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ABSTRACT

Title of thesis: A Comparative Study of Cadmium Uptake
By Cells of Gram-Positive and Gram
-Negative Bacteria.

Shubhangi Bhende: Master of Science in Environmental
Science, 1990

Thesis directed by: Sam S. Sofer, professor
Sponsored Chair in Biotechnology

Cadmium uptake by cells of Gram-positive and Gram-negative bacteria was studied. Gram-positive and Gram-negative bacterial cells were separated from activated sludge collected from the Parsippany Troy Hills Water Pollution Control Plant (NJ) by isolating them on agar plates. These were grown separately and used for uptake experiments.

Gram-positive bacterial cells showed 17% more uptake of cadmium as compared to Gram-negative cells at 30°C and pH 6.6. More than 95% of the total cadmium removal from the solution was observed during first 5 min. of cell-metal

contact time. In case of Gram-positive bacteria cadmium uptake decreased by 5-10% after cells were inhibited by contacting with 1M sodium azide for 45 min. In the case of Gram-negative bacteria, uptake decreased by 4-5% under same conditions.

Cadmium uptake increased by 13% in Gram-positive bacteria after addition of nutrients, suggesting some metabolic uptake of cadmium. Although cadmium uptake decreased by 4-5% after inhibition of cells with sodium azide, there was no significant increase in cadmium uptake by Gram-negative bacteria after nutrient addition.

2) A COMPARATIVE STUDY OF CADMIUM UPTAKE BY CELLS OF GRAM-
POSITIVE AND GRAM-NEGATIVE BACTERIA.

by
Shubhangi ^{Rajan} Bhende

Thesis submitted to the Faculty of the Graduate school of
the New Jersey Institute of Technology in partial
fulfillment of the requirements for the degree of
Master of Science in Environmental Science.

1990

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APPROVAL SHEET

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CHAPTER I
INTRODUCTION

Industrial development throughout the world has resulted in increased circulation of heavy metals in the environment. Heavy metals are present at high concentration in some industrial effluents and also appear as side pollutants at low concentration in many wastes. These pollutants are highly toxic even at very low concentration and many living organisms are capable of accumulating them. Thus, these metals enter the human food chain. Heavy metal uptake by biological cells is known as biosorption. This phenomenon includes both passive adsorption of heavy metals at binding sites present on the cell surface and metabolically mediated uptake which implies any metabolic response of the microorganism to the presence of metal ion in the solution.

Efforts have been made to reduce the emission of heavy metals. New, cleaner technologies may partly allow us to reach this goal by minimizing the production of metal laden industrial effluents. The removal of heavy metals from liquid effluents is a major concern. A large number of physical and chemical methods are presently employed by certain industries, which include ion exchange on natural and synthetic polymers, hydroxide or sulfide precipitation

and membrane processes, but biological methods investigated present many potential advantages and seem to be promising.

Many investigators have carried out studies on the mechanism of heavy metal uptake using pure microbial species. [Horikoshi et al. (1981); Higham et al.(1985); Scott et al. (1986); Nakajima and Sakaguchi (1986)]. However, the use of activated sludge appears to be more convenient for field application since it is used widely in sewage treatment processes and can be used as an inexpensive source of microorganisms.

Earlier studies carried out at the NJIT Biotechnology Laboratory in this area have indicated that uptake of cadmium is largely a passive phenomenon. This implies that cadmium gets attached to the cell wall components. Gram-positive and Gram-negative bacteria have significant differences in their cell wall structure. Therefore, Gram-positive and Gram-negative species of bacteria from activated sludge have been chosen to evaluate differences in cadmium uptake properties. Cadmium has been chosen as a model because it is found in the effluents of many industries and is one of the most toxic heavy metals (Nriagu and Pacyna 1988; Murphy and Spiegel 1982). Toxicity to man results in the itai-itai disease which causes severe bone troubles. Cadmium is largely retained in humans and disease may occur several years after intoxication.

CHAPTER II

LITERATURE SURVEY

Cadmium is a relatively rare element with no known biological function. It is normally found in the natural environment at low levels. Cadmium can enter the environment from variety of anthropogenic sources, such as by-products from coal combustion, mine wastes, fertilizers and pesticides, electroplating processes, and iron and steel production. Industrial utilization of cadmium has increased the rates of biological mobilization and transportation of this element, as the input exceeds the amounts involved in natural abiotic cycling processes.

Babich and Stotzky (1978) in their studies have noted that cadmium is a potent toxicant to microorganisms such as bacteria, fungi and algae and there are some microbial strains which are resistant to this metal. They also noted that the toxicity of cadmium is highly dependent on speciation of the metal, as well as various physical, chemical and biological factors.

Wood and Wang (1983) reported that a number of chemical parameters must exist before a metal ion can be taken up by a living cell. These include the charge on the metal, its ionic radius, preference of metals for certain

organic ligands, available concentration of metals in both polluted and non-polluted environments, chemical reactions of metal ion in solution and the existence of metal uptake and efflux pumps in organisms.

Titus and Pfizer (1982) concluded that the toxicity of cadmium to microorganisms depends on its uptake which is affected by pH and temperature. Gauthier and Flatau (1980) showed that it also depends on salinity, cadmium and peptone concentration.

Steeman et al.(1973), Gonye et al.(1970), Jackson et al.(1978) reported that free metal ions are often more toxic. Lindberg et al.(1974) showed that their toxicity can be reduced in the presence of organic matter.

Mitra et al. (1978) reported that cadmium causes single stranded breakage in Escherichia coli DNA. Weiss et al. (1978); Silver et al.(1982); and Foster(1983) reported that cadmium ions are taken into sensitive bacterial cells via energy dependent Mn transport system where they cause immobilization of respiration by binding the sulfhydryl groups in proteins. Higham et al. (1984) noted that CdCl₂-resistant Pseudomonas putida synthesizes cadmium binding proteins.

Resistance to cadmium in bacteria is genetically determined. It is shown that penicillinase plasmids found

in Staphylococcus aureus also encode for resistance to Hg^{++} and Cd^{++} . Chopra (1971) reported an uptake of cadmium by resistant strains of Staphylococcus aureus and there was a significant decrease in cadmium uptake in resistant cells when compared with cells that did not contain the cad-v gene.

Silver et al. (1984) in their paper reported that two distinct genes are responsible for cadmium resistance in Staphylococcus aureus the cad A which encodes for high resistance and cad B which encodes for small increase in resistance. When both genes are present in a single strain, the cad A resistance masks the cad B gene effect. It was also noted that a plasmid-determined cadmium resistant strain of Staphylococcus aureus removed less cadmium from the solution than a cadmium sensitive strain, and it was suggested that the plasmid in the resistant cells blocked an energy dependent process of cadmium transport which may be functional in many sensitive cells. Surowitz et al. (1984) showed that a cadmium sensitive strain of Bacillus subtilis accumulated 10-fold more cadmium than a resistant strain of the same spp.

Higham et al. (1984) noted that $CdCl_2$ resistant Pseudomonas putida synthesizes Cd-binding proteins. They also reported that cadmium resistance of Pseudomonas putida

strain was quickly lost when cadmium was not included in the growth medium and cadmium uptake was not observed at 4°C suggesting that metabolic energy was required for cadmium transport.

Horikoshi et al. (1981); Higham et al. (1986) have reported that cadmium concentration in the test solution decreases tremendously within the initial 5-10 minutes. According to some published papers metal uptake is a passive phenomenon where metal ions get attached to the extracellular polymers produced by microorganisms. These polymers can be negatively charged sugar units of polysaccharide chains extending from the microbial cell wall or may be polymers of uronic acid, hexosamines or organically bound phosphates. Cell surface accumulation is thus the result of complexation reaction between metal ions and charged receptive constituents of the cell wall. The composition of the cell wall of microorganisms is highly spp. dependent and differs considerably among Gram-positive and Gram-negative bacteria.

According to some researchers metal uptake is an active phenomenon where intracellular accumulation of a metal results from the lack of specificity in the normal metal transport system and at the right concentration, metals may act as competitive substrates in the transport system. In

many cases their uptake has been shown to be controlled by plasmid linked genes.

CHAPTER III

STRUCTURE OF BACTERIAL CELL WALL

The cytoplasmic membranes of many cells are protected by a rigid external structure called the cell wall that surrounds the cell. Generally, the cell wall is relatively porous so that it does not greatly restrict the flow of small molecules to or from the cytoplasmic membrane, although very large molecules usually are unable to pass across it.

The cell wall protects the bacterial cell against osmotic pressure. It is the rigidity of the cell wall surrounding the cytoplasmic membrane that prevents the cytoplasmic membrane of the cell from expanding and bursting because of the pressure placed on it. The cell wall is also responsible for the shape of a bacterial cell.

The cell wall of almost every eubacterial cell contains peptidoglycan, which is also known as murein or mucopeptide. As the name implies, there are two parts to the peptidoglycan molecule- a peptide portion, which is composed of amino acids connected by peptide linkages, and a glycan or sugar portion. The glycan portion, which forms the backbone of the molecule, is composed of alternately repeating units of the amino sugars N-acetylglucosamine and

N-acetylmuramic acid. Short peptide chains with four amino acids are attached to some of the N-acetylmuramic acid molecule.

Some of the amino acids occurring in the peptide portion of the molecule are relatively unique in biological systems. These include D-amino acids and diaminopimelic acids. All the tetrapeptide sequences include L-alanine, D-glutamic acid, either L-lysine or diaminopimelic acid, and D-alanine. The major variation that occurs is the substitution of lysine and diaminopimelic acid, with lysine occurring in most Gram-positive bacteria and diaminopimelic acid occurring in all Gram-negative bacteria.

The tetrapeptide chains are interlinked by a peptide bridge between the carboxyl group of an amino acid in one tetrapeptide chain and the amino group of an amino acid in another tetrapeptide chain. When this cross-linkage is disrupted, the cell wall is defective and cannot adequately protect the bacterial cell against osmotic shock.

The difference between Gram-positive and Gram-negative bacteria is due to the difference in cell wall structure. This difference is critical because it distinguishes the major taxonomic groups of eubacteria and has practical importance with regard to the survival of bacteria in nature.

The Gram-positive cell wall has a peptidoglycan layer that is relatively thick and comprises 90 percent of the cell wall. The rigid peptidoglycan acts to fulfill the primary protective function of the cell wall. In addition to peptidoglycan, Gram-positive cell walls generally have teichoic acids which are acidic polysaccharides. They contain a carbohydrate such as glucose phosphate and an alcohol-either glycerol or ribitol. Teichoic acids can bind protons, maintaining the cell wall at relatively low pH so that self produced enzymes do not degrade the cell wall. They also have several other functions, such as binding metals and acting as receptor site for some viruses.

The Gram-negative cell wall is biochemically more complex. The peptidoglycan layer of the Gram-negative cell wall is very thin and often comprises only 10 percent or less of the cell wall. Teichoic acids generally do not occur in Gram-negative bacteria, but lipoproteins are bonded to the peptidoglycan, forming an integral part of the Gram-negative bacterial cell wall. Additionally there are layers of lipopolysaccharide, phospholipids, and proteins outside the peptidoglycan layer. In addition to the peptidoglycan layer Gram-negative bacteria also have a more complex structure called the cell envelope. The cell en-

velope extends outwards from the cytoplasmic membrane to a second membrane-the outer membrane. The outer membrane is a bilipid layer containing phospholipids and proteins, but it also contains lipopolysaccharide. Functionally, the outer membrane of the Gram-negative bacterial cell is a coarse molecular sieve that allows the diffusion of both hydrophilic and hydrophobic molecules depending on the species. Permeability of the outer membrane to nutrients is provided in part by proteins collectively called porins, which, in aggregates usually of three, form cross membrane channels through which certain small molecules can diffuse.

CHAPTER IV

OBJECTIVE

This study is primarily aimed at determining differences in the metal uptake properties of cells of Gram-positive and Gram-negative bacteria separated from activated sludge.

From the earlier studies carried out using activated sludge cadmium uptake was found to be largely passive. This indicates that cell wall plays an important role in cadmium uptake. The phenol degradation studies carried out earlier also showed that there are some differences in the degradation capabilities of these bacteria. The first step has been separation and purification of cells of Gram-positive and Gram-negative bacteria from activated sludge. The next step involves determination of effects of pH, inhibition of respiratory activity, and effect of nutrient addition on cadmium uptake property of Gram-positive and Gram-negative bacteria.

CHAPTER V
MATERIALS AND METHODS

5.1 MICROORGANISMS

Activated sludge (a mixed microbial community) from the Parsippany-Troy Hills Water Pollution Control Plant (NJ) was used in this study. It was passed through a 500 μm sieve to remove inert gross particles and kept overnight at room temperature with good aeration. Then it was allowed to settle for 5-10 minutes and the supernatant was replaced by tap water. The sludge was then mixed, settled, and washed again with tap water. This step was repeated twice with distilled water. After this pre-treatment pellets were prepared by centrifuging the sludge at 1500 rpm for 5 minutes. Determination of dry cell concentration in the pellets gave an average value of 58.2 \pm 0.2 mg/g wet pellets (measured by weighing a given amount of sludge before and after drying at 105°C overnight).

5.2 ISOLATION OF BACTERIAL CELLS

The pellets obtained after washing and centrifuging the sludge were used for isolation studies. One gram of wet pellets was suspended in 10 ml of 0.8% sterile saline solution. Following this, serial 1:10 dilutions were done and

0.1 ml of each dilution was surface spread on agar plates (in triplicates). The selection of different agar plates used was based on Prakasam and Dondero (1967) which included, floc agar (FA), plate count agar (PCA), trypticase soy agar (TSA), nutrient agar (NA) and casitone glycerol yeast extract agar (CGY).

The highest number of characteristically different colonies grew on nutrient agar plates. Following this further isolation was done using sterile nutrient agar plates. Colonies showing the same characteristics on the agar plate were all considered as being of the same type. Sterile nutrient broth was used as the storage medium for the isolated colonies. Following the transfer of the isolated colonies, tubes were incubated at room temperature in order to allow growth and subsequently the broth was stored at 4°C.

5.3 GRAM STAINING

Staining is one of the most important methods used for identification of bacteria. There are certain staining methods which are used specially for certain organisms. Gram staining is used for most of the organisms. This method has been developed empirically by the Danish bacteriologists Christian Gram in 1884. The crystal violet

which is used initially is a basic dye which is taken up in similar amounts by all bacteria. Iodine which is used next acts as a mordant. Alcohol acetone mixture is used as decolorizer and then safranin is used as counter stain. Gram-positive organisms retain the initial violet stain, whereas Gram-negative organisms are decolorized by the organic solvent and therefore show the counter stain. It was later on shown by Salton that the Gram-positive cell wall is not stained itself but the presence of permeability barrier to elution of the dye-iodine complex by alcohol causes stain retention. This mechanism accounts for the observation that Gram-positive cells often become Gram-negative in aging cultures (in which autolytic enzyme attack the wall). The isolates were checked for their Gram nature using the Gram staining method as follows :

1. A loopful of isolate was smeared on the greasefree slide. The smear was fixed by passing it through flame.
2. Smear was stained with Gram's crystal violet for 1 minute. The stain was washed with water. The slide was covered with Gram's iodine for 1 minute. It was washed with water. Then the smear was decolorized using the alcohol acetone mixture and again stained with safranin. On the basis of Gram's staining the isolates were divided into two categories. Gram-positive and Gram-negative and were collected accordingly and stored in sterile nutrient broth.

5.4 GROWING OF CELLS

The separated cells were grown using double strength nutrient broth by inoculating 5 ml nutrient broth stock culture in 250 ml of double strength sterile nutrient broth under sterile conditions. The flasks were incubated on a shaker at a speed of 120 rpm and at 28°C - 30°C for 24 hours. The cells were inoculated in series of shaker flasks in order to get sufficient biomass. The cells were collected by centrifuging the medium for 15 minutes at 28°C and 10,000 rpm using a Sorvall Ultracentrifuge (Dupont). The cells were washed with sterile 0.5% NaCl followed by sterile d/w, and pellets were collected and stored.

5.5 STORAGE

Pellets were frozen and stored at 20°C in 5 ml sterile screw cap glass tubes to prevent biological evolution. Skim milk was used as a protective agent on the basis of 25 grams of wet pellets suspended in 100 ml of 12.5% (w/v) skim milk solution. Dry cell concentration in this suspension was measured by washing a known amount of suspension as detailed below and weighing the cells after drying at 105°C overnight.

5.6 PREPARATION OF CELL SUSPENSION FOR EXPERIMENTS

Before starting any experiment, skim milk was washed from the suspension as follows :

One or more tubes of frozen cells were thawed by placing them in water at room temperature. Then the desired amount of suspension was weighed to get the desired weight of the cells given the ratio dry cells weight/total weight of suspension which was previously determined. 1-5 ml of suspension were then poured in a centrifuge tube in which 35 ml of 0.5% W/V NaCl solution at 5°C was added and mixed. Centrifugation was carried out at 1500 rpm at 5°C for 5 minutes. Supernatant was removed and the cells were resuspended in 35 ml of 0.5% W/V NaCl solution at 5°C. This step was repeated 4 times. The use of sodium chloride in the step as well as in the uptake experiment was meant to prevent cell damage due to osmotic pressure. Finally the cells were suspended in an appropriate volume of 0.5% W/V NaCl solution (at room temperature) so as to get a dry cell concentration of 2000 mg/liter or as required for the experiment.

5.7 CHEMICALS

All chemicals used were analytical grade reagents. Cadmium was generally used in the form of a 1000 ppm Cd

solution prepared by dissolving cadmium chloride in 0.5% W/V NaCl solution. The pH was adjusted using a 0.01 N or 0.1 N NaOH solution in 0.5% W/V NaCl. Nutrients were added in the form of a solution of glucose 1000 ppm, $(\text{NH}_4)_2\text{SO}_4$ 946 ppm, KH_2PO_4 17.6 ppm, yeast extract 1 ppm and NaCl 5000 ppm.

5.8 STERILIZATION

Sterilization of all the plates, tubes, nutrient medium and skim milk was carried out by autoclaving at 120°C and 15 psi for 15 minutes. All the material was incubated overnight in order to check for sterility.

5.9 METABOLIC INHIBITION OF MICROORGANISMS USING SODIUM AZIDE

Inhibition by sodium azide (NaN_3) was performed after washing the cells as detailed above. The cells were suspended in 1 M NaN_3 solution and mixed on a magnetic stirrer plate at room temperature. Cell concentration in the suspension was 2000 mg/l. After 45 min. the cells were washed again and resuspended in a volume of 0.5% NaCl solution at room temperature to get a biomass concentration of 2000 mg/l. The 2000 mg/l suspension of inhibited cells was used to test cadmium uptake and the respiratory activity was measured at the same time.

5.10 DETERMINATION OF RESPIRATORY ACTIVITY

The microassay reactor was used to check the activity of the cells used in the experiment. The experimental set up of the reactor is as shown in the Figure 1. The bioreactor was a small jacketed vessel of 1.8 ml capacity with a provision for a Clark type dissolved oxygen probe. Water at 30°C was recirculated through a water bath to the reactor jacket. The reactor was mounted on a stirrer plate and a small magnetic bar which maintained it at a uniform temperature. Before the start of each run the reactor was washed with tap water followed by methanol and d/W. 1.18 ml of CdCl₂ solution (at the desired cadmium concentration between 0 and 500 ppm) in 0.5% w/v NaCl was added and saturated with oxygen by bubbling air through it. When the saturation level was reached aeration was stopped and 20 µl of 0.01 N NaOH was added. Then 0.6 ml of 2000 ppm cell suspension prepared as detailed above was added to get a final pH of 6.6 +/- 0.1 in the final volume of 1.8 ml. The reactor was plugged in and the decrease in oxygen concentration due to endogenic respiration was recorded. A linear response was obtained between 90% and 60% of oxygen saturation and the rate of oxygen consumption could therefore be easily calculated in that range.

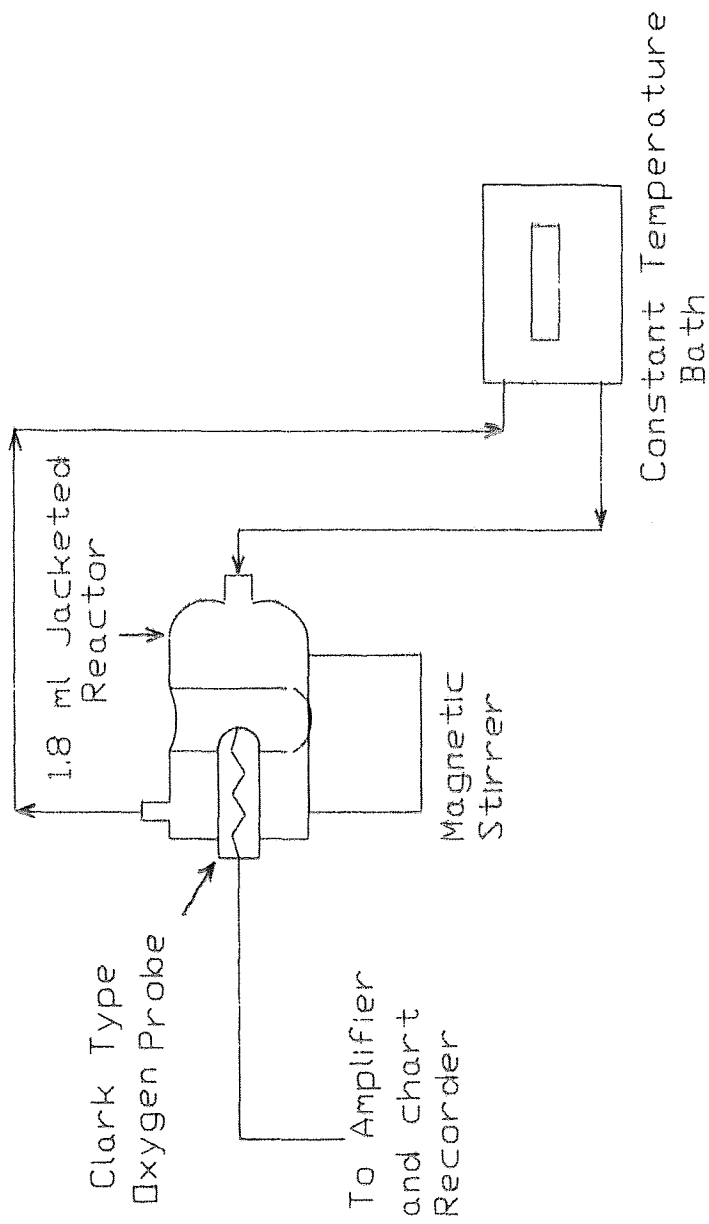


Fig. 1 Microassay Reactor

5.11 UPTAKE EXPERIMENTS

Runs were carried out in 50 ml shaker flasks with 25 ml of medium. The medium consisted of a 0.5% w/v NaCl solution in which the cells and cadmium was added successively. The pH was adjusted with 0.1 N NaOH. Since it was not easy to adjust the pH with good accuracy because no buffer solution was used, it was decided to bracket the desired pH by testing 5 to 6 different pH values between 6.2-6.8 and subsequently determining cadmium uptake at pH 6.6 by interpolation. This procedure increased the number of runs to be performed considerably but rendered the experimental results more reliable. The 2000 mg/l cell suspension was prepared as detailed above and used as the source of microorganisms to study the uptake by free cells. Dry cell concentration in all runs was 200 mg/l. A series of blanks was also carried out for each series of experiments. One series was with cadmium but no cells and other with cells but no cadmium. Initial cadmium concentration was 10 mg/l in all runs. When nutrients were added, final C, N, S, and P concentrations in the medium were 4.00 ppm, 0.20 ppm, 0.23 ppm, and 0.04 ppm respectively. These low concentrations were meant to keep biomass growth at an insignificant level. The purpose for low phosphorous concentrations was also to avoid precipitation of cadmium

phosphate. All the studies were carried out at 30°C. All the data presented were obtained at pH 6.6 +/- 0.1 in order to avoid chemical precipitation of cadmium hydroxide or carbonate (due to CO₂ dissociation in the medium during incubation period). The shaker flasks were placed in an incubator where they were stirred permanently at the desired temperature. The incubation time necessary to reach equilibrium was determined in preliminary runs and was found to be 2 h for free cells. After incubation, pH was measured in each flask and the suspension was filtered using syringe filters with 0.45-μm pore size cellulose acetate millipore membrane. The liquid phase was analyzed for cadmium concentration. Most of the runs were repeated at least three times.

5.12 ANALYTICAL METHODS

Cadmium concentrations were determined by atomic absorption with a Perkin Elmer spectrophotometer model 305 B. The operating conditions were as recommended in the Perkin Elmer instruction manual. The accuracy with these standard conditions was estimated to be +/- 2%.

TABLE 5.1: COMPOSITION OF NUTRIENT AGAR

Pancreatic digest of gelatin peptone....	5.0 g
Beef Extract.....	3.0 g
Agar.....	15.0 g

TABLE 5.2: COMPOSITION OF NUTRIENT MEDIUM USED FOR UPTAKE
EXPERIMENTS

Glucose	1000 mg
Ammonium sulfate.....	94.6 mg
Potassium phosphate.....	17.6 mg
Yeast extract.....	1.0 mg
Sodium chloride (0.5%).....	1000 ml

TABLE 5.3: COMPOSITION OF GRAM'S STAIN

1. GRAM'S CRYSTAL VIOLET

Solution A

Crystal violet..... 2 g
Ethyl alcohol..... 20 ml

Solution B

Ammonium oxalate..... 0.8 g
Distilled water..... 80 ml

Mix solution A and B and store for 24 hours before use.
Filter through a filter paper into a staining bottle.

2. GRAM'S IODINE

Iodine..... 1 g
Potassium iodide..... 2 g
Distilled water..... 300 ml

Grind the dry iodine and potassium iodide in a mortar.
Add water and grind thoroughly after each addition.
Rinse the solution into an amber glass bottle with
remainder of the distilled water.

3. DECOLORIZER

Decoloriser used is a acetone alcohol mixture with 100

ml of 95% ethyl alcohol and 100 ml of acetone.

4. COUNTER STAIN

Safranin O..... 2.5 g

Ethyl alcohol..... 100 ml (95%)

Working solution

Stock solution..... 10 ml

Distilled water..... 90 ml

CHAPTER VI

RESULTS AND DISCUSSION

6.1 COMPARISON OF MEDIA

Different agar media were used in order to choose the agar that offered the maximum number of characteristically different colonies of bacteria. The highest number of different colony types were found on nutrient agar medium. Thus, nutrient agar medium was chosen as the isolation medium.

6.2 IDENTIFICATION OF THE GRAM'S NATURE

A number of different colonies obtained from the plates were gram stained with a view to determine their Gram's nature. Out of the 15 different colonies obtained on the nutrient agar plates 9 were found to be Gram-positive and 6 were found to be Gram-negative.

6.3 DIFFICULTIES OBSERVED DURING GROWING OF GRAM-NEGATIVE CELLS

Double strength nutrient broth was used as a growth medium for both Gram-positive and Gram-negative cells. It was found that Gram-positive cells were easy to grow and gave thick growth after 24 h incubation. However, Gram-

negative cells gave gelatinous growth after 24 h incubation. Therefore, biomass obtained was less as compared to that obtained from Gram-positive cells. In order to get sufficient biomass for uptake experiments more shaker flasks were inoculated as compared to that for Gram-positive cells.

6.4 EFFECT OF pH

The effect of pH on biosorption is illustrated by Figures 2 and 3. It was seen that the chemical precipitation of cadmium ions did not occur below pH 7.0 under these conditions for both the types of cells. In the preliminary runs it was observed that with higher initial cadmium concentration or low temperature chemical precipitation did occur below pH 7.0. It was also observed that pH had a very high effect on cadmium uptake. This is why pH 6.6 was taken as a reference pH for all over the study, at which no chemical precipitation was ever observed.

Table 6.1 shows that at pH 6.6 Gram-positive cells show higher uptake of cadmium as compared to Gram-negative cells. Gram-positive cells show 17% more cadmium uptake than Gram-negative cells. Figure 8 clearly shows the difference between cadmium uptake by both the types of cells at different pH values.

Effect of pH on cadmium uptake can be explained as follows :

Surfaces of cells suspended in solution have a net surface charge and a corresponding electrical potential. As pH changes, H^+ and OH^- equilibrate with the surface and if no other strong adsorbates are present, the balance between bound H^+ and OH^- controls the surface charge. At some pH, designated the point of zero charge (PZC), the concentration of surface bound H^+ and OH^- are equal. When $pH < PZC$ the surface is positively charged and when $pH > PZC$ it is negatively charged. As surface charge changes, coulombic attraction force between the surface and ions in the solution also changes. Thus for example, an increased negative charge favors electrochemical attraction and adsorption of cations. So adsorption of cations is increased as pH is increased.

Since metabolically mediated biosorption was found to be low under the conditions used for the experiments, the effect of pH was most likely due to competition between protons and cadmium ions at the binding sites on the cell surface.

6.5 EFFECT OF METABOLIC INHIBITION

This study was performed at $30^{\circ}C$ and under the same conditions used for other experiments. Table 6.2 shows

that metabolic inhibition of both types of cells had a very high effect on respiratory activity, on the other hand the inhibition had little effect on cadmium uptake.

This shows that uptake of cadmium by both types of cells was largely passive. However, the decrease in cadmium uptake after metabolic inhibition indicated that 5-10% of global cadmium uptake at 30°C and pH 6.6 was metabolically mediated. The cells after treatment with sodium azide (1M) for 45 min. lost 90% of their respiratory activity. Figures 4 and 5 show experimental results obtained after metabolic inhibition of Gram-positive and Gram-negative cells respectively.

6.6 EFFECT OF NUTRIENT ADDITION

Table 6.3 gives the effect of nutrient addition on cadmium uptake by both types of cells. This study was performed at 30°C. It was observed that in the case of Gram-positive cells cadmium uptake increased by 13% after addition of nutrients in 2 h of incubation, indicating that some metabolic uptake did occur where cadmium was likely taken into bacterial cells via energy-dependent transport system. Another possibility is biosynthesis of intracellular polymers that would serve as traps for the removal cadmium ions from the solution. These metabolic

processes have been described for cadmium removal by microorganisms (Trevors et al 1986; Higham et al 1985; Wood and Wang 1983). The release of polysaccharide has also been reported (Higham et al 1985; Sterritt and Lester 1980; Brown and Lester 1979).

The study with Gram-negative cells did not give any conclusive results. It is possible that these cells had enough carbon source within themselves that they could use during 2 h incubation time and therefore did not show any significant change in cadmium uptake. However, these cells did show a slight decrease in cadmium uptake after inhibition by sodium azide indicating that some cadmium uptake was indeed metabolically mediated.

TABLE 6.1: TEST RESULTS OF CADMIUM UPTAKE BY GRAM-POSITIVE
AND GRAM-NEGATIVE BACTERIAL CELLS AT pH 6.6.

pH	Cadmium uptake ^a	Cell Type
6.6	16.25	Gram-positive
6.6	13.50	Gram-negative
6.6	13.50	Activated Sludge

a - As μg of cadmium, mg^{-1} dry cells in 2 h contact time
at 30°C.

Initial cadmium concentration was 10 $\mu\text{g}/\text{l}$

TABLE 6.2: EFFECT OF METABOLIC INHIBITION ON CADMIUM UPTAKE BY GRAM-POSITIVE AND GRAM-NEGATIVE CELLS OF BACTERIA.

	% Activity ^a	Cd Uptake ^b	Cell Type
Untreated	100	16.25	Gram-positive
Untreated	100	13.50	Gram-negative
NaN ₃ treated ^c	10	14.50	Gram-positive
NaN ₃ treated ^c	10	12.75	Gram-negative
NaN ₃ treated ^c	20	13.0	Activated sludge

a - As measured by the rate of endogenous O₂ consumption.

b - As ug cadmium.mg⁻¹ dry cells in 2 h at 30°C at pH 6.6.

c - With 1M NaN₃ for 45 min.

TABLE 6.3: EFFECT OF NUTRIENT ADDITION ON CADMIUM UPTAKE
BY GRAM-POSITIVE AND GRAM-NEGATIVE CELLS OF
BACTERIA.

Cadmium Uptake ^a		Cell Type
No nutrients	Nutrients	
16.25	18.50	Gram-positive
13.50	13.50	Gram-negative
13.50	13.50	Activated sludge

a- As μg of cadmium. mg^{-1} dry cells in 2 hour contact time
at 30°C and pH 6.6.

Initial cadmium concentration was 10 $\mu\text{g}/\text{l}$.

Fig.2 Effect of pH on uptake of cadmium by Gram-positive bacterial cells.

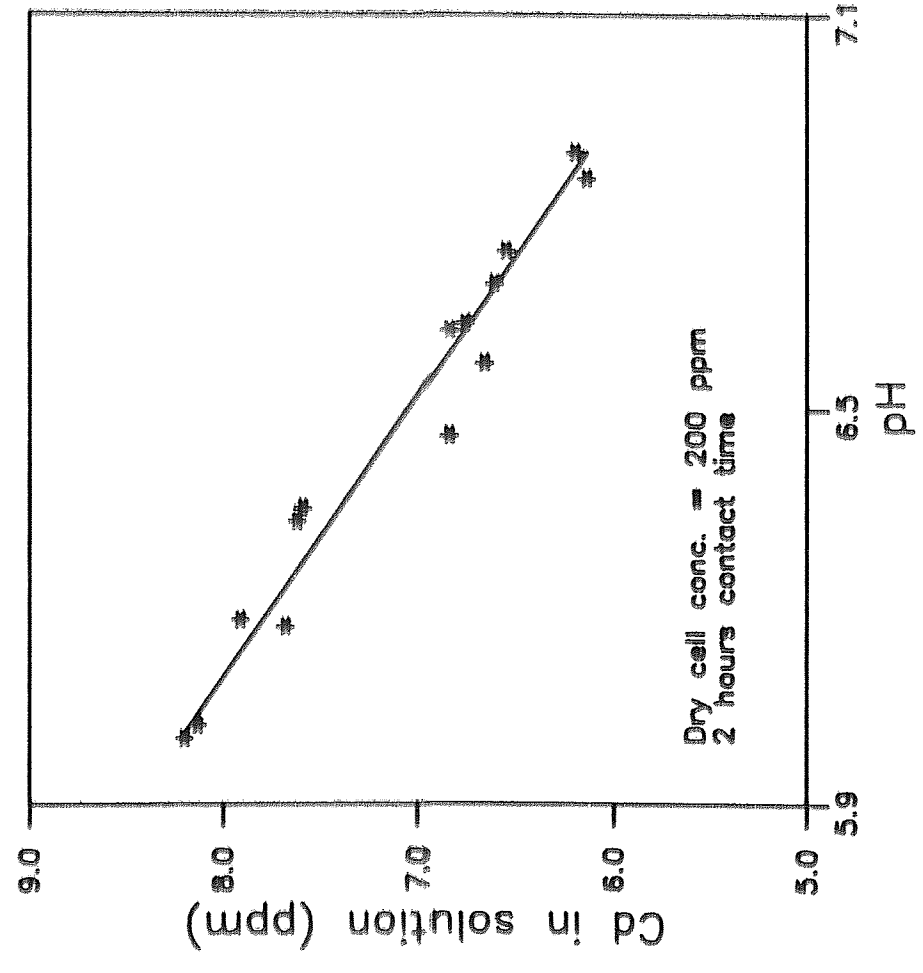


Fig.3 Effect of pH on cadmium uptake by Gram-negative bacterial cells.

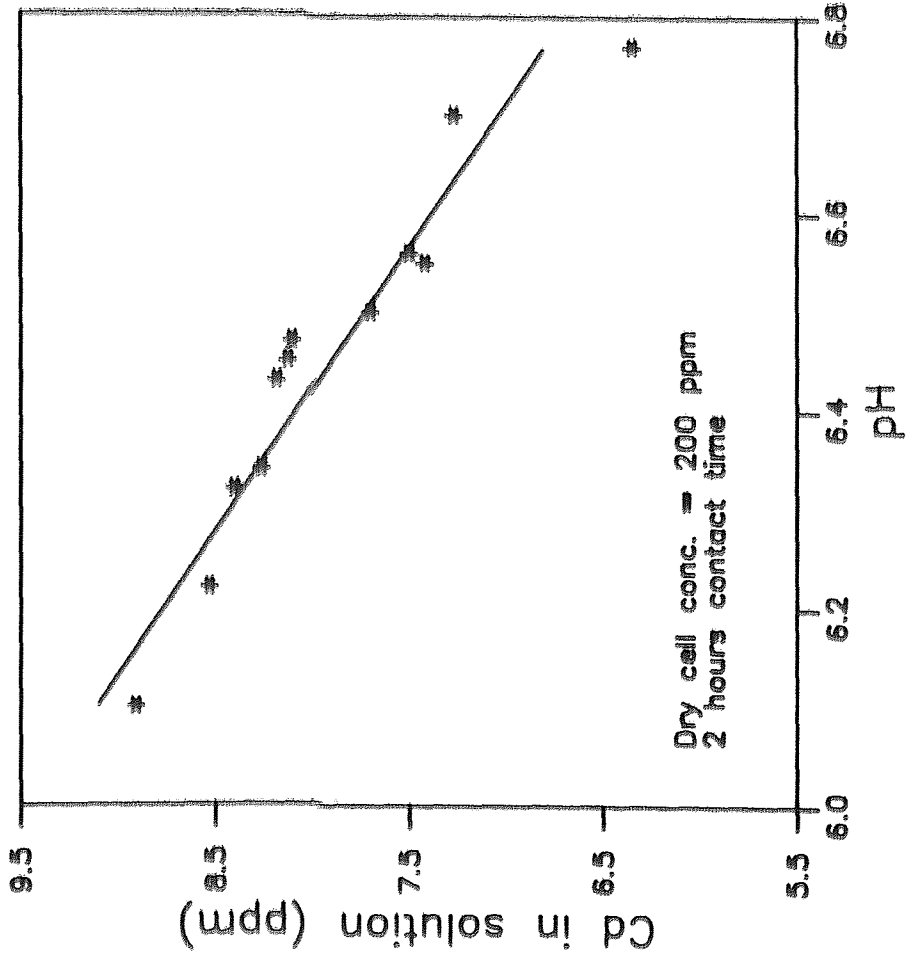


Fig.4 Effect of inhibition on cadmium uptake by Gram-positive bacterial cells.

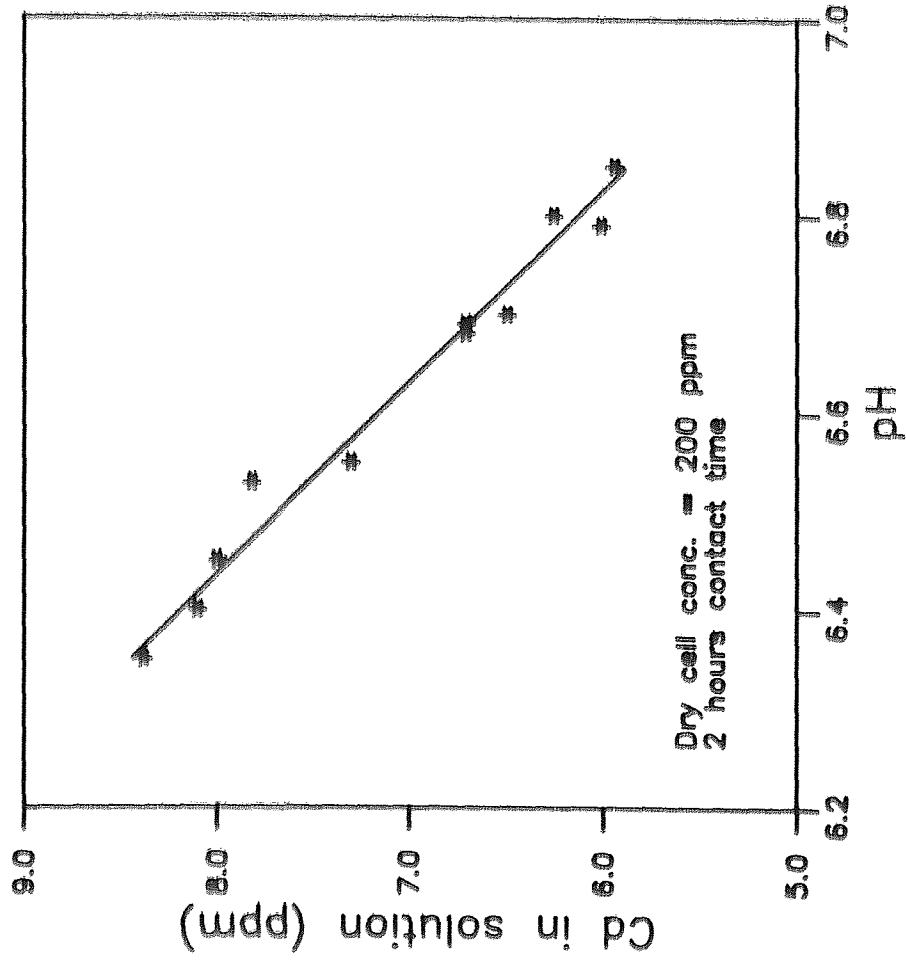


Fig.5 Effect of inhibition on cadmium uptake by Gram-negative bacterial cells.

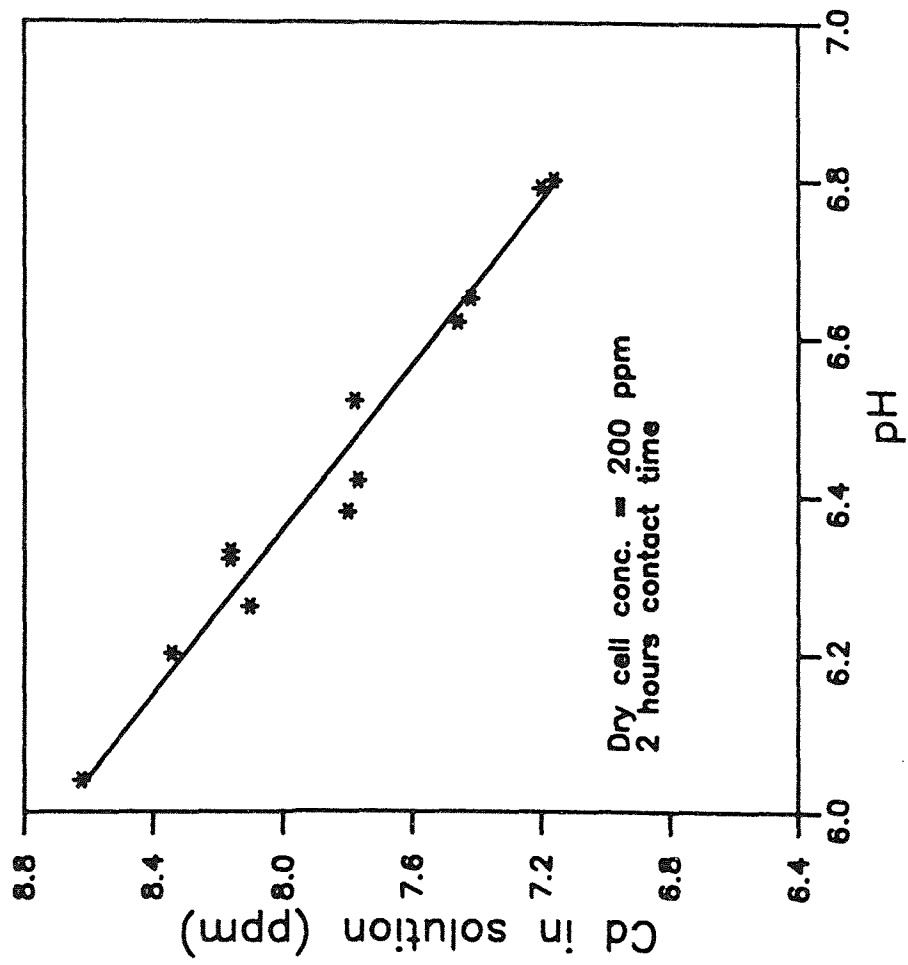


Fig.6 Effect of nutrient addition on cadmium uptake by Gram-positive bacterial cells.

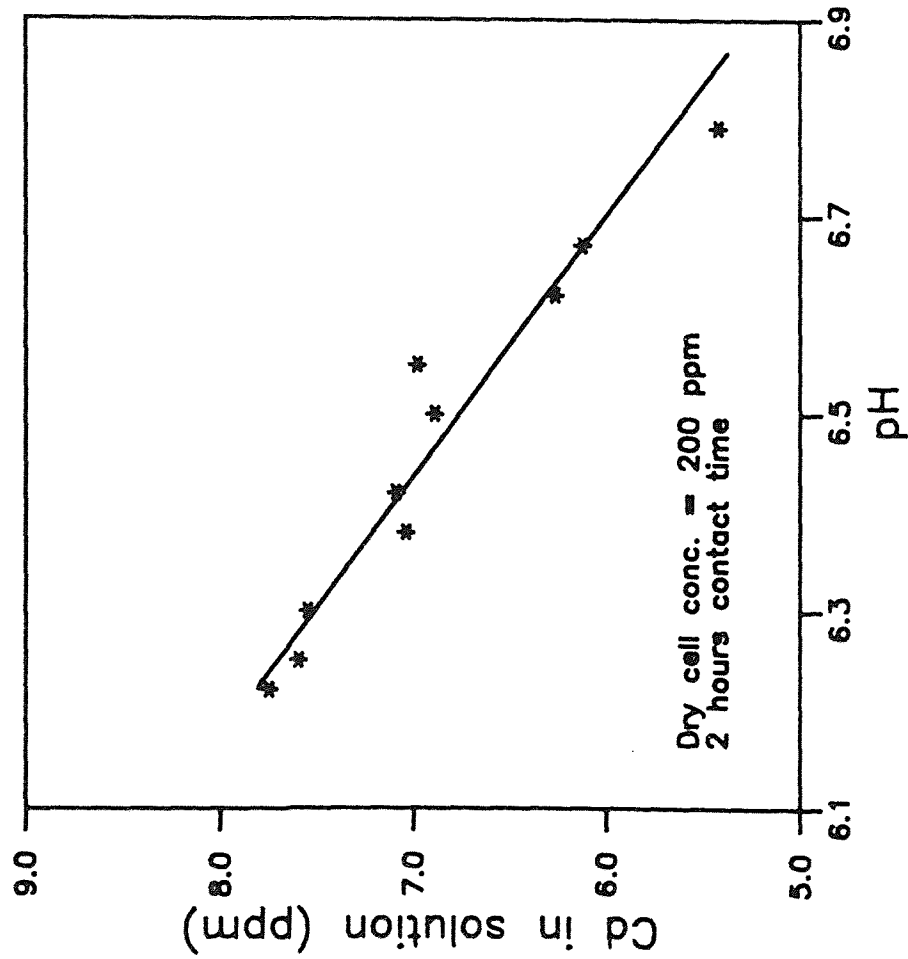


Fig.7 Effect of nutrient addition on cadmium uptake by Gram-negative bacterial cells.

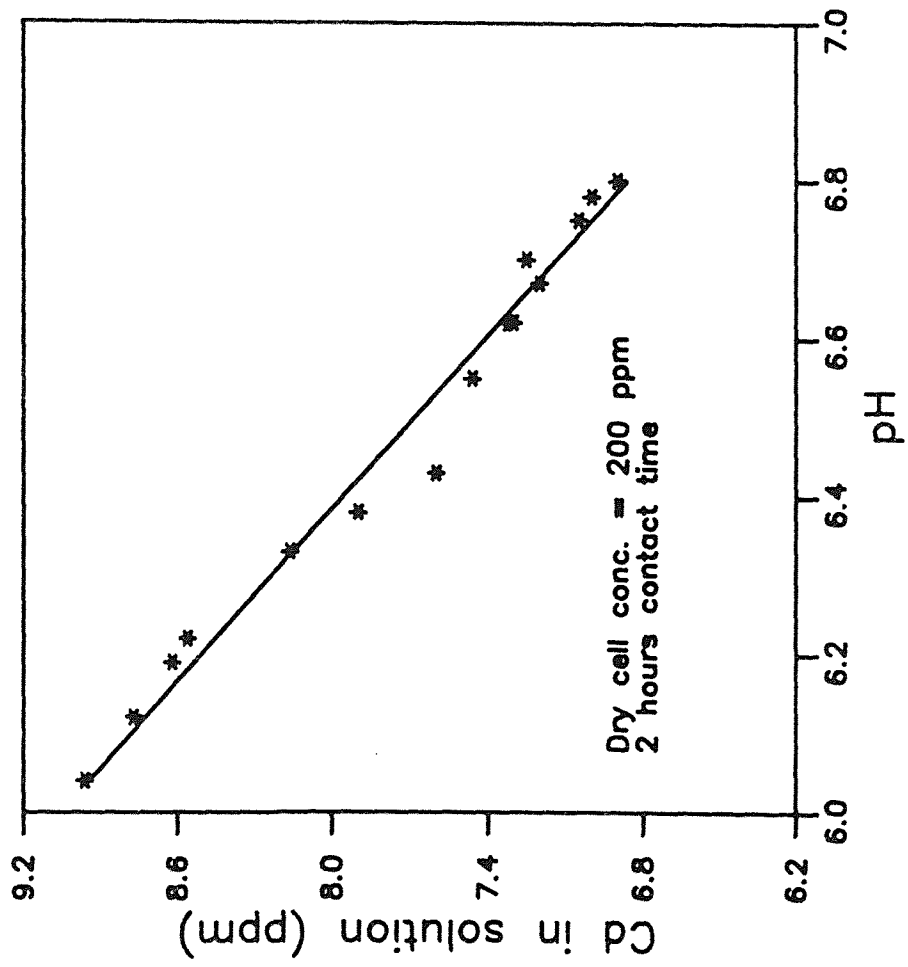
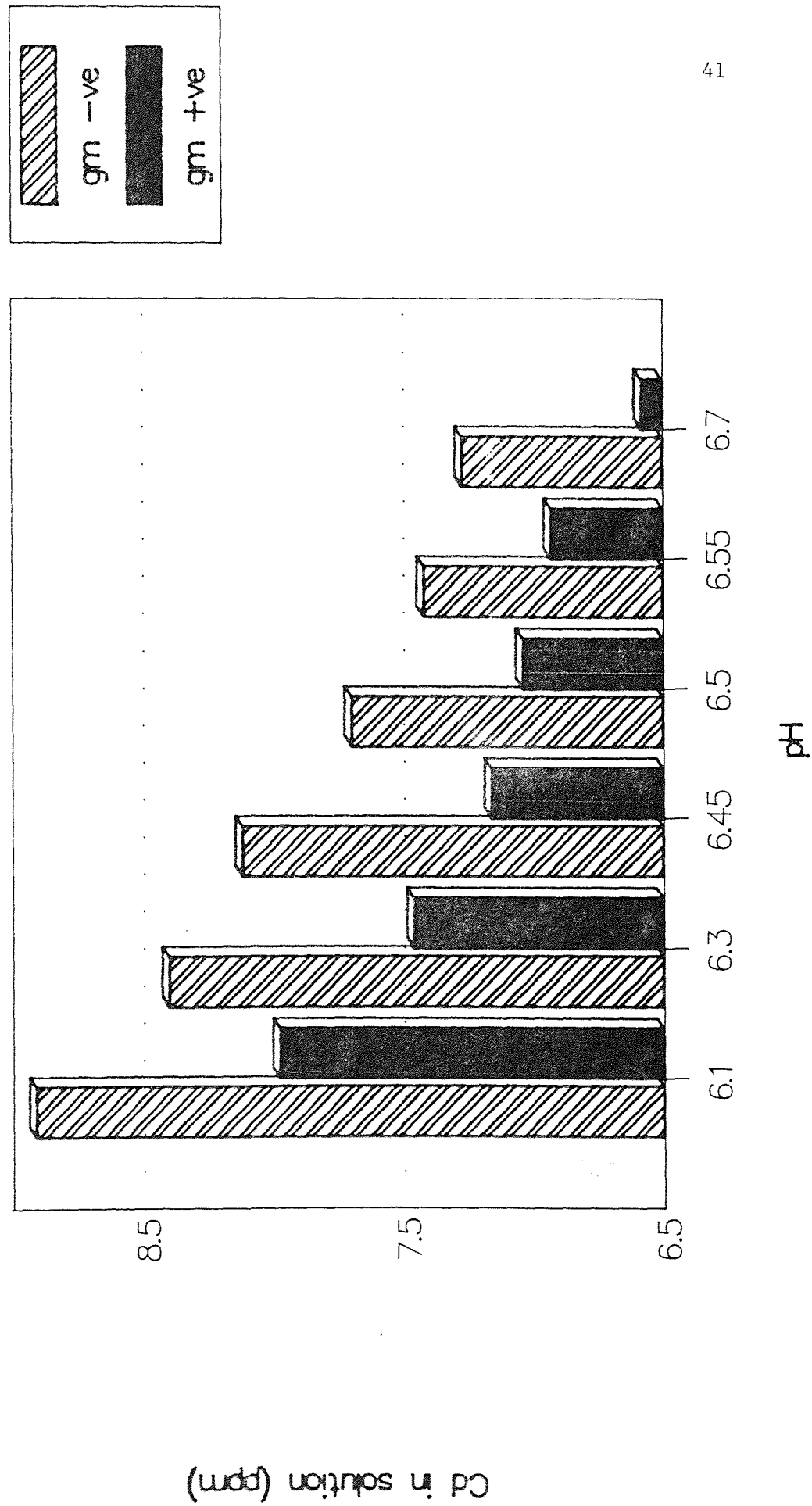


Fig.8 Cadmium uptake by Gram positive and Gram negative bacterial cells.



CHAPTER VII

CONCLUSIONS

Gram-positive and Gram-negative cells separated from activated sludge were studied with respect to cadmium uptake. Based on the results obtained, the following conclusions were derived.

Nutrient agar was found to be the best medium for growth of different types of bacteria.

Gram-negative cells were found to be difficult to grow under the same conditions used for the growth of Gram-positive cells.

Experimental results showed that Gram-positive cells remove cadmium more efficiently from the solution as compared to the Gram-negative bacterial cells. Gram positive bacterial cells showed 17% more uptake of cadmium than Gram-negative bacterial cells at pH 6.6. Gram-positive bacterial cells showed 11-12% decrease in cadmium uptake when the cells were inhibited using sodium azide. In case of Gram-negative bacteria this decrease was only 4-5%. Gram-positive bacterial cells showed a 13% increase in cadmium uptake when nutrients were added to the medium. However, these nutrients were not sufficient for biomass increase since the concentration of carbon in the nutrient

medium was kept very low. Gram-negative bacterial cells did not show any significant increase in cadmium uptake after addition of nutrients. More than 95% of the total cadmium removal from solution was observed within first 5 min. of cells-metal contact time.

These results suggest that cadmium uptake occur mainly through a passive, physico-chemical mechanism of adsorption at binding sites on the surface of the cells.

The results also indicate that Gram-positive cells show more metabolic uptake as compared to Gram-negative cells. This may be due to the fact that Gram-positive cells separated from activated sludge are more sensitive. This agrees with the results obtained by Babich and Stotzky that Gram-negative bacteria are more tolerant to cadmium than Gram-positive bacteria which are sensitive to cadmium. They also found that sensitive bacteria accumulate more amount of metal as compared to tolerant bacteria.

CHAPTER VIII

SUGGESTIONS FOR FURTHER WORK

In the present study Gram-positive and Gram-negative cells separated from activated sludge are used for cadmium uptake studies. The most eminent suggestions that fall out from the study are discussed below.

The study suggests that metal uptake was largely passive. Therefore, it will be worthwhile to study the attachment sites for cadmium on the bacterial cell. Some studies can also be carried out to determine if there is any competition when cells are subjected to two different metals.

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