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Effect of Zinc and Calcium on the Intracellularly Uptake of Cadmium and Growth of *Escherichia coli*

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***E. coli* was tested for their ability to uptake cadmium intracellularly, and the effect of zinc and calcium on cadmium toxicity to *E. coli* was observed. In addition, the effect of zinc and calcium on the uptake of cadmium was also studied. This study showed that living *E. coli* cells took up more cadmium than the dead cells. *E. coli* in the log phase uptake cadmium more actively than *E. coli* in the stationary phase. These results suggested that there may be metabolic reactions or compounds which encourage the uptake of cadmium. This study also showed that cadmium was sequestered by cell components of which molecular weight is about 30,000. Adding of zinc and calcium chloride reduced cadmium toxicity in *E. coli* and encouraged intracellular uptake by *E. coli*. However adding of heavy metal solutions helped the microorganisms to adsorb more cadmium. Extremely high or low concentrations of zinc, however, did not affect cell viability.**

Key words: cadmium, zinc, calcium, *E. coli*

Cadmium is a non-essential element to living things. It is acutely and chronically toxic in microorganisms, plants and animals including humans. However, the introduction of cadmium into the environment has been increasing through mining operation, industrial pollution, and agricultural run-off. Therefore, removing heavy metals from running water and farmland is becoming an important subject. In recent years there have been a lot of reports on the use of microorganisms to remove toxic heavy metals from environments (7, 9, 14, 21). Although cadmium is one of the most hazardous metals which inhibits the growth of microorganisms, some microorganisms are known to be able to concentrate cadmium, intracellularly (2, 9, 12, 18). For example, microorganisms have shown to remove cadmium in a continuous culture in two-stage chemostat (14). Free bacteria accumulated 1,200 ppm of cadmium whereas attached bacteria concentrated 6,000 ppm of cadmium from water at a steady state (21).

Mclean *et al.* (1972) found that when *E. coli* was grown up in appropriate media with cadmium chloride and cells took up 80% of the cadmium. Even in concentrations of cadmium that inhibited the growth of other microorganisms, *E. coli* grew well (8). *Chlorella pyrenoidosa* is capable of concentrating cadmium, and the amount accumulated is directly proportional to the initial concentration of metal present. The ability of *C. pyrenoidosa* to accumulate cadmium before showing adverse effects may be related to the presence of cadmium sequestering mechanism within the cell (13).

A few other metals and compounds are known to reduce the toxicity of cadmium to microorganisms (1, 3, 5, 6, 11, 16). The toxicity of cadmium towards bacteria and fungi could be reduced by incorporating zinc into the growth medium (1, 10).

Study of the influence of the simultaneous administration of zinc on the toxicity of cadmium showed that zinc could prevent this toxicity in rats tested (17).

Other study showed that calcium in water could prevent the toxicity to microorganisms. The observed differences in cadmium toxicity in water were shown to be caused by the degree of Cd^{2+} binding to inorganic anions, and the amount of binding varied directly with the hardness of the water (3).

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Materials and Methods

Materials and cultures

The 250-ml kimax flasks and other glassware were obtained from Fisher Scientific Company. The petri plated, Liqui-Nox^R, spectrophotometer tubes, and 13×100 mm screw capped tubes were obtained from Baxter Healthcare Corporation. The Baush and Lomb Spectronic 20 spectrophotometer, Fisher 915 pH meter, Jarrell Ash series 82~516 maximum versatility atomic adsorption flame emission spectrophotometer, cadmium hollow cathode lamp, precision drying, and centrifuge concentrator MWCO-50,000 were purchased from Fisher Scientific Company. Other centrifuge concentrators of which the MWCO (Molecular Weight Cut off) are 5,000, 30,000 and 100,000 were purchased from Millipore Corporation. Acetylene was obtained from Standard Welders. $CaCl_2$ (anhydrous), $HClO_4$ and HNO_3 were obtained from J.T baker chemical Co. $CdCl_2$ and $ZnCl_2$ were obtained from Sigma Chemical Co. Bacto-nutrient broth and Bacto Nutrient-agar were purchased from Difco laboratories. *Escherichia coli* was obtained from the stock culture collection at Mississippi State University. Cultures were grown in Bacto-Nutrient broth at 35℃, stored at 4℃ and subcultured every 10 days.

Determination of the Cd concentration

The samples to be analyzed were dried at approximately 90℃ in a drying oven for 1 hour. One ml of HNO_3 was added to dissolve the residues, and the temperature was increased to 150℃ and kept till the acid was evaporated. After cooling to room temperature, digestion was accomplished by adding 0.25 ml of $HClO_4$, and then the acid was evaporated, again. During these procedures, the temperature was increased from 89℃ to 240℃ using a heating block on a cooling hot plate. After cooling down to room temperature, the samples were diluted to a final volume (5 ml 1% nitric acid). The cadmium concentration was determined with an atomic adsorption spectrophotometer at 299 nm.

Preparation of heavy metal stock solutions

A stock solution of cadmium chloride was prepared by adding 0.01 g of cadmium chloride to 10 ml of distilled water. The solution was sterilized by autoclaving. Stock solutions of other heavy metals were prepared by adding 0.01 g of the heavy metal chloride salts to 10 ml of distilled water and sterilized by autoclaving.

Cadmium accumulation by dead and alive *E. coli* cells

One hundred microliters of cadmium solution were added to 10 ml of an overnight culture of *E. coli* (0.95 O.D at 550 nm). Five ml of this culture were dispensed into screw capped test tubes (13×10 mm). Another 10 ml of the overnight culture of *E. coli* was washed with potassium phosphate buffer three times, and one hundred microliters of cadmium solutions were added; then five ml of this culture were also dispensed into screw capped tubes. Cells in half of the tubes were killed by heating (90℃ for 20 min). All the tubes were incubated for twenty hours at 35℃ on rotary shaker at 96 rpm. After incubation, these samples were filtered by Gelman Science Membrane. One ml of nitric acid was added to one ml of the filtered solution and incubated at 90℃ for two hours, and analyzed for cadmium (final volume=1 ml). To study the effect of zinc and calcium chloride on cadmium accumulation by dead and live cells, these two metal solutions were added to both dead and live cells, respectively, and incubated under the same conditions and for the same length of time before cadmium analysis. In this experiment, an overnight culture is defined as "grown" with an O.D. of about 0.95 and a start culture is defined as a "growing" culture with an O.D. of about 0.1. One hundred microliter of cadmium solutions were added to 10 ml of "grown" and "growing" cultures, and the culture was washed three times with potassium phosphate buffer, and resuspended in 10 ml of potassium phosphate buffer. These cultures were analyzed for cadmium using the same methods mentioned to determine of the Cd concentration.

Determination of molecular weight

E. coli cells of the overnight cultures in a nutrient broth were washed with potassium phosphate buffer three times. The pellet was resuspended with 5 ml of potassium phosphate buffer, and sonicated for approximately 5 min (50 seconds exposure+15 seconds rest). Four hundred μl of cell free solution was transferred to Ultra-Filtration membrane centrifuge concentrators which can isolate cell compounds according to molecular weight (5,000, 30,000, 50,000 and 100,000). These were centrifuged for 5 min at 6,000 rpm. Five hundred μl of nitric acids were added to the collected solution. The samples were exposed to H_2S gas in a gas chamber for 5 min. The gas was produced by adding hydrochloric acid to zinc sulfide. The reactions can be recognized by the solution color becoming yellow.

Effect of zinc and calcium to cell viability

Varying amounts of metal solutions (10, 50, 100 μl of 0.004 M metal solutions) were added to the washed overnight culture (0.95 O.D. at 550 nm) and the log phase culture (0.1 O.D. at 550 nm) containing 0.004 M

of cadmium. These cultures were incubated for 20 hours at 35℃ on a rotary shaker. The incubated cultures were filtered by using a Gelman Science· Membrane and a 100 ml syringe. One ml of this solution was transferred to a 13×10 mm tube, and digested by nitric acids and analyzed for cadmium. The number of viable cells was determined by the conventional spread plate technique.

Results

In order to determine the amount of given metallic ion intracellularly taken up by *E. coli*, the amount of cadmium taken up by dead cells was subtracted from that of live cells. In the first experiment, *E. coli* was tested for their ability to accumulate cadmium in both nutrient broth and potassium phosphate buffer. The results from the nutrient broth suggested that the complicated media did not affect the accumulation of cadmium by the cells. Table 1 shows that more cadmium was taken up by the live cells. This result suggested that live *E. coli* cells have a mechanisms to uptake cadmium into the cells. In addition to this experiment, amount of uptake in *E. coli* cells in the log phase (Growing) was compared to cells in the stationary phase (Grown). Although the optical density of "growing" cells was lower than "grown" cells, "growing" cells took up more cadmium than "grown" cells. However, this study also showed that the total amount of cadmium taken up was dependent on the total number of cells (Fig. 1).

Then, the molecular weight of cell compounds sequestering cadmium was determined by a membrane centrifuge concentrator and identified by hydrogen sulfide gas. The solution containing the compounds of which the molecular weight is over 30,000 to 50,000 showed a stronger yellow color than those of the other two solutions.

It is known that some heavy metals and compounds can prevent cadmium toxicity to microbial cells. This study also showed that calcium and zinc have the ability to prevent cadmium toxicity at certain concentrations. Higher the concentration of calcium at a constant cadmium concentration (0.004 M), the more cells survived (Fig. 2). Zinc also was able to reduce the toxicity of cadmium. However, in low concentrations or in very high concentrations, the cell numbers was approximately the same as the culture containing the cadmium alone (Fig. 3). Although both calcium and zinc helped "growing" *E. coli* cells take up more cadmium than "grown" *E. coli* cells (Fig. 3), these two metals inhibited *E. coli* cells to accumulate cadmium.

Table 1. Cadmium up taken up by dead and living *E. coli* cells

Type of broth	Condition of cell	Mean of Cd conc. (PPM)
P.P buffer	Dead	0.3
	Live	0.8
Nutrient broth	Dead	0.3
	Live	0.8

P.P is potassium phosphate buffer. Mean of Cd conc is the amounts of cadmium in the filtered buffer or broth.

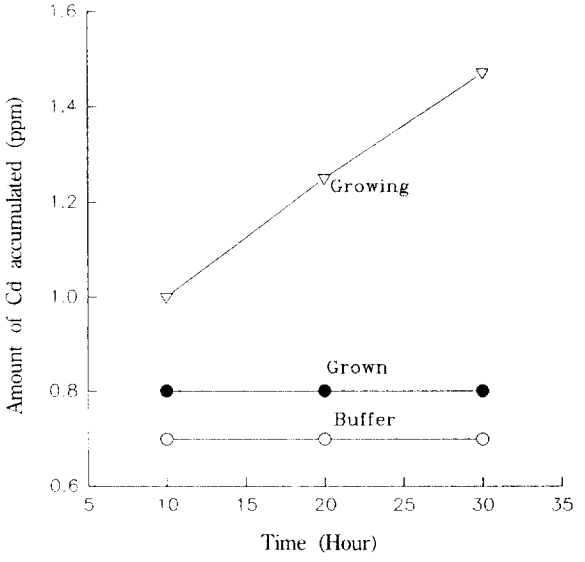

Fig. 1. Comparision of Cd amounts accumulated by *E. coli* cells. Growing=Cells in log phase, Grown=Cells in stationry phase Buffer=Buffer solution.

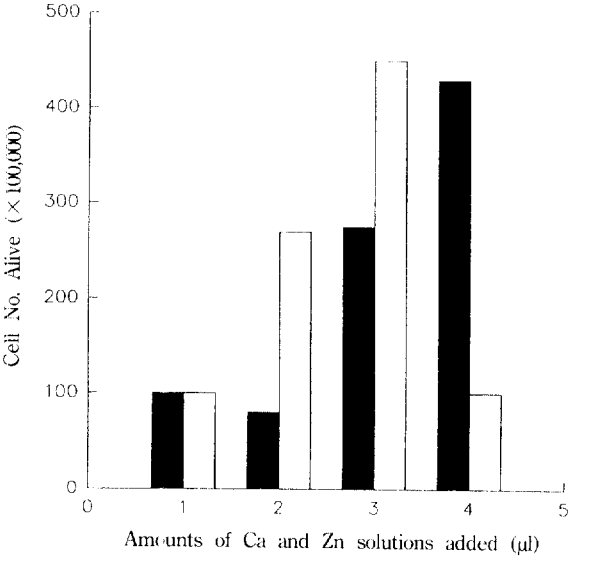

Fig. 2. Effect of Ca and Zn to cell viability of the cultures containing Cd. 1=Cd alone (0.004 M), 2=Adding 10 μl of 0.04 M Ca and Zn solution, 3=Adding 50 μl of 0.04 M Ca and Zn solution, 4=Adding 100 μl of 0.004 M of Ca and Zn solution ■ Ca □.

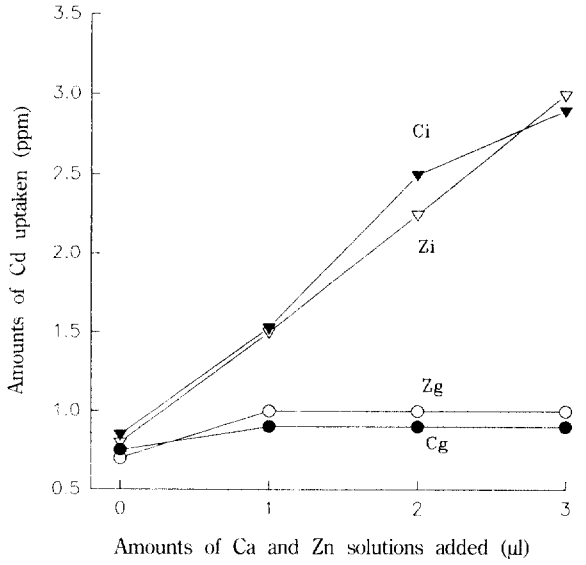

Fig. 3. The effect of different concentrations of Ca and Zn on Cd uptake by Growing and Grown *E. coli* cells. 0=Cd alone 1=Adding 10 μl of Ca and Zn solutions, 2=50 μl, 3=100 μl Ci=Growing (log phase) cells with Ca solution, Cg=Grown (Stationary phase) cells with Ca solution, Zi=Growing cells with Zn solution, Zg=Grown cells with Zn solution.

Table 2. Approximate molecular weight of cell compound sequestering cadmium

Moleular weight	Depth of color (yellow)	Mean of Cd. conc. (PPM)
100,000	+ − −	<0.1
50,000	+ + −	<0.2
30,000	+ + +	>0.2
5,000	− − +	<0.1

+; more recognizable yellow color, −; less recognizable yellow color (There was no change of color in the samples.)

Table 3. Effect of calcium and zinc on cadmium uptake by *E. coli*

Solution	Condition of cell	Mean of Cd. conc (PPM)
Cd alone (0.004 M)	Dead	0.3
	Live	0.8
Cd+Ca (0.004 M)	Dead	1.1
	Live	1.1
Cd+Zn (0.004 M)	Dead	1.3
	live	1.2

Mean of Cd conc. is the amounts of cadmium in the filtered buffer.

Discussion

The ability of microorganisms to accumulate certain heavy metals has been widely recognized (7, 13, 14, 20). *Micrococcus luteus* and *Azotobacter* sp. immobilized large quantities of lead when these organisms were "grown" in a media containing lead salts (26). *Actinomyces niger* also showed a significant accumulating ability of cadmium in very high concentrations, though not in low concentration (8). *Chlorella pyrenoidosa* is capable of concentrating cadmium, and the amount taken up is directly proportional to the concentration of heavy metals present initially (13). The study of six species of microorganisms, *Escherichia coli, Bacillus cereus, Lactobacillus acidophilus, Staphylococcus aureus, Entercoccus faecalis,* and *Actinomyces niger* showed that Cd absorbtion patterns were significantly different among the organisms. Three organisms (*E. coli, B. cereus* and *S. aureus*) demonstrated the ability to accumulate cadmium (data not shown). However, since *E. coli* had the greatest accumulating ability of the organisms tested, it was selected for further study. There are reports showing that *E. coli* has the ability to accumulate cadmium, and has tolerance to toxic levels of cadmium (8, 18). In this study, living *E. coli* cells took more cadmium than dead *E. coli* cells (Table 1); and "growing" cells took up more cadmium than "grown" cells (Fig. 1). These results indicate that *E. coli* takes up cadmium metabolically into the cell. There are various mechanisms which exist where organisms can tolerate and accumulate cadmium (7). These are shown in the concentration of metals in the cell wall and chelation or transformation of the elements. When *E. coli* was inoculated into a defined medium, 75% of Cd^{2+} were associated with the cell membrane and 23% in cytoplasm (18). Molecular sieve chromatography of cell extracts showed that the Cd^{2+} is associated with two classes of macromolecules. The study also showed that the ability of *C. pyrenoidosa* to accumulate large concentrations of cadmium before showing adverse effects may be related to the cadmium sequestering agent(s). Our study also suggests that some cadmium sequestering agents exist. Table 2 shows that the molecular weight of the compounds is from 30,000 to 50,000., and could not be dissolved by methanol-chloroform solvents (data not shown) suggesting these compounds to be hydrophilic. Many environmental factors influence the uptake of trace elements by microorganisms. Important factors are temperature, pH, soil constituents, and other heavy metals (4, 11, 15, 16). Among them, the reduction of heavy metal toxicity by other heavy metals has been widely recognized (1, 19). Magnesium at proper concentration are shown to prevent cadmium toxicity to *E. coli* (1). It is reported that selenium also could protect rat from damage by cadmium (17). A study of the influence of the simultaneous administration of zinc on the toxicity of cadmium showed that zinc could prevent this toxicity. Cadmium caused a significant decrease in oxidative phosphoryla-

tion in the mithochondria, and zinc prevented decrease in oxidative phosphorylation in the mithochondria. The toxicity of cadmium towards bacteria and fungi could be lessened by incorporating zinc and magnesium into the growth medium (1, 18). The toxicity of cadmium towards *E. coli* was also prevented by proper concentration of zinc (Fig. 2). However, higher concentrations of zinc increased cadmium absorption by *E. coli* cells (Fig. 3). In addition to zinc, few other heavy metals and compounds are known to reduce the toxicity of cadmium to microorganisms. Uptake of cadmium also is affected by the hardness of water. The observed differences in cadmium toxicity in water are shown to be caused by the degree of Cd^{2+} binding with inorganic anions, and the amount of binding varied directly with the hardness of water. Calcium also prevented cadmium toxicity to *E. coli* (Fig. 2). However, the higher the concentration of calcium, the more effectively it prevented cadmium toxicity to *E. coli*. Calcium also increased the uptake of cadmium by *E. coli*. There is a possibility that the increase in the uptake is related to multiplying of cell number. These results indicate that the presence of other metallic ions in the environment have major impact on adsorption and accumulation of Cd by *E. coli*.

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