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# Uptake of metal cations by fibroblasts *in vitro*

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Atomic absorption spectroscopy was used to assess uptake of  $\text{Ag}^{+1}$ ,  $\text{Au}^{+4}$ ,  $\text{Cd}^{+2}$ ,  $\text{Cu}^{+2}$ ,  $\text{In}^{+3}$ ,  $\text{Ni}^{+2}$ ,  $\text{Pd}^{+2}$ , and  $\text{Zn}^{+2}$  by *in vitro* cultures of Balb/c 3T3 fibroblasts as a function of concentration of the cations in cell-culture medium. Reversibility of this uptake was also measured. Metal cations exhibited a 400-fold difference in their tendency to accumulate in the cells;  $\text{In}^{+3}$  tended to accumulate the most, whereas  $\text{Pd}^{+2}$  accumulated the least. Uptake of the cations in the cells increased linearly with the concentration of the cation in the medium for all cations up to their 50% toxicity concentrations. Reversal of this uptake was slower than that of the initial uptake

for three cations studied in more detail ( $\text{Cd}^{+2}$ ,  $\text{In}^{+3}$ , and  $\text{Ni}^{+2}$ ). The duration of the initial exposure affected the proportion of the metal cations that were retained by the cells 5 h after the cations were removed from the medium. The proportion of retained  $\text{Cd}^{+2}$  did not change when the initial exposure was increased from 2 h to 6 h, whereas the proportion of retained  $\text{In}^{+3}$  decreased and  $\text{Ni}^{+2}$  increased over the same period. The tendency of the cells to accumulate these cations correlated with their cytotoxic potency (measured previously). © 1993 John Wiley & Sons, Inc.

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## INTRODUCTION

The release of metal cations from a variety of dental biomaterials has been well documented, and the toxicities of these cations have also been measured *in vitro* and *in vivo*.<sup>1,2</sup> However, there is uncertainty about how important such a release is clinically,<sup>3</sup> and how its importance might be estimated by *in vitro* methods. Metals are normally bound to enzymes and have been shown to bind to other macromolecules.<sup>4,5</sup> Furthermore, binding characteristics of some metallo-drugs have been shown to affect their cytotoxicity, mutagenicity, and antitumor activity.<sup>6</sup>

The uptake of several metal cations in cells has been previously studied. For example, the biosorption of copper by yeasts has been reported by Junghans to be rapid, independent of the yeast species, and pH-dependent.<sup>7</sup> McGregor et al. have reported that the uptake of aluminum bound to transferrin by erythroleukemia cells is dose-dependent and continues with time, but does not affect proliferation of the cells.<sup>8</sup> The uptake of copper, zinc, and silver by oyster tissues has been shown to be a function of the salinity of the water, but silver uptake is apparently

governed by a different mechanism than that which governs copper or zinc.<sup>9</sup>

Cellular uptake of some metal cations that are released from dental materials has not been studied in detail by means that permit direct comparisons in cells relevant to the oral cavity. Study of the uptake of these cations is a necessary precursor to investigations into specific binding characteristics and mechanisms of toxicity of these metal cations. Metal cations released from dental materials may cause local deleterious effects if they accumulate in cells adjacent to the biomaterials because they are less likely to be washed away by saliva.<sup>10</sup> Thus, the characteristics of the uptake of these metal cations in cells may be important in determining whether a released metal cation is likely to cause deleterious effects. Specifically, the efficiency with which different cations accumulate in tissues, concentrations of cations at which uptake occurs, and the reversibility of the uptake are all important factors to consider.

The purpose of this study was to measure the uptake of eight metal cations by cells in culture as a function of concentration of the cations in the medium, to assess the reversibility of this uptake, and to attempt to correlate the tendencies of these metal cations

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to accumulate in cells with previously determined cytotoxicity data for these cations.

## MATERIALS AND METHODS

### 1. Cell culture

Balb/c 3T3 fibroblasts (ATCC CCL 163) were used. Cells were maintained in Dulbecco's Modified Eagle Medium (without glutamine), 3% NuSerum, penicillin (125 units/mL), streptomycin (125  $\mu\text{g}/\text{mL}$ ), gentamycin (10  $\mu\text{g}/\text{mL}$ ), and glutamine (2 mmol/L). HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 28 mmol/L, pH = 7.2) was added to cultures during the experiments to control pH when metal cations were added to the medium.

The uptake of metal cations by cells was determined by exposing cells to six concentrations of the cations; the duration of exposure was adjusted for each cation. Ranges of concentrations and durations of exposure are shown in Table I. The concentrations of metal cations were selected to be at or below the 50% cytotoxic concentrations for these cations.<sup>11</sup> The durations of exposure for the cations were selected to provide a maximum amount of exposure during which increases in cell number from cell growth or decreases from detachment caused by toxicity were minimal as monitored by cell counting (Coulter, Luton, Beds., UK). Metal cations were prepared from their chloride or sulfate salts as described previously.<sup>11</sup> Cells were plated in 75-cm<sup>2</sup> polystyrene flasks (Costar, Cambridge, MA) at 25,000 cells/cm<sup>2</sup> in 15 mL of medium, and were incubated at 37°C in 5% CO<sub>2</sub> and 95% air for 48 h. This plating density provided approximately 35,500 cells/cm<sup>2</sup> in a nonconfluent culture at 48 h.

After 48 h, a small volume (<100  $\mu\text{L}$ ) of the metal cation in aqueous solution was added to each flask and mixed thoroughly. A separate flask was used to test each metal cation concentration. Pilot studies established that the pH of the medium was unchanged by the addition of these concentrations of metal cations. After the exposure time (Table I), the medium was removed and the cell monolayer was washed six times with 2 mL phosphate-buffered saline solution. Pilot studies showed that this washing procedure effectively removed soluble metal cations from the monolayer to levels below atomic absorption (AA) detection limits. The final washing solution was retained for AA analysis in each experiment to confirm the efficacy of the washing procedure. Cells were released from the flask using 1.5 mL of a 0.05% trypsin solution in 0.02% EDTA for 4 min at 37°C. An equal volume of medium was added to quench the trypsinization, and the cells were dispersed into the medium with a Pasteur pipette. A 50- $\mu\text{L}$  aliquot was then removed for cell counting, and the num-

ber of cells was determined by means of a Coulter counter. The remainder of the suspension was centrifuged at 200g for 4 min to pellet the cells. The supernatant liquid was carefully removed and retained for atomic absorption analysis. Five hundred microliters of double-distilled water was added to the pellet, and this suspension was vortexed for 30 s, then treated ultrasonically for 60 s (Kontes, Vineland, NJ). This solution was analyzed by means of AA.

In experiments that assessed reversibility of the uptake, 15 mL of cell-culture medium was added to the flasks after the washing procedure. The flasks were then incubated for an additional time, after which the cells were trypsinized and dispersed as outlined previously. Reversibility experiments were performed for Cd<sup>+2</sup>, In<sup>+3</sup>, and Ni<sup>+2</sup>. Cd<sup>+2</sup> was chosen because it was the most potent toxin of the cations studied previously.<sup>11</sup> In<sup>+3</sup> was chosen because it showed no toxicity at the concentrations studied, and Ni<sup>+2</sup> was chosen because it is a common component of many dental and medical alloys with a history of cytotoxicity and allergenicity.<sup>12,13</sup> Furthermore, each of these cations could be added to cell cultures for at least 6 h without significant cell detachment. In these experiments, the cells were first exposed to the maximum concentrations of the metal cations (Table I) for 2, 4, or 6 h. Reversibility of the uptake was then assessed after 5 h (for all exposure times) and 10 h (for the 2-h exposure time). It was not possible to study 10-h reversibility after initial exposures of 4 or 6 h because cell growth and cell detachment were too great in this period and would have biased the results.

Controls were assessed in each experiment by adding water in place of the metal cation solutions to a separate flask at 48 h, and then processing the flask as outlined previously. These flasks were also used to establish the concentrations of these metal cations normally found in the cells.

TABLE I  
Concentration Ranges and  
Durations of Exposures Used

Metal Cation	Concentration Range ( $\mu\text{M}$ )	DOE <sup>a</sup> (h)
Ag <sup>+1</sup>	0-6.9	4
Au <sup>+4</sup>	0-90	4
Cd <sup>+2</sup>	0-0.90	8
Cu <sup>+2</sup>	0-240	8
In <sup>+3</sup>	0-440	8
Ni <sup>+2</sup>	0-200	8
Pd <sup>+2</sup>	0-280	8
Zn <sup>+2</sup>	0-31	5

<sup>a</sup>Duration of exposure = amount of time cells were exposed to the cations.

**2. Atomic absorption**

Flame atomic absorption was used to assay solutions for the metal cations (Perkin Elmer, Norwalk, CT). Details of parameters for the assay have been published previously.<sup>13</sup> Standard solutions for calibration of the instrument were purchased (Aldrich, Milwaukee, WI). Detection limits, adjusted to reflect the minimum amount of metal cation detectable per cell, are listed in Table II. Phosphate-buffered saline solutions, distilled water, and trypsin solutions were all tested to ensure that the levels of metal cations in these solutions were below detection limits.

**3. Calculations and statistics**

The average quantity of a metal cation per cell was determined by dividing the quantity of the cation in the cell pellet by the number of cells in the pellet. This calculation assumed minimal cell loss during centrifugation. To verify this assumption, the supernatant liquids were counted in the Coulter counter in pilot experiments; these liquids showed counts equivalent to background. The average quantity of a metal cation per cell was then expressed in femtomoles/cell (fmol = 10<sup>-15</sup> moles) The quantity of metal cations in normal cells was calculated from the control flasks (Table II). Uncertainty in the metal cation levels was 10% (at *p* = .05) based on pilot experiments that used replicate flasks.

Least-squares linear regression was used to determine slopes of plots of the average quantities of metal cations accumulated per cell vs. metal cation concentrations in the medium. Each slope was then divided by the duration of exposure in hours. This calculation yielded an adjusted slope that represented the uptake efficiency of the metal cation by the cell and had

units of fmol/cell/μM/h. These adjusted slopes were used because the metal cations had diverse cytotoxic potencies. For example, if similar concentrations were used to study Cd<sup>+2</sup> and Pd<sup>+2</sup>, the concentration of Cd<sup>+2</sup> would be so high as to cause rapid detachment of the cells from the flask. If the concentrations were reduced until the Cd<sup>+2</sup> effect was tolerable, the Pd<sup>+2</sup> would not be detectable and would be far below (>100 times) its toxicologically relevant concentration. The best strategy appeared to be to study a range of toxicologically relevant concentrations with durations of exposure that limited cell loss from detachment and then to normalize for both concentration and exposure time.

Reversibility of the metal cation uptake was determined by comparing the maximum uptake of a cation per cell to the amount of the cation that remained after additional incubation in uncontaminated medium. Student *t* tests were used to compare the levels (*p* = .05).

**RESULTS**

**1. Metal cation-cell uptake**

The detection limits and the normal quantities of metal cations per cell are listed in Table II. If a cation was not detectable in the control cells, the normal amount was listed as less than the detection limit. Only Zn<sup>+2</sup> and Cu<sup>+2</sup> had normal cell levels above detection limits. Table II also lists the maximum quantities of the cations that accumulated in cells under the conditions shown in Table I. Metal cations were present at levels 1.2 to 80 times normal or AA detection limits, depending on the metal.

Plots of the uptake of a metal cation per cell vs. the amount of the metal cation in the medium were linear

**TABLE II**  
**Normal, Maximum, and Reversal Quantities of Metal Cations in Cells and Uptake Efficiencies**

Metal Cation	Detection Limit (fmol/cell)	Normal <sup>a</sup> (fmol/cell)	Maximum <sup>b</sup> (fmol/cell)	Uptake Efficiency <sup>c</sup> (fmol/cell/μM/h)	R <sup>2</sup>
Ag <sup>+1</sup>	0.020	<0.020	0.65	23.8	0.98
Au <sup>+4</sup>	0.028	<0.028	0.39	1.0	0.98
Cd <sup>+2</sup>	0.019	<0.019	0.30	38.0	0.84
Cu <sup>+2</sup>	0.037	0.094	0.61	0.26	0.97
In <sup>+3</sup>	1.60	<1.60	132	45.3	0.97
Ni <sup>+2</sup>	0.077	<0.077	0.33	0.21	0.99
Pd <sup>+2</sup>	0.090	<0.090	0.27	0.11	0.99
Zn <sup>+2</sup>	0.020	1.02	1.20	0.73	0.22

<sup>a</sup>Average quantity of a metal cation in a control cell. Units are femtomoles (10<sup>-15</sup> moles)/cells. Uncertainty in these and other concentrations are 10% at *p* = .05 based on experiments designed to test precision.

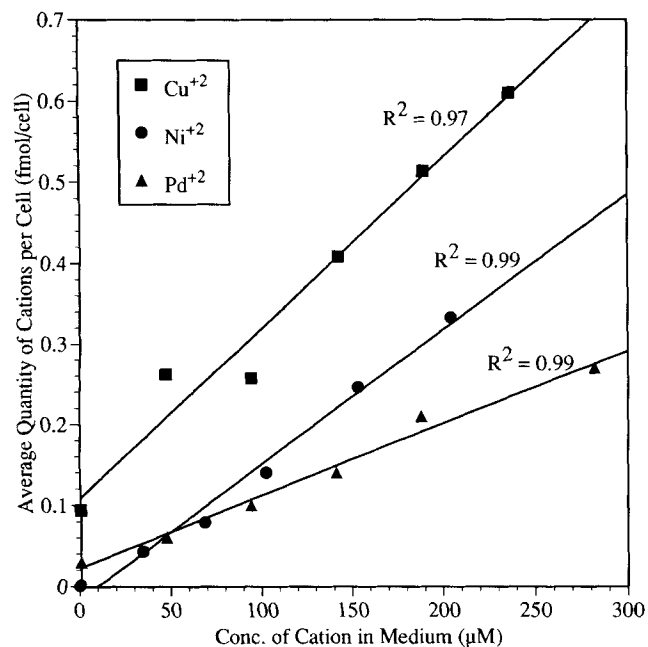
<sup>b</sup>Average quantity of a metal cation accumulated after exposure to the highest concentration of cation.

<sup>c</sup>Slope of the quantity of metal cation in the cell after cation-cell incubation vs. the concentration of the cation in the medium. Values are normalized for the different durations of exposures (Table I), and are multiplied by 1000 to reduce the number of decimal places. Determined using least squares linear regression model. R<sup>2</sup> (next column) indicates the percentage variability explained using this model (1.0 = 100%).

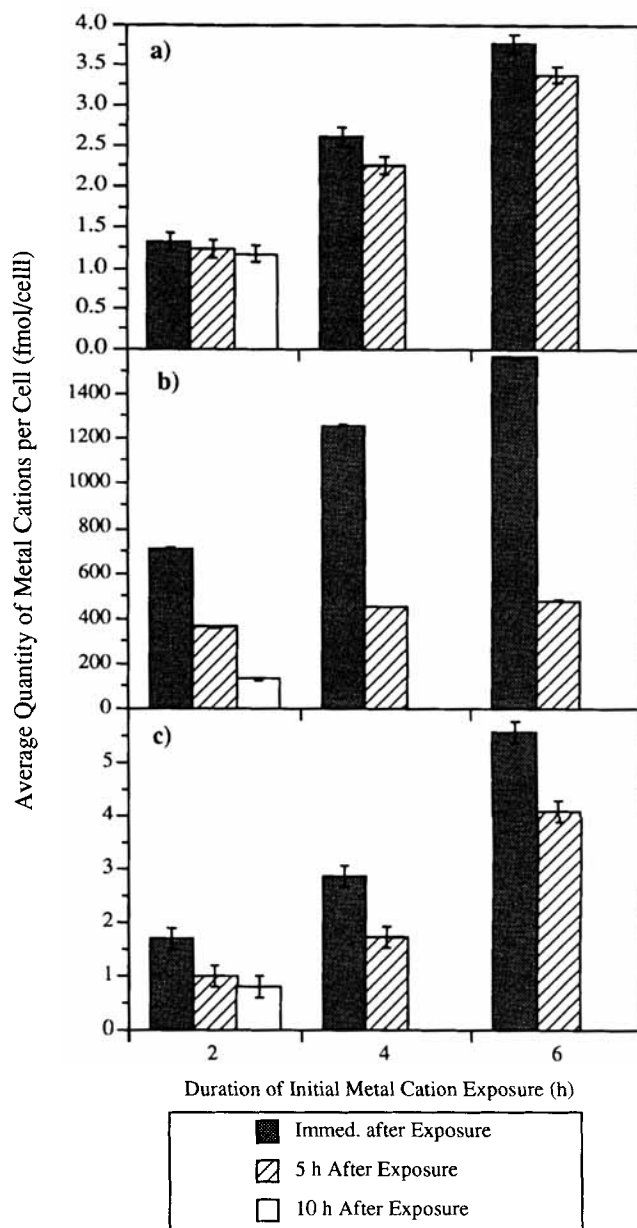
for all cations studied. Typical examples are shown in Figure 1 for  $\text{Cu}^{+2}$ ,  $\text{Ni}^{+2}$ , and  $\text{Pd}^{+2}$ . The adjusted slopes derived from these plots (adjusted for the duration of exposure) allow comparisons of the uptake efficiency of the metal cations (Table II). A broad range of uptake efficiencies was observed; the smallest (for  $\text{Pd}^{+2}$ ) was over 400 times smaller than the largest (for  $\text{In}^{+3}$ ).

## 2. Reversibility of metal cation-cell uptake

The results from reversibility experiments for  $\text{Cd}^{+2}$ ,  $\text{Ni}^{+2}$ , and  $\text{In}^{+3}$  are shown in Figure 2. Each of these cations behaved differently in its reversibility. Uptake of  $\text{Cd}^{+2}$  reversed relatively slowly [Fig. 2(a)], and as the initial exposure time increased, the percentage of  $\text{Cd}^{+2}$  retained with the cells remained at about 90%.  $\text{In}^{+3}$  uptake reversed significantly over the 5-h reversal time [Fig. 2(b)], and increases in the initial exposure increased the percentage during the reversal period from 50% at 2 h to almost 70% at 6 h. The reversibility of  $\text{Ni}^{+2}$  uptake was intermediate [Fig. 2(c)], but the percentage of  $\text{Ni}^{+2}$  that remained with the cells with longer initial exposures increased from 60% at 2 h to almost 75% at 6 h. In each case, cells continued to release the metals for at least 10 h [Figs. 2(a)–(c)], although the decrease observed in the second 5-h interval appeared smaller than that in the first 5-h interval.



**Figure 1.** Plots of the average uptake of metal cations per cell vs. concentration of the metal cation in the medium for  $\text{Cu}^{+2}$ ,  $\text{Ni}^{+2}$ , and  $\text{Pd}^{+2}$ . The least-squares method was used to fit linear curves to the points.



**Figure 2.** Graphs of the quantity of metal cation per cell as a function of initial exposure times of 2, 4, or 6 h followed by reincubation of cells without metal cations for 5 or 10 h for  $\text{Cd}^{+2}$  (a),  $\text{In}^{+3}$  (b), and  $\text{Ni}^{+2}$  (c). These three cations exhibited reversibility of uptake, but at different rates. Error bars represent 3 standard errors of the mean ( $p = .05$ ). Reincubation of cells for 10 h was only possible for the cells exposed initially for 2 h because total incubation times of greater than 12 h allowed too much cell growth or detachment and biased the results.

## DISCUSSION

Detection levels were not equivalent for all cations, and were not especially sensitive for several cations ( $\text{In}^{+3}$  and  $\text{Pd}^{+2}$ , Table I). Although more sophisticated techniques, such as flameless atomic absorption, are available to improve these sensitivities, it was not necessary to use these because the uptake of these

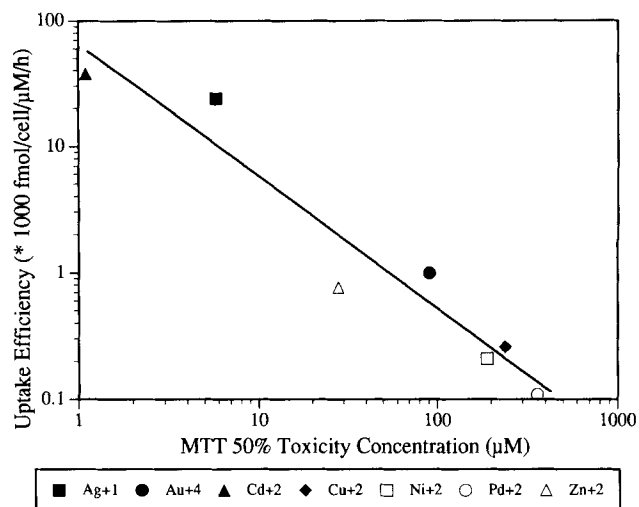
metal cations in cells was always great enough easily to allow detection of cations.

These results are highly dependent upon the assumption that the washing procedure removed all unaccumulated cations from the monolayer. To support this assumption, pilot experiments monitored the concentration of cations removed by successive washings; these experiments established that five washings reduced the metal cation concentrations to levels below detection limits. A sixth washing was added and was assayed in every experiment to ensure that the washing procedure was effective.

The use of adjusted slopes (Table II) to quantify the uptake efficiencies of metal cations was more complex than using identical concentrations and durations of exposures for all cations. However, the diverse cytotoxic potencies of these cations made the use of identical concentrations and durations of exposure impractical. Furthermore, it seemed desirable to study the uptake of these metal cations in a range of concentrations that were toxicologically relevant. Therefore, concentrations were selected that were at or below the 50% cytotoxic concentrations for these metals. The use of different durations of exposure ensured that cell detachment during the exposure did not affect the results. In addition, pilot studies established that uptake of these cations by cells increased linearly with time if the durations of exposure were  $\leq 8$  h. Therefore, dividing the slopes by the duration of exposure was a legitimate means of normalizing the data in these experiments.

Although linear statistical models were used to fit all uptake curves (Fig. 1), it is possible that other models may have been applicable as well. Linear models were adopted because they accounted for a large percentage of the variation in the dependent variable (Table II) and allowed a single slope to represent the efficiency of metal-cell uptake. The curves shown in Figure 1 do not support the existence of threshold concentrations, below which metals do not accumulate in cells, or maximum concentrations, above which metal cations will not continue to accumulate. It appeared from these studies that uptake was directly proportional to the amount of cation in the medium within these concentration ranges. However, it is possible that threshold or maximum concentrations exist outside these concentrations. Maximum concentrations are less likely under the conditions of these experiments, since toxic effects would occur rapidly above the concentrations used in Table I.

Uptake efficiencies (Table II) correlated inversely with the 50% cytotoxicity concentrations of these cations measured by MTT-formazan production (a measure of mitochondrial succinic dehydrogenase activity), as determined previously (Fig. 3).<sup>11</sup>  $\text{In}^{+3}$  was not included in this analysis because it exhibited no cytotoxicity at these concentrations and thus could



**Figure 3.** Inverse correlation between cellular uptake efficiencies of seven of the cations measured in this experiment and the cytotoxicities of these cations as determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide)-formazan production (a measure of mitochondrial succinic dehydrogenase activity) in a previous study [J.C. Wataha, C.T. Hanks, and R.G. Craig, "The *in vitro* effects of metal cations on eukaryotic cell metabolism," *J. Biomed. Mater. Res.*, **25**, 1133–1139 (1991)]. The potency of a cation was greater if the uptake efficiency was high.  $\text{In}^{+3}$  is not shown because it exhibited no cytotoxicity. Least-squares linear regression yielded an  $R^2$  of 0.93 for these points. Uptake efficiencies were normalized to incubation times and medium concentrations and thus have units of  $\mu\text{M}^{-1}\text{h}^{-1}$ .

not be plotted properly. The observation that  $\text{In}^{+3}$  had a large relative slope with no cytotoxicity implies that uptake of metals by cells is not sufficient by itself to cause cytotoxicity. Figure 3 does support the hypothesis that if cations are toxic, more efficient uptake is associated with a greater potency.

The reversibility of the uptake of metal cations by cells is important because of its ramifications for the *in vivo* release of these cations from biomaterials. If uptake is reversible, then short-term release of metals from biomaterials might be less disturbing. These experiments indicated that the reversibility of the uptake is dependent upon the cation, and that some cations such as  $\text{Cd}^{+2}$  are not readily released from cells once accumulated (Fig. 2).  $\text{Cd}^{+2}$ , the most potent cation, was slow to release from the cells, whereas  $\text{In}^{+3}$ , which exhibited no cytotoxicity in previous studies, showed significant reversibility of uptake. These observations should be taken into consideration when designing *in vitro* tests for biomaterials and when assessing the importance of the release of elements from biomaterials.

The reversibility of metal-cell uptake could be construed as an artifact if some cells preferentially absorbed metal cations in the initial exposure, and then died and detached from the flask during the reversal period. Subsequent analysis of the monolayer would

then show an apparent decrease in average cation levels per cell. It is not likely that this situation occurred, since cell yields from reversal flasks were greater than or equal to those before reversal. Furthermore, the medium that covered the cells during the reversal period did not contain large numbers of detached cells.

The uptake of a metal cation in a cell does not demonstrate that it is bound to the cell. Metal-DNA binding or metal-enzyme binding has been well documented by other researchers,<sup>4,5</sup> but these studies cannot determine if this type of binding occurred in these experiments. The consequences of uptake of metal cations that occur naturally may be different from those that do not, since existing cellular pathways for their uptake, distribution, and excretion are in place; whereas nonnative cations may or may not interact with existing pathways or may act in other ways. Of the cations used in this study,  $\text{Cu}^{+2}$ ,  $\text{Ni}^{+2}$ , and  $\text{Zn}^{+2}$  are known to occur naturally,<sup>14</sup> and these tended to accumulate less efficiently than most other nonessential elements (Table II). In addition, the induction of metallothioneins by elements, particularly  $\text{Cd}^{+2}$ ,  $\text{Cu}^{+2}$ , and  $\text{Zn}^{+2}$ , might influence the consequences of cation accumulation. Previous research has shown that metallothioneins exist in most cells, but are expressed to different levels depending on the cell and organism.<sup>15</sup> The binding of the metal cations to these proteins would influence their uptake and the reversibility of the uptake. Elevated levels of hepatic metallothioneins have been suspected to influence the selective accumulation of copper into hepatocytes over fibroblasts.<sup>16</sup>

These experiments do not delineate the locus of the accumulation of the metal cation in the cells. Additional studies that analyze cell fractions would help provide this information. In addition, since these experiments measured metal-cell uptakes in cell populations, it is not known whether all cells accumulate these cations to equal degrees, or whether uptake varies among cells. If metal-cell uptake varies among cells, methods of determining metal-cell uptake in individual cells might provide insight into the *in vivo* importance of this phenomenon, as well as the mechanisms by which these cations act. Finally, studies that assess the rates of metal-cell uptake and attempt to correlate these rates with cell metabolic disruption would be of value.

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