The *in vitro* effects of metal cations on eukaryotic cell metabolism

J.C. Wataha,* C.T. Hanks, and R.G. Craig
The University of Michigan School of Dentistry, Ann Arbor, Michigan 48109-1078

The *in vitro* cytotoxicity of nine metal cations common in dental casting alloys was evaluated using Balb/c 3T3 fibroblasts and four toxicity parameters: total protein production, ^{3}H-leucine incorporation, ^{3}H-thymidine incorporation, and MTT-formazan production. Concentrations causing 50% toxicity compared to controls (TC50's) and reversibility of these effects were determined. The range of potency of the metal cations was 2–3 orders of magnitude, with Cd^{2+} showing the greatest potency and In^{3+} showing the least. Potency did not correlate with atomic weight for these metals. For each metal cation, the TC50's of the various toxicity parameters were similar in most cases. However, several cations (Cu^{2+}, Ga^{3+}) showed greater potency with ^{3}H-thymidine incorporation. Reversibility of the toxic effects was observed for all cations; the effects generally became irreversible at concentrations in the range of the TC50 value for each cation. Several stimulatory effects were seen. Small but statistically significant stimulations were observed after 24 h of metal exposure for Ag^{1+}, Au^{4+}, Cu^{2+}, Ga^{3+}, and Ni^{2+}. Residual stimulations 24 h after removal of the metal cations were observed for Au^{4+}, Cd^{2+}, Ni^{2+}, and Zn^{2+}. Stimulations always occurred at concentrations below the TC50 concentrations. This study should be useful in evaluating the potential cytotoxic effects of metal cations released from dental alloys.

INTRODUCTION

In vitro testing methods have come into focus as the cost and controversy associated with animal testing have increased. *In vitro* testing of dental alloys has followed this trend, and has become particularly important with the development of new alloys. In recent years, a number of researchers have investigated the *in vitro* performance of dental casting alloys. In 1982, the *in vitro* toxicities of ternary alloys of Au-Cu-Ag were evaluated by Wright et al. using ^{51}Cr release, and the toxicity of these alloys appeared to be correlated with the atomic abundance of copper in the alloys.[1] Niemi and Hensten-Pettersen evaluated the toxicity of Ag-Pd-Cu alloys in 1985 using agar overlays and neutral red, and found that alloys with less than 30 wt% copper were less toxic.[2] Exbrayat et al. showed that gingival explants could adhere and grow on Ni-Cr-Mo alloys, but that these cells showed some morphological aberrations (compared to glass) and had decreased type III collagen production.[3] Bumgardner et al. tested Cu-based alloys using trypan blue ex-

*To whom correspondence should be addressed.

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clusion and ^{3}H-thymidine uptake.[4] They found that these alloys released significant amounts of copper and zinc, that some altered cellular morphologies, and that most inhibited ^{3}H-thymidine uptake. Most recently, Craig and Hanks[5] tested a wide variety of casting alloys and pure metals, and found that succinic dehydrogenase activity could discriminate differences in toxicities of these alloys. Thus, past research has established that some types of alloys show significant *in vitro* toxicity, and that elements of these alloys are often released in significant quantities.

Other research has focused on the toxicity of individual metal cations. As early as 1954, Heath showed that cobalt caused abnormal mitoses.[6] In 1977 Jacobsen used primary gingival cells to show that as little as 2.5 ppm of Ni^{2+} would inhibit cell number, glucose consumption, and lactate production.[7] Costa used ornithine decarboxylase activity and incorporation of ^{3}H-leucine, ^{3}H-thymidine, and ^{3}H-uridine to study the toxicities of Ca, Cr, Co, Cu, Ni, Cd, Mn, and Sn cations. He found that the ornithine decarboxylase test generally correlated well with the other methods of evaluation.[8] Babich et al. used fish cell lines and neutral red uptake to evaluate the cations of Cd, Cu, Ni, and Zn.[9] Recently, Jowett et al. used mouse embryo tooth bud explants to study nitrate salts of Cd, Zn, Cu, and Sn.[10] They were able to show differences in the effect of these cations on viability, dentin matrix secretion, and enamel epithelium differentiation. Finally, Itakura et al. tested the *in vitro* toxicity of Pt cations using osteogenic cell lines and alkaline phosphatase activity.[11]

Although many metal cations have been tested *in vitro*, comparisons among these studies are nearly impossible due to the diversity of testing methods. Variations in cell lines, method of toxicity assessment, time of exposure to cations, target cell number, and other variables can affect toxicity measurements. Furthermore, the toxicities of cations of several common dental casting alloy elements (such as Au, Ag, Pd, Ga, and In) have been investigated minimally, and the methods of toxicity assessment in many previous studies have utilized uptake or release of foreign molecules which are difficult to correlate with intracellular biochemical events. The purposes of this study were to: (1) evaluate the *in vitro* cytotoxicity of nine metal cations which occur as components of dental casting alloys, (2) measure toxicity with tests indicative of cellular metabolism, (3) evaluate the reversibility of toxic effects with these cations, and (4) use these data to compare toxicity of the cations and formulate a basic understanding of the biochemical basis for their differences in toxicity.

METHOD AND MATERIALS

Metal cation solutions

Solutions of metal cations of Ag, Au, Cd, Cu, Ni, Pd, and Zn were prepared volumetrically from Ag_2SO_4, $HAuCl_4 \cdot 3H_2O$, $CdCl_2$, $CuCl_2$, $NiCl_2 \cdot 6H_2O$, $PdCl_2$, and $ZnCl_2$ by dilution with double distilled water. Purities of the solids were better than 99.99%. A minimum volume of 0.1 mol/L HCl was first

used to dissolve Ag^{1+}, Cd^{2+}, and Pd^{2+}. Ga^{3+} and In^{3+} solutions were prepared by volumetric dilution of purchased atomic absorption standard solutions with double-distilled water. All solutions were checked for actual metal content atomic absorption spectroscopy. Control solutions for the anions were prepared volumetrically from $NaNO_3$, NaCl, and Na_2SO_4.

Cell culture

Balb/c 3T3 mouse fibroblasts were used in these experiments (ATCC CCL 163: anchorage dependent, aneuploid, and contact inhibited fibroblasts). Experimental cell culture medium consisted of Dulbecco's Modified Eagle Medium (DMEM) without glutamine, 3% NuSerum, 28 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH = 7.2), penicillin (125 units/mL), streptomycin (125 μg/mL), gentamycin (10 μg/mL), and glutamine (2 mmol/L). HEPES was added to control pH when metal cations were added. The fibroblasts were maintained in the same medium without HEPES. Cells were grown to approximately 90% confluence prior to harvest, and were harvested using a sterile trypsin/EDTA solution (0.05% trypsin-0.02% EDTA in normal saline) for 4 min at 37°C. After trypsinization, the cells were harvested, resuspended in cell culture medium, and diluted to 32,000 cells/mL with medium. One milliliter of this suspension was added to each well of a 24-well sterile polystyrene cell culture tray, and these trays were incubated at 37°C in 95% air, 5% CO_2, and 100% relative humidity for 24 h. A 20.4-μL drop of metal cation solution at 50 times the final desired concentration was added to each well after 24 h. The cells were then incubated for another 24 h. At this time, the toxic effect was evaluated by processing the trays. To test reversibility of the toxicity, medium containing the metal cations was removed in some trays, the cells washed with sterile phosphate buffered saline, and fresh medium was applied. These trays were allowed to incubate another 24 h and were then processed. Six replicates were prepared for each concentration/condition tested.

Toxicities were determined in two separate experiments. The first experiment tested a broad range of cation concentrations (from 0.01 ppm to 100.0 ppm for all elements except Ga^{3+} or In^{3+} where the maximum was 50 ppm). The second experiment tested a range of concentrations which focused on concentrations eliciting the toxic effects. Results from the two experiments were compared to evaluate the repeatability of the assay.

Measurements of cytotoxicity

Four toxicity parameters were used: total protein mass (TP), ^{3}H-Leu incorporation (^{3}H-Leu), ^{3}H-Tdr incorporation (^{3}H-Tdr), and MTT-formazan production (MTT-f). Total protein mass was measured using a commercially available assay which employed bicinchoninic acid/Cu^{1+} capture (Pierce, Rockford, IL). This method is described in detail elsewhere.[12] Briefly, 100-μL ali-

quots of cell monolayers solubilized with 0.25% v/v Triton X 100 in 0.1 mol/L NaOH were added to 1.5 mL of working solution, mixed, and incubated for 30 min at 60°C. The resulting solutions were checked for absorbance at 562 nm using a Beckmann DU-64 spectrophotometer. Standards were mixed using bovine serum albumin from 10 to 300 μg/mL.

^{3}H-Leu incorporation was measured using standard techniques described in detail elsewhere.[13] ^{3}H-Leu was purchased at 1 μCi/μL and 65 Ci/mmol (ICN, Irvine, CA). Cells were pulsed with 5 μCi/well for 90 min at the end of the experiment. Washed and solubilized monolayers were applied to Whatman cellulose filters (Maidstone, England, 3 MM, 2.5 cm diameter). The filters were then washed with trichloroacetic acid (TCA), dried, and evaluated for β-emission in a nonaqueous scintillant (Biosafe NA, RPI, Mount Prospect, IL).

The procedure for ^{3}H-Tdr incorporation is described in detail elsewhere.[14] ^{3}H-Tdr was purchased at 1 μCi/μL and 65 Ci/mmol (ICN, Irvine, CA). Cells were pulsed for 45 min at the end of the experiment with 5 μCi/well. Acid insoluble material was extracted from saline and TCA washed monolayers into 6.7 N perchloric acid at 60°C for 30 minutes. Aliquots of 50 μL of the resulting extractants were evaluated for β-emission in aqueous scintillant (Biosafe II).

MTT-f production was measured using histochemical staining and disodium succinate as a substrate; the procedures are described in detail elsewhere.[15] At the end of the experiment, cells were washed with saline, then incubated with the staining solution for 90 min. The formazan dye was extracted in 6.25% v/v 0.1 mol/L NaOH in dimethylsulfoxide (DMSO) from washed and dried monolayers. The amount of formazan was quantified spectrophotometrically at 560 nm. Standards were prepared at concentrations between 0.5 μg/mL and 20 μg/mL from the formazan.

Calculations

(1) Each of the toxicity parameters (total protein, ^{3}H-leu incorporation, ^{3}H-Tdr incorporation, and MTT-f production) were plotted against concentrations of metal cations. The concentration at which the activity of a parameter was reduced to 50% of the control was determined graphically (TC50 values-concentration causing 50% toxicity, see Fig. 1).

(2) A numerical value for the relative 'broadness' of the concentration range over which the toxic effect occurred was developed to allow comparisons between the cations and was determined as follows. The concentration at which 10% depression occurred was subtracted from the concentration at which 90% depression occurred (see Fig. 1). This gave the concentration range over which most of the toxic effect was observed (from 10% to 90%). To allow comparisons between metal cations of different potencies, this number was then divided by the TC50. The resulting unitless number gave an estimate of the relative broadness of the concentrations over which toxicity was seen. For example, a value of 2.1 means that the concentration range over

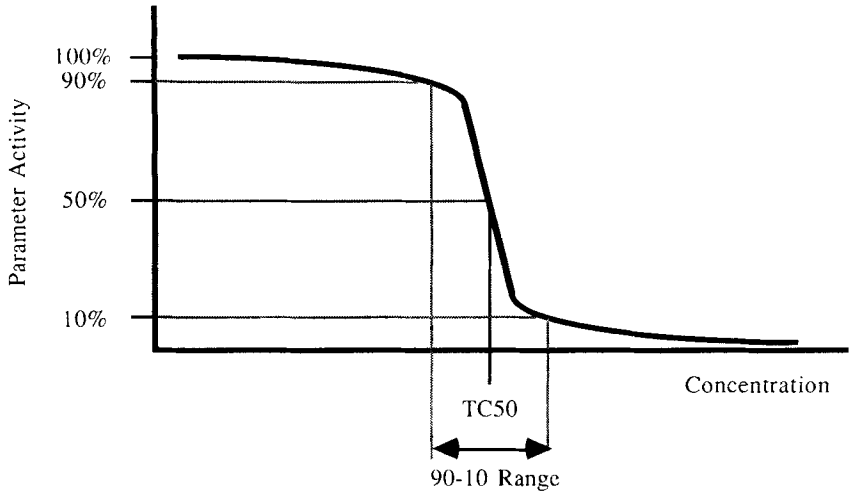

Figure 1. Diagram showing methods for determining TC50 values and relative broadness of toxic effect values.

which most of the toxicity occurred is 2.1 times the 50% inhibitory concentration for that metal cation and that toxicity parameter.

(3) The activity of a parameter was plotted against time for each concentration of metal cation. Each graph had six lines plotted, one for each of the metal cation concentrations tested (including the controls, see Fig. 2). From these graphs, the concentration range in which irreversibility occurred was determined by evaluating the parameter activity between 24 and 48 h. If the activity increased during that period, the effect was defined as reversible. If the activity of the parameter decreased or stayed the same, the effect was

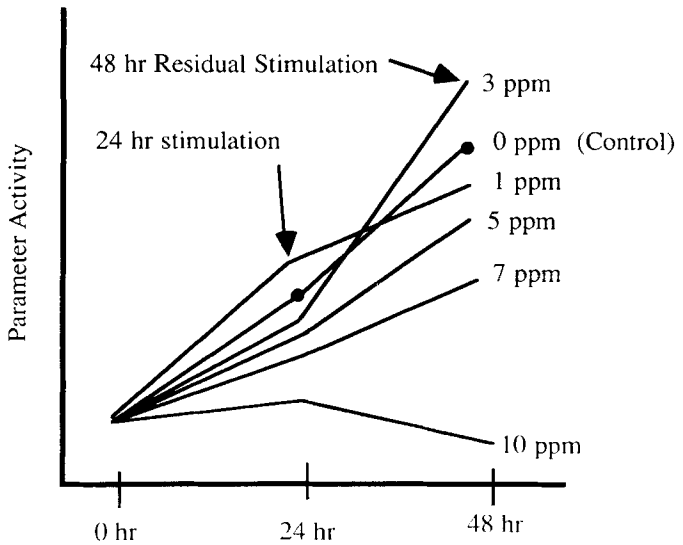

Figure 2. Diagram showing methods for determination of reversibility and stimulation effects.

defined as irreversible. It was not possible to determine a specific concentration at which an effect became irreversible because a finite number of concentrations were used. Thus, the narrowest concentration range possible from the plot was selected. For example, in Figure 2 the range for irreversibility is 7–10 ppm, since there was some reversibility at 7 ppm but none at 10 ppm.

(4) The activity vs time plots were also used to determine if 24 h stimulation or 48 h residual stimulation occurred. Twenty-four-hour stimulation was defined as an increase in parameter activity between 0 and 24 h greater than that for the controls. In Figure 2, this effect occurred at 1 ppm. Forty-eight-hour residual stimulation was defined as a recovery from 24 to 48 h greater than that seen in the controls. In Figure 2, this effect occurred at 3 ppm.

(5) Error bars for graphs were determined by computing three standard errors of the mean for the six replicates. These error bars represented approximate 95% confidence intervals based on t statistics for these sample sizes. Errors for TC50 values were determined graphically, and represent approximate 95% confidence intervals. Errors for relative broadness calculations were estimated using propagation of error techniques (from TC10, TC50, and TC90 errors). Twenty-four-hour stimulations and 48 h residual stimulations were labeled significant if the 95% confidence interval did not include the control values; they were labeled slight if they were elevated above control values but did not exhibit statistical identity.

RESULTS

50% toxicity concentrations (TC50)

Figure 3 shows an example of the activity–concentration plots. Table I lists the 50% toxicity concentrations for the metal cations. Four TC50 values are shown for each cation, one for each toxicity parameter. The TC50 values are shown in ppm of the metal cation and μM concentrations; errors are approximately 10%. The focused range experiments were generally used to determine TC50 values since they had the most data points in the areas of rapid change. Specific values for In^{3+} are not shown because In^{3+} exhibited no toxicity up to 50 ppm.

Table I shows that the potency of metal cations varied by 2–3 orders of magnitude. The most potent cation appeared to be Cd^{2+}, which exhibited a TC50 of about 0.9 μM (0.10 ppm) on the average. The least potent cation was In^{3+}, which exhibited no toxicity at concentrations below 435 μM (50 ppm). All potencies were determined based upon a cell target of approximately 32000 cells. The ranking of the potencies depended upon the unit of concentration used for establishing rank; if the cations were ranked by TC50's in ppm (using total protein), then the ranking for the elements was (most potent to least potent):

$$Cd^{2+} > Ag^{1+} > Zn^{2+} > Ni^{2+} > Au^{4+} > Cu^{2+} > Ga^{3+} > Pd^{2+} > In^{3+}.$$

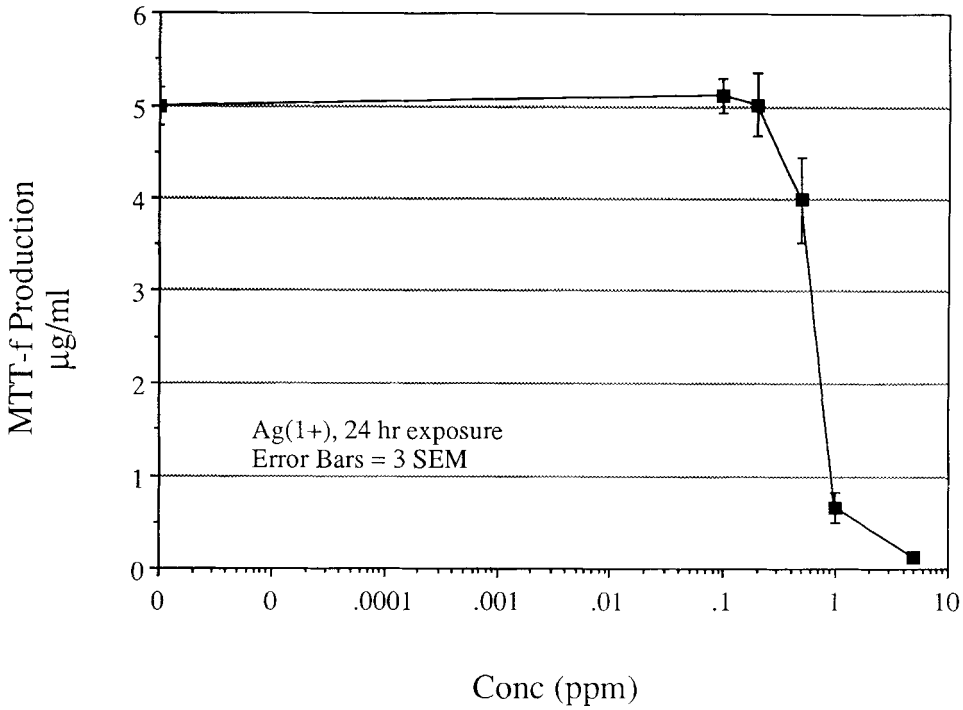

Figure 3. Example of an activity–concentration plot (Ag^{1+} using MTT-formazan production to measure activity). This type of plot was used to assess the 50% toxicity values (TC50s) and broadness of toxic effect values. Here Ag^{1+} exhibited a TC50 of 0.63 ppm (5.8 μM) and a relative broadness value of 1.9.

If, however, μM was used as the unit of concentration, then the ranking was:

$$Cd^{2+} > Ag^{1+} > Zn^{2+} > Au^{4+} > Ni^{2+} > Pd^{2+} > Cu^{2+} > Ga^{3+} > In^{3+}.$$

The change in potency rank was a result of differences in atomic weights (AW) of the metals and the fact that ppm is a unit based on mass, while μM is a unit based on numbers of atoms. The latter unit is probably the one of choice because it represented a more realistic assessment of the toxicity of each atom of a metal. The ranks also depended somewhat upon which toxicity parameter was used (TP, ^{3}H-Leu, etc.). The above rankings were based upon total protein, but if ^{3}H-Tdr incorporation were used, several of the metals (Cu^{2+} or Ga^{3+}) would rank as more potent toxins. The best approach was to use the lowest concentration (in μM) of the four parameters as a measure of potency, since this was the lowest concentration which would cause some disturbance of cell metabolism. When this was done, the ranking was:

$$Cd^{2+} > Ag^{1+} > Zn^{2+} > Cu^{2+} > Ga^{3+} > Au^{4+} > Ni^{2+} > Pd^{2+} > In^{3+}.$$

The relative toxicity broadness values for the cations are shown in Table II. These values were also determined from the activity-concentration curves (as in Fig. 3), and had an approximate error of 20–30%.

TABLE I
50% Toxicity Concentrations in ppm and μM, 24-h Exposure

		Total Protein		^{3}H-Leu Incorporation		^{3}H-Tdr Incorporation		MTT-f Production	
Cation	AW	(ppm)	(μM)	(ppm)	(μM)	(ppm)	(μM)	(ppm)	(μM)
Ag^{1+}	107.87	0.67	6.2	0.68	6.3	0.67	6.2	0.63	5.8
Au^{4+}	196.97	21	106	17	86	19	96	18	91
Cd^{2+}	112.41	0.13	1.2	0.07	0.7	0.05	0.4	0.12	1.1
Cu^{2+}	63.56	25	390	25	390	2.8	44	15	240
Ga^{3+}	69.72	32	458	9.8	143	4.5	65	14	200
In^{3+}	114.82	>50	>435	>50	>435	>50	>435	>50	>435
Ni^{2+}	58.69	11	190	10	170	11	190	11	190
Pd^{2+}	106.42	39	370	36	340	32	300	38	360
Zn^{2+}	65.39	1.8	28	1.6	24	1.8	28	1.8	28

Errors (95% confidence intervals) are approximately 10%.
Values are per 32000 cells at 16000 cells/cm^2.

TABLE II
Relative Broadness of Toxicity Range, 24 h Exposure

Cation	Tot. Prot.	^{3}H-Leu	^{3}H-Tdr	MTT-f
Ag^{1+}	1.2	2.9	0.7	1.9
Au^{4+}	1.9	2.0	1.9	2.0
Cd^{2+}	3.7	4.4	4.6	3.6
Cu^{2+}	1.6	1.8	2.8	2.4
Ga^{3+}	(6)	3.9	3.6	(6)
In^{3+}	NA	NA	NA	NA
Ni^{2+}	1.6	2.7	1.9	2.7
Pd^{2+}	1.1	0.8	1.1	1.5
Zn^{2+}	2.2	2.6	1.9	1.9

Values are unitless; errors (95% confidence intervals) are approximately 20–30%.
NA = not applicable
(6) = possibly inaccurate due to extrapolated activity–concentration curve.

The relative toxicity ranges were interesting in several respects. If the 20–30% error is taken into consideration, the magnitudes of these ranges did not vary much for the four toxicity parameters for Au^{4+}, Cd^{2+}, Pd^{2+}, or Zn^{2+}. Differences did occur from parameter to parameter in some elements (Ag^{1+}, Cu^{2+}, Ga^{3+}, and Ni^{2+}), but no overall pattern to these variations was evident. When comparisons are made between metals, differences can also be seen. For example, the relative broadness for Pd^{2+} averaged about 1.2 (no units), while for Cd^{2+} it was about 4.0. Thus, the range over which a metal cation caused the precipitous drop in cellular functions did seem to depend upon the cation involved. It is interesting to note that this range did not appear to correlate with the potency of the cation's toxicity. Cd^{2+}, which had relatively high potency, had a broader range; Ag^{1+}, which was also quite potent, had a narrower relative range.

Reversibility

Figure 4 shows an example of the activity time plots used to assess reversibility of the toxic reactions, and Table III lists the reversibility ranges determined from these curves. The reversibility ranges shown in Table III generally encompassed the TC50 values. Within each element they were largely the same for all toxicity parameters, except for Cu^{2+} and Ga^{3+}, which both showed lower reversibility ranges for ^{3}H-Tdr incorporation. These cations also exhibited lower TC50 values for this toxicity parameter.

Stimulation effects

Two stimulatory effects were noted. The first effect was a stimulation of the activity of a toxicity parameter in the presence of the metal cation (between 0 and 24 h), usually at concentrations below the TC50. Figure 5 shows an example of such an effect for Ni^{2+} (^{3}H-Tdr incorporation). Table IV lists the

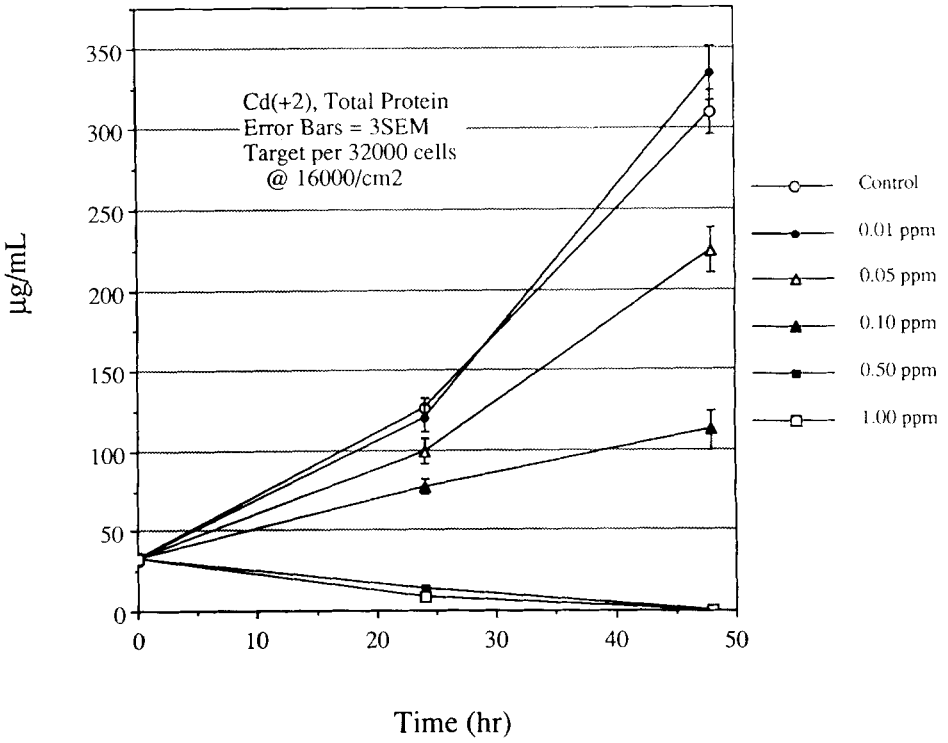

Figure 4. Example of an activity–time plot (Cd^{2+} using total protein to measure activity). This type of plot was used to assess reversibility and stimulation effects. The point of irreversible cytotoxicity was between 0.1 and 0.5 ppm. Forty-eight-hour stimulation effect evident at 0.01 ppm; the effect was small but statistically significant ($p = 0.05$). Error bars for 0.50 and 1.00 ppm concentrations were too small to illustrate.

TABLE III
Reversibility Concentration Ranges 24-h Exposure, 24-h Recovery, in µM

	Tot. Prot.	^{3}H-Leu	^{3}H-Tdr	MTT-f
Cation	µM	µM	µM	µM
Ag^{1+}	NA	NA	NA	NA
Au^{4+}	50–250	50–250	50–250	50–250
Cd^{2+}	0.9–4.4	0.9–4.4	0.9–4.4	0.9–4.4
Cu^{2+}	160–790	160–790	16–79	160–790
Ga^{3+}	140–215	140–215	70–140	140–215
In^{3+}	NA	NA	NA	NA
Ni^{2+}	85–260	85–260	85–260	85–260
Pd^{2+}	230–470	230–470	230–470	230–470
Zn^{2+}	15–75	15–75	15–75	15–75

NA = not available.
Values are in µM, per 32000 cells at 16000 cells/cm^2.

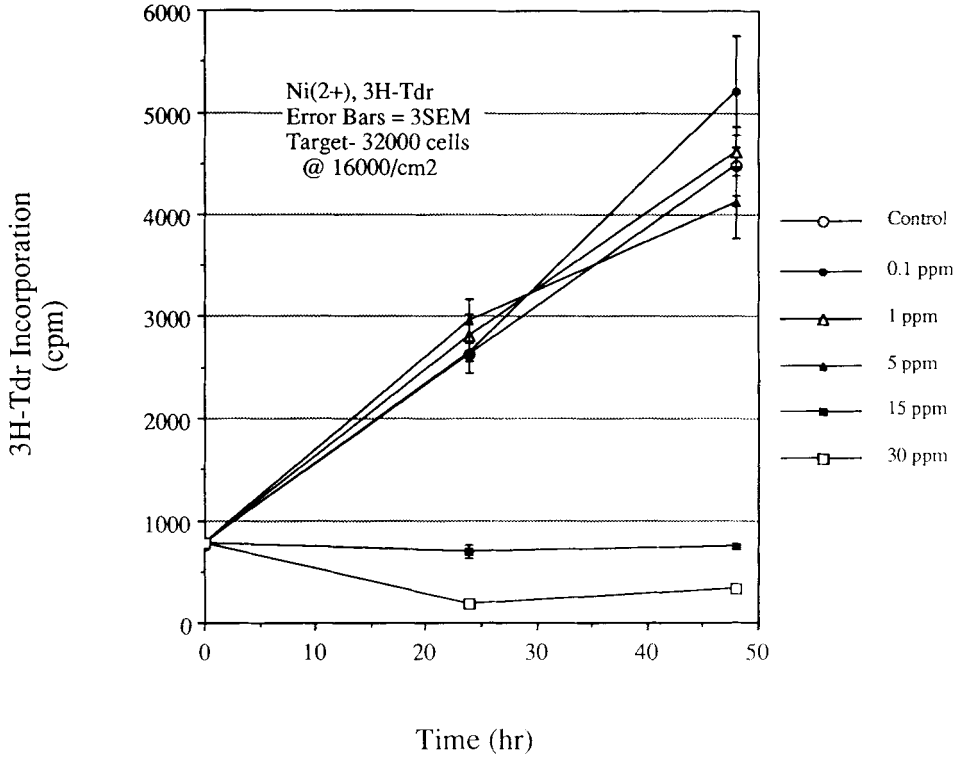

Figure 5. Example of 24-h stimulation effect for Ni^{2+} using ^{3}H-Tdr incorporation to measure activity. Small but statistically significant ($p = 0.05$) stimulations above controls were evident at 1.0 and 5.0 ppm at 24 h. Error bars for 15- and 30-ppm concentrations were too small to illustrate.

24-h stimulation effects observed in these experiments. The effects are listed at the concentrations at which they occurred. Generally, these effects immediately preceded the precipitous drop in activity which followed at higher concentrations.

The second stimulatory effect was a residual stimulation which some metallic cations seemed to exhibit after the medium containing the metal cations had been removed from the medium. An example of this type of effect is shown in Figure 4. Table IV lists all of the 48-h residual stimulation effects which were observed.

The 24-h stimulation and 48-h residual stimulation values shown in Table IV were the least certain parameters. They were probably not artifacts since the effects were seen in a number of experiments with a number of different cations. However, there appeared to be little pattern to their occurrence from metal to metal or within the toxicity parameters of a given metal. The only generalization which appeared to be apt was the fact that concentrations of metal cations which caused the 24-h stimulatory effects were always slightly lower than TC50 values, and often just preceded the concentrations causing precipitous toxicity. Concentrations which caused 48-h

TABLE IV
24-h Stimulation Effects and 48-h Residual Stimulation Effects 24-h Exposure, 24-h Recovery, in μM

		24-h Stimulation				48-h Residual Stimulation			
Cation	AW	TP	^{3}H-Leu	^{3}H-Tdr	MTT-f	TP	^{3}H-Leu	^{3}H-Tdr	MTT-f
Ag^{1+}	107.87	N	N	0.9 sl	N	NA	NA	NA	NA
Au^{4+}	196.97	5, 25 sl	N	N	N	N	5, 25 sig	N	5 sl, 25 sig
Cd^{2+}	112.41	N	N	N	N	0.1 sig	0.1 sl	N	0.1 sig
Cu^{2+}	63.56	15, 80 sig	15, 80 sig	15 sig	N	N	N	N	N
Ga^{3+}	69.72	5, 15 sl	N	5 sl	N	N	N	N	N
In^{3+}	114.82	NA	NA	NA	NA	NA	NA	NA	NA
Ni^{2+}	58.69	N	N	15, 85 sig	15 sig	15 sig	N	2 sig	N
Pd^{2+}	106.42	N	N	N	N	N	N	N	N
Zn^{2+}	65.39	N	N	N	N	2, 7.5, 15 sig	2, 7.5, 15 sig	N	N

Values are in μM.
N = not observed, NA = not available.
sl = Slight effect (not statistically significant at $p = 0.05$), sig = significant effect ($p = 0.05$).

residual stimulations were usually significantly lower than the TC50 concentrations.

DISCUSSION

Adequacy of experiments

Baseline levels of all the toxicity parameters were assessed at 0 h to assure that cells had unrestricted growth potential during the entire experiment. By establishing these baselines, the activity of the controls could be followed throughout the experiment to confirm that potential for increase existed. Figure 5 was typical of most experiments in that it established that growth of controls was unrestricted for 72 h. It is impossible to tell if a growth restriction had started at 72 h without readings after 72 h. Preliminary experiments had established that growth could occur somewhat longer than 72 h at these plating densities. Thus, it appeared that the growth potential of the controls was adequate.

A second major area of concern was reproducibility. Reproducibility of the experiments was assessed by comparing the activity–concentration curves (as a percentage of the control values for each experiment) for both broad and focus range experiments on the same graph. By using percentages, variations caused by differences in cell populations could be minimized. The example in Figure 6 was typical of such a comparison in that it showed adequate agreement.

The potential toxicity of the anions associated with the metal cations was also a concern. Anion concentrations reached levels of 500 ppm in the In^{3+} and Ga^{3+} experiments which used atomic absorption standards as a source of metal cations. The toxicity of the anions was tested by applying solutions of the sodium salts as potential toxins. Chloride, sulfate, and nitrate anions at concentrations of 1000, 50, and 1000 ppm, respectively, were tested for their effect on all toxicity parameters. These experiments showed no evidence of anion toxicity at these concentrations. Since anion concentrations were below these concentrations in all metal cation experiments, any toxic effects observed were not attributed to the anions.

TC50 values/reversibility

The ranking of metal cation potency (Table I) was somewhat arbitrary depending upon the parameter and units chosen. The absolute toxicities are probably more important than rankings, since it is absolute TC50, rather than its rank among other metals, which would be important in determining whether ions released would cause a problem.

It was interesting that potency did not correlate well with the AW (Table I). For example, Au^{4+}, the heaviest metal (AW = 196), was not nearly as potent as Cd^{2+} (AW = 112) or Zn^{2+} (AW = 65). Also, In^{3+} and Cd^{2+}, which had very

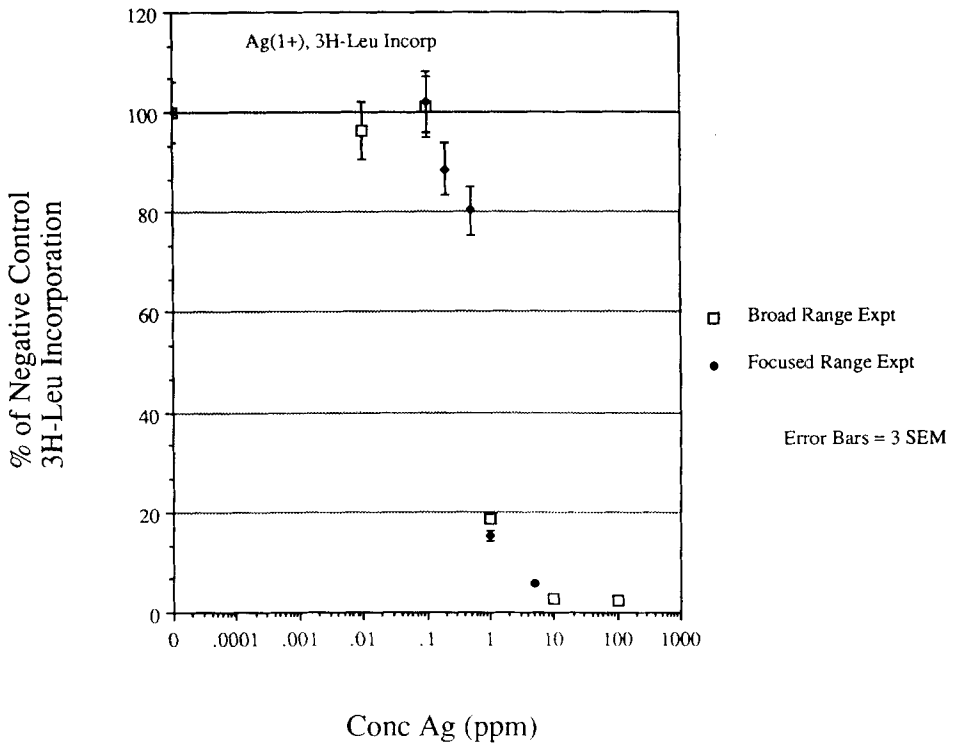

Figure 6. A typical example of comparison of broad range and focused range experimental results for Ag^{1+} using ^{3}H-Leu incorporation to measure activity. The results for the two experiments are in agreement.

similar atomic weights had different potencies. Thus, the common belief that "heavy metals" intrinsically exhibit highly potent toxic effect did not appear to be true for these metals.

In most instances, the TC50's for the four toxicity parameters were roughly equivalent for a given metal. Thus, the TC50 for Ni^{2+} appeared to be 170-190 μM regardless of which parameter was used as an indicator (Table I). However, for Cd^{2+}, Cu^{2+}, and Ga^{3+}, there were significant differences in the magnitudes of the TC50's for the different toxicity parameters. In these latter cases, ^{3}H-Tdr incorporation appeared to be the most sensitive of the parameters. The cause of this disparity is unknown, but it might have been simply a function of the rate of the toxic effect and the time of exposure.

The observation that all of the toxicity parameters ultimately reacted negatively to the metal cations supported the idea that the ions exerted nonspecific toxicity in the cell. Unlike a pharmacological agent, which might exert an effect on a specific aspect of cellular metabolism, these ions affected several aspects of cellular metabolism, including the ability to attach to the polystyrene surface. This observation was not surprising, since these ions probably exerted their toxic effects via a multitude of nonspecific binding sites, as well as some specific binding sites.[16] It is possible that the potency of a metal cation depended upon the nature and number of these interactions.

It may be that there was some relation between the TC50 and the reversibility of a toxic effect since the reversibility ranges generally encompassed the TC50's. These measurements were made on a population of cells and thus it was difficult to know what reversibility meant in terms of a single cell exposed to a cation. Thus, these population reversibility ranges did not provide information on single cell reversibility. Individually, some cells may have reacted quite irreversibly to even very low concentrations of these metals. If individual cells reacted to the cations at different rates (because of differences in cell cycle, division rate, or degree of spreading), this individual effect would be masked because other cells which reacted more slowly to the cation would continue to multiply once the cation was removed; the net effect observed would then be reversibility. However, these reversibility values are informative in that they do show that, as a population, the cells can recover from an exposure to a metal cation.

The explanation for the apparent random occurrence of the 24-h stimulation and 48-h residual stimulation values may have stemmed from their ephemeral nature. It is possible that such stimulation effects were either an attempt to combat toxicity or a consequence of a loss of metabolic balance resulting from the initial stages of toxicity. In either case, the effects would have been transient as the toxic effect gradually pervaded other aspects of cellular metabolism. Given the relatively simple population assessments used in these studies, such ephemeral effects would only be observed if the toxicity assessment caught the effect at its peak.

Comparisons with previous investigations were of limited value because of the diverse experimental conditions employed. For example, Jowett et al. found that Zn^{2+} did not cause significant cell death at 20 ppm in an organ culture model,[10] but Leirskar found a 96% decrease in cell number with 10 ppm Zn^{2+} (human epithelial cells),[17] and this study found a 50% decrease in total protein with as little as 1.8 ppm and 100% decrease with 2.5 ppm. One of the reasons for performing this study was test common casting alloy elements under similar conditions to allow valid comparisons.

CONCLUSIONS

(1) All metal cations tested (Ag^{1+}, Au^{4+}, Cd^{2+}, Cu^{2+}, Ga^{3+}, In^{3+}, Ni^{2+}, Pd^{2+}, and Zn^{2+}) except In^{3+} exhibited toxicity as measured by total protein, ^{3}H-leucine incorporation, ^{3}H-thymidine incorporation, and MTT-formazan production. Toxicity was nonspecific in that all toxicity parameters were ultimately affected by all toxic metal cations. Indium was not toxic below 435 μM (50 ppm).

(2) Potency range of metal cation toxicity spanned 2–3 orders of magnitude. The rank for potency (most toxic to least toxic; based on the most sensitive cellular parameter) was:

$$Cd^{2+} > Ag^{1+} > Zn^{2+} > Cu^{2+} > Ga^{3+}, > Au^{4+} > Ni^{2+} > Pd^{2+} > In^{3+}.$$

Cd^{2+} showed a 50% toxicity (TC50) of 0.4 μM (0.05 ppm) while Pd^{2+} showed a TC50 of 300 μM (32 ppm). In^{3+} was not toxic at 435 μM (50 ppm).

(3) All toxic effects were reversible at concentrations near the TC50 concentrations. Reversibility was assessed on a population of cells, not on individual cells.

(4) At concentrations below the TC50s, several cations showed stimulation effects on the cell populations. Stimulations occurred either during exposure to the cations (24-h stimulations), or during the 24-h period after removal of the cations (48-h residual stimulations).

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