruland, K. W. 1989. Trace elements in sea-water, p.194-196, Chemical Oceanography, Vol. 8. Academic Press, London.

Chalkey, H. W. 1943. Methods for the quantitative morphologic analysis of tissues. J. Natl. Cancer Inst. 4:47-53.

Codd, G. A., and W. J. N. Marsden. 1984. The carboxysomes (polyhedral bodies) of autotrophic prokaryotes. Biol. Rev. 59: 389-422.

Coleman, J. R., J. R. Nilsson, R. R. Warner, and P. Batt. 1972. Qualitative and quantitative electron probe analysis of cytoplasmic granules in *Tetrahymena pyriformis*. Exptl. Cell Res. 74: 207-219.

Corpe, W. A., and T. E. Jensen. 1992. An electron microscopic study of picoplanktonic organisms from a small lake. Microb. Ecol. 24:181-197.

Courgeon, A. M., C. Maisonhaute, and M. Best-Belpomme. 1984. Heat shock proteins are induced by cadmium in *Drosophila* cells. Exp. Cell. Res. 153:515-521.

Doonan, B. B., R. E. Crang, T. E. Jensen, and M. Baxter. 1979. In Situ X-ray energy dispersive microanalysis of polyphosphate bodies in *Aureobasidium pullulans*. J. Ultrastruct. Res. 69:232-238.

Doyle, J. J., R. T. Marshall, and W. H. Pfander. 1975. Effects of cadmium on the growth and uptake of cadmium by microorganisms. Appl. Microbiol. 29:562-564.

Dubois, M. F., C. Ferrieux, N. Robert, P. Lebon, and A. G. Hovanessian. 1987. Modification, after heat shock, of the antiviral state induced by interferon in murine L cells. Ann. Inst. Pasteur/Virol. 138:345-353.

Ebel, J. P., J. Colas et S. Muller. 1958. Rechercher cytochimiques sur les polyphosphates inorganiques contenus dans les orgainismes vivants. II. Mise au point de methodes de detection cytochimiques specifiques der polyphosphates. Exp. Cell Res. 15:28-36.

Ellis, R. J., and S. M. van der Vies. 1991. Molecular chaperones. Annu. Rev. Biochem. 60:321-347.

Fairlamb, A. H., and A. Cerami. 1992. Metabolism and functions of trypanothione in the kinetoplastida. Annu. Rev. Microbiol. 46:695-729.

Finney, D. J. 1964. Probit Analysis. Cambridge Univ. Press, England, p318.

Fitzgerald, G. P., G. C. Gerloff, and F. Skoog. 1952. Studies on chemicals with selective toxicity to blue-green algae. Sewage Ind. Wastes 24:888-896.

Fogg, G. E., W. D. P. Stewart, P. Fay, and A. E. Walsby. 1973. The blue-green algae. Academic Press-London and New York.

Friedberg, I., and G. Avigad. 1968. Structures containing polyphosphate in *Micrococcus lysodeikticus.* J. Bacteriol. 96: 544-553.

Gadd, G. M., and A. J. Griffiths. 1978. Microorganisms and heavy metal toxicity. Microb. Ecol. 4:303-317.

Gekeler, W., E. Grill, E-L. Winnacker, and M. H. Zenk. 1988. Algae sequester heavy metals via synthesis of phytochelatin complexes. Arch. Microbiol. 150:197-202.

Ghoshroy, S., and M. J. Nadakavukaren. 1990. Influence of cadmium on the ultrastructure of developing chloroplasts in soybean and corn. Environ. and Exp. Bot. 30: 187-192.

Gipps, J. F. and B. A. W. Coller. 1980. Effect of physical and culture conditions on uptake of cadmium by *Chlorella pyrenoidosa.* Aust. J. Mar. Freshwater Res. 31:747-755.

Glagoleff, A. A. 1933. On the geometrical methods of quantitative mineralogical analysis of rocks. Tr. Inst. Econ. Min. and Metal. Moskau, 59.

Goff, S. A., and A. L. Goldberg. 1985. Production of abnormal proteins in *E. coli* stimulates transcription of lon and other heat shock genes. Cell 41;587-595.

Grill, E., E-L. Winnacker, and M. H. Zenk. 1985a. Phytochelatins: the principle heavy-metal complexing peptides of higher plants. Science 230:674-676.

Grill, E., M. H. Zenk, and E-L. Winnacler. 1985b. Induction of heavy metal-sequestering phytochelatin by cadmium in cell cultures of *Rauvolfia serpentina.* Naturwissenschaften 72:432-433

Hahn, G. M., and G. C. Li. 1990. Thermotolerance, thermoresistance, and thermosensitization, p. 79-100, *In* R. I. Morimoto, A. Tissieres, and C. Geogopoulos (eds.), Stress proteins in biology and medicine. Cold spring Harbor Laboratory Press.

Hamer, D. H. 1986. Metallothioneins. Annu, Rev. Biochem. 55:913-952.

Harold, F. M. 1966. Inorganic polyphosphate in biology: structure, metabolism, and function. Bacteriol. Rev. 30:772-794.

Hightower, L. E. 1980. Cultured cells exposed to amino acid analogues and puromycin rapidly synthesize several polypeptides. J. Cell Physiol. 102:407-427.

Hiromi, Y., and Y. Hotta. 1985. Actin gene mutations in *Drosophila*, heat shock activation in the indirect flight muscles. EMBO J. 4:1681-1687.

Inouye, M., and J. P. Guthrie. 1969. A mutation which changes a membrane protein of *E. coli*. Proc. Natl. Acad. Sci. 64:957-961.

Jensen, T. E. 1968. Electron microscopy of polyphosphate bodies in a blue-green alga, *Nostoc pruniforme*. Arch. Mikrobiol. 62:144-152.

Jensen, T. E. 1969. Fine structure of developing polyphosphate bodies in a blue-green alga, *Plectonema boryanum*. Arch. Mikrobiol. 67:328-338.

Jensen, T. E. 1990. Inclusion bodies in the Cyanobacteria, p.1-35. *In* H. D. Kumar (ed.), Phycotalk Vol. 2-1990. Rastogi & Company, India.

Jensen, T. E., and C. C. Bowen. 1961. Organization of the centroplasm in *Nostoc pruniforme*. Proc. Iowa Acad. Sci. 68:86-89.

Jensen, T. E., and J. W. Rachlin. 1984. Effect of varying sulphur deficiency on structural components of a cyanobacterium *Synechococcus leopoliensis*: a morphometric study. Cytobios 41:35-46.

Jensen, T. E., and L. M. Sicko. 1973. The fine structure of *Chlorogloea fritschii* cultured in sodium acetate enriched medium. Cytologia 38:381-391.

Jensen, T. E., and W. A. Corpe. 1988. Utilization of morphometric analysis in evaluating analysis the ultrastructure of various methylotrophic bacteria. Cytobios 53:159-171.

Jensen, T. E., and W. A. Corpe. 1993. Picoplanktonic cyanophytes from three small lakes. (in press)

Jensen, T. E., J. W. Rachlin, V. Jani, and B. Warkentine. 1982b. An X-ray energy dispersive study of cellular compartmentalization of lead and zinc in *Chlorella saccharophila* (Chlorophyta), *Navicula incerta* and *Nitzschia closterium* (Bacillariophyta). Environ. Exp. Bot. 22:319-328.

Jensen, T. E., J. W. Rachlin, V. Jani, and B. Warkentine. 1986. Heavy metal uptake in relation to phosphorus nutrition in *Anabaena variabilis* (Cyanophyceae). Environ. Pollut. 42:261-271.

Jensen, T. E., L, Sicko-Goad, and R. P. Ayala. 1977. Phosphate metabolism in Blue-green algae III. The effect of fixation and post-staining on the morphology of polyphosphate bodies in *Plectonema boryanum*. Cytologia 42: 357-369.

Jensen, T. E., M. Baxter, J. W. Rachlin, and V. Jani. 1982a. Uptake of heavy metals by *Plectonema boryanum* (cyanophyceae) into cellular components,

especially polyphosphate bodies: an X-ray energy dispersive study. Environ. Pollut. 27: 119-127.

Johnson, P. W., and S. Mc. N. Sieburth. 1979. Chroococcoid cyanobacteria in the sea: a ubiquitous and diverse photo biomass. Limno. Oceanogr. 24:928-935.

Kägi, J. H. R. 1991. Overview of metallothionein, p. 613-626, *In* J. F. Riordan (ed.), Methods in enzymology. V205. Academic Press, Inc.

Kellenberger, E., A. Ryter, and J. Sechand. 1958. Electron microscope study of DNA containing plasma. II. Vegetative and mature phage DNA as compared with normal bacterial nucleotides in different physiological states. J. Biophys. Biochem. Cyto. 4:671-678.

Kojima, Y., C. Berger, B. L. Vallee, and J. H. R. Kagi. 1976. Amino-acid sequence of equine renal metallothionein-1B. Proc. Natl. Acad. Sci. 73: 3413-3417.

Lanaras, T., and G. A. Codd. 1981. Ribulose 1, 5-biphosphate carboxylase and polyhedral bodies of *Chlorogloeopsis frischii.* Planta 153: 279-285.

Lawry, N. H. 1979. The ultrastructural effects of exogenous sulfur compound on condensed phosphate deposition in *Synechococcus* sp. (*Anacystis nidulans*). A thesis for the degree of Doctor of Philosophy, The City University of New York.

Lawry, N. H., and T. E. Jensen. 1979. Deposition of condensed phosphate as an effect of varying sulfur deficiency in the Cyanobacterium *Synechococcus sp.* (*Anacystis nidulans*). Arch. Microbiol. 120:1-7.

Levinson, W., H. Oppermann, and J. Jackson. 1980. Transition series metals and sulfhydryl reagents induce the synthesis of four proteins in eukaryotic cells. Biochim. Biophys. Acta 606:170-180.

Li, G. C., D. C. Shrieve, and Z. Werb. 1982. Heat shock and other inducers, p. 395-404. *In* M. J. Schlesinger, M. Ashburner, and A. Tissieres (eds.), Heat shock from bacteria to man. Cold Spring Harbor Laboratory.

Lindquist, S., and E. A. Craig. 1988. The heat-shock proteins. Annu. Rev. Genet. 22:631-677.

Luderitz, V., and A. Nicklisch. 1989. The effect of pH on copper toxicity to blue-green algae. Int. Revue ges. Hydrobiol. 73:283-291.

Luft, J. H. 1961. Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytol. 9: 409-414.

Macaskie, L. E., A. C. R. Dean, A. K. Cheetham, R. J. B. Jakeman, and A. J. Skarnulis. 1987. Cadmium accumulation by a *Citrobacter* sp.: the chemical

nature of the accumulated metal precipitate and its location on the bacterial cells. J. Gen. Microbiol. 133:539-544.

Mantis, N. J., and S. C. Winans. 1992. Characterization of the *Agrobacterium tumefaciens* heat shock response: evidence for a σ^{32} like sigma factor. J. Bacteriol. 174:991-997.

Margoshes, M., and B. L. Vallee. 1957. A cadmium protein from equine kidney cortex. J. Am. Chem. Soc. 79:4813-4814.

McCormick, C. C., and L. Y. Lin. 1991. Quantification and identification of metallothioneins by gel electrophoresis and silver staining, p. 71-77. *In* J. F. Riordan (ed.) Methods in enzymology. V205. Academic Press, Inc.

McLean, M. W., and F. B. Williamson. 1977. Cadmium accumulation by the marine red alga *Porphyra umbilicalis.* Physiol. Plant. 41:268-272.

Merian, E. 1990. Environmental chemistry and biological effects of cadmium compounds. Toxicol. Environm. Chem. 26:27-44.

Mori, H. and A. K. Christensen. 1980. Morphometric analysis of Leydig cells in the normal rat testes. J. Cell Biol. 84:340-354.

Morimoto, R. I., A. Tissieres, C. Geogopoulos. 1990. The stress response, function of the proteins, and perspectives, p. 1-36. *In* R. I. Morimoto, A. Tissieres, and C. Geogopoulos (eds.), Stress proteins in biology and medicine. Cold spring Harbor Laboratory Press.

Morimoto, R. I., K. D. Sarge, and K. Abravaya. 1992. Transcriptional regulation of heat shock genes. J. Biol. Chem. 267:21987-21990.

Mullen, M. D., D. C. Wolf, E. G. Ferris, T. J. Beveridge, C. A. Flemming, and G. W. Bailey. 1989. Bacterial sorption of heavy metals. Appl. Environ. Microbiol. 55:3143-3149.

Munro, S., and H. Pelham. 1985. What turns on heat shock genes? Nature 317:477-478.

Mushrifah, I., and P. J. Peterson. 1991. Uptake and accumulation of cadmium and tin to the insoluble fractions of *Anabaena flos-aquae.* Biomed. Lett. 46:189-198.

Nakahara, H., and H. Kozukue. 1982. Volatilization of mercury determined by plasmids in *E. coli* isolated from an aquatic environment, p. 337-340. *In* S. Mitsuhashi (ed.), Drug resistance in bacteria: Genetics, Biochemistry, and Molecular Biology. Tokyo: Jpn. Sci. Soc.

Neidhardt, F. C., R. A. VanBogelen, and V. Vaughn. 1984. The genetics and regulation of heat-shock proteins. Ann. Rev. Genet. 18:295-329.

Nguyen, V. T., M. Morange, and O. Bensaude. 1989. Protein denaturation during heat shock and related stress. J. Biol. Chem. 264:10487-10492.

Olafson, R. W. 1991. Purification of prokaryotic metallothioneins, p. 283-285. *In* J. F. Riordan (ed.), Methods in enzymology. V205. Academic Press, Inc.

Ord, M. J., and G. R. Al-Atia. 1979. The intracellular effects of cadmium: an experimental study using *Amoeba proteus* as a single cell model, P. 141-173. *In* M. Webb (ed.), The chemistry, biochemistry and biology of cadmium. Elsevier/North-Holland Biomedical Press.

Pankratz, H. S., and C. C. Bowen. 1963. Cytology of blue-green algae. I. The cells of *Symploca muscorum.* Am. J. Bot. 50: 387-399.

Peterson, H. G., F. P. Healey, and R. Wagemann. 1984. Metal toxicity to algae: a highly pH dependent phenomenon. Can. J. Fish. Aquat. Sci. 41:974-979.

Pettersson, A., L. Hällbom, and B. Bergman. 1986. Aluminium uptake by *Anabaena cylindrica.* J. Gen. Microbiol. 132:1771-1774.

Pettersson, A., L. Kunst, B. Bergman, and G. M. Roomans. 1985. Accumulation of aluminium by *Anabaena cylindrica* into polyphosphate granules and cell wall: an X-ray energy-dispersive microanalysis study. Journal of General Microbiology 131:2545-2548.

Peverly, J. H., J. Adamec, and M. V. Parthasarathy. 1978. Association of potassium and some other monovalent cations with occurrence of polyphosphate bodies in *Chlorella pyrenoidosa.* Plant Physiol. 62:120-126.

Pohl, T. 1990. Concentration of protein and removal of solutes, p. 68-83. *In* M. P. Deutscher (ed.), Methods in Enzymology. Vol. 182. Academic Press Inc.

Preiss, J. 1989. Chemistry and metabolism of intracellular reserves, p.189-258. *In* J. S. Poindexter and E. R. Leadbetter (eds.), Bacteria in nature, vol 3. Plenum Publishing Corporation.

Rachlin, J. W., B. Warkentine, and T. E. Jensen. 1982b. The growth response of *Chlorella saccharophila, Navicula incerta* and *Nitzschia closterium* to selected concentration of cadmium. Bull. Torry bot. Club. 109: 129-135

Rachlin, J. W., T. E. Jensen, and B. Warkentine. 1984. The toxicological response of the alga *Anabaena flos-aquae* (Cyanophyceae) to cadmium. Arch. Environ. Contam. Toxicol. 13:143-151.

Rachlin, J. W., T. E. Jensen, and B. Warkentine. 1985. Morphometric analysis of the response of *Anabaena flos-aquae* and *Anabaena variabilis* (Cyanophyceae) to selected concentrations of Zinc. Arch. Environ. Contam. Toxicol. 14:395-402.

Rachlin, J. W., T. E. Jensen, M. Baxter, and V. Jani. 1982a. Utilization of morphometric analysis in evaluating response of *Plectonema boryanum* (Cyanophyceae) to exposure to eight heavy metals. Arch. Environm. Toxicol. 11:323-333.

Rai, L. C., J. P. Gaur, and H. D. Kumar. 1981. Phycology and heavy metal pollution. Biol. Rev. 56:99-151.

Rai, L. C., T. E. Jensen, and J. W. Rachlin. 1990. A morphometric and X-ray energy dispersive approach to monitoring pH-altered cadmium toxicity in *Anabaena flos-aquae.* Arch. Environ. Contam. Toxicol. 19:479-487.

Rauser, W. E. 1990. Phytochelatins. Annu. Rev. Biochem. 59:61-86.

Reed, R. H. and G. M. Gadd. 1989. Metal tolerance in eukaryotic and prokaryotic algae. CRC Press Inc. Boca Raton. Florida.

Remacle, J. 1990. Culture conditions and biomass metal-binding properties, p. 293-301. *In* B. Volesky (ed.), Biosorption of heavy metals. CRC Press.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning, a laboratory mannual. 2nd Ed., Cold Spring Harbor Laboratory Press.

Schiering, N., W. Kabsch, M. J. Moore, M. D. Distefano, C. T. Walsh, and E. F. Pai. 1991. Structure of the detoxification catalyst mercuric ion reductase from *Bacillus* sp. strain RC607. Nature 352:168-172.

Schlesinger, M. J. 1990. Heat shock proteins. J. Biol. Chem. 265:12111-12114.

Schmidt, G., L. Hecht, and S. J. Thannhauser. 1946. The enzymatic formation and the accumulation of large amounts of a metaphosphate in bakers' yeast under certain conditions. J. Biol. Chem. 166:775-776.

Shehata, F. H. A., and B. A. Whitton. 1981. Field and laboratory studies on the blue-green algae from aquatic sites with high levels of zinc. Verh. Int. Ver. Theor. Angew. Limno. 21: 1466-1471.

Shively, J. M. 1988. Inclusions: Carboxysomes, p. 204-206. *In* L. Packer, A. N. Glazer (eds.) Methods in Enzymology. Vol 167, Cyanobacteria. Academic Press, Inc..

Shively, J. M., and R. S. English. 1991. The carboxysome, a prokaryotic organelle: a mini-review. Can. J. Bot. 69: 957-962.

Sicko-Goad, L. M., R. E. Crang, and T. E. Jensen. 1975. Phosphate metabolism in blue-green algae. IV. *In situ* analysis of polyphosphate bodies by X-ray energy dispersive analysis. Cytobiol. 11:430-437.

Sicko-Goad, L. S. 1982. A morphometric analysis of algal response to low dose, short-term heavy metal exposure. Protoplasma 110:75-86.

Sicko-Goad, L., and E. F. Stoermer. 1979. A morphometric study of lead and copper effects on *Diatoma tenue var.* elongatum (Bacillariophyta). J. Phycol. 15:316-321.

Sicko-Goad, L., E. F. Stoermer, and B. G. Ladewski. 1977. A morphometric method for correcting phytoplankton cell volume estimates. Protoplasma. 93:147-163.

Silver, S., and T. K. Misra. 1984. Bacterial transformations of and resistances to heavy metals, p. 23-46. *In* G. S. Omenn and A. Hollaender (eds.), Genetic Control of Environmental Pollutants. New York: Plenum.

Silver, S., and T. K. Misra. 1988. Plasmid-mediated heavy metal resistances. Ann. Rev. Microbiol. 42:717-743.

Silverberg, B. A., P. M. Stokes, and L. B. Ferstenberg. 1976. Intranuclear complexes in a copper tolerant green alga. J. Cell Biol. 69:210

Singhal, R. K., M. E. Anderson, and A. Meister. 1987. Glutathione, a first line of defense against cadmium toxicity. FASEB J. 1:220-223.

Skowronski, T. 1984. Uptake of cadmium by *Stichococcus bacillaris.* Chemosphere 13:1384-1389.

Sokal, R. R. and F. J. Rohlf. 1981. Biometry. 2nd Ed. W. H. Freeman and Company, New York.

Sorokin, C. 1975. Dry weight, packed cell volume and optical density, p. 321-344. *In* J. Stein (ed.), Phycology handbook, 1975. Cambridge University Press.

Squibb, K. S., J. M. O'Connor, and T. J. Kneip. 1991. New York/ New Jersey Harbor Estuary program. Module 3.1: Toxics characterization report (Environmental Protection Agency)

Starodub, M. E., P. T. S. Wong, C. I. Mayfield, and Y. K. Chau. 1987. Influence of complexation and pH on individual and combined heavy metal toxicity to a freshwater green alga. Can. J. Fish. Aquat. Sci. 44:1173-1180.

Starr, R. C., and J. A. Zeikus. 1987. UTEX-the culture collection of algae at the University of Texas at Austin. J. Phycol. 23:32.

Steffens, J. C. 1990. The heavy metal-binding peptides of plants. Annu. Rev. Plant Physiol. Plant Mol. Bio. 41:553-575.

Stempak, J. F., and R. T. Ward. 1964. An improved staining method for electron microscopy. J. Cell Biol. 22: 697-701.

Stokes, P. M. 1983. Responses of freshwater algae to metals, p. 87-112. *In* Round and Chapman (eds.), Progress in phycological research. Vol. 2. Elsevier Science Publishers B. V.

Stratton, G. W., and C. T. Corke. 1979. The effect of cadmium ion on the growth, photosynthesis, and nitrogenase activity of *Anabaena inaequalis.* Chemosphere 5:277-282.

Teige, M., B. Huchzermeyer, and G. Schultz. 1990. Inhibition of chloroplast ATPsynthase/ATPase is a primary effect of heavy metal toxicity in Spinach plants. Biochem. Physiol. Pflanzen 186:165-168.

Tissieres, A., H. K. Mitchell, and V. M. Tracy. 1974. Protein synthesis in salivary glands of *Drosophola melanogaster*: relation to chromosome puffs. J. Mol. Biol. 84:389-398.

Toledo, A. G., H. Babich, and G. Stotzky. 1985. Training of *Rhizopus stolonifer* and *Cunninghamella blakesleeana* to copper: cotolerance to cadmium, cobalt, nickel, and lead. Can. J. Microbiol. 31:485-492.

Trevors, J. T., and C. M. Cotter. 1990. Copper toxicity and uptake in microorganisms. J. Indus. Microbiol. 6:77-84.

Trevors, J. T., K. M. Oddie, and B. H. Belliveau. 1985. Metal resistance in bacteria. FEMS Microbiol. Rev. 32:39-54.

Valdovinos, J. G., T. E. Jensen, and S. J. Lieberman. 1985. An ultrastructural and physiological study of the effects of actinomycin D and cycloheximide on abscission of tobacco flower pedicels. Bot. Gaz. 146:308-314.

Vallee, B. L. 1991. Introduction to metallothionein, p. 3-7. *In* J. F. Riordan (ed.), Methods in enzymology. V205. Academic Press, Inc.

Vallee, B. L., and D. D. Ulmer. 1972. Biochemical effects of mercury, cadmium, and lead. Ann. Rev. Biochem. 41:91-128.

Van Bogelen, R. A., P. M. Kelley, and F. C. Neidhardt. 1987. Different induction of heat shock, SOS, and oxidation stress regulons and accumulation of nucleotides in *Escherichia coli.* J. Bacteriol. 169:26-32.

van Groenestijn, J. W., G. J. F. M. Vlekke, D. M. E. Anink, M. H. Deinema, and A. J. B. Zehnder. 1988. Role of cations in accumulation and release of phosphate by *Acinetobacter* strain 210A. Appl. Environ. Microbiol. 54:2894-2901.

Vasák, M. 1991. Standard isolation procedure for metallothionein. P. 41-44. *In* J. F. Riordan (ed.) Methods in enzymology. V205. Academic Press, Inc.

Verbeke, J. A. 1989. Stereological analysis of ultrastructural changes during induced epidermal cell redifferentiation in developing flowers of catharanthus roseus (Apocynaceae). Amer. J. Bot. 76:952-957.

Visviki, I., and J. W. Rachlin. 1991. The toxic action and interactions of copper and cadmium to the marine alga *Dunaliella minuta*, in both acute and chronic exposure. Arch. Environ. Contam. Toxicol. 20:271-275.

Wang, W. S., and R. G. Tischer. 1973. Study on the extracellular polysaccharides produced by a blue-green alga *Anabaena flos-aquae* A-37. Arch. Microbiol. 91:77-81.

Watanabe, M., T. Takamatsu, K. Kohata, and M. Kunugi. 1989. Luxury phosphate uptake and variation of intracellular metal concentrations in *Heterosigma Akashiwa* (Raphidophyceae). J. Phycol. 25:428-436.

Weckesser, J., and U. J. Jurgens. 1988. Cell walls and external layers, p. 173-188. *In* L. Packer (ed.), Methods in enzymology, Vol. 167. Academic Press, Inc.

Wehr, J. D., L. M. Brown, and I. E. Vanderelst. 1986. Hydrogen ion buffering of culture media for algae from moderately acidic, oligotrophic waters. J. Phycol. 22:88-94.

Weibel, E. R., and R. P. Bolender. 1973. Stereological techniques for electron microscopic morphometry. *In* M. A. Hayat (ed.), Principles and Techniques of Electron Microscopy. Van Nostrand-Reinhold, New York.

Weibel, E. R., G. S. Kistler and W. F. Scherle. 1966. Practical stereological methods for morphometric cytology. J. Cell Biol. 30, 23-38.

Wiame, J. M. 1947a. The metachromatic reaction of hexametaphosphate. J. Am. Chem. Soc. 69:3146-3147.

Wiame, J. M. 1947b. Etude d'une substance polyphosphoree, basophile et metachromatique chez les levures. Biochim. Biophys. Acta 1:234-255.

Wiame, J. M. 1949. The occurrence and physiological behavior of two metaphosphate fractions in yeast. J. Biol. Chem. 178:919-929.

Wood, H. G., and J. E. Clark. 1988. Biological aspects of inorganic polyphosphates. Ann. Rev. Biochem. 57:235-260.

Wood, J. M., and H. K. Wang. 1983. Microbial resistance to heavy metals. Environ. Sci. Technol. 17:582-590.

Yanagimoto, M., H. Saitoh, and N. Kakimoto. 1983. Alkaline shift effect on the uptake of germanium by algae, *Chlorella ellipsoideae, Oscillatoria* sp. and *Spirulina platensis*. J. Ferment. Technol. 61:233-238.

Yoon, K. P., and S. Silver. 1991. A second gene in the *Staphylococcus aureus cadA* cadmium resistance determinant of plasmid pI258. J. Bacteriol. 173:7636-7642.

Zender A., and P. R. Gorham. 1960. Factors influencing the growth of *Microcystis aeruginosa* Kutz. Amend. Elenkin. Can. J. Microbiol. 6:645-660.