Toxicity, Uptake, and Mutagenicity of Particulate and Soluble Nickel Compounds

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Toxicity testing in AS52 cells (24-hr exposures) gave LC_{50} values of 2 to 130 μg Ni/ml for particulate nickel compounds and 45 to 60 μg Ni/ml for water-soluble salts ($NiCl_2$, $NiSO_4$, $Ni(CH_3COO)_2$). The $Ni(OH)_2$, $NiCO_3$, and sulfides (Ni_3S_2, Ni_7S_6, "amorphous NiS") exhibited similar toxicities (LC_{50}'s of 2 to 8 μg Ni/ml), while three nickel oxides were more variable and less toxic (LC_{50}'s of 18 to 130 μg Ni/ml). Most compounds displayed nuclear to cytoplasmic nickel ratios of ≈ 1:1.5 to 1:5 (except ≈ 1:20 for nickel salts). At the LC_{50}'s, a 75-fold range in exposure levels occurred compared to a 10-fold range in cytoplasmic and nuclear nickel concentrations, [Ni]. Cellular nickel distribution indicated three groupings: inert compounds (green NiO, lithium nickel oxide, relatively low nuclear and cytosolic [Ni]); water-soluble salts (very low nuclear [Ni]; high cytosolic [Ni]), and slightly soluble compounds (relatively high cytosolic and nuclear [Ni]). Nickel compounds are considered to be only weak or equivocal mutagens. In this study, a low but significant increase in mutation rate at the *gpt* locus was shown. Although the results would not be sufficient to deem nickel compounds mutagenic by traditional criteria, characterization by PCR analysis indicated that the spontaneous and nickel-induced mutants exhibited different and compound-specific mutational spectra (thus confirming nickel compound involvement). The results reported illustrate some of the methodologic problems involved in testing "weak" mutagens and indicate that alternative approaches may be necessary in classifying the mutagenicity of nickel and other compounds. — Environ Health Perspect 102(Suppl 3):69–79 (1994).

Key words: nickel compounds, AS52 (CHO) cells, intracellular uptake, cytotoxicity, mutagenicity, mutant characterization, PCR

Introduction

Single injections of experimental animals with crystalline nickel compounds such as Ni_3S_2 and NiS have been found to result in a 90 to 100% incidence of tumors at the exposure site (*1–3*). By contrast, water soluble compounds ($NiCl_2$, $NiSO_4$) do not induce tumors, while amorphous NiS does so weakly. More recently, however, the repeated administration of nickel salts has produced a low incidence of malignancies in rats (*4*). In cell culture both crystalline and soluble compounds induce transformation, though soluble compounds must be present at much higher levels (*2,5–7*). Amorphous NiS has not exhibited transforming activity. Cellular transformation and toxicity have been found to depend on the ability of nickel to enter the cell by phagocytosis (*8–10*). Attempts to explain the mechanism of action and potency differences between various nickel compounds have lead to the "Nickel-Ion Hypothesis" (*11,12*). This hypothesis suggests that the nickel(II) ion is the active agent in nickel toxicity, mutagenesis and carcinogenesis, and that its intracellular concentration is a major determinant, irrespective of the nickel compound to which an organism is exposed.

Despite their tumorigenicity in man and animals, nickel compounds and many other metal compounds have not been found to be mutagenic in traditional *in vitro* test systems. In the established CHO/*hprt* mutation assay large deletions and rearrangements extending past the *hprt* locus may affect or delete neighboring genes necessary for survival, thus decreasing the ability to detect or quantify certain types of mutagens (*13*). An approach to circumvent this problem was to use AS52 cells, which are CHO cells modified by deleting most of the *hprt* gene and adding the corresponding bacterial *xprt* gene (*gpt*) to a somatic chromosome (*14,15*). Thus, in addition to base substitutions, frame shifts, small deletions, additions, and chromosome breakage and rearrangements normally measured in CHO cells (*16*), the new location of this gene is known to allow detection of mutations caused by X-irradiation (which causes large deletions). With this modification we hoped to be able to detect nickel-induced mutations, which are suspected to involve large DNA deletions or rearrangements.

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Materials and Methods

Preparation and Characterization of Nickel Compounds

Nickel hydroxide was synthesized by slowly mixing 0.6 M $NiSO_4.7H_2O$ and hot 1.4 M NaOH followed by centrifugation and filtration to collect the precipitate [modified from Kasprzak et al. (*17*)]. Heating at 80°C for 20 hr to dry and improve crystallinity gave a dark-green product. The sample was ground in a Spex 8000 Mixer/Mill (Spex Industries, Metucher, NJ) with tungsten carbide grinding vial and the light green powder was dry-sieved through a series of wire mesh sieves (#100, #200, #325, #400; respectively, 150, 75, 45, 38 μm openings). Particles not passing through the last sieve were reground and sieved, then wet sieved by suspending in water and filtering through 25 μm and 5 μm sieves. Particles passing through the final 5 μm sieve were collected by centrifugation. The $Ni(OH)_2$ was dried at room temperature and then at 80°C for 5 hr.

$NiCO_3$, which was to be essentially free of nickel hydroxide, was prepared by a procedure suggested by V. J. Zatka (per-

Table 1. Identification of compounds.

Sample	Name and formula in text	Formula from chemical analysis[a]	Species from X-ray diffraction analysis
1	Nickel hydroxide, $Ni(OH)_2$	$Ni(OH)_2.0.04\ NiCO_3.0.6\ H_2O$	$Ni(OH)_2$
2A	Nickel carbonate, $NiCO_3$	$NiCO_3.0.55\ Ni(OH)_2.3.7\ H_2O$	
2D	Nickel carbonate, $NiCO_3$	$NiCO_3.0.58\ Ni(OH)_2.4.2\ H_2O$	$NiCO_3.6H_2O$
3	Black nickel oxide, NiO	NiO	NiO
4	Green nickel oxide, NiO	NiO	NiO
5	Lithium nickel oxide	$Li_{2.23}Ni_8O_{10.22}$	$Li_2Ni_8O_{10}$ or NiO
6	Amorphous nickel sulfide, NiS	$NiS_2.2.25\ NiSO_4.6H_2O.4.76\ NiS$	$NiS_2 + NiSO_4.6H_2O$
7	α-Ni_7S_6	α-Ni_7S_6 + 5% NiS + 1% $NiSO_4$	α-Ni_7S_6 (major); NiS + $NiSO_4$ +Ni_3S_2 (minor)
8	Nickel subsulphide, Ni_3S_2	Ni_3S_2	Ni_3S_2
9	Nickel chloride, $NiCl_2$	$NiCl_2.6\ H_2O$	
10	Nickel sulfate, $NiSO_4$	$NiSO_4.7\ H_2O$	
11	Nickel acetate, $Ni(CH_3COO)_2$	$Ni(CH_3COO)_2.4\ H_2O$	

[a] Nickel salts (nos. 9 –11) were identified by product labels and suppliers' documentation.

sonal communication). $(NH_4)_2CO_3$ and $Ni(NO_3)._26H_2O$ solutions were cooled to 0°C. The $Ni(NO_3)_2$ solution was slowly added to the CO_2-saturated $(NH_4)2CO_3$ solution, then the mixture let stand for 6 days at room temperature to allow formation of blue-green $NiCO_3$ crystals. The solution plus cold water used to rinse the crystals were poured through a 5-µm sieve. The combined filtrates were centrifuged to collect the ≤ 5 µm fraction of $NiCO_3$ particles. These particles were left to dry at room temperature, then collected for in vitro testing. This light green product is referred to as $NiCO_3$ sample 2A (<10 µm without grinding). Larger particles (>25 µm) air dried and ground were kept for characterization but not used in the *in vivo* tests.

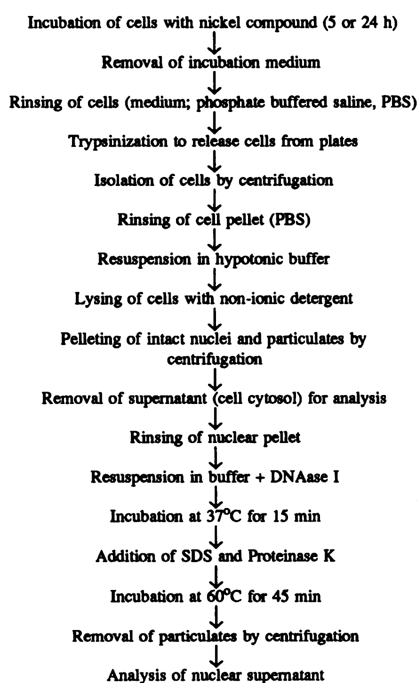

Figure 1. Nuclear isolation flow chart.

Amorphous NiS was prepared using a 15% $NiCl_2$ solution and excess 22% $(NH_4)_2S$ (*10*) in an acetate buffered system. The pH was measured periodically and glacial acetic acid added as required to keep the pH in the range of 4.0 to 6.0. The product was filtered and rinsed with water and acetone, dried at 110°C for 17 hr, then ground in the Spex grinder. Particles sieved and collected as described for the nickel hydroxide were sterilized in acetone and dried for 5 hr at 80°C.

Black NiO, $Li_2Ni_8O_{10}$ (nickel oxide in which 2.39% Li stabilizes a Ni(III) content of 19.2%), crystalline α-Ni_7S_6, and crystalline nickel subsulphide (Ni_3S_2) were obtained from INCO Limited (Toronto, ON) and green NiO was purchased from Johnson Matthey Chemicals (Brampton, ON) (puratronic grade). These compounds were wet-sieved through a 25-µm sieve and 10-µm nylon Spectra/mesh disposable filter. Particles were collected by centrifugation, sterilized in acetone, centrifuged, and then dried for 5 hr at 80°C.

High purity nickel chloride and nickel sulfate were purchased from BDH (Toronto, ON) (analar grade) and nickel acetate from Johnson Matthey (puratronic grade). Stock solutions were made in water, filter-sterilized through 0.22-µm filters, and stored in sterile glass bottles. $NiCl_2$ and $NiSO_4$ were prepared as 500 mM stock solutions, while $Ni(CH_3COO)_2$ was made as a 100 mM stock solution.

The nickel, sulfur, and minor constituent contents of the compounds provided by INCO Ltd. (Toronto, ON) were determined by them. The amorphous NiS, $Ni(OH)_2$, and $NiCO_3$ samples were analyzed chemically at INCO's Sudbury or Mississauga facilities. Formulas of the nickel salts (water soluble compounds) were assumed to be accurate as reported on the labels and in the suppliers' catalogues.

X-ray crystal powder diffraction patterns were obtained for all of the particulate compounds. Diffraction analysis was performed at the Institute for Materials Research, McMaster University, using Cu-K_α radiation at 1.5405Å. Samples were then identified (Table 1) by comparing lattice spacings and peak intensities to standard spectra available in the JCPDS data base (*18*).

Cell Culture

AS52 cells derived by modification of the CHO-K1 line were kindly provided by K. R. Tindall, National Institute of Environmental Health Sciences (Research Triangle Park, NC). These cells were grown in Ham's F12 medium (F12) with 5 to 10% fetal bovine serum (FBS). Dialyzed FBS was used in mutation testing procedures. Prior to mutagenicity studies, the medium was supplemented with 10 µg/ml mycophenolic acid, 25 µg/ml adenine, 50 µM thymidine, 250 µg/ml xanthine, and 3 µM aminopterin (MPA medium), to eliminate spontaneous mutants. Antibiotics were not added for routine culturing, though 100 U penicillin G, 100 µg streptomycin, and 0.25 µg amphotericin B per ml medium were employed in individual experiments. Cells were grown in a 37°C, 95 to 100% humidity, 5% CO_2 (continuous flow) incubator, and routinely subcultured every 3 to 4 days. A doubling time of 14 to 18 hr and plating efficiencies of 50 to 70% were observed. A Coulter Model Z_F particle counter was used for cell-number determinations.

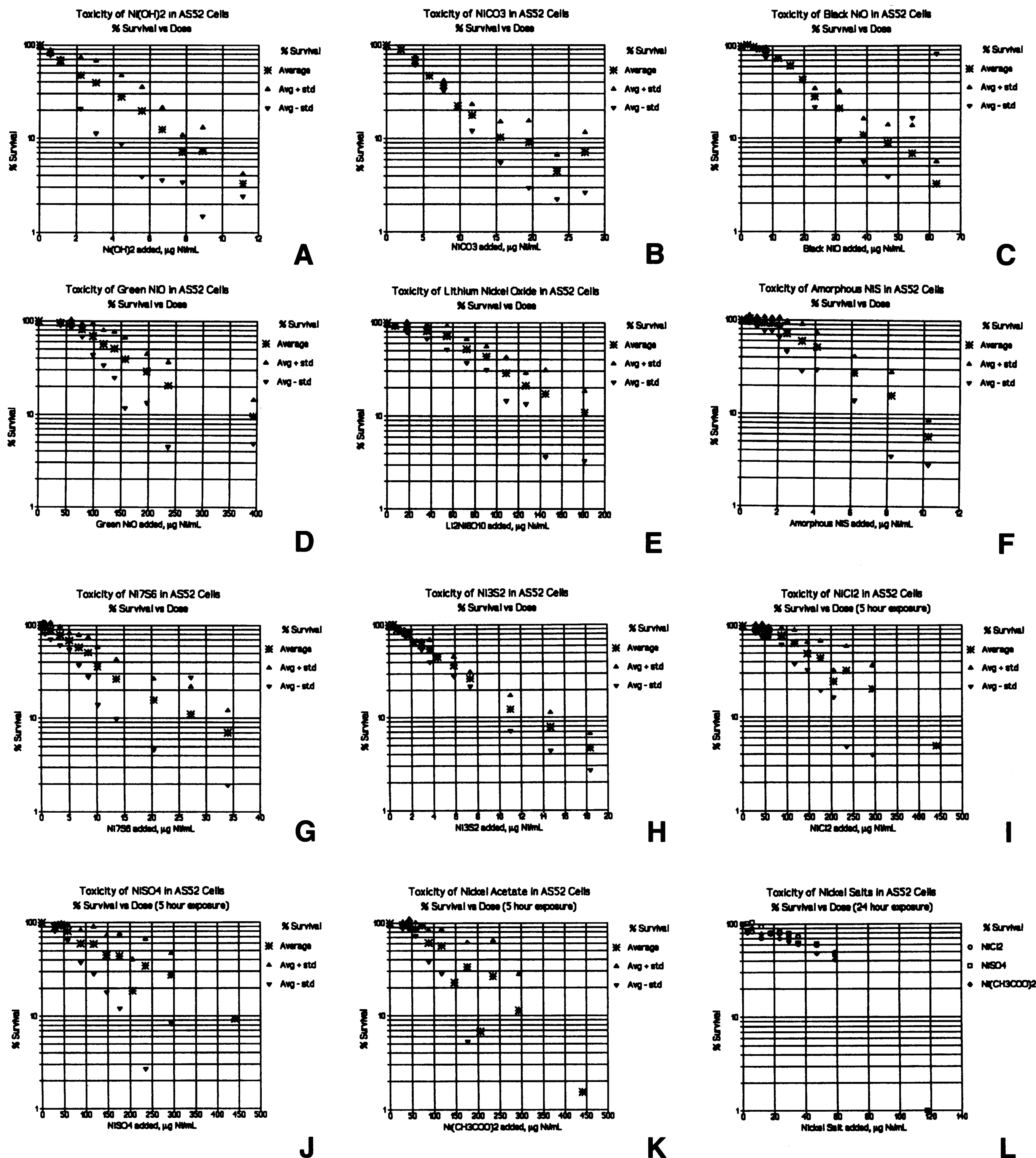

Figure 2. Nickel toxicity curves in AS52 cells. AS52 cells were seeded in F12/10% FBS medium at 500 cells per 60 mm diameter dish 2 days before treatment. The cells were exposed to the range of nickel compounds in serum-free medium at the concentrations indicated for 24 hr (or 5 hr exposure for nickel salts, as noted). The cells were rinsed to remove extracellular nickel, then incubated in fresh F12/10% FBS medium until colonies formed (7 to 9 days). The percent survival was determined by comparing the number of colonies counted to the number obtained in control (nonexposed) dishes. Averages are based on three to five replicate experiments (three dishes per experiment per test condition) for each compound. (To facilitate semilogarithmic plotting of the data, survival values and standard deviations (std) less than 1 have been either omitted or plotted as 1% survival, as appropriate.)

Toxicity Testing

AS52 cells from a near confluent culture dish were released with trypsin, counted, diluted, and replated at 500 cells per 60 mm diameter dish in F12 medium with 10% fetal bovine serum (F12/10). One to two days later, the medium was replaced with serum-free medium (F12/0) and the test compound was added. After an exposure period of 24 hr, or 5 hr for nickel salts, the medium was removed and the cells rinsed to remove nickel not taken up by the cells. The F12/10 medium was renewed and then the cells were incubated for 6 to 8 days until colonies of sufficient size to be counted developed. Colonies were stained using a solution of 0.5% crystal violet in ethanol, thereby producing clearly visible, circular colonies of a deep violet color. These colonies were then counted using an automated colony counter and the number per dish relative to the number in untreated control culture dishes (relative % survival) was reported.

Analysis of Nickel Content in Cells, Nuclei, and Cytosol

To get a measure of the biologically available nickel levels (as opposed to total nickel present), we set out to determine the dissolved nickel (nickel ion) levels in the cytosol and nuclei. The final procedure used to assess the cytosolic fraction and a nuclear fraction free of particulates is outlined in Figure 1. To assess possible contributions to the measured nickel levels from particulate dissolution during the cellular fractionation protocol outlined in Figure 1, simulation experiments were conducted for green NiO and Ni_3S_2. All samples were analyzed by electrothermal atomic absorption spectrometry (EAAS). A Perkin Elmer Atomic Absorption Spectrometer Model 703 (Rexdale, ON) furnished with a graphite furnace Model HGA-500 was used.

Mutagenicity Testing

Cells growing in MPA medium were replated in F12/10% FBS on day −2. On day 0 the medium was replaced with serum-free F12 and the nickel compound or ethylmethanesulfonate (EMS, positive control) was added. Test doses for particulate nickel compounds were chosen to give 20 to 80% survival based on previously determined toxicity curves. After an exposure period of 24 hr for particulates or 5-hr for nickel salts and EMS, the medium was removed and the cells rinsed with F12/5% FBS to remove nickel not taken up by the cells. For 5-hr exposures, fresh F12/10%

Table 2. Nickel compound LC_{50} values in AS52 and CHO cells and corresponding cytosolic and nuclear nickel levels.

	Compound	LC_{50}[a] compound, µg/ml or mM	LC_{50}[a] nickel, µg Ni/ml	Nickel µg/10^6 cells, cytosol[b,f]	Nuclei[b,g]	Dissolution half time (T_{50})[c] (rat or human serum
1	$Ni(OH)_2$	3.6 µg/ml	2.0	0.16	0.08	< 1 day
2	$NiCO_3$	14.9	5.8	0.80	0.07	
3	Black NiO	23.3	18.1	0.11	0.045	0.8 year
4	Green NiO	165.4	130.0	0.035	0.007	> 11 years
5	$Li_2Ni_8O_{10}$	103.6	75.0	0.06	0.018	
6	Amorphous NiS	10.0	4.1	0.25	0.07	24 days[d],<96 days[e]
7	Ni_7S_6	12.1	8.2	0.08	0.055	
8	Ni_3S_2	5.6	4.1	0.19	0.08	28 days
9	$NiCl_2$ (5 hr)	2.6 mM	150	0.22	0.006	
10	$NiSO_4$ (5 hr)	2.1	125	0.17	0.007	
11	$Ni(CH_3COO)_2$ (5 hr)	2.0	120	0.16	0.008	
12	$NiCl_2$ (24 hr)	1.0	60	0.18		
13	$NiSO_4$ (24 hr)	1.0	60	0.17		
14	$Ni(CH_3COO)_2$ (24 hr)	0.8	45	0.15		

[a]LC_{50} is the concentration at which the survival of exposed cells is 50% of that of nonexposed control cells as determined in colony forming assays. Values were determined from Figures 2A to 2L. [b]Dissolved cytosolic and nuclear nickel levels determined from plots of the data in Appendix 1. [c]From reference Kasprzak et al. (*17*); Kuehn et al. (*20*); Thornhill et al. (*21*). [d]Value for amorphous NiS. [e]Value for NiS_2 determined in water. [f]For plot of LC_{50} vs cytosolic nickel, $LC_{50} = 75-87\ Ni_{cytosol}$, $r = -0.29$, $p > 0.05$, $n = 14$. [g]For plot of LC_{50} vs nuclear nickel, $LC_{50} = 129-1810\ Ni_{nuclei}$, $r = -0.93$, $p < 0.01$, $n = 11$.

Table 3. Nickel compound mutagenicity in AS52 cells.[a]

Compound	Amount of nickel µg Ni/ml[b]	Mutation frequency/ 10^6 cells	Confidence interval, 95% level[c]	Dose–response correlation coefficient, r[c]
$Ni(OH)_2$	1.1	25	10 – 40	0.974[e]
	1.7	48[d]	39 – 58	
	2.4	43[d]	27 – 58	
	3.4	75[d]	55 – 95	
	5.5	84[d]	66 –103	
$NiCO_3$	2.5	33	24 – 43	0.626
	4.4	18	7 – 30	
	6.2	23	10 – 35	
	8.2	58[d]	38 – 79	
	11.2	44[d]	26 – 62	
NiO, black	9.5	51[d]	41 – 61	0.695
	14.0	30	8 – 52	
	18.1	42[d]	32 – 51	
	22.2	28	17 – 38	
	31.8	79[d]	61 – 98	
NiO, green	85	38[d]	26 – 51	0.826
	144	40[d]	20 – 60	
	180	38	24 – 51	
	282	42[d]	20 – 64	
$Li_2Ni_8O_{10}$	38	30	21 – 39	0.822
	60	35	29 – 41	
	76	20	11 – 29	
	101	33	17 – 48	
	146	50[d]	44 – 56	
NiS, amorphous	1.8	20	11 – 29	0.953[e]
	2.8	43[d]	30 – 57	
	3.9	33	28 – 37	
	5.2	64[d]	43 – 86	
	7.1	83[d]	53 –114	
Ni_7S_6	3.2	53[d]	37 – 69	0.623
	5.7	18	5 – 30	
	8.6	39[d]	22 – 56	

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Table 3 *(continued)*. Nickel compound mutagenicity in AS52 cells.[a]

Compound	Amount of nickel μg Ni/ml[b]	Mutation frequency/ 10^6 cells	Confidence interval, 95% level[c]	Dose–response correlation coefficient, r[c]
Ni_7S_6	10.5	32	4 – 59	
	19.3	63[d]	54 – 73	
Ni_3S_2	1.6	33	19 – 47	0.932[e]
	3.0	38	22 – 53	
	4.3	44[d]	26 – 62	
	6.2	62[d]	47 – 76	
	9.1	49[d]	35 – 63	
$NiCl_2$ 5 hours	59	25	19 – 31	0.130
	117	58[d]	39 – 76	
	176	60[d]	45 – 75	
	235	8	3 – 14	
	294	47[d]	33 – 61	
$NiSO_4$ 5 hours	59	49[d]	41 – 58	0.570
	117	56[d]	39 – 73	
	176	68[d]	46 – 89	
	235	59[d]	45 – 74	
	294	67[d]	52 – 81	
$Ni(CH3COO)_2$ 5 hr	59	12	4 – 20	0.632
	117	33	15 – 50	
	176	57[d]	44 – 70	
	235	20	10 – 30	
	294	57[d]	49 – 65	
$NiCl_2$ 24 hr	14.7	72[d]	56 – 87	0.246
	29.4	18	10 – 27	
	44.0	30	15 – 45	
	58.7	15	10 – 20	
	73.4	55[d]	49 – 61	
$NiSO_4$ 24 hr	14.7	51[d]	36 – 66	0.886[e]
	29.4			
	44.0	45[d]	32 – 58	
	58.7	44[d]	20 – 68	
	73.4	46[d]	20 – 72	
$Ni(CH_3COO)_2$ 24 hr	14.7	24	15 – 34	0.637
	29.4	32	20 – 43	
	44.0	23	15 – 30	
	58.7	53[d]	37 – 68	
	73.4	37	26 – 47	
Positive controls (EMS)	50 μg/ml	121[d]	102 –139	0.969[e]
	100 μg/ml	208[d]	195 –220	
	200 μg/ml	273[d]	253 –292	
	300 μg/ml	444[d]	428 –460	
	400 μg/ml	352[d]	321 –383	
Negative controls (no addition)		33	19 – 48	
		28	12 – 43	
		37	26 – 47	
		31	21 – 40	
		16	11 – 21	
Average		29	24 – 34	

[a]AS52 cells grown in MPA medium to eliminate spontaneous mutations or loss of the *gpt* gene were plated in F12/10% FBS 2 days (≈ 5.10^5 cells/100 mm dish) prior to treatment. Cells were exposed to the test compounds for 24 hr (or 5 hr for nickel salts, as indicated) in serum-free medium, then rinsed, trypsinized, and replated in F12/10% FBS. The cells were subcultured every 3 days as required during the expression period of 8 days. After this period, 6-thioguanine resistant mutants were selected by plating at $2x10^5$ cells/100 mm dish in 10 ml F12/10% dialyzed FBS plus 10 μM 6-thioguanine. At this same time 500 cells/60 mm dish were plated in medium without thioguanine for determination of plating efficiency. After 10 to 15 days, dishes were rinsed with PBS and colonies stained and counted. The mutation frequency is expressed as the number of mutant colonies per million cells plated. Results are reported in this table for a single experiment based on 5 to 6 dishes per treatment condition with further replicates for negative controls as indicated. [b]Test doses were selected to correspond to formal survival rates of 80%, 65%, 50%, 35%, and 20%. See Table A1 for amounts of the compound added. [c]Two-tailed significance test. [d]Significantly different from control rates by one-tailed *t*-test, at the 0.05 probability level or better. [e]Significant at the 0.05 probability level or better.

FBS was added, the cells were incubated 19 to 24 hr, and were then rinsed once with phosphate-buffered saline (PBS). For 24-hr exposures, the cells were rinsed with PBS. Subcultures were made on day 1 and every 2 to 3 days thereafter to allow expression of the altered gene, with a larger number of cells replated from cultures in which a lower survival was expected, so that at the next replating approximately equal densities were reached. On day 9 the cells were replated at 2×10^5 cells/100 mm dish (5 or 6 dishes per treatment condition) in 10 ml F12/10% dFBS (or 5% dFBS and 5% dialyzed calf serum) plus 10 μM 6-thioguanine (6-TG) to select for *gpt* mutants. At the same time, 500 cells/60 mm dish (3 dishes per treatment condition) were plated in the same medium without 6-TG for a plating-efficiency (PE) determination. After 8 days (day 17), the PE dishes were rinsed with PBS and stained with crystal violet. Following a selection period of 10 to 12 days the 6-TG-resistant colonies (loss of xanthine-guanine phosphoribosyl transferase (XPRT) activity at the *gpt* locus) were stained with crystal violet and counted.

Characterization of Mutants

Colonies were picked off plates and clonally expanded to approximately 10^6 cells. Approximately 0.5 to 1 μg of mutant DNA was amplified by the polymerase chain reaction (PCR) employing suitable primers encompassing the *gpt* gene. These primers constituted the starting points for the DNA synthesis (polymerization). The products were separated by electrophoresis on a 1% agarose gel, which was subsequently stained with ethidium bromide. Additional details are provided in Rossetto et al. (*19*).

Results

Characterization of Nickel Compounds

Results of the chemical and X-ray diffraction analysis of the nickel compounds are given in Table 1. The percent nickel was used to convert from μg compound/ml (as weighed out) to μg nickel/ml, the units reported in the tables and figures.

For Sample 1 (nickel hydroxide), the X-ray diffraction peaks were too broad to allow an accurate measurement of d values or the diffraction angles (2θ), though the pattern was qualitatively consistent with the $Ni(OH)_2$ standard. Chemical analysis was compatible with $Ni(OH)_2{\cdot}0.6H_2O$, with possibly a very small amount of

$NiCO_3 \cdot (0.04$ per $Ni(OH)_2$ unit). Sample 2A showed no observable diffraction planes, though Sample 2D (larger particles) corresponded to the $NiCO_3.6H_2O$ standard. The chemical analyses for Samples 2A and 2D were similar, indicating the same chemical make-up, although differences in the degree of crystallinity existed. Though none is indicated in the X-ray pattern, chemical analysis suggested a significant amount of nickel hydroxide might have been contained in the carbonate samples. The hydroxide, if present, was expected to be in a noncrystalline (amorphous) form. The black and green nickel oxides were indistinguishable by their diffraction patterns and corresponded to the NiO standard. Sample 5 exhibited characteristics of both the NiO and $Li_2Ni_8O_{10}$ diffraction patterns. Chemical analysis, however, indicated a formula of $Li_{2.23}Ni_8O_{10.22}$. Sample 6, expected to be amorphous NiS, according to the diffraction pattern contained a mixture of NiS_2 and $NiSO_4.6H_2O$. These are possible oxidation products in the formation of NiS, though they do not account for the analyzed nickel and sulfur contents. The high background in the diffraction pattern obtained suggested that a noncrystalline component was present, the most likely species being amorphous NiS ($\approx$ 60 mole percent). For Sample 7, the complexity of the pattern prevented a definite identification, although α-Ni_7S_6 appeared to be the major component. Other nickel/sulfur compounds were probable, with NiS, anhydrous $NiSO_4$, and Ni_3S_2 implicated. Sample 8 was definitively identified as Ni_3S_2 by both X-ray diffraction and chemical analysis.

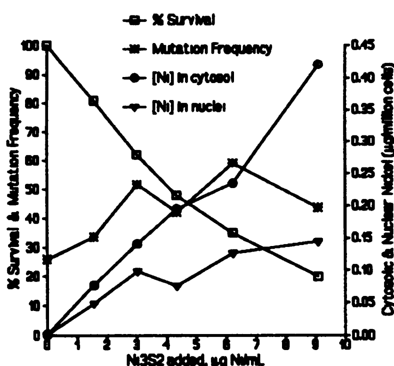

Figure 3. Toxicity, mutagenicity, and uptake of Ni_3S_2 in AS52 cells after 24 hr exposure. The data plotted in this summary figure were derived from those depicted in figure 2H or compiled in Tables 3 and A1.

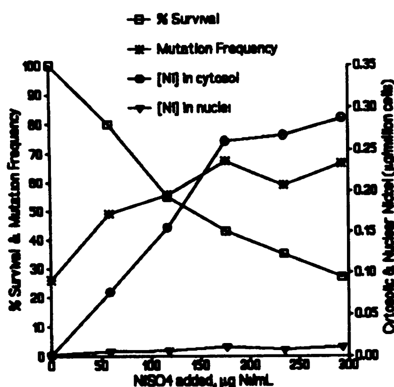

Figure 4. Toxicity, mutagenicity, and uptake of $NiSO_4$ in AS52 cells after 5 hr exposure. The data plotted in this summary figure were derived from those depicted in Figure 2J or compiled in Tables 3 and A1.

Toxicity of Nickel Compounds

Dose-response curves showing the percent survival of the exposed cells (with respect to untreated control cells) versus the amount of nickel added to the culture medium are given in Figure 2 (2A to 2L) for the 11 compounds tested. The average percent survival and standard deviation depicted were calculated from the results of at least 3 replicate experiments. LC_{50} values (concentration which kills 50% of the exposed cells) were obtained from the respective graph for each compound tested and are given in Table 2 along with cellular nickel levels. To facilitate comparison of the various compounds, concentrations are expressed in units of µg Ni/ml.

Nickel Uptake

Appendix Table A1 summarizes the nonparticulate ("dissolved") nickel contents of the cell cytosol (average of 4 experiments) and of the cell nuclei (average of 2 experiments). Exposure doses are the same as those used in the mutation experiments. For most of the compounds, a significant ($p<0.05$) relationship exists between administered dose and observed intracellular nickel concentrations.

Mutagenicity Testing

Representative mutation frequencies for 6-thioguanine (6-TG) resistance induced by the various nickel compounds are summarized in Table 3. The mutation frequency represents the number of mutations observed per million cells plated. At the time of selection, the cells had recovered such that the PE of treated cultures were approximately the same (40 to 50%) as the negative (nonexposed) controls, regardless of the exposure dose of the nickel compound. The plating efficiencies of the EMS-treated cells (17 to 34% depending on dose) were significantly lower than the controls.

The mutation frequency of nonexposed cells observed in separate experiments appears to adhere to a normal statistical distribution. In 4 mutation experiments, 12 independent assessments (69 dishes total) yielded a spontaneous mutation rate of 26 ± 2.5 (mean ± sd; range 0–55). The statistical significance of the increase in mutation frequency after nickel exposure was assessed by comparison to the negative control data (no nickel added). Since the mutation frequency reported for the test compounds is based on the average of 5 to 6 replicate dishes in the same experiment, the standard deviation and the 95% confidence interval for each exposure condition were calculated; and are denoted as upper and lower limits in Table 3. A comparison of the mean mutation frequency with that of nonexposed (negative) control cultures using the one-tailed Student's *t*-test was employed to determine the significance of the nickel-induced mutation rates. For each compound, the results were also tested for the significance of the correlation between the administered nickel dose and the induced mutation rate. For this comparison, the correlation coefficient (r) was calculated and the significance determined using standard two-tailed statistical tables.

In Figures 3 and 4, the mutagenicity and nickel uptake data are plotted in conjunction with the cytoxicity results for Ni_3S_2 and $NiSO_4$. As is evident from the data in Table 3, the increase in mutation frequency with intracellular nickel uptake is significant ($p<0.05$) only for $Ni(OH)_2$, "amorphous NiS", Ni_3S_2, and $NiSO_4$.

Mutant Characterization

Sequencing and restriction analysis of the two PCR products observed revealed that the smaller (lower relative molecular mass) band corresponds to the functional *gpt* locus, while the larger band is functionally inactive [designated as a pseudogene, (*19*)]. Northern blot analysis of mRNA and reverse transcriptase PCR (RT-PCR) analysis of both mRNA and total RNA confirms this (data not shown). Analysis of the amplification products on an agarose gel (1%) revealed 3 distinct alterations at the *gpt*/pseudogene loci: both PCR DNA bands remain intact; the smaller DNA band (corresponding to the functional *gpt* gene) is absent; or both bands are absent. Repetition of the PCR work has confirmed

the qualitative characterization of the observed mutants reported in Figure 5. In addition, re-evaluation of the mutants with a second primer set encompassing the *gpt* gene and larger stretches of DNA both upstream and downstream of the gene has also corroborated the depicted mutation profiles. Details are given in Rossetto et al. (*19*).

Discussion and Conclusions

Major Findings

X-ray diffraction characterization of "amorphous NiS" synthesized from $NiCl_2$ and $(NH_4)_2S$ in the presence of air illustrates the difficulty of preparing a pure product without impurities such as NiS_2 and $NiSO_4$, at least under the conditions employed. The synthesis procedure used differed from that of Abbracchio et al. (*10*) only in our use of an acetate buffered medium. Both the cytotoxicity (LD_{50} = 4.1 μg Ni/ml) and mutagenicity of "amorphous NiS" suggest that crystalline NiS_2 is a sulfide of high cytotoxic and genotoxic potency. Carcinogenicity studies of NiS_2 in rats support this conclusion (*3*). In view of the X-ray diffraction evidence for the presence of NiS_2, the alternate explanation that the unexpected activity of amorphous nickel sulfide was due to negative surface charges, which is known to make it biologically active (*22*), is less likely.

Contributions to the measured nickel levels from particulate dissolution during the cellular fractionation process is judged to be small. Simulation experiments incorporating the protocol outlined in Figure 1 (i.e., same buffers, incubation periods, and temperatures) indicated that little of the particulates dissolved (0.01% for green NiO and 0.9% for Ni_3S_2), in agreement with the known serum dissolution half-times (T_{50}) of >11 years and 28 days, respectively (Table 2). In contrast to the other particulate nickel compounds studied, there was no visible evidence of residual particulate material during the manipulations summarized in Figure 1 for $Ni(OH)_2$ (T_{50}<1 day, Table 2) and $NiCO_3$, both of which are reported to be slightly soluble in water, rather than insoluble (*23*).

As illustrated by the toxicity curves of Figure 2A to 2L and the LC_{50} data in Table 2, there are significant differences in the toxicity of the various nickel compounds tested. For particulate compounds, the range of LC_{50} values is from 2 μg Ni/ml for $Ni(OH)_2$ to 130 μg Ni/ml for the green NiO. For the water-soluble salts, the LC_{50} increased from 45 to 60 μg Ni/ml for 24-hr exposures to 120 to 125 μg Ni/ml for 5-hr exposure. The ($Ni(OH)_2$, carbonate, and sulfides show similar toxicities, while the nickel oxides are shown to be less toxic, with the potency depending on the compound tested (green, black, or containing Ni(III)/Li). The cytosolic and nuclear nickel contents of the compounds at a given cytotoxicity level show considerably less variation than the administered dose. Thus at LC_{50}, the cytosolic nickel levels observed were in the range of 35 to 250 ng/10^6 cells ($NiCO_3$ exempted), while levels of 6 to 80 ng/10^6 cells were observed in the nuclear fraction (Tables 2,A1). The particulate compounds with the lowest toxicity (green NiO and NiO with Ni(III)/Li), and therefore necessitating the highest extracellular nickel concentrations to give measurable cell toxicity, show the lowest intracellular nickel levels. Because considerable cytotoxicity occurred for these 2 compounds, an influence other than dissolved nickel in cells appears to contribute to the loss of cell viability.

The compounds exhibiting the strongest mutagenic responses—$Ni(OH)_2$ "amorphous NiS," and Ni_3S_2, have the highest nuclear nickel levels and the lowest administered doses. Although nickel compounds appear to be only weak mutagens, the present results do indicate a significant increase ($p<0.05$) in mutation rate for the nickel compounds, although not consistently at all doses tested. The Ni(OH)2, Ni3S2, and "amorphous NiS," which not only exhibited evidence for a significant dose–response ($p<0.01$ or $p<0.05$) but also increased mutation frequencies relative to control cultures ($p<0.025$), might be termed mutagenic by generally accepted criteria requiring both observations to occur. The water soluble nickel salts at the majority of the administered doses produced significant increases ($p<0.05$) in mutation rate and raised cytosolic nickel levels, but had relatively low nickel concentrations in the nuclear compartment. [Relatively low nuclear concentrations of nickel have also been observed by T. Malinski (Oakland University, Rochester, MI) when cells were exposed to nickel salts (personal communication)]. A number of interpretations of these observations are possible.

From the PCR characterization, three qualitative classes of mutations were observed (Figure 5). Sequence analysis of a number of EMS mutants has shown that point mutations are members of this first family (both PCR gpt bands present). In addition, small deletions of several base pairs or frameshift mutations theoretically would also be scored in this first class. Deletions of all or part of the *gpt* locus are assigned to the second empirical type (small band absent), while multilocus deletions or damage are believed to correspond to the third category of mutants (both bands absent). Although it can be postulated that small mutations (i.e., point, frameshift, or deletion) in critical regions of primer annealing may result in similar profiles, the corroboration of mutation profiles utilizing a second distinct set of PCR primers argues against this conclusion. (Note that in this discussion gene deletion denotes an operational definition, i.e., the inability of a DNA segment to be amplified by PCR, and does not necessarily imply the total physical absence of a gene.) The profile of damage inflicted was found to depend on the nature of the nickel compound. $Ni(OH)_2$ and $NiSO_4$ induced substantial increases in deletions in all or part of the *gpt* gene compared to EMS and spontaneous mutants. Thus, although the mutagenicity testing showed the nickel compounds to be weakly mutagenic, they were able to induce mutations with molecular lesion profiles distinct from spontaneous changes. Perhaps, as in wild-type CHO cells, cytotoxicity associated with the clastogenic properties of nickel compounds

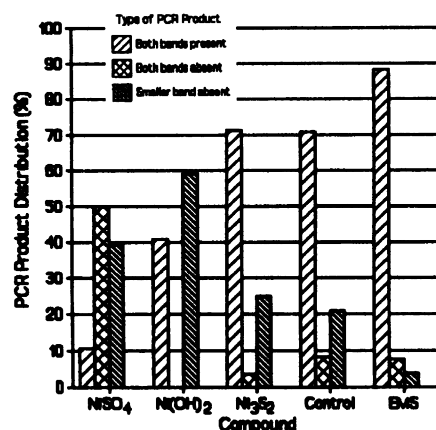

Figure 5. PCR analysis of AS52 *gpt* mutants: distribution of PCR products determined by electrophoresis. Designation of mutant types was based on an examination of PCR amplification products on standard 1% agarose gels. Three types of gel patterns were observed as indicated in the key shown above: (a), both PCR bands present (point mutation, small deletion, or frameshift) or (b), the smaller band not observed (active gene absent) or (c), both PCR bands not observed (active gene and pseudogene absent). Control mutants denote those arising spontaneously in the absence of any test chemical.

circumvents the observation of strongly positive mutagenic responses in AS52 cells.

Interpretations

An examination of the nickel uptake and cytotoxicity data suggests that, as a first approximation, the "Nickel-Ion Hypothesis" holds. While administered LC_{50} doses differed by a factor of 75 for the 11 nickel compounds studied, the corresponding intracellular (cytosolic and nuclear), nonparticulate nickel concentrations varied only by about a factor of 10. (The cytosolic nickel levels for $NiCO_3$ are excluded in this analysis; below.) The observed cytosolic nickel levels did not correlate with the LC_{50} values, while the nuclear concentrations did ($p<0.01$) (Tables 2,A1).

By comparing the administered doses with the measured nickel levels in the cytosol and nucleus, one can see that the $Ni(OH)_2$, $NiCO_3$, "amorphous NiS" and Ni_3S_2 were the most efficient in delivering nickel to cells (measured as nonparticulate or "dissolved" form) and were also the most toxic. This observation is consistent with the Nickel-Ion Hypothesis. The fact that all the nickel compounds produce mutagenic responses ($p<0.05$) supports the notion of the Nickel-Ion Hypothesis that all nickel compounds are potentially genotoxic if they release nickel(II) ions.

It is reasonable to suggest that the measured, nonparticulate (dissolved) cytosolic nickel represents different compartments for the uptake from salt solutions than for the incorporation of particulate nickel compounds. It is known that phagocytized particulates of Ni_3S_2 are often present in cytoplasmic vacuoles (*5,24*). It is hypothesized that within vacuoles particulates undergo dissolution and that nickel(II) ions can be released from them to the cytosol (*12,25*). Presumably, the lysing step employed in releasing cytosolic nickel in the present study (see Figure 1) makes available for measurement nickel(II) ions present in both vacuoles and the cytosol proper. On the other hand, nickel(II) ions taken up from salt solutions [perhaps via calcium channels, (*26*)] may be expected to occur only in the cytosol proper, likely bound to proteins and low-molecular-mass ligands. In this case, only this pool is suspected of being released in the cell-membrane lysing step.

It is obvious from the present study that factors other than intracellular nickel concentration contribute to the cytotoxicity. Nickel compounds are known to exhibit differential surface activities which, for example, result in quite different abilities to induce haemolysis in human erythrocytes (*27*). Consequently, physicochemical surface or cell interactions probably contribute to cell injury. Such an effect may be especially important when relatively large doses are required, such as for the green and Ni(III)/Li nickel oxides.

Nickel-induced mutagenicity also seems to have a number of determinants. This can be seen by comparing the mutagenicity and the cytosolic/nuclear nickel levels for the particulate compounds with the water-soluble nickel salts (Tables 3,A1). Consequently, low nickel levels relative to those found in the cytosol fraction are observed in the nuclei of cells exposed to the chloride, sulfate, or acetate salts (and perhaps also to the carbonate, which appears to be the most soluble of the particulate compounds). In contrast, the cytosolic concentrations are substantially elevated for these compounds. A number of implications and interpretations flow from this observation.

The simplest interpretation is that intracellular compartmentalization of nickel results in different mechanisms of genotoxicity. It is tempting to assign the mutagenicity of nickel salts to high levels of nickel in the cytoplasm, which, through indirect effects damage the genome. One such indirect mechanism might be related to the demonstrated ability of certain nickel(II) complexes of natural ligands to participate in active oxygen biochemistry (*12,28–31*). Such processes generate radical species that are known to damage DNA. By contrast, high concentrations of nickel(II) ions in the nucleus are capable, on binding to DNA and nuclear proteins, of damaging DNA [e.g., depurination, (*26*)], or inducing changes in chromosomal conformation that may result in permanent changes in gene expression (*12,29,32,33*). These interpretations receive strong support from the work of Sen and Costa (*25*), who demonstrated that the pathway of delivery of nickel to CHO cells determined the type of interaction with chromosomes. Thus nickel(II) salts, like crystalline NiS, induced chromosomal aberrations (gaps, breaks, and exchanges), but only the particulate compounds produced X-chromosome fragmentation. However, when nickel(II) was delivered to the cell in liposomes (as $NiCl_2$ or the nickel(II) complex of bovine serum albumin), X-chromosome fragmentation was also observed. Our work corroborates these observations by Sen and Costa (*25*). The measured nonparticulate nickel concentrations support the notion that differential intracellular compartmentalization of nickel determines such differences in genotoxic responses.

The response to $NiCO_3$ warrants additional comment. Crystallinity has generally been accepted as a prerequisite for the uptake of nickel compounds by phagocytosis. Although poorly crystalline by X-ray diffraction analysis, the $NiCO_3$ used in our cytotoxicity and mutagenicity studies was considerably more efficient than the water-soluble salts in delivering nickel to the cells. Of course, microcrystallinity cannot be ruled out. Like the salts, the nuclear fraction of nickel in this case was also found to be small compared to the cytosolic levels observed, which is consistent with its solubility. This suggests that the $NiCO_3$ induced mutagenicity, at least in part, in a fashion similar to the salts. Perhaps on uptake, the degree of vacuolation is less, or the extent of dissolution in vacuoles greater, than for the other solids. Nonvacuolized Ni_3S_2 has been reported in CHO cells (*5,24*).

Conclusions

In conclusion, our work has demonstrated that nonparticulate, intracellular nickel(II) concentrations, irrespective of the parent compound, constitute an important determinant in the cytotoxicity and mutagenicity responses by AS52 CHO cells to nickel compounds. This finding is consistent with the Nickel-Ion Hypothesis. However, this hypothesis must be modified to allow for differential compartmentalization of nonparticulate dissolved nickel within the cell that is dependent on the nickel-uptake pathway, as well as mechanical cell surface effects in case of relatively nontoxic compounds. The uptake of particulates by phagocytosis appears to elevate the measured, nonparticulate nickel levels in both the cytosol and the nucleus, while nickel from salts accumulates preferentially in the cytosol. These results suggest that cytosolic and nuclear nickel accumulations (in nonparticulate form) appear to produce mutations by different mechanisms. The mutant profile analysis suggests that induction of mutations at levels only slightly above background may indeed be indicative of the mutagenic potential of nickel compounds. It appears that there are methodologic problems involved in testing "weak" mutagens and that alternative approaches are necessary in classifying the mutagenicity of nickel and other compounds. The PCR analysis reported illustrates the potential of employing molecular biology techniques in a complementary manner.

APPENDIX

Table A1. Nickel content in the cytosol and nuclei of AS52 cells.[a]

	Amount added[b] µg/ml		Cytosol µg Ni/10^6 cells			Nuclei µg Ni/10^6 cells			Correaltion coefficient, (*r*)	
Compound	Compound	Ni	Avg	Std	Range	Avg	Std	Range	Cytosol	Nuclei
$Ni(OH)_2$	1.9	1.1	0.088	0.035	0.059 – 0.135	0.113	0.065	0.067 – 0.159	0.999[c]	0.891[d]
	3.1	1.7	0.136	0.043	0.099 – 0.181	0.085	0.065	0.039 – 0.131		
	4.3	2.4	0.202	0.059	0.149 – 0.280	0.084	0.028	0.064 – 0.104		
	6.2	3.4	0.274	0.079	0.191 – 0.355	0.133	0.060	0.091 – 0.175		
	9.9	5.5	0.421	0.105	0.361 – 0.578	0.292	0.11	0.208 – 0.375		
$NiCO_3$	6.4	2.5	0.260	0.198	0.091 – 0.478	0.022	0.000	0.022 – 0.022	0.966[c]	0.916[d]
	11.2	4.4	0.536	0.163	0.402 – 0.718	0.051	0.001	0.050 – 0.051		
	16.0	6.2	0.854	0.281	0.554 – 1.112	0.067	0.026	0.049 – 0.086		
	21.1	8.2	1.014	0.371	0.585 – 1.230	0.104	0.018	0.092 – 0.117		
	28.8	11.2	1.161	0.011	1.153 – 1.170	0.097	0.044	0.066 – 0.128		
$NiCO_3$ crystalline	2.5	1.0	0.077	0.022	0.061 – 0.093	0.020	0.006	0.015 – 0.024	0.988[c]	0.882[d]
	5.0	1.9	0.146	0.006	0.142 – 0.150	0.026	0.001	0.025 – 0.027		
	7.5	2.9	0.204	0.007	0.198 – 0.209	0.045	0.038	0.018 – 0.071		
	10.0	3.8	0.246	0.042	0.217 – 0.276	0.064	0.047	0.031 – 0.097		
	15.0	5.7	0.444	0.045	0.412 – 0.475	0.059	0.045	0.027 – 0.090		
	20.0	7.6	0.444	0.148	0.340 – 0.549	0.099	0.042	0.069 – 0.129		
	30.0	11.4	0.764	0.146	0.661 – 0.867	0.285	0.222	0.128 – 0.442		
	40.0	15.2	0.640	0.299	0.428 – 0.852	0.262	0.253	0.082 – 0.441		
NiO, black	12.2	9.5	0.076	0.038	0.044 – 0.131	0.039	0.015	0.028 – 0.050	0.970[c]	0.858
	18.0	14.0	0.098	0.034	0.064 – 0.132	0.042	0.014	0.032 – 0.052		
	23.3	18.1	0.094	0.020	0.072 – 0.120	0.046	0.003	0.044 – 0.049		
	28.5	22.2	0.129	0.038	0.099 – 0.181	0.041	0.034	0.017 – 0.065		
	40.9	31.8	0.181	0.054	0.121 – 0.251	0.057	0.039	0.029 – 0.085		
NiO, green	109	85	0.029	0.012	0.018 – 0.047	0.007	0.004	0.004 – 0.010	0.973[c]	0.939[d]
	183	143	0.035	0.009	0.027 – 0.043	0.005	0.000	0.005 – 0.006		
	229	180	0.062	0.029	0.028 – 0.098	0.017	0.006	0.013 – 0.022		
	358	281	0.094	0.071	0.046 – 0.175	0.045				
$Li_2Ni_8O_{10}$	52	38	0.024	0.014	0.015 – 0.040	0.009	0.006	0.005 – 0.013	0.943[d]	0.951[d]
	83	60	0.060	0.028	0.040 – 0.080	0.010				
	104	76	0.061	0.022	0.037 – 0.080	0.017	0.009	0.011 – 0.023		
	139	100	0.062	0.010	0.055 – 0.069	0.027				
	201	145	0.116	0.049	0.082 – 0.151	0.031				
NiS amorphous	4.4	1.8	0.108	0.044	0.056 – 0.162	0.072	0.003	0.069 – 0.074	0.983[c]	0.539
	6.9	2.8	0.145	0.089	0.075 – 0.263	0.121	0.077	0.066 – 0.175		
	9.5	3.9	0.267	0.091	0.182 – 0.346	0.070	0.012	0.061 – 0.079		
	12.7	5.2	0.290	0.106	0.178 – 0.428	0.086	0.008	0.080 – 0.092		
	17.4	7.1	0.420	0.136	0.260 – 0.569	0.133	0.025	0.115 – 0.150		
Ni_7S_6	4.7	3.2	0.043	0.016	0.026 – 0.061	0.027	0.009	0.021 – 0.033	0.994[c]	0.336
	8.3	5.7	0.068	0.024	0.046 – 0.099	0.040	0.001	0.039 – 0.041		
	12.7	8.6	0.082	0.015	0.072 – 0.093	0.072	0.056	0.032 – 0.111		
	15.5	10.5	0.099	0.008	0.093 – 0.105	0.045				
	28.4	19.3	0.189	0.001	0.188 – 0.189	0.016				
Ni_3S_2	2.1	1.6	0.076	0.023	0.058 – 0.108	0.048	0.004	0.046 – 0.051	0.985[c]	0.912[d]
	4.1	3.0	0.141	0.044	0.104 – 0.200	0.098	0.059	0.057 – 0.139		
	5.9	4.3	0.195	0.032	0.165 – 0.234	0.076	0.013	0.067 – 0.085		
	8.5	6.2	0.235	0.159	0.093 – 0.406	0.127	0.045	0.095 – 0.159		
	12.4	9.1	0.421	0.194	0.216 – 0.638	0.145	0.047	0.111 – 0.178		
$NiCl_2$ 5 hr	1.0	59	0.079	0.014	0.060 – 0.091	0.003	0.002	0.002 – 0.005	0.970[c]	0.930[d]
	2.0	117	0.182	0.064	0.123 – 0.270	0.006	0.002	0.005 – 0.008		
	3.0	176	0.250	0.115	0.127 – 0.404	0.005	0.001	0.005 – 0.005		
	4.0	235	0.269	0.101	0.169 – 0.400	0.013	0.005	0.009 – 0.016		
	5.0	293	0.324	0.099	0.211 – 0.438	0.016	0.003	0.014 – 0.018		
$NiSO^4$ 5 hr	1.0	59	0.077	0.025	0.060 – 0.114	0.006	0.004	0.003 – 0.009	0.935[d]	0.723
	2.0	117	0.155	0.080	0.093 – 0.272	0.007	0.001	0.006 – 0.007		
	3.0	176	0.260	0.119	0.169 – 0.435	0.012	0.008	0.006 – 0.017		
	4.0	235	0.267	0.103	0.186 – 0.407	0.008	0.000	0.008 – 0.008		
	5.0	293	0.288	0.061	0.235 – 0.374	0.011	0.003	0.009 – 0.013		
$Ni(CH_3COO)_2$ 5 hr	1.0	59	0.090	0.022	0.068 – 0.110	0.005	0.001	0.005 – 0.006	0.997[c]	0.991[c]
	2.0	117	0.158	0.083	0.064 – 0.266	0.008	0.003	0.006 – 0.010[c]		
	3.0	176	0.253	0.104	0.154 – 0.399	0.011	0.004	0.008 – 0.014		
	4.0	235	0.312	0.158	0.138 – 0.515	0.016	0.009	0.010 – 0.022		
	5.0	293	0.374	0.147	0.182 – 0.495	0.020	0.007	0.015 – 0.025		

(*continued*)

Table A1. (*continued*). Nickel content in the cytosol and nuclei of AS52 cells.[a]

Compound	Amount added[b] μg/ml Compound	Ni	Cytosol μg Ni/10^6 cells Avg	Std	Range	Nuclei μg Ni/10^6 cells Avg	Std	Range	Correaltion coefficient, (*r*) Cytosol	Nuclei
$NiCl_2$	0.25	14.7	0.053						0.961[c]	
24 hr	0.50	29.4	0.077							
	0.75	44.0	0.180							
	1.00	58.7	0.184							
	1.25	73.4	0.231							
$NiSO_4$	0.25	14.7	0.048							
0.958[d]	0.50	29.4	0.095							
24 hr	0.75	44.0	0.154							
	1.00	58.7	0.146							
	1.25	73.4	0.238							
$Ni(CH_3COO)_2$	0.25	14.7	0.048							
0.798	0.50	29.4	0.160							
24 hr	0.75	44.0	0.111							
	1.00	58.7	0.287							
	1.25	73.4	0.219							
Negative controls (no addition)	0		0.002	0.001	0.001 – 0.003	0.002	0.001	0.001 – 0.003		

[a]AS52 cells were seeded in F12/10% FBS medium at ≈ 0.5–1.0*10^6 cells per 100-mm diameter dish 1 to 2 days before treatment. The cells were exposed to the range of nickel compounds in serum-free medium at the concentrations indicated for 24 hr (or 5-hr exposure for nickel salts as noted). The cells were rinsed to remove extracellular nickel and then trypsinized, pelleted, rinsed, and counted. The cells were lysed in hypotonic buffer + Nonidet P-40, and centrifuged to pellet the nuclei and particulates. The supernatant (referred to as the cytosol) was kept for analysis. The pellet was digested with DNAse I followed by Proteinase K/SDS and the particulates removed by centrifugation. The supernatant consisting of the nuclear nickel was analyzed for nickel content. Averages and standard deviations (std) were obtained based on four replicate experiments for the cytosolic nickel and two experiments for the nuclear nickel content.. [b]Test doses were selected to correspond to formal survival rates of 80%, 65%, 50%, 35%, and 20%. [c]Significant at the .01 probability level [d]Significant at the .05 probability level.

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