THE NICKEL-ION HYPOTHESIS OF CYTOTOXIC RESPONSES

EVALUATION OF THE "NICKEL-ION HYPOTHESIS" OF CYTOTOXIC RESPONSES IN AS52 CHO CELLS

by

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ABSTRACT

Eleven nickel compounds representing a range of solubilities and biological activities were tested for toxicity, mutagenicity, and cytosolic and nuclear nickel uptake in AS52 cells. LC_{50} values ranging from 2-130 µg Ni/mL for particulates and 120-150 µg Ni/mL for the water-soluble salts ($NiCl_2$, $NiSO_4$, $Ni(CH_3COO)_2$) were determined. The $Ni(OH)_2$, $NiCO_3$, and nickel sulphides (Ni_3S_2, Ni_7S_6, amorphous NiS) exhibited similar toxicities (LC_{50}'s of 2.0, 5.8, 4.1, 8.2, 4.1 µg Ni/mL respectively), while the nickel oxides were less toxic and showed large variations between the black, $Li_2Ni_8O_{10}$, and green NiO forms (LC_{50}'s of 18.1, 75, 130 µg Ni/mL). Concentrations reducing survival to the range 20-80% were tested for mutagenicity and degree of nickel uptake. Although nickel compounds have been reported to be only weak or equivocal mutagens, the results indicate a low but significant increase in mutation rate at the *gpt* locus induced by all the nickel compounds tested.

The majority of compounds displayed nuclear to cytoplasmic nickel ratios of ≈ 1:4 to 1:2, though this was ≈ 1:20 for nickel salts. $NiCO_3$ appeared to be intermediate in behaviour with a ratio of ≈ 1:12. Comparison of the eleven compounds at the same toxicity level (LC_{50}) showed a 75-fold difference in exposure levels but about a 10-fold difference in cytoplasmic and nuclear nickel levels. There appears to be a very good correspondence between previously reported dissolution half times (T_{50}'s) of the compounds tested and the cytosolic nickel levels at a given

toxicity level. For the water-soluble salts, previous reports have shown that cellular distribution varies from that of particulates due to differences in the manner of uptake. The present work confirms this and suggests that the compounds can be divided into three classes: water-soluble salts producing very low nuclear levels and high cytosolic levels, inert nickel oxides (green NiO and lithium nickel oxide) with relatively low nuclear and cytosolic nickel levels, and the remaining compounds (the major class) with relatively high cytosolic levels and nuclear nickel levels. Overall, the data supports the Nickel-Ion Hypothesis which suggests that the Ni^{2+} ion is the active agent in nickel toxicity and mutagenicity, and that, as a first approximation, its intracellular concentration is responsible for the observed effects, irrespective of the nickel compound.

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I. INTRODUCTION

Nickel, widely distributed in nature mainly as oxide and sulphide ores, composes 0.008% of the earth's crust and 8.5% of the earth's core (Mastromatteo, 1986, 1988). Since the isolation of impure nickel from niccolite in 1751, and its purification and investigation in 1804, extensive use has been made of this metal. Up to 50% of the world's nickel production (excluding the USSR) is obtained from nickel/iron sulphide ore deposits (pentlandite and pyrrhotite) in the Sudbury region of Ontario (Weast, 1983-84). The majority of nickel is used in the production of stainless steels and other corrosion resistant alloys and as pure nickel or protective coatings applied by electrodeposition from salt solutions (nickel sulphate, nickel chloride, nickel sulfamate). Nickel is also used as a catalyst in the hydrogenation of vegetable oils and other reactions, in production of alkaline (nickel/cadmium) batteries, and in pigmentation for enamels, ceramics, and glass.

A. Environmental and Non-Occupational Exposure to Nickel

Atmospheric nickel levels in the United States have been reported to average 6 ng Ni/m^3 in rural areas and 20 ng Ni/m^3 in general, with considerably higher levels around locations where nickel is mined, smelted, refined, or alloys are produced (Mastromatteo, 1986). Léonard et al (1981) reported air concentrations of 70 and 80 ng/m^3 for rural and

urban areas, with levels to 200 ng/m^3 near nickel emitting sources. Most of the atmospheric nickel comes from the combustion of fossil fuels (Nieboer et al., 1988a; Costa, 1980b), and is generally in the form of oxides and nickel sulphate. Assuming an inhalation rate of 20 m^3/day, total lung exposure would be 0.12-1.6 µg Ni/day in a non-industrial area.

Sea water contains .1-.5 µg Ni/L (Mastromatteo, 1986), while public water supplies have been reported to range from 0.3 µg/L in Finland to 4.8 µg/L in the United States (Léonard et al., 1981). Calabrese et al (1985), describing a study of United States waters, reported an average of 19 µg Ni/L, with a low of 5 µg/L in the Missouri River and Western Gulf area and a high of 130 µg/L in the Cuyahoga River in Ohio. Léonard reported that surface water nickel levels can reach 960 µg/L and as much as 400 mg/L in waste water. In general though, the majority of nickel uptake comes from food. An average dietary nickel content has been reported as 160-250 µg/day, though earlier reports stated levels of 300-600 µg/day were consumed (Nieboer et al., 1988b). Some foods such as soya products, grains, cocoa, and dried legumes have a relatively high nickel content (2-10 µg/g), so that for certain diets intakes of 900 µg Ni/day may be reached. Since only a small portion of ingested nickel (≈ 5%) is absorbed, these levels are generally considered to be safe. Though no specific function for nickel in humans and no deficiency disorder have been identified, it appears that nickel may have a role as an essential trace element. Nickel-containing enzymes in plants and bacteria have been isolated, and dietary-induced nickel deficiency symptoms in several animal species including goats, pigs, chickens, and rats have been observed

(Coogan et al., 1989). The fact that nickel has been found in low concentrations in all human tissues and fluids, including fetal tissue, and the generally narrow ranges, renal reabsorption, and relatively rapid excretion of water soluble nickel would suggest some form of nickel regulation exists (Nieboer et al., 1988b).

The major problem with exposure to nickel in the general population is allergic reactions (immune responses), which result in contact dermatitis (Menné & Nieboer, 1989). Nickel is one of the most common skin allergens, with sensitivity in ≈ 5-10% of the population. A 10-fold higher incidence in women than in men is thought to be due to the greater likelihood of exposure to nickel-containing objects such as jewellery, cutlery, cosmetics, detergents, and clothing fasteners (Nieboer et al., 1988a). High environmental exposure or ingestion of nickel in sensitive patients has been found to exacerbate the dermatitis, while lowering dietary intake and chelation therapy have had some success in treatment of this condition. Nickel containing medical implants and dental fillings have also been found to have potentially adverse effects on health. Common #316 stainless steel, for example, contains about 8% nickel and 18% chromium, and is often used in implants such as heart valves and orthopaedic implants (Costa, 1980b). Development of sarcomas following stainless steel implants and hemangioendothelioma in the tibia of a patient receiving a steel plate implant have been documented. Subdermal implants of nickel-gallium dental filling was also found to cause sarcomas in 9 of 10 rats studied.

B. Occupational Nickel Exposure

In comparison to the non-occupational exposure levels of nickel (20 ng/m^3 in air), workers in nickel-related industry may face extremely elevated nickel concentrations (Mastromatteo, 1986, 1988). Airborne exposures of 0.025 - 0.050 mg Ni/m^3 have been reported for nickel miners and grinders. Exposure levels of 0.05 - 1.0 mg/m^3 in smelting, < 1.0 mg/m^3 in refinery work and packaging of nickel powders, 0.004 - 0.01 mg/m^3 in electroplating, and 0.01 - 0.3 mg/m^3 in production of nickel alloys, welding stainless steel, and in nickel foundries have been reported. Earlier operations involving calcining and sintering of nickel sulphides to produce nickel oxide created large quantities of dust and nickel exposure levels of 25-30 mg/m^3 (1 million times normal atmospheric levels). These operations were improved or eliminated from the nickel refineries after several studies showed workers were at a much higher risk of developing cancer of the lung and nasal sinus.

The first links between nickel exposure and respiratory tract cancers were observed by Grenfell in 1932 at a nickel refinery in Clydach, Wales (Nelson, 1985; Mastromatteo, 1986). The first epidemiological study was carried out at this plant by Hill in 1939. He found a relative risk of 22 for nasal cancer and 16 for lung cancer among the refinery workers. Extensive studies of the workers have been undertaken since that time (eg., Doll et al., 1977; Kaldor et al., 1986; see EPA, 1986 for compilation of epidemiological studies). Follow up on 968 workers employed on at least two occasions five or more years apart before 1945 showed the highest excess risk in workers first employed before 1925,

though some increased risk remained in workers first employed as late as about 1930. It was found that the decrease in risk corresponded to improvements in the refining process. The mortality of workers due to lung and nasal sinus cancers is shown in the table below (from Kaldor et al., 1986).

Period First Employed	# of Men	Lung Cancer		Nasal Sinus Cancer	
		Observed	Expected	Observed	Expected
Before 1925	679	137	26.9	56	0.21
1925-1929	97	11	5.5	0	0.03
1930-1944	192	11	9.1	0	0.05

Risks similar to those described in South Wales have also been reported in mining and refinery workers in Canada and Norway, with increases in larynx cancer also noted (Magnus et al., 1982; Shannon et al., 1984; Nieboer et al., 1988a). Increased incidence of respiratory tract cancers have also been observed in nickel workers in the German Democratic Republic, Japan, and the USSR (Doll et al., 1977; EPA, 1986). Examination of lung specimens obtained during autopsies from subjects originating in an area of particularly high atmospheric nickel levels has revealed a significant increase in the concentration of nickel (and chromium) in lung tissue with increasing age (Kollmeier et al., 1985, 1987). Similar study of lung specimens from 39 autopsied nickel refinery workers showed enhanced nickel levels in the exposed individuals (Andersen & Svenes, 1989). Tissues from the 15 workers in the Roasting and Smelting Department (mainly nickel-copper oxides, Ni_3S_2, and metallic dust exposure) contained an average nickel level (± standard deviation) of 330 ± 380 µg/g dry weight, while tissues from 24 Electrolysis Department workers (mainly water soluble $NiSO_4$ and $NiCl_2$ exposure) contained 34 ± 48 µg Ni/g tissue.

These levels were statistically elevated ($p \leq 0.01$) compared to individuals not connected with the refinery (0.76 ± 0.39 µg/g). It was concluded that exposure in electrolysis and in roasting/smelting constitute distinct groups with respect to accumulation of nickel in the lungs. No difference in nickel levels between workers who died from cancer and other nickel workers was noted.

As confirmed by the analytical measurements just presented, inhaled nickel particles may continue to accumulate in the lungs throughout life. Clearance of relatively inert nickel particulates from the respiratory tract is very slow, with particulates in the nasal mucosa of nickel-smelting operators estimated to have a half-life of 3.5 y (Nieboer et al., 1988b). Particle size is known to determine the site of deposition within the respiratory system and therefore the biological effect. Larger particles ($\geq$ 10 µm diameter) are predominantly deposited in the nasopharyngeal and tracheobronchial regions (Nieboer & Sanford, 1985). The greatest deposition occurs in the alveolar regions (lower lung) when particles are 2-3 µm in diameter. The upper regions of the respiratory tract are cleared by cilia covering the epithelial cells and the movement of mucous. Macrophages are also involved in clearing particles from the alveoli. As lungs become overloaded with dusts/particulates, as typified by a progressive reduction in particle clearance from the deep lung, it appears there may be a breakdown or cessation of alveolar macrophage mobility and dust removal (Morrow, 1988). At this point, lung dust burdens increase at a rate approximating the rate of deposition. In contrast to the long clearance time of nickel particulates, solutions of

nickel salts inhaled as aerosols have been found to be rapidly cleared, with a half-life of 1-1½ d.

In addition to the well substantiated risk of respiratory cancers, other reported or potential occupational effects of nickel inhalation exposure include chronic irritation of the upper respiratory tract (manifested by rhinitis, sinusitis, perforation of the nasal septum, loss of smell), pulmonary irritation and fibrosis, pneumoconiosis, increased susceptibility to respiratory infections, and bronchial asthma (Mastromatteo, 1986). There is also some evidence, though inconsistent, of increased risk of stomach and intestinal cancer in nickel electroplaters. Accidental ingestion of large amounts of nickel salts (0.5 - 2.5 g of nickel as nickel chloride and nickel sulphate) by 32 workers was reported to cause symptoms such as nausea, vomiting, abdominal discomfort, diarrhea, giddiness, lassitude, headache, cough, and shortness of breath generally lasting a few hours but also up to 1-2 d in 7 workers (Sunderman et al., 1988). No long term effects were noted. A 2.5 year old girl, who consumed nickel sulphate crystals (2.2-3.3 g of nickel), developed nuchal rigidity, erythema, dilated pupils, tachycardia, pulmonary congestion, acute hemorrhagic gastritis, several cardiac arrests, and death after 8 h. Apart from a few acute incidents, exposure to nickel by ingestion has not been reported to be a health concern. Nickel carbonyl ($Ni(CO)_4$) is a highly toxic and volatile liquid or gas (bp 43°C) used in some refining operations (Mastromatteo, 1986; Nieboer et al., 1988a). Exposure to levels of 30 ppm in air for 30 min may be lethal. Initial symptoms include headache, fatigue, weakness, nausea,

vomiting, and influenza-type symptoms. These symptoms may clear or be followed in 12-36 h by severe pneumonia-like symptoms and possible death due to respiratory failure (severe pneumonitis).

C. Animal Studies of Nickel Carcinogenicity

Injection of nickel compounds has been found to produce tumours, generally at the injection site, in a range of animal species including rats, mice, hamsters, rabbits, and guinea pigs (Costa, 1980b; Sunderman, 1981, 1985). Some nickel compounds have been found to be carcinogenic even in species generally resistant to chemical carcinogens. For example, a single 100 µg intraocular injection of Ni_3S_2 into the Japanese common newt (*Cynops pyrrhogaster*) produced tumours in 7 out of 8 animals, while there is no other known carcinogen for this species (Coogan et al., 1989). The degree of response varies among species, with hamsters being less responsive than the more commonly used rat and mouse species. In a comparison of 18 nickel compounds at equivalent doses (intramuscular injection of male Fischer rats, 14 mg Ni/rat) Sunderman (1984a, 1985) grouped the compounds into 5 categories: Class A compounds (Ni_3S_2, βNiS, Ni_4FeS_4) induced sarcomas at the injection site in 100% of the rats; Class B compounds (NiO, NiS_2, Ni_3Se_2, NiAsS, Ni_5As_2) induced sarcomas in 85-93% of the rats; Class C compounds (Ni dust, NiSb, NiTe, NiSe, $Ni_{11}As_8$) induced sarcomas in 50-65% of the rats; Class D compounds (amorphous NiS, $NiCrO_4$) induced sarcomas in 6-12% of the rats; and Class E compounds (NiAs, $NiTiO_3$, NiFe alloy) along with control vehicle injections induced no

sarcomas. Whereas amorphous NiS had previously been considered non-carcinogenic (eg Sunderman & Maenza, 1976; Costa 1980b), this extensive study shows its ability to induce tumours, though at a much lower incidence than the crystalline NiS or Ni_3S_2. Other studies have found crystalline $Ni(OH)_2$ to cause tumours by im injection (8/40 rats; Kasprzak et al., 1983). $Ni(CO)_3$ has also been reported to be carcinogenic (Léonard et al., 1981). In general, Ni_3S_2 is the most potent compound, producing a high rate of tumours by intramuscular (im), intrarenal (ir), intratesticular, intraocular, and subcutaneous (sc) injection (Sunderman, 1981). Sunderman also reported a dose-response relationship for induction of sarcomas in male Fischer rats by single im injections of Ni_3S_2, covering the range from 24% induction (.63 mg) to 100% induction (20 mg). A study of tumours after im implantation of Ni_3S_2 in Fischer rats traced the earliest changes and sequence of development of tumours through a series of biopsies (Lumb and Sunderman, 1988). By contrast to the insoluble or particulate compounds, soluble nickel salts ($NiCl_2$ and $NiSO_4$) are not considered tumorigenic in animals by ingestion or injection, probably due to their rapid clearance from the body (Sunderman, 1976; Léonard et al., 1981). Daily injections of $NiCl_2$, however, (500 or 750 µmol/kg, sc in male Fisher rats) were found to cause severe lung damage including hyperplasia, cellular atypia, and frequent mitoses (Coogan et al., 1989). These lesions, though the result of subcutaneous exposure, are similar to those produced by direct inhalation. Repeated intraperitoneal (ip) injection (3 times a week for 8 weeks in mice) of nickel acetate, a relatively soluble nickel salt, gave a 3-fold increase in lung tumours

compared to controls (Sunderman, 1981). It appears that nickel may target the lungs following exposure to soluble/slightly soluble nickel compounds by various routes.

Of more immediate relevance to nickel workers may be the direct inhalation and intratracheal administration of nickel compounds. In a study of several dusts, fibres, and metal compounds, Pott et al (1987) found repeated exposure to nickel powder, nickel oxide, or nickel subsulphide by intratracheal instillation (10-20 times, female Wistar rats) produced a 25-30% incidence of lung tumours. Other studies have shown Ni_3S_2, $NiSO_4$, and $Ni(CO)_4$ to be tumorigenic by inhalation exposure in rats and mice (Coogan et al., 1989). Inhalation of Ni_3S_2 by male or female Fisher rats has been shown to cause hyperplasia, metaplasia, adenomas, and adenocarcinomas in both bronchiolar and alveolar regions. A synergistic action between Ni_3S_2 and benzo(a)pyrene has been observed after exposure in rats by intratracheal injection (Nordberg & Pershagen, 1985). A mixture of the two compounds produced an increased incidence of premalignant changes in the lungs compared to rats receiving one of these compounds at a time. Administration of Ni_3S_2 and benzo(a)pyrene by intramuscular injection also showed a synergistic effect in significantly shortening the lag time for induction of sarcomas. Although carcinogenicity has been the focus of this section, several other systemic toxicological effects are produced by nickel exposure. These effects have been reviewed recently (Nieboer et al., 1988a; Coogan et al., 1989) and include nephrotoxicity, embryotoxicity and teratogenicity, hepatotoxicity, cardiovascular toxicity, pulmonary toxicity, and immunological responses.

D. Nickel Induced Transformation in Tissue Culture

The use of experimental animals to evaluate various compounds may give the best indication of their carcinogenic potential but also has a number of limitations. Animal studies are generally long-term, with a single experiment spanning a 2 y period using rats (longer for more complex species) from exposure to evaluation of the tumorigenic response. For validity, several animals need to be tested for each condition (dose). Such long term experiments, sometimes necessitating hundreds of animals, are very expensive to perform. The development of cancer in animals is also a very complex process to study. Therefore tissue culture systems have been developed to study toxicity and carcinogenesis, and provide a simpler, more economical, and more rapid method for screening and investigation.

As cells progress from normal to neoplastic, there are a number of changes in the cell growth parameters. Morphological transformation assays detect changes in the target cell culture induced by exposure to the test substance. These changes include loss of contact inhibition, disordered growth, development of the ability to grow in semi-solid medium, and immortalization (no longer have limited life-span). Cultures in which changes in morphology are measured are termed (morphologically) transformed. The ultimate test of neoplastic transformation is the ability of these cells to form tumours when implanted in a host. The morphologically transformed cells are therefore not necessarily tumorigenic but have undergone some of the changes involved in the process; changes only observed following exposure to carcinogens (Costa,

1979).

The two general classes of transformation assays are those that involve an established cell line and those that use newly isolated primary cultures of embryonic mammalian cells (Heck & Costa, 1982a). Established cell lines have undergone partial transformation as indicated by their immortality. Included in this category are the C3H/10T½ and Balb 3T3 cells in which transformation is detected by the appearance of dense foci on a confluent nontransformed monolayer. The BHK-21 (baby hamster kidney) line detects transformed cells which have developed the ability to grow in semisolid growth medium (agar). Primary Syrian hamster embryo cells (SHE cells) represent the second class, with mutations being detected based solely on subtle differences in colony morphology.

Using SHE cells, DiPaolo & Casto (1979) found Ni_3S_2 and $NiSO_4$, but not amorphous NiS, induced morphological transformation. Costa also reported transformation in SHE cells by Ni_3S_2 and αNiS but not amorphous NiS (Costa, 1979; Costa et al., 1979; Costa, 1980a). Implantation of Ni_3S_2 transformed cells into nude mice resulted in sarcomas in 26 out of 27 mice, but 0 out of 19 control mice injected with non-transformed SHE cells developed tumours. Pretreatment of SHE cells for 24 h with benzopyrene (3 µg/mL), followed by Ni_3S_2 exposure (2 µg/mL), produced 22% transformed colonies, while exposure to only benzopyrene or Ni_3S_2 produced 3.6% and 3.2% transformed colonies respectively (Costa et al., 1980). As in the animal studies discussed previously, benzopyrene and nickel compounds (Ni_3S_2) appear to act synergistically in transformation of SHE cells. A later report by Costa & Heck (1982) indicated that Ni_3S_2 and Ni_3Se_2 exhibit

the strongest transforming ability; crystalline NiS exhibits 85% of this level, $NiCl_2$ 35%, metallic nickel and Ni_2O_3 15%, and NiO and amorphous NiS 7% of the Ni_3S_2 transforming ability. In another study, crystalline NiS and Inco black NiO were found to produce the same rate of morphological transformation, while the green NiO produced ≈ 1/3 this rate (Sunderman et al., 1987). Using the BHK-21 cell line, Ni_3S_2 and a nickel oxide catalyst were found to have the greatest transforming activity, followed by a black NiO, then nickel acetate, then nickel powder (Hansen & Stern, 1984). Choosing doses to give 50% survival, the above compounds produced approximately equal transformation. In cell culture, both crystalline and soluble compounds are found to induce transformation, though soluble compounds must be present at much higher levels (Costa & Mollenhauer, 1980; Costa, 1980a; Sunderman, 1984b). It is to be noted that the soluble nickel salts appear to be more active in the tissue culture transformation assay than in the induction of tumours in experimental animals. As mentioned previously, this may be explained by the relatively rapid clearance of the soluble salts from the animal, while exposure is continuous in the culture dish.

E. Mutagenicity of Nickel Compounds

Bacterial mutation assays have been found to have a reasonably high predictive value in determining carcinogenicity of several classes of compounds (Tennant et al., 1987). Since uptake of particulates does not occur in bacteria, insoluble nickel compounds can not be tested in these

bacterial systems. Several studies using the customary *Salmonella typhimurium* tester strains, *E. Coli*, and *B. subtilis* have found no significant mutagenicity for the nickel salts $NiCl_2$ or $NiSO_4$ (Tso & Fung, 1981; Biggart & Costa, 1986). One study using a homoserine-dependent Corynebacterium strain indicated $NiCl_2$ to be mutagenic in this particular system, though results need to be confirmed (EPA, 1986). Although nickel compounds have generally been found to give negative results, synergistic responses with alkylating agents or 9-aminoacridine (a frameshift mutant) have been observed in both *E. coli* and *S. typhimurium* (Coogan et al., 1989).

Using mammalian cells, the toxicity of both soluble and insoluble compounds can be determined. Results of most of these mutagenicity tests indicate that nickel compounds are weak mutagens. Amacher & Paillet (1980) measured trifluorothymidine-resistant mutants in L5178Y/$TK^{+/-}$ mouse lymphoma cells and found $NiCl_2$ (3-h exposure) to cause a 4-fold increase in mutation frequency (7-fold at one dose giving 5% survival). Miyaki et al (1979) measured 8-azaguanine (AG) mutations at the hypoxanthine-guanine phosphoribosyl transferase (HPRT or HGPRT) locus in Chinese hamster lung V79 cells. Nickel chloride (20 h exposure) induced a slight increase in mutation rate (per 10^6 cells) significant only at very low survival. Mutation rates of 7.1 at 55% survival and 15.6 at 0.4% survival, versus 5.8 for the negative control were reported. Costa et al (1980) also found very weak mutagenicity of Ni_3S_2 and amorphous NiS at the HPRT locus in Chinese hamster ovary (CHO) cells, with Ni_3S_2 exerting a stronger effect. Results for a single experiment, 4 dishes per dose

(maximum 10 colonies total) were presented. Hartwig & Beyersmann (1987, 1989) measured a mutation rate at the V79 HPRT locus of 35.2 after 5 h exposure to 2 mM $NiCl_2$, (54% survival) versus 2.4 for non-treated controls. Combined treatment consisting of 19 h $NiCl_2$ exposure (1.0 mM), UV irradiation (5 J/m^2), then a further 5 h $NiCl_2$ exposure in serum free medium produced a mutation rate of 100 compared to 12 (nickel only) and 8 (UV only) under the same conditions. The increase in mutagenicity was attributed to inhibition of DNA repair after UV damage by the presence of nickel. A direct comparison of mutation induction at the HPRT locus in the V79 and CHO-AT3-2 lines after ethylmethanesulfonate (EMS) or potassium dichromate treatment illustrates the necessity to compare results obtained using the same cell lines and conditions (Paschin et al., 1983). After a 2 h EMS treatment (400 µg/mL), mutation frequencies of 140 and 18 per 10^5 survivors were measured in the V79 and CHO cells respectively. Potassium dichromate at .5 µg/mL produced similar toxicity (75% survival) but mutation frequencies of 17 and 6 (control = 2) per 10^5 survivors in the V79 and CHO cells.

F. Other Effects of Nickel

Injection of $NiCO_3$ in rats (ip) has been found to cause DNA lesions (Ciccarelli & Wetterhahn, 1982). Single-strand breaks were detected in lung and kidney nuclei and both DNA-protein and DNA interstrand cross-links were detectable in kidney nuclei. Tissue and intracellular nickel levels were found to correlate with the levels of DNA damage and repair.

Treatment of CHO cells with nickel compounds has also been found to cause DNA-protein cross-linking as detected by alkaline-elution methods (Patierno & Costa, 1985). Both crystalline NiS and soluble $NiCl_2$ induced rapidly repaired single strand breaks at non-toxic and toxic levels, whereas the more stable DNA protein cross-links (persisting at least 24 h after exposure) were detected only at non-toxic nickel levels comparable to those that reversibly inhibit cellular replication. Closer examination indicated that DNA-protein cross-linking appeared within 1 h of 2.5 mM $NiCl_2$ addition, with additional cross-links forming in a time-dependent manner (Patierno et al., 1985). Cross-linking occurred preferentially in late S-phase and required active cell cycling to occur. Crystalline NiS and $NiCl_2$ were also found to cause other chromosomal aberrations including gaps, breaks and sister chromatid exchanges (Sen & Costa, 1985, 1986a, 1986b). $NiCl_2$-induced chromosomal aberrations occurred randomly among the autosomal arms, but there was preferential damage to the heterochromatic regions. In addition, NiS caused selective fragmentation/decondensation of the heterochromatic long arms of the X-chromosomes. This added effect was attributed to different mechanisms of uptake since liposome-mediated $NiCl_2$ delivery also caused fragmentation of the long arm of the X-chromosomes. Investigation of the nickel-DNA-protein complexes indicated that the most tightly bound proteins were nonhistone chromosomal proteins and possibly histone 1 (Patierno & Costa, 1987). The stability of the ternary DNA-protein-nickel complex was reported greater than either DNA-nickel or protein-nickel complexes. Since DNA-protein binding is involved in the regulation of DNA replication and gene expression, nickel's effect

may interfere with the regulation and contribute to the carcinogenic activity of nickel compounds.

Other effects of nickel include the blocking of cell growth in S phase in CHO cells by $NiCl_2$, Ni_3S_2, Ni_3Se_2, and NiO (Nieboer et al., 1988a). DNA repair synthesis of strandbreaks (which primarily involving the action of DNA ligases) is induced in CHO, SHE, and cultured human fibroblast cells by $NiCl_2$, Ni_3S_2, and crystalline NiS; whereas DNA excision repair (which requires coordinated actions of endonucleases and DNA polymerases) is inhibited in CHO, V79, SHE, HeLa cells, and rat hepatocytes (Sunderman, 1985, 1989). Reduction in the fidelity of DNA synthesis and transcription by Ni^{2+} has been reported. Working with synthetic poly-d(G-C) and $NiCl_2$, $NiSO_4$, $NiCO_3$, and Ni_3S_2 or poly-d(A-C)·poly-d(G-T) and Ni^{2+}, a conversion from B-DNA (normal right-handed double helix) to Z-DNA (left-handed double helix) has been found to occur on nickel addition. Stabilization of Z-DNA by low nickel concentrations in the nuclei may distort transcription and be involved in carcinogenesis (Sunderman, 1989).

G. Nickel Uptake and Distribution

Costa et al. (1981a) found, by light and electron microscopy observation, that crystalline nickel particles (Ni_3S_2, NiS, Ni_3Se_2) were actively phagoctyized by cultured CHO and SHE cells. Particles of amorphous NiS and metallic nickel were phagocytized to a much smaller extent (.3% uptake vs 13-23% for crystalline compounds at the same dose).

Measurement of nickel levels by x-ray fluorescence spectrometry indicated that the nickel content in whole cells, cytoplasm, and nuclei of amorphous NiS treated cells was generally less than 10% of the nickel levels for crystalline NiS or Ni_3S_2 cells. Although nickel particles were only observed in the cytoplasm (light and electron microscopy), significant nickel levels were also measured in the nuclei, suggesting that the particles were broken down in the cytoplasm before entering the nuclei.

Subsequent studies showed a correlation between the phagocytosis of particulate nickel compounds in CHO cells and the induction of transformation in SHE cells (Costa et al., 1981b). Crystalline Ni_3S_2, NiS, and Ni_3Se_2 had considerably more transforming activity and were more actively phagocytized than the amorphous NiS, metallic Ni, NiO, or Ni_2O_3. The addition of amorphous NiS, Mn, or $MnCl_2$ inhibited the uptake of crystalline NiS particles. In a comparison of amorphous and crystalline NiS, both dissolved slowly in complete medium (half life in serum 34 d and 2.6 y respectively); by contrast the crystalline NiS dissolved rapidly in the cytosol after phagocytosis (Abbracchio et al., 1982). ^{63}NiS particles disappeared from almost half the cells in 2 d, while the total radioactivity associated with the cells and the cell number in the monolayer remained the same. It appears that once phagocytized, rapid dissolution of crystalline NiS provides high intracellular nickel levels, whereas the amorphous NiS is not readily taken up by the cells and undergoes slower dissolution in the extracellular medium. A comparison of amorphous and crystalline NiS revealed a difference in surface charge and structure (Abbracchio et al., 1982; Heck & Costa, 1982b). The

amorphous NiS was found to have a positive surface charge (Zeta potential of + 9 mV) while the crystalline form had a negative surface charge (Zeta potential of - 27 mV). The outermost surface (1-4 nm) of the nickel sulphides showed striking differences in the Ni/S ratios and their sulphur oxidation state. Reduction of the positive surface charge of the amorphous NiS with $LiAlH_4$ increased the degree of phagocytosis by CHO cells and the level of induced morphological transformation in SHE cells.

Observation by video intensification microscopy (VIM) studies of living cells showed that crystalline NiS particles interact with the cell membrane, binding and entering in areas of active cell ruffling (Costa, 1983). Amorphous NiS particles interact less avidly with the cell membrane. After phagocytosis, the particles move about the cell by saltatory motion and lysosomes repeatedly interact with them. Following containment of the particles in an endocytic vacuole and repeated lysosomal interactions, the particles tend to accumulate around the nucleus in the perinuclear region. Dissolution of the particles, enhanced by the acidic environment of the vacuole, allows release of dissolved nickel in close proximity to the nucleus. As mentioned previously, no nickel particles have been observed in nuclei though significant nickel levels have been measured. The nickel is therefore thought to enter the nucleus in the form Ni^{2+} and interact with the DNA, RNA, and protein. Measurement of the amount of ^{63}Ni bound to the RNA, DNA, and protein isolated from CHO cells after exposure to crystalline NiS or $NiCl_2$ has been reported (Harnett et al., 1982). NiS treatment of 10 µg/mL for 3 d resulting in a binding in the range of 100 ng nickel per mg macromolecule

for each of these 3 components. $NiCl_2$ treatment of 10 µg/mL for 1 or 5 d gave 48 pg (1 d) and 286 pg Ni/mg RNA (5 d), while the protein levels were 4 ng and 12.2 ng Ni/mg protein. DNA nickel levels were reported to be comparable to the RNA nickel levels. Therefore, after $NiCl_2$ treatment, nickel was bound preferentially to protein at 6 to 16 times lower than the level after NiS treatment, while DNA and RNA binding was much lower, 400-2400 times less than after NiS treatment. Difference in cellular distribution of dissolved nickel after exposure to water soluble salts compared to insoluble particulates are therefore apparent and could account for some of the differences in cellular activity. A later report (Patierno et al., 1987) noted that after a 6 h $^{63}NiCl_2$ exposure, 10-20% of the total radioactivity counts were associated with the nuclear fraction. By 24 h after treatment, 60% of the total nickel counts were lost, with an additional 20% lost after 48 h. By comparison, after 24 h 50% of the nuclear counts were lost, with no further decrease up to 48 h. Although the total nickel decreased, the proportion of the nickel in the nucleus relative to the whole cell increased with the length of time after exposure.

By contrast to the uptake of particulates by phagocytosis, water soluble nickel salts such as $NiCl_2$ may enter cells by other mechanisms. For example, nickel salts have been found to cross the cell membrane as Ni^{2+} via Ca^{2+} channels; Ni^{2+} evidently competes with Ca^{2+} for specific receptors (Sunderman, 1989). The nickel ion is thought to become bound to proteins and other cytoplasmic constituents, and is thus not as readily available to enter the nucleus as is the Ni^{2+} released from vacuoles of

dissolving particulates near the nuclear membrane. As mentioned previously, enclosing $NiCl_2$ in liposomes to be taken up by phagocytosis appears to alter the cellular distribution of the nickel ion such that the chromosomal effects are more similar to those caused by particulate nickel compounds.

H. The Nickel Ion Hypothesis and Research Objectives

Various nickel compounds demonstrate considerable differences in their *in vivo* and *in vitro* biological activities including tumour induction in humans and animals, transformation potential, toxicity, and chromosomal interactions. Properties of particulate compounds such as crystallinity, surface charge, particle size, and solubilization kinetics are thought to influence or determine the degree of cellular uptake and the intracellular availability of Ni^{2+}. A correlation has been reported between the degree of phagocytosis and the transformation potential of several nickel compounds at equal doses. Differences in the activities of soluble and particulate compounds *in vivo* appears due to the vast differences in clearance from the body (half life of $NiCl_2$ is 1-2 d compared to 3-4 y for particulates in nickel workers). *In vitro*, nickel salts appear less toxic at equal doses due to lesser uptake (eg via Ca^{2+} channels, diffusion, etc) compared to the phagocytosis and dissolution of particulates. Uptake of $NiCl_2$ in liposomes by phagocytosis was seen to eliminate observed differences in DNA binding, suggesting that the level of <u>available</u> Ni^{2+} is crucial in determining the cellular effect.

To explain the mechanism of action and the potency differences of nickel compounds, the "Nickel-Ion Hypothesis" has been proposed (Hansen & Stern, 1983; Nieboer et al., 1986, 1988a). This hypothesis suggests that the Ni^{2+} ion is the active agent in nickel toxicity, mutagenicity and carcinogenicity, and that its intracellular concentration, irrespective of the extracellular nickel compound, is responsible for the observed effects. In the study by Hansen & Stern (1983), exposure of BHK-21 cells to nickel compounds at the same toxicity level (50% survival) was found to induce the same level of transformation. Equal toxicity was assumed to infer equal cellular nickel concentrations, though no nickel analysis was performed. It was the goal of this present research to evaluate further the Nickel-Ion Hypothesis by exposing AS52-CHO cells to a range of nickel compounds and concentrations, followed by evaluation of the toxicity and corresponding cytosolic and nuclear nickel levels. Comparison of the intracellular/nuclear nickel levels resulting from exposure to various compounds at the same degree of toxicity would allow assessment of this hypothesis. As an additional measure, the level of 6-thioguanine resistant mutants at the *gpt* locus after exposure to equitoxic concentrations of nickel compounds was also determined.

II. MATERIALS AND METHODS

A. Water

Ultrapure water was used in the preparation of solutions, nickel compounds, culture medium, sample dilutions, and all other procedures unless otherwise stated. Ultrapure water, hereafter referred to as water or ddw (distilled double-deionized water), was prepared by passing centrally deionized water through a Corning MP-3A water purification system. This system consists of 2 filter cartridges to remove ions and organic impurities, followed by distillation in an all glass still and collection and storage in Nalgene LDPE carboys.

B. Nickel Compounds Tested

A summary of the nickel compounds tested and their sources is given in Table 1; additional details are given in Tables 2 and 3. Details of synthesis and/or particle size preparation are furnished in the following sections. Researchers have found that the particle size of water-insoluble nickel compounds is important to the toxic and transforming effects observed (Costa et al., 1981b). Particles with a diameter of less than 5 μm possess activity, with 1-4 μm being optimum. This is also physiologically relevant, since particles > 10 μm do not penetrate to the narrow passages of the lungs, while particles less than 1 μm are largely

Table 1: List and Sources of Compounds Tested

Compound as Referenced in Report #	Formula[a]	Name	Source	Comments, Sample Treatment[b]
1	$Ni(OH)_2$	Nickel Hydroxide	synthesized	
2	$NiCO_3$	Nickel Carbonate	synthesized	A. particles < 10 µm without grinding, batch 2 B. particles ground to < 10 µm, batch 2 C. particles ground to < 10 µm, batch 1 D. particles ground but > 10 µm, batch 2
3	NiO, black	Black Nickel Oxide	INCO INCO	A. INCO soluble black NiO, previously obtained B. as above but received Nov/88
4	NiO, green	Green Nickel Oxide	Johnson Matthey Chemicals	A. Puratronic, batch S.82912.B, received May/81
			INCO	B. received Nov/88
5	$Li_2Ni_8O_{10}$	Lithium Nickel Oxide	INCO	A. received Nov/88
6	NiS, amorphous	Amorphous Nickel Sulphide	synthesized	A. amorphous NiS + $NiSO_4 \cdot 6\ H_2O$ + NiS_2 B. as 6A, but ground finer
7	Ni_7S_6	Nickel Sulphide	INCO	A. received Nov/88
8	Ni_3S_2	Nickel Subsulphide	INCO	A. received Nov/88
9	$NiCl_2$	Nickel Chloride	BDH	A. AnalaR grade, lot 6872030J, received Apr/89
10	$NiSO_4$	Nickel Sulphate	BDH	A. AnalaR grade, lot 6394100J, received May/89
11	$Ni(CH_3COO)_2$	Nickel Acetate	Johnson Matthey Chemicals	A. Puratronic, lot S92038, received Apr/89

[a]Major species only. A more detailed description, including waters of hydration, is included in Tables 2 and 3.

[b]For each of compounds #1-11, only one sample (indicated as "A" in the comments column) was employed in the biological testing procedures. X-ray diffraction patterns of other listed compounds were obtained only to assist in verifying the sample identity.

Table 2: Chemical Analysis of Nickel Compounds[a]

	Percent Species by Weight						Calculated Values			
Sample	Ni	S	O	C	Li	Ni as Ni(III)	CO_2[b]	L.O.I. (1050°C)[c]	$NiCO_3$[d]	$Ni(OH)_2$[e]	H_2O[f]
1	55.6						1.7	27.4	4.59	84.24	9.33
2A	38.9						18.8	51.8	50.71	21.84	28.76
2D	37.2						17.6	52.3	47.47	21.68	30.49
3B	77.7		22.2[g]	0.15		0.32					
4B	78.6		21.4[g]	0.006		< 0.03					
5	72.4		25.2[g]		2.39	19.2					
6	41.02	25.2									
7	67.87	31.96	0.38	0.01							
8	73.4	26.5	0.09								
9[h]	24.7										
10	20.9										
11	23.59										

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[a]The analytical data were kindly provided by D Maskery, JS Warner, and N Zelding of INCO Ltd, Toronto, Ontario

[b]By gasometry, 1 $NiCO_3$ yields 1 CO_2
[c]Loss on ignition, 1 $NiCO_3$ yields 1 CO_2, 1 $Ni(OH)_2$ yields 1 H_2O, all H_2O released
[d]Assume all CO_2 comes from $NiCO_3$
[e]Assume all Nickel is as either $NiCO_3$ or $Ni(OH)_2$
[f]Calculated from LOI
[g]Assumed balance is O (oxygen)
[h]Fraction nickel in salts calculated from formulas given on labels

Table 3: Identification of Compounds[a]

Sample	Formula from Chemical Analysis	Species from X-ray Diffraction Analysis
1	$Ni(OH)_2 \cdot 0.04NiCO_3 \cdot 0.6H_2O$	$Ni(OH)_2$
2A	$NiCO_3 \cdot 0.55Ni(OH)_2 \cdot 3.7H_2O$	
2D	$NiCO_3 \cdot 0.58Ni(OH)_2 \cdot 4.2H_2O$	$NiCO_3 \cdot 6H_2O$
3	NiO	NiO
4	NiO	NiO
5	$Li_{2.23}Ni_8O_{10.22}$	$Li_2Ni_8O_{10}$ or NiO
6	$NiS_2 \cdot 2.25NiSO_4.6H_2O \cdot 4.76NiS$	$NiS_2 + NiSO_4.6H_2O$
7	Ni_7S_6 + 5% NiS + 1% $NiSO_4$	Ni_7S_6 (major); NiS + $NiSO_4$ + Ni_3S_2 (minor)
8	Ni_3S_2	Ni_3S_2
9	$NiCl_2 \cdot 6H_2O$	
10	$NiSO_4 \cdot 7H_2O$	
11	$Ni(CH_3COO)_2 \cdot 4H_2O$	

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[a]Nickel Salts (#9-11) were identified by product labels and material data sheets obtained at the time of purchase.

exhaled. Consequently, samples of nickel compounds tested in cell culture were ground and separated according to particle size using a series of sieves ending with a sieve of 5-10 μm mesh size.

1. Synthesis and Preparation of $NiOH_2$

Nickel hydroxide was prepared according to previously reported procedures (Kasprzak et al., 1983) but with slight modifications. The method involved mixing 200 mL of 0.6 M $NiSO_4 \cdot 7H_2O$ (BDH AnalaR, 33.70 g in warm ddw) and 200 mL of 1.4 M NaOH (BDH AnalaR, 8.00 g in warm ddw). The NaOH solution was first heated to 78°C; the $NiSO_4$ was then added from a burette over 70 min (approximately 3 mL/minute). The NaOH solution was stirred and kept at 75-80°C throughout the addition of $NiSO_4$ and for 15 min afterwards. The mixture was then left to cool for 70 min, with stirring for the first 40 min. The suspension was poured into eight 50 mL polypropylene tubes and centrifuged for 5 min at 800g. The supernatant was discarded and the precipitate rinsed 7 times by resuspending in hot water (85-95°C) followed by centrifugation as above. During the rinsing, the number of tubes was reduced to 2 by combining the precipitate and rinsing the tubes as appropriate. After the last centrifugation, the supernatant was discarded and the precipitate transferred to Whatman #541 filter paper. The precipitate was washed with small amounts of hot water (total volume ≈ 200 mL) and partially dried with the aid of a water aspirator to pull air through the sample. The $Ni(OH)_2$ product at this stage was a thick paste, lime green in colour. To dry the $Ni(OH)_2$ and produce a more crystalline form, the filter paper with the $Ni(OH)_2$ was

placed in a recrystallization dish and heated at 80-82°C for 20 h. The product at this stage was a dark-green colour. After an initial 10 min grinding of the sample in a Spex 8000 Mixer/Mill with tungsten carbide grinding vial and ball, the sample was again a light green colour. The sample was dry sieved through a series of wire mesh sieves #100, #200, #325, #400 (150, 75, 45, 38 μm openings); particles not passing through the last sieve were ground twice more in the Spex mill and sieved. Particles were then wet sieved by suspending the particles in water and filtering through 25 μm and 5 μm sieves. Particles passing through the final 5 μm sieve were collected by centrifugation at 200g for 5 min. Repeated centrifugation and rinsing was performed until all the particles were combined into one 50 mL centrifuge tube. The $Ni(OH)_2$ was dried at room temperature and then dried at 80°C for 5 h.

2. Synthesis and Preparation of $NiCO_3$

$NiCO_3$, which was essentially free of nickel hydroxide, was prepared by the procedure suggested by Dr VJ Zatka (personal communication). Into a 2-L Nalgene LDPE jugs, 25 g $(NH_4)_2CO_3$ (Fluka Chemicals) was dissolved in 1.7 L ddw; similarly 60 g $Ni(NO_3)_2 \cdot 6H_2O$ (BDH AnalaR) was dissolved in 2.0 L ddw. These solutions were cooled to 6°C in the refrigerator and subsequently to 0°C by placing the jugs in a -10°C mixture of ice/acetone. The $(NH_4)_2CO_3$ was saturated with gaseous CO_2 and poured into a 4 L flask resting in a dishpan containing ice/acetone and sitting on a magnetic stirrer. The solution was stirred and CO_2 bubbled in continuously while the $Ni(NO_3)_2$ solution was added slowly and for 45 min thereafter. The CO_2

flow and stirring were stopped and the reaction flask covered. On sitting at room temperature, blue-green $NiCO_3$ crystals formed and adhered to the sides and bottom of the flask. After 6 d, the reaction mixture was poured through a 5 µm sieve; the crystals remaining in the reaction flask were washed with cold water until the washings were clear. The washings were also poured through the 5 µm sieve. The combined filtrates were then centrifuged at 2000g for 5 min to collect the $\leq$ 5 µm fraction of $NiCO_3$ particles. These particles were transferred to a watch glass and permitted to air dry at room temperature. This fraction was used for *in vitro* testing and is referred to as Compound 2A, or $NiCO_3$ particles < 10 µm without grinding (batch 2). These particles were a light green colour.

Particles that would not pass through a 25 µm sieve were air-dried and then ground for 10 min in a Spex grinder. On grinding, the particles changed from aqua blue to light green and were similar in colour to Compound 2A, though they exhibited a slight blackish tinge. This material was wet sieved through a 10 µm nylon Spectra/mesh disposable filter; particles passing through the filter were collected by centrifugation and air dried. These particles are referred to as Compound 2B, or $NiCO_3$ particles ground to < 10 µm (batch 2). Particles not passing through the 10 µm filter were collected, dried, and designated as Sample 2D--$NiCO_3$ particles ground but > 10 µm (batch 2). Sample 2C is comparable to sample 2B, but from a previously prepared batch of $NiCO_3$.

3. Preparation of Black NiO

A sample of black NiO was obtained from Dr. JS Warner, INCO Limited. Under microscopic observation on a stage micrometer, the particles appeared to be ≈ 2-20 μm in diameter. Particles were wet-sieved (particles suspended in water) through 25 μm and 5 μm sieves. Particles passing through the final 5 μm sieve were collected by centrifugation at 200g for 5 min. Repeated centrifugation and rinsing was performed until all the particles were combined into one 50 mL centrifuge tube. The particles were sterilized by suspending in 20 mL acetone, centrifuged, and then dried for 5 h at 80°C. This black NiO sample is referred to as #3B. Due to the small initial sample and losses during particle sizing, not enough sample was available for *in vitro* testing.

Another sample of black NiO previously obtained from INCO was prepared in a similar manner except that a 10 μm nylon Spectra/mesh disposable filter was used in the final separation instead of the 5 μm sieve. This black NiO sample is referred to as sample 3A and was used for all cell culture testing.

4. Preparation of Green NiO

An initial sample of green NiO was obtained from Dr. JS Warner, INCO Limited. This sample had been prepared by heating the Inco black nickel oxide to 1045°C for 1 h. Under microscopic observation on a stage micrometer, the particles appeared to be ≈ 10-20 μm in diameter. Particles were wet-sieved through 25 μm and 5 μm sieves. Particles passing through the final 5 μm sieve were collected by centrifugation at

200g for 5 min. Repeated centrifugation and rinsing was performed until all the particles were combined into one 50 mL centrifuge tube. It was noted that the green NiO was very difficult to sieve or filter since the particles tended to clump; they quickly clogged the sieve/filter, and strongly adhered to the surface of tubes or flasks. The particles were sterilized by suspending in 20 mL acetone, centrifuged, and then dried for 5 h at 80°C. This green NiO sample is referred to as #4B. Due to the small initial sample and losses during particle sizing, not enough sample was available for *in vitro* testing.

Another sample of green NiO previously purchased from Johnson Matthey Chemicals, indistinguishable in appearance from sample 4B, was prepared in a similar manner. This green NiO sample is referred to as sample 4A and was used for all cell culture testing.

5. Preparation of $Li_2Ni_8O_{10}$

A sample of nickel oxide in which 2.39% Li stabilizes a Ni(III) content of 19.2% was obtained from Dr. JS Warner, INCO Limited. Particles were wet-sieved through a 25 μm sieve and a 10 μm Spectra/mesh filter. Particles passing through the final 10 μm filter were collected by centrifugation at 200g for 5 min. Repeated centrifugation and rinsing was performed until all the particles were combined into one 50 mL centrifuge tube. The particles were sterilized by suspending in 20 mL acetone, centrifuged, and then dried for 5 h at 80°C. This NiO with high Ni(III) content is also referred to as Sample 5.

6. Synthesis and Preparation of Amorphous NiS

Amorphous NiS was prepared using a 15% $NiCl_2$ solution and excess 22% $(NH_4)_2S$ (Abbracchio et al., 1982) in an acetate buffered system. 75 g of $NiCl_2 \cdot 6H_2O$ (BDH AnalaR), 7.7 g CH_3COONH_4 (BDH ACS Assured), 5.8 mL glacial acetic acid (Baxter ACS), and 145 mL water were mixed in a 2 L flask (pH = 3.9). In a fume hood 220 mL of 22% $(NH_4)_2S$ (Fisher Certified) was added by burette (≈3 mL/min) to the $NiCl_2$ solution with continuous stirring. 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 mixture was stirred for an additional 15 min after the $(NH_4)_2S$ had been added, then the product collected by filtration on a Whatman #541 filter. Twice the product was resuspended in ≈ 600 mL water and filtered, resuspended in smaller volumes of water, filtered, and given a final acetone rinse. The product was collected, dried at 110°C for 17 h, then ground for 10 min in the Spex grinder. Particles were dry-sieved through #100, #200, #325, and #400 sieves, then wet-sieved and collected as for $Ni(OH)_2$. The product was sterilized by suspending in 20 mL acetone, centrifuged, and then dried for 5 h at 80°C. This product is referred to as Compound 6, or amorphous NiS.

7. Preparation of Crystalline Ni_7S_6

A sample of crystalline Ni_7S_6 was obtained from Dr. JS Warner, INCO Limited. Particles were wet-sieved through a 25 µm sieve and a 10 µm Spectra/mesh filter. Particles passing through the final 10 µm filter were collected by centrifugation at 200g for 5 min. Repeated

centrifugation and rinsing was performed until all the particles were combined into one 50 mL centrifuge tube. The particles were sterilized by suspending in 20 mL acetone, centrifuged, then dried 5 h at 80°C. This crystalline Ni_7S_6 is referred to as Sample 7A.

8. Preparation of Ni_3S_2

A sample of crystalline nickel subsulphide, Ni_3S_2, was obtained from Dr. JS Warner, INCO Limited. Particles were wet sieved through a 25 µm sieve and a 10 µm Spectra/mesh filter. Particles passing through the final 10 µm filter were collected by centrifugation at 200g for 5 min. Repeated centrifugation and rinsing was performed until all the particles were combined into one 50 mL centrifuge tube. The particles were sterilized by suspending in 20 mL acetone, centrifuged, and then dried for 5 h at 80°C. This sample is also referred to as Sample 8.

9. Soluble Nickel Compounds

High purity nickel chloride, nickel sulphate, and nickel acetate were obtained from the sources indicated in Table 1. 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.

C. Characterization of Nickel Compounds

1. X-Ray Diffraction

X-ray crystal powder diffraction patterns were obtained for all of the particulate compounds mentioned in the previous section. Diffraction analysis was performed at the Institute for Materials Research, McMaster University, using Cu-K_α radiation at 1.5405 Å.

2. Chemical Analysis

The nickel and sulphur contents as well as minor constituents of the nickel compounds provided by INCO were certified by them. Amorphous NiS was analyzed for total nickel, sulphur, and other metal content at INCO's laboratory in Sudbury, Ontario. Analysis of $Ni(OH)_2$ and $NiCO_3$ samples were arranged by Dr. JS Warner at INCO's J Roy Research Laboratory, Mississauga, Ontario.

D. Growth of CHO and AS52 Cells

1. Source and Characteristics of Cells Used

Initial work was done using wild type CHO (Chinese hamster ovary) cells kindly provided by Dr. R Gupta, McMaster University. These cells and their growth characteristics and requirements have been previously described (Gupta, 1984). Due to difficulty in detecting nickel-induced mutations at the HGPRT (hypoxanthine-guanine phosphoribosyl transferase) locus, a line derived by modification of the CHO-K1 line and designated as AS52 was substituted in later experiments. These AS52 cells were

kindly provided by Dr. KR Tindall, National Institute of Environmental Health Sciences, NC. Further description of the mutation assay and the AS52 line is given in Section F.

2. Routine Culture Medium and Solutions

Medium

CHO cells were grown in αMEM medium containing all 4 ribonucleosides and deoxyribonucleosides (MEM^+, GIBCO). This medium was prepared from powdered concentrate by the addition of water and 2.2 g/L $NaHCO_3$ (Caledon, certified ACS) and was supplemented with 5% FBS (fetal bovine serum, GIBCO), 100 U/mL penicillin G, and 100 µg/mL streptomycin (10 mL/L of 100X concentrated antibiotic solution, GIBCO). The pH of the medium with supplements was adjusted to 6.9 with HCl, then the medium was filter sterilized through a 0.22 µm Falcon Bottle Top Filter (Becton Dickinson) and stored at 4°C. For mutation experiments, αMEM medium without nucleosides (MEM^-) was prepared as above, but substituting dialysed FBS (dFBS, GIBCO) for the regular FBS. Note that FBS was heat-inactivated (30 min at 56°C), filtered (.22 µm), aliquotted in 50 mL tubes, and stored frozen (-20°C) until needed.

AS52 cells were grown in Ham's F12 medium (F12, Sigma) prepared from powdered concentrate by the addition of water and 2.2 g/L $NaHCO_3$, and routinely supplemented with 5-10% dialysed FBS (dFBS, GIBCO) and MPA additives (10 µg/mL mycophenolic acid (Sigma), 25 µg/mL adenine (Sigma), 50 µM thymidine (GIBCO), 250 µg/mL xanthine (Sigma), 3 µM aminopterin (Sigma)). Medium with MPA additives will be referred to as MPA medium.

During experiments (toxicity, uptake, mutagenicity) or if no mutagenicity testing with a particular cell batch was anticipated, MPA additives were omitted and regular (non-dialysed) FBS (Flow) was used. This medium will be referred to as F12 medium. Antibiotics were not added for routine culturing but an antibiotic/antibiotic lyophilized powder (Sigma) was used during individual experiments (100 U penicillin G, 100 µg streptomycin, 0.25 µg amphotericin B per mL medium). After all additions were made, the pH was adjusted to ≈ 7.1 with HCl. The medium was then filter sterilized and stored at 4°C.

PBS (Phosphate Buffered Saline)

PBS was initially made up as a 10X solution containing 80g NaCl, 2 g KCl, 2g KH_2PO_4, and 21.6 g $Na_2HPO_4 \cdot 7H_2O$ (or the equivalent $Na_2HPO_4 \cdot H_2O$ or Na_2HPO_4) in 1 L water. The pH was adjusted to 7.2-7.4 and the solution was filter sterilized (.22 µm). Before use, the 10X PBS was diluted with water to give 1X PBS (referred to as PBS; 136 mM NaCl, 2.68 mM KCl, 1.47 mM KH_2PO_4, 8.06 mM $Na_2HPO_4 \cdot 7H_2O$), the pH was adjusted to 7.2-7.4, and the PBS was then filter sterilized. Both the 10X and 1X PBS were stored at 4°C.

Trypsin

Trypsin solution was prepared by dissolving 0.5 g of 1:250 trypsin (GIBCO) in 400 mL PBS to give a final concentration of 0.125% (w/v). The solution was filter sterilized (.22 µm), aliquoted in 15 or 50 mL tubes, and stored frozen (-20°C).

Stains

A 0.5% methylene blue solution was prepared by dissolving 5 g of methylene blue (Sigma) in 500 mL 50% methanol and 500 mL water. This solution was filtered through Whatman No 1 chromatography (filter) paper and stored in glass bottles at room temperature.

A 0.5% crystal violet solution was prepared by dissolving 5 g of crystal violet (Sigma) in 1 L of 95% ethanol.

A 0.5% solution of acridine orange was prepared by dissolving 1 g of acridine orange (Sigma) in 200 mL water.

3. Routine Cell Growth and Culturing Procedures

General Growth

Cells were grown in a 37°C, 95-100% humidity, 5% CO_2 (continuous flow) incubator, normally in 100 mm diameter culture dishes (Falcon) with 10 mL of medium per dish. For toxicity and plating efficiency determinations, cells were grown in 60 mm diameter dishes (Falcon) with 4 mL medium per dish. All cell culture manipulations were performed in a laminar flow hood. Cells were routinely subcultured every 3-4 d by splitting the cells at 1:2, 1:4, or 1:8 (i.e. cells from 1 dish were replated in 2, 4, or 8 dishes). Cells were replated by removing the culture medium by aspiration, rinsing the cells with PBS, removing the PBS, and then adding 1 mL of trypsin solution per dish. The dishes were tilted and rotated to distribute the trypsin; they were then allowed to stand for 3-5 min until cells became rounded and started to detach from the plates. Medium with 5-10 % FBS was added to stop the trypsin action.

Cells were dislodged by pipetting and transferred to new dishes or polypropylene tubes. When the cell number was required, cells were diluted in Isoton II saline solution and counted using a Coulter Model Z_F particle counter.

Long-Term Storage of Cells

Cell stocks were stored frozen in NUNC 2 mL cryovials at -78°C. Trypsinized cells were concentrated by centrifuging at low speed (400g) for 5-10 min and were then suspended at $0.5-1.0x10^7$ cells per mL in growth medium plus 10% sterile DMSO (Sigma). Cells were aliquotted at 1 mL per cryovial and frozen. When required, cells were thawed by immersing vials in a 37°C water bath until ice crystals had just melted. The cells were subsequently added to 20 mL of cold medium, centrifuged 5 min at 400g, resuspended in fresh medium, and plated.

Sterilization

Only sterile glass and plasticware were allowed to contact cells and culture medium or solutions. All bottles (borosilicate glass), beakers, stir bars, flasks for solution preparation, 1-10 mL glass pipettes, pasteur pipettes, plastic pipette tips, and Eppendorf tubes were sterilized by heating at 150-160°C for 2 h. Culture dishes and centrifuge tubes were purchased sterile. Repeater pipette tips were sterilized by storage in 95% ethanol. All solutions were made with purified water (described previously) and sterilized by filtering through .22 µm filters into sterile bottles or tubes.

E. Toxicity Testing of Nickel Compounds

Cells from a near confluent culture dish were released with trypsin, counted, diluted, and replated at 250 cells per 60 mm dish in MEM^+ + 5% FBS (CHO cells) or at 500 cells per 60 mm dish in F12 medium + 10% FBS (AS52 cells). One to two days later, medium was removed from the dishes and replaced with 4 mL serum free medium and an appropriate aliquot of freshly prepared nickel compound solution. Nickel particles were weighed out (15-50 mg), transferred to sterile 15 mL polystyrene tubes, medium + 5% FBS was added, and then the solutions were sonicated (for AS52 cells only; 2 x 10-20 s) to break up aggregates. The small amount of serum in the medium was necessary in order to prevent significant particle adherence to the stock solution tubes. Working dilutions were made by vortexing the stock solutions and adding a portion to another tube containing medium. The working dilution of a nickel compound was vortexed and then an aliquot (20-100 µL of the appropriate dilution) was added to the culture dishes. After an exposure period of 24 h for particulates and 5 (or 24) h for nickel salts, medium was removed and the cells rinsed with medium + 5% FBS and PBS to remove nickel not taken up by the cells. Fresh medium + 10% FBS was added and the cells incubated for 6-8 d until colonies of sufficient size to be counted developed. Colonies were stained using a solution of methylene blue (CHO cells) or crystal violet (AS52 cells), thereby producing clearly visible, circular colonies of a deep blue/violet colour. These colonies were counted using an automated colony counter (Biotran II Automated Colony Counter, NBS Model CIII, New Brunswick Scientific) and the number per dish relative to that in

untreated control culture dishes (relative % survival) recorded.

F. Mutagenicity Testing

Mutations were quantified by counting colonies showing 6-thioguanine (6-TG) resistance, representing loss of hypoxanthine-guanine phosphoribosyl transferase (hprt) activity (CHO cells) or the analogous xanthine-guanine phosphoribosyl transferase (xprt) activity in AS52 cells (Committee on Chemical Environmental Mutagens, 1983). In wild type CHO cells, HPRT is a salvage enzyme for purines and pyrimidines after nucleic acid degradation. It is coded on the X-chromosome and catalyzes the conversion of hypoxanthine and guanine to the corresponding nucleoside-5'-monophosphates (Cole & Arlett, 1984). HPRT also normally converts added 6-TG and other analogues to lethal products. Since HPRT activity, unlike that of most other enzyme markers, is not necessary for growth under normal conditions, all classes of mutations including base substitutions, frame shifts, deletions, additions, and chromosome breakage and rearrangements should be able to be detected (Gupta, 1984).

A problem with the CHO/HPRT assay is that nickel is thought to cause large deletions and rearrangements extending past the HPRT locus. Neighbouring genes necessary for survival are suspected of being deleted/affected, giving low survival and thus low detection of HPRT mutants. 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

(Stankowski & Hsie, 1986; Stankowski et al., 1986). The new location of this gene is known to allow detection of mutations caused by X-irradiation (which causes large deletions) not detectable in the CHO cells.

For mutagenicity testing, confluent/almost confluent dishes of AS52 cells growing in MPA medium were trypsinized, then the cells plated with 10 mL F12/10% FBS in 100 mm dishes at 1:4 to 1:8, so as to be almost confluent 3 d later ($\approx 5*10^5$/dish). Two days after plating (Day 0), the medium was replaced with 10 mL of F12 without serum. Nickel compound stock solutions and dilutions were made in F12/5% FBS as for toxicity testing and aliquots (20-100 µL of the appropriate dilution) were added to the culture dishes. Ethyl methanesulfonate (EMS) was employed as a positive control. Test doses for particulate nickel compounds were chosen, based on toxicity curves, giving 80% (A), 65% (B), 50% (C), 35% (D), and 20% (E) survival. For the nickel salts, doses covering this range were also used. After an exposure period of 24 h for particulates or 5 h 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 h exposures, fresh F12 + 10% FBS was added, the cells were incubated 19-24 h, and were then rinse once with 8 mL PBS. For 24 h exposures, the cells were rinsed with 8 mL PBS. The cells were trypsinized and replated after PBS rinsing and again every 2-3 d to allow expression of the altered gene. On Day 9, the cells were replated at $2x10^5$ cells/100 mm dish (5-6 dishes per treatment condition) in 10 mL F12/10% dFBS (or 5% dFBS and 5% dialysed fetal calf serum) plus 10 µM 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 d (Day 17), the PE dishes were rinsed with PBS and stained with crystal violet. After a selection period of 10-12 d, the resistant colonies were stained with crystal violet and counted.

G. Analysis of Nickel Content in Cells

1. Nickel Analysis in CHO Cells

Total Cell-Associated Nickel

Cells exposed to $NiCl_2$ as described in the previous section were rinsed, trypsinized, and collected in 15 or 50 mL centrifuge tubes. The cells were counted, after which an aliquot was removed and placed in a 5 mL pointed glass centrifuge tube. The cells were then centrifuged for 10 min at 700g. After removal of the medium the cells were washed with buffer and recentrifuged. The cells were treated and analyzed employing a procedure in which nitric acid plus heat is used for deproteinization prior to analysis (Sunderman et al., 1984). After removal of the buffer from the pelleted cells, 1 mL water and 50 µL concentrated HNO_3 (Ultrex, JT Baker) were added. HNO_3 lyses the cells, releases nickel from all binding sites, and precipitates the protein fraction. After these additions, the tubes were vortexed for 1 min, heated at 70°C for 5 min, and then vortexed for 10 s. Before analysis, the samples were centrifuged at 800g for 10 min and the supernatant was poured into an atomic absorption (AA) spectrometry sample cup.

Ni_3S_2 exposed cells were treated in a similar matter, though with

a more vigorous acid treatment and heating to dissolve the Ni_3S_2 particles. To pelleted cells, 100 µL water and 50 µL concentrated HNO_3 were added. The mixture was vortexed for 45 s, immersed in boiling water for 10 min, vortexed 10 s, and then 900 µL water and 20 µL of 0.1% Triton X-100 (Bio Rad) were added. The mixture was vortexed, centrifuged at 900g for 10 min, and the supernatant analyzed.

Fractionation of Cells

Some initial work at separating nuclear and non-nuclear (cytosolic) cell fractions was performed in CHO cells with methods adapted from published procedures (Borun et al., 1967; Gurley et al., 1973; Prentice & Gurley, 1983). Cells were collected as described in the previous section and $0.5\text{-}1.5\text{x}10^7$ cells suspended in 4 mL of hypotonic buffer (10 mM NaCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2 \cdot 6H_2O$, pH 7.4) by vortexing 1-3 min at full speed. Cells were put on ice for 5 min, after which 0.5 mL of 10% Triton solution (5 mL Triton X-100, 5 mL Triton X-114 in 100 mL water) was added; subsequently the cells were vortexed for 30 s. After sitting on ice for 15 min, 0.5 mL of 5% sodium deoxycholate solution (2.5 g in 50 mL water) was added, the solution was vortexed for 30 s, and was placed on ice for 15 min. After vortexing the suspension for 30 s, the nuclei were pelleted by centrifugation (1000g, 10 min). The pellet was resuspended in hypotonic buffer supplemented with 1 mM $CaCl_2$. In an attempt to separate Ni_3S_2 particles from nuclei (Costa et al., 1981a), 1 mL of the suspended pellet was layered on 5 mL of 97 % sucrose (97 g sucrose + 38.7 g water) and centrifuged for 15 min at 50000g.

2. Non-particulate Nickel in Cytosol and Nuclei of AS52 Cells

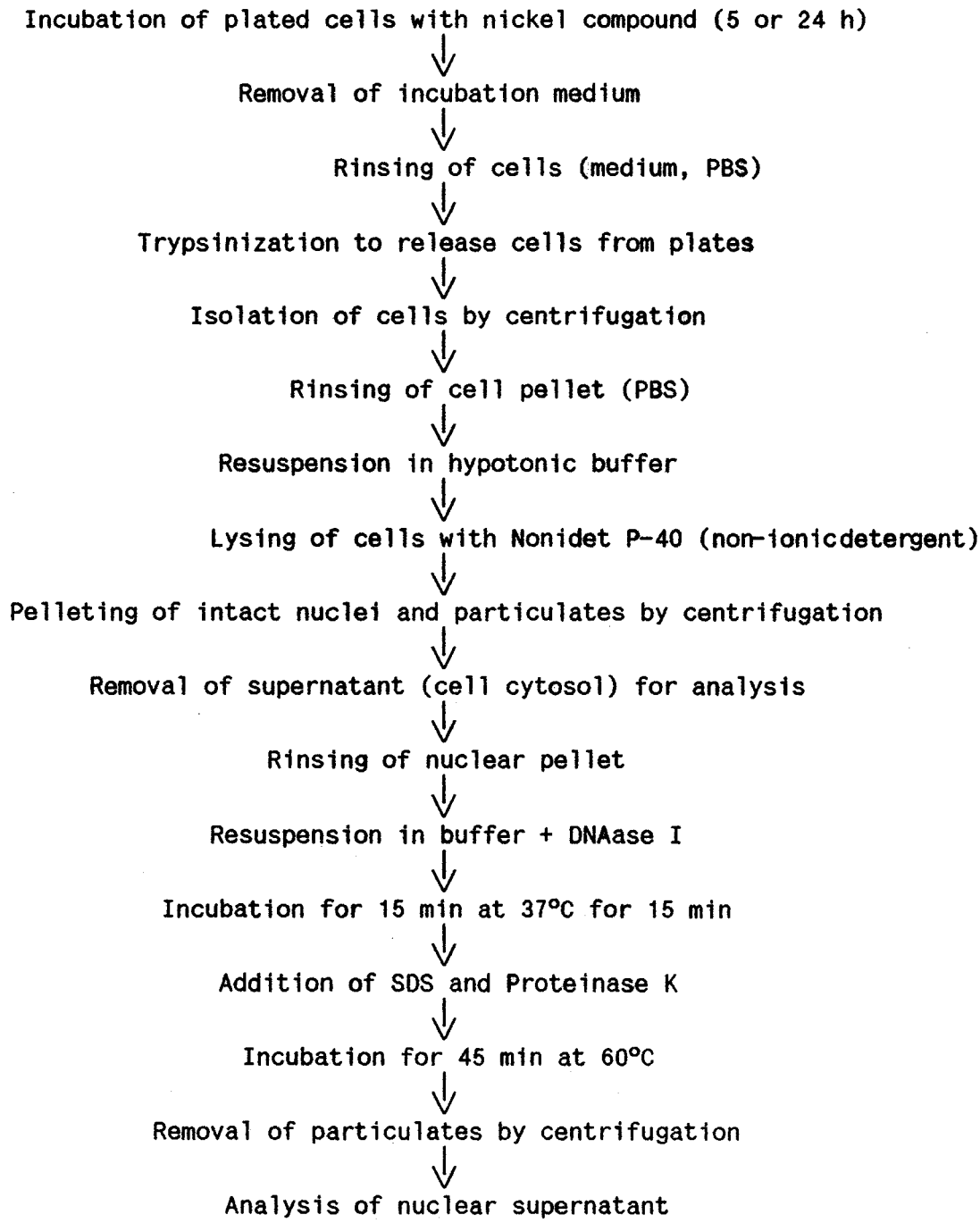
Optimization of Cell Fractionation Procedure

Very low recovery of nuclei, complicated further by difficulties in suspending cells and nuclei, necessitated revising the procedure of nuclear isolation described in the previous section. Major changes included elimination of the sodium deoxycholate addition, replacing pointed centrifuge tubes with rounded tubes to facilitate resuspension of cell and nuclear pellets, and using gentler vortexing (½ speed instead of full speed). Microscopic observation at several stages of the procedure with the aid of a haemocytometer was used to develop and evaluate the procedure with respect to yield and nuclear purity. For additional confirmation of purity, nuclei were stained with acridine orange and observed by fluorescent microscopy (Gurley et al., 1973). Four drops of nuclei suspended in hypotonic buffer plus $CaCl_2$ and 2 drops of 0.5% acridine orange solution were added to 5 mL of 0.14 M NaCl. After vortexing, the nuclei were left to stain for 2 min and were recovered by centrifuging for 5 min at 1000g; they were then resuspended in 0.5 mL fresh 0.14 M NaCl. Stained nuclei were observed by fluorescence microscopy under conditions routinely used for Q-banding in the Cytogenetics laboratory.

Isolation of Nuclei

An outline of the revised nuclear isolation procedure in given in Figure 1 and is described below. After exposing cells to nickel compounds (5 or 24 h), the cells were rinsed with F12/5% FBS and PBS, trypsinized,

Figure 1: Nuclear Isolation Flow Chart



resuspended in F12/5% FBS, and then collected by centrifugation at 500g for 5 min. Cells were resuspended in 4 mL Buffer 1, consisting of hypotonic buffer + 1 mM monosodium p-chloromercuriphenylsulfonate ($ClHgPhSO_3$, Sigma), to remove medium/serum. Cells were then transferred to 5 mL rounded polystyrene centrifuge tubes and centrifuged for 5 min at 500g. The cells ($.5\text{-}1x10^7$) were suspended in 1 mL Buffer 1 by vortexing at 1/2 speed for 20 s and the number of cells determined by counting an aliquot of 100 µL by Coulter counter. Cells were left to swell at 4°C for at least 10 min. The suspension was vortexed for 20 s, 100 µL of 10% Nonidet P-40 (Sigma) added, and the suspension again vortexed for 30 s. This caused the cell membranes to disintegrate and allowed intact nuclei to be pelleted by immediately centrifuging for 10 min at 1000g. At this stage, any particulates would also sediment out along with the nuclei. The supernatant (cytosol) was then removed, placing 800 µL in a 1.5 mL Eppendorf tube to keep for analysis of dissolved or non-particulate nickel content. The nuclear pellet was rinsed by adding 3 mL of Buffer 2 (Buffer 1 + 1 mM $CaCl_2$) and repelleted by centrifuging for 5 min at 1000g.

Nuclear Digestion and Particulate Separation

Nuclei ($\approx 7.5x10^6$) were suspended in 900 µL of Buffer 2 and heated to 37°C for 5 min. DNAase I was then added to digest some of the DNA, which aided in solubilization of the suspension and facilitated the release of bound nickel. DNase I stock solution (Deoxyribonuclease I, Type II, EC 3.1.21.1, Sigma), previously made at 2000 units/mL in 0.15 mL NaCl/50% glycerol and stored at -20°C, was diluted in Buffer 2 to 333

units/mL and 100 µL was added to each nuclei sample. The solution was vortexed immediately and incubated at 37°C for 15 min. The anionic detergent SDS (sodium dodecyl sulphate) plus Proteinase K were then added to disrupt the nuclear membrane and cleave proteins further. One mL of 2X Proteinase K mixture, containing 10 mM HEPES (Sigma), 0.8% SDS (Bio Rad electrophoresis grade), and .25 mg/mL Proteinase K (Boehringer Mannhein GmbH), was added and the mixture was incubated for 45 min at 60°C. Nickel particulates were separated by centrifugation at 2000g for 10 min. One mL of supernatant was placed in a 1.5 mL Eppendorf tube and kept for analysis.

3. Atomic Absorption Spectrometry Determination of Nickel

Acid Washing of Glass and Plasticware

All containers or labware in contact with solutions involved in nickel analysis were acid washed before use. Pipette tips, AA sample cups, and centrifuge tubes were placed in polyethylene canisters and rinsed with water. After decanting the water, the containers were filled with ≈ 1 M double distilled HCl (ddHCl, prepared in lab by distillation of reagent grade HCl) and let sit at least 24 h. Similarly, volumetric flasks, beakers, and polyethylene bottles were rinsed and filled with ddHCl. The HCl was poured off and collected and the glass/plasticware rinsed at least 3 times with water. The containers were refilled with water and let stand at least 24 h. The water was poured off and the glass/plasticware rinsed once more with water and dried at 70°C.

Preparation of Standards

Nickel stock standard (100 mg/L) was prepared by adding 50 mg nickel powder (99.999% pure, 5µm spheres, Alpha products) to 5 mL of water and 5 mL of concentrated HNO_3 (Ultrex, JT Baker) in a 25 mL beaker and warming cautiously until the nickel was dissolved. The solution was transferred to a 500 mL volumetric flask, filled with water, then stored in a polyethylene bottle. Nickel intermediate standard (400 µg/L) was prepared by diluting 2 mL nickel stock and 2 mL concentrated HNO_3 to 500 mL and storing in a polyethylene bottle (up to 3 months). Working standards of 0, 4, 8, 12, 16, and 20 µg/L were made by adding 0 to 5 mL intermediate standard plus dilute HNO_3 (1 mL conc HNO_3 diluted to 250 mL) to give 5 mL total stock plus acid then diluting to 100 mL. Working standards were replaced weekly when required.

Atomic Absorption Conditions and Settings

Samples were analyzed using a Perkin Elmer Atomic Absorption Spectrometer (model 703) furnished with graphite furnace (HGA-500) and using pyrolytic graphite tubes, an automatic sampling system, ultrapure Argon gas, and a chart recorder and digital printer for data recording. A wavelength of 232.0 nm, lamp current of 25 mA, slit width setting of .3, and a scan speed of .1 were used. Peak area of the absorbance signals was recorded, with the chart recorder set to a range of 2 mV and a speed of 60 mm/minute. The following temperature program was used: (1) 10 s ramp to 100°C, (2) 20 s ramp to 140°C, (3) 10 s ramp to 190°C, (4) 40 s ramp to 1200°C with the temperature held at this level for 10 s,

(5) atomization at 2600°C for 5 s, and (6) 1 s ramp to 2700°C and holding at this temperature for 2 s. The argon flow was 300 mL per min, except during the atomization (step 5) during which it was reduced to 30 mL per min. The baseline was adjusted 6 s before atomization and the absorption signal integrated from 2 s before atomization to the end of the atomization step.

Preparation and Analysis of Samples

The sample preparation involving CHO cells was described in Section G1. For AS52 cells, a small volume (100 µL) of concentrated perchloric acid (Ultrex, JT Baker) was added to the cell cytosol and nuclear fractions collected, in order that a final concentration of approximately 1 N $HClO_4$ was obtained. This allowed release of bound nickel and the precipitation of any remaining organic matter (proteins, DNA, detergent). After vortexing, cooling (15 min at 4°C), and centrifugation (10 min at full speed in microfuge), the samples were analyzed for nickel content by Electrothermal Atomic Absorption Spectrometry (EAAS). All samples were diluted with water as required to give an absorbance reading in the range covered by the standards.

III. RESULTS

A. Characterization of Nickel Compounds

Results of the chemical analysis of the nickel compounds are given in Table 2. The nickel content was determined for all particulate (insoluble) compounds, with other elements or components measured as indicated. Formulas of the nickel salts (water soluble compounds) were assumed to be accurate as reported on the labels and in the suppliers' catalogues. The percent nickel was used to convert from µg compound/mL as weighed out and applied to the cells to µg nickel/mL reported in the tables and figures, allowing better comparison of the compounds.

X-ray powder diffraction spectra are plotted as relative intensity (I/I_1 or I/I_{max}) versus angle (2θ) in Figures 2-1 to 2-8, while the corresponding lattice spacings (d values) and intensities for samples and standards are tabulated in Tables A1-A7 (see Appendix; JCPDS-International Centre for Diffraction Data, 1988). Plotted below each sample spectrum is the most closely matching pattern from the available on-line JCPDS data base. Sample identification based on the X-ray diffraction patterns is summarized in Table 3.

For Sample 1 (nickel hydroxide), the peaks are too broad to allow an accurate measurement of d (or 2θ) values, though the pattern is qualitatively consistent with the nickel hydroxide standard. Chemical analysis is compatible with $Ni(OH)_2 \cdot 0.6H_2O$, with possibly a very small amount of $NiCO_3$ (0.04 per one $Ni(OH)_2$ unit). Sample 2A shows no observable

diffraction planes, though Samples 2B-D correspond to the $NiCO_3 \cdot 6H_2O$ standard, with varying degrees of resolution. The chemical analysis for Samples 2A and 2D is similar, indicating the same chemical make-up though differences in the degree of crystallinity exist. Though none is indicated in the x-ray pattern, chemical analysis suggests a significant amount of nickel hydroxide may be contained in the carbonate samples. The hydroxide, if present, is expected to be in a non-crystalline (amorphous) form. Samples 3A, 3B, 4A, and 4B (black and green nickel oxides) are indistinguishable by their diffraction patterns. All correspond to the NiO standard. The peaks in Sample 5 are similar but slightly shifted from the other nickel oxides. Sample 5 has characteristics of both the NiO and $Li_2Ni_8O_{10}$ diffraction patterns. Chemical analysis, however, indicates a formula of $Li_{2.23}Ni_8O_{10.22}$ and a Ni(III) content of 19.2% stabilized by the presence of the lithium. Sample 6, expected to be amorphous NiS, is clearly indicated by the diffraction pattern to contain a mixture of NiS_2 and $NiSO_4 \cdot 6H_2O$. These are possible oxidation products in the formation of NiS, though they do not account for the analyzed nickel and sulphur contents. The high background in the diffraction pattern obtained suggests that a non-crystalline component is present, with the most likely species being amorphous NiS ($\approx$ 60 mole percent). For Sample 7, the complexity of the pattern prevents a definite identification, though Ni_7S_6 appears the major component. The JCPDS standards for other possible minor constituents are tabulated with the data for this compound. Other nickel/sulphur compounds are 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.

B. Toxicity of Nickel Compounds

The average results obtained from several toxicity experiments in CHO cells are reported in Table 4. Only $NiCl_2$ and Ni_3S_2 were tested for 24 h exposure periods in the CHO cells. Graphical representation in the form of toxicity curves plotting % survival versus dose (reported as µg nickel per mL medium) is given in Figures 3-1 and 3-2. The results of toxicity testing in AS52 cells are reported in Table 5, with the data presented graphically in Figures 4-1 to 4-12. At least 3 sets of results from separate experiments (3 dishes per experiment) were obtained for each compound tested, with additional replicates performed for some of the compounds when data reliability was suspect. The average values and the standard deviation (std) are reported for each compound tested. The omission of a standard deviation in the table indicates that results from only one experiment were available for the dose. This occurred because slightly different doses were tested or bacterial/fungal contamination was encountered.

Figure 2: X-Ray Powder Diffraction Patterns of the Nickel Compounds Employed in the Present Study

The x-ray powder diffraction patterns for each of the compounds tested, as identified in Table 3, are given in Figures 2-1 to 2-8. The bottom frame in each case shows the identity and normalized line pattern of the standard most closely matching the compound tested. In these figures the diffraction angle 2θ is indicated on the x-axis while the relative intensity (I/I_{max} or I/I_1, i.e. intensity of the peak compared to that of the strongest peak observed) is displayed on the y-axis. Crystal lattice d-spacings and relative intensities for both samples and standards have been compiled in Tables A-1 to A-5 located in the Appendix. The experimental diffraction patterns were obtained using CuK_α radiation at 1.5405 Å.

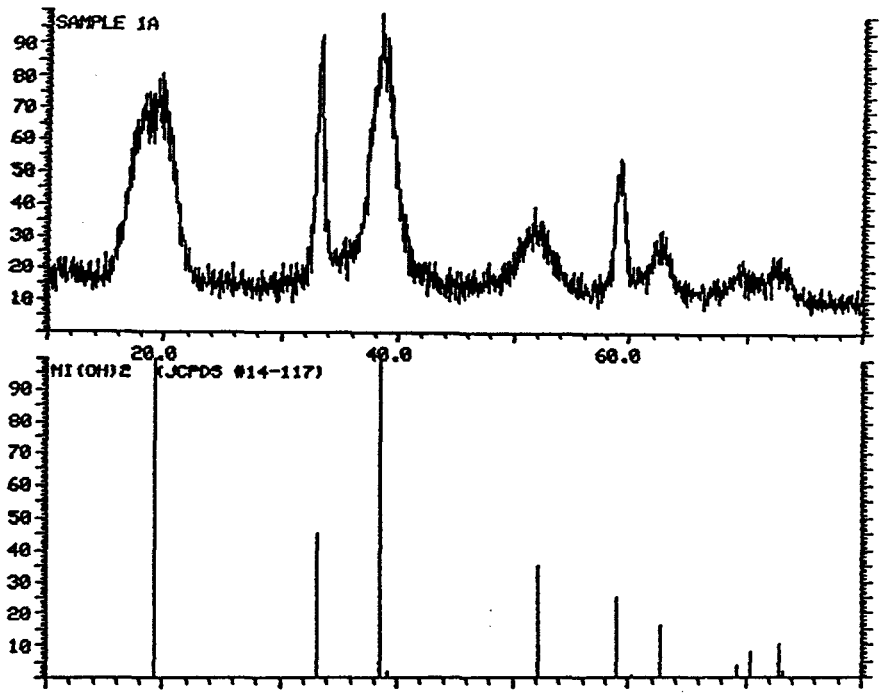


Figure 2-1: X-Ray Diffraction Pattern of $Ni(OH)_2$ (Sample 1)

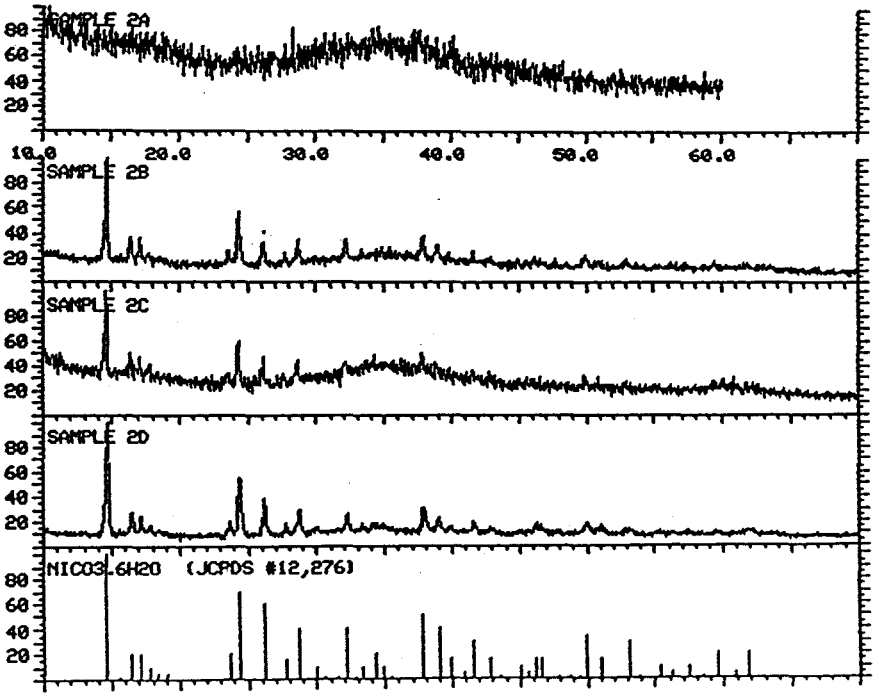


Figure 2-2: X-Ray Diffraction Pattern of $NiCO_3$ (Sample 2)

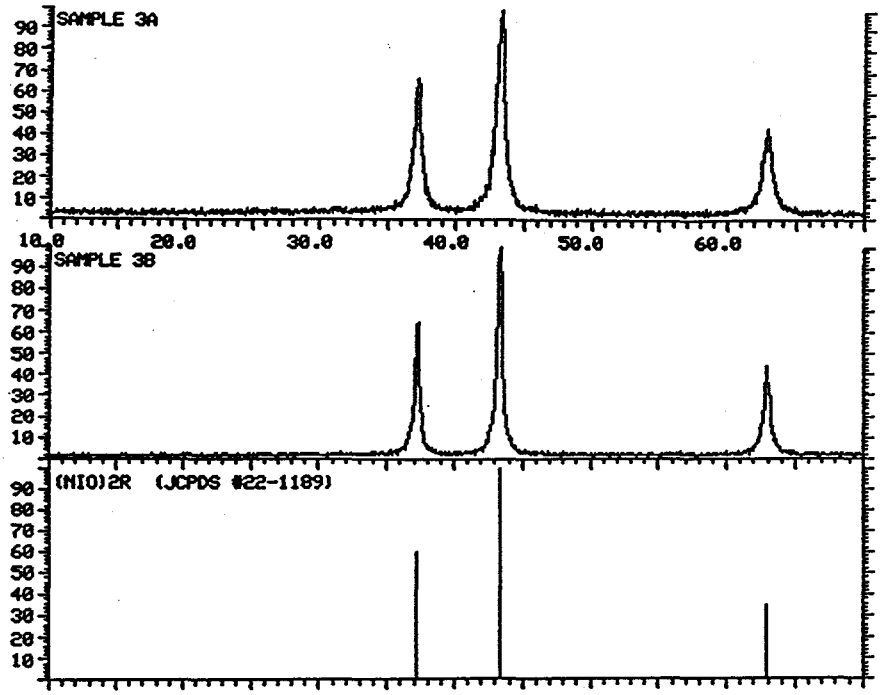


Figure 2-3: X-Ray Diffraction Pattern of Black NiO (Sample 3)

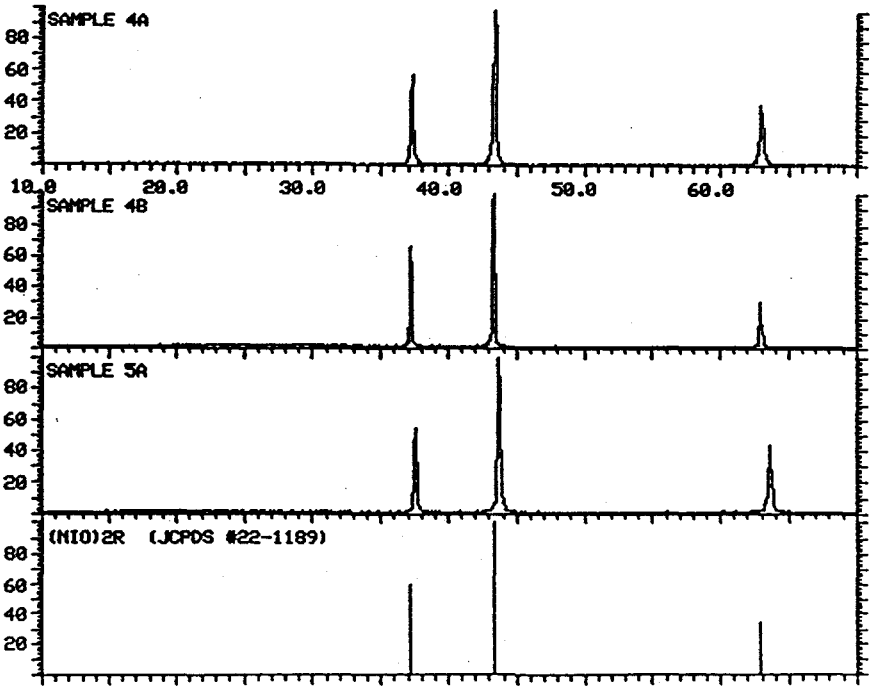


Figure 2-4: X-Ray Diffraction Pattern of Green NiO and $Li_2Ni_8O_{10}$ (Samples 4 & 5)

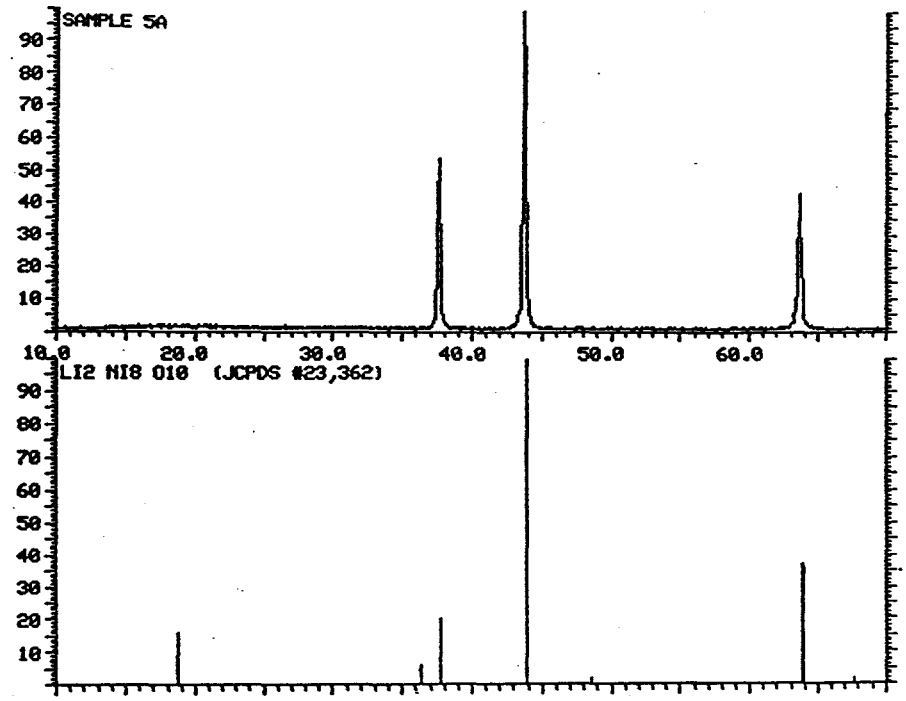


Figure 2-5: X-Ray Diffraction Pattern of $Li_2Ni_8O_{10}$ (Sample 5)

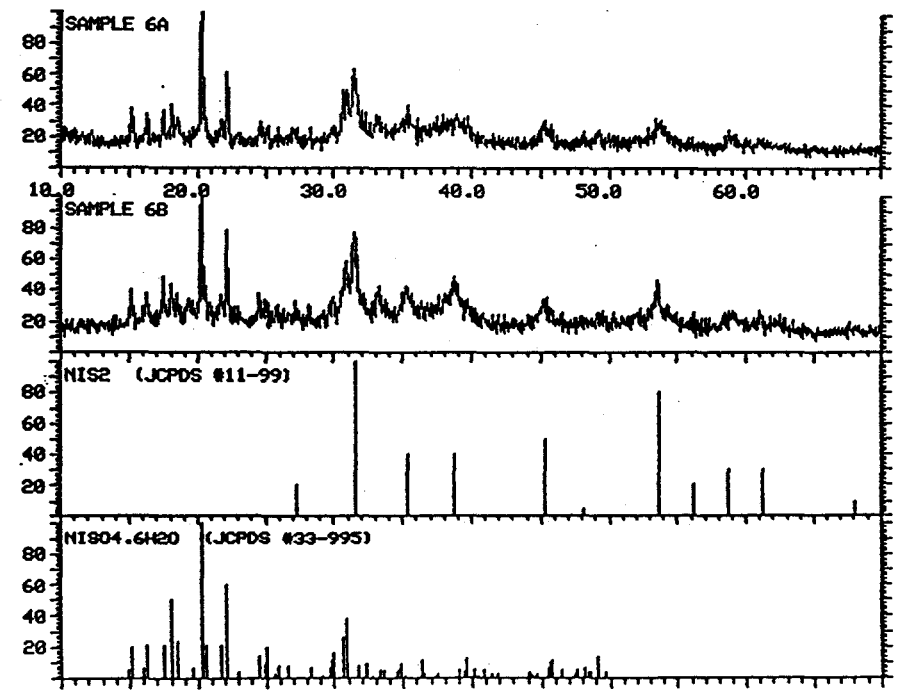


Figure 2-6: X-Ray Diffraction Pattern of "Amorphous NiS" (Sample 6)

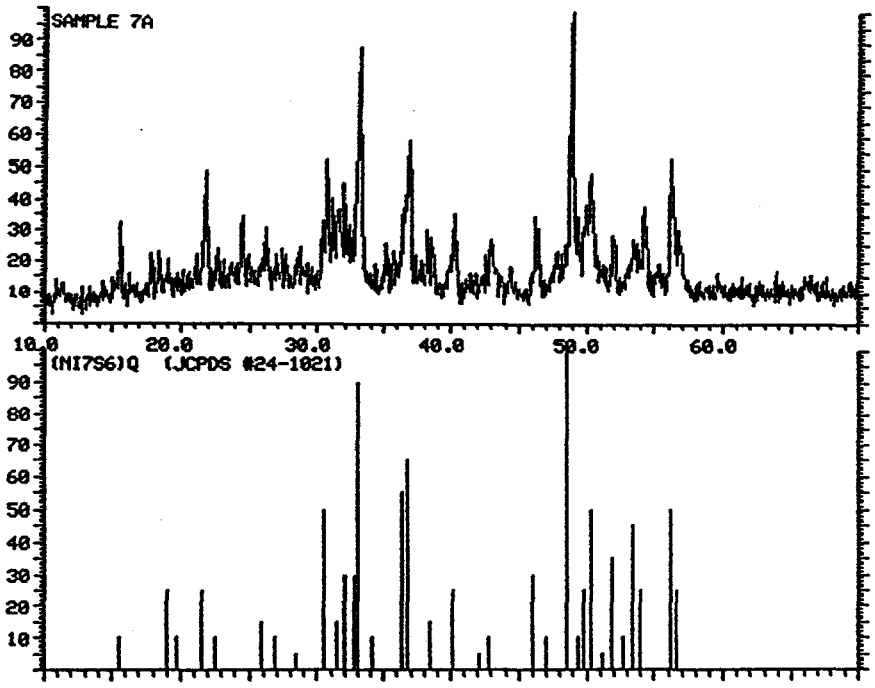


Figure 2-7: X-Ray Diffraction Pattern of Ni_7S_6 (Sample 7)

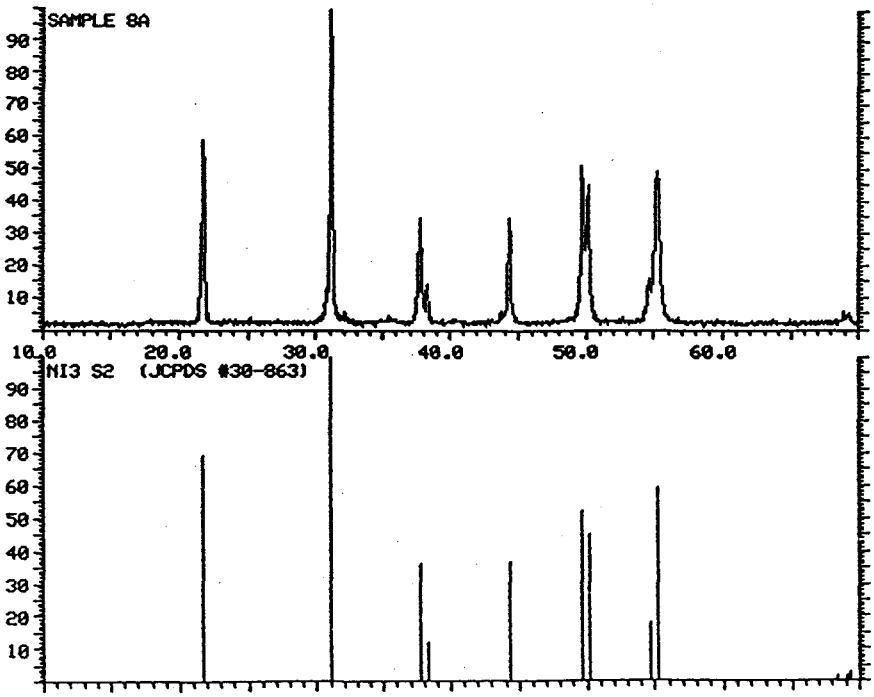


Figure 2-8: X-Ray Diffraction Pattern of Ni_3S_2 (Sample 8)

Table 4: $NiCl_2$ and Ni_3S_2 Toxicity in CHO Cells, 24 h Exposure[a]

$[NiCl_2]$ (µg/mL)	(µg Ni/mL)	% Survival avg	std	$[Ni_3S_2]$ (µg/mL)	(µg Ni/mL)	% Survival avg	std
0	0.0	100	0	0.0	0.0	100	0
10	4.5	104	10	0.5	0.4	103	15
20	9.1	101	9	1.0	0.7	96	6
30	13.6	78	16	2.0	1.5	84	20
40	18.1	77	10	3.0	2.2	79	26
50	22.6	74	8	4.0	2.9	65	25
60	27.2	71	8	5.0	3.7	50	26
70	31.7	63	15	6.0	4.4	48	35
80	36.2	51	1	7.5	5.5	41	25
90	40.8	45	0	10.0	7.3	23	14
100	45.3	30	7	15.0	11.0	12	4
120	54.3	26		20.0	14.7	8	2

[a]CHO cells, seeded at 250 cells per 60 mm diameter dish (αMEM/5% FBS) 1 d before treatment, were exposed to either $NiCl_2$ or Ni_3S_2 at the concentrations indicated for 24 h in serum free αMEM media. The cells were rinsed to remove extracellular nickel and then incubated in fresh medium (αMEM/5% FBS) until colonies formed (≈ 7-9 d). The % survival was determined by comparing the number of colonies counted to the number obtained in control (non-exposed) dishes. Averages are based on at least 3 replicate experiments (3 dishes per experiment per test condition) for each compound.

Figure 3: Nickel Toxicity Curves in CHO Cells

CHO cells, seeded at 250 cells per 60 mm diameter dish (αMEM/5% FBS) 1 d before treatment, were exposed to either $NiCl_2$ or Ni_3S_2 at the concentrations indicated for 24 h in serum free αMEM media. The cells were rinsed to remove extracellular nickel and then incubated in fresh medium (αMEM/5% FBS) until colonies formed (≈ 7-9 d). The % survival was determined by comparing the number of colonies counted to the number obtained in control (non-exposed) dishes. Averages are based on at least 3 replicate experiments (3 dishes per experiment per test condition) for each compound. These figures have been plotted from the data compiled in Table 4.

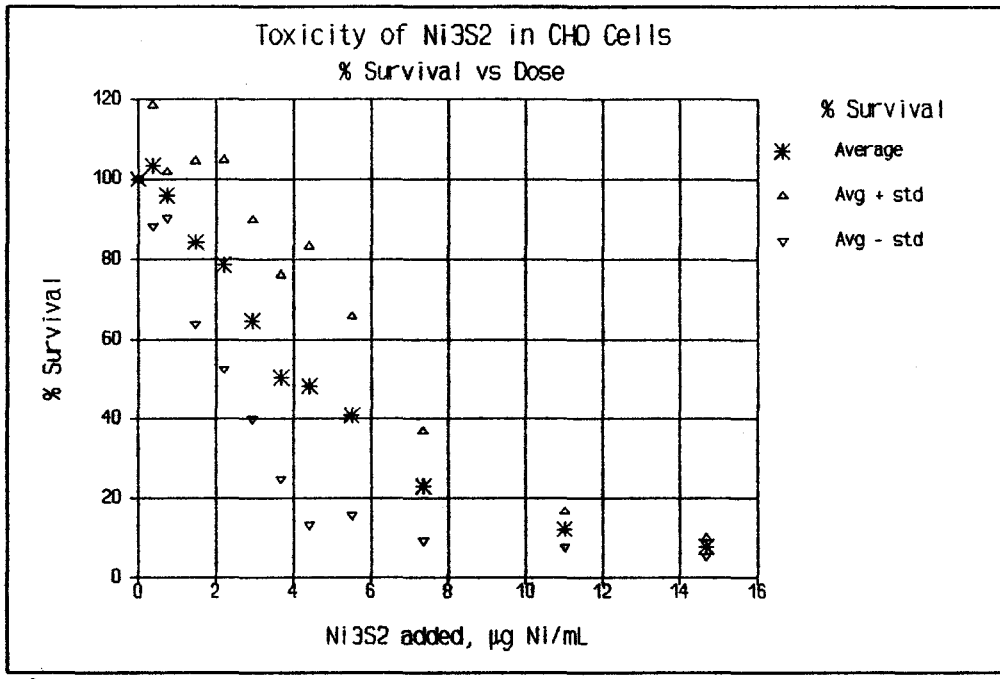


Figure 3-1

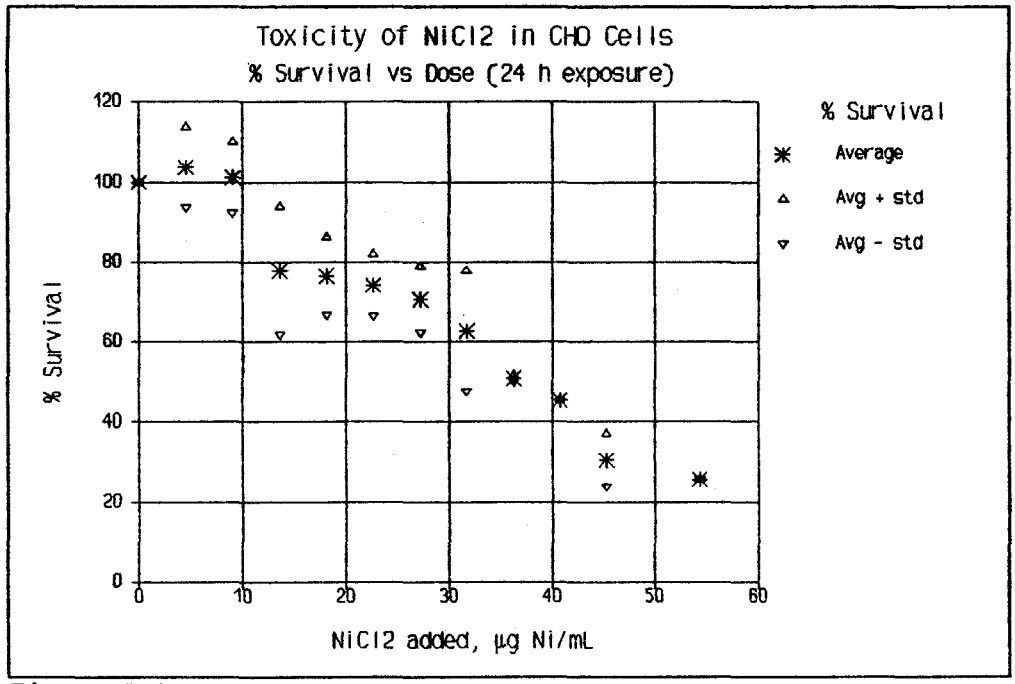


Figure 3-2

Table 5: Toxicity Testing of Nickel Compounds in AS52 Cells[a]

1. $Ni(OH)_2$

µg/mL	µg Ni/mL	% Survival average	std
0.0	0.0	100	0
1.0	0.6	83	5
2.0	1.1	68	3
4.0	2.2	47	26
5.5	3.1	39	28
8.0	4.4	28	19
10.0	5.6	20	16
12.0	6.7	12	9
14.0	7.8	7	4
16.0	8.9	7	6
20.0	11.1	3	1
25.0	13.9	0	

2. $NiCO_3$

µg/mL	µg Ni/mL	% Survival average	std
0	0.0	100	0
5	1.9	90	
10	3.9	69	7
15	5.8	47	
20	7.8	37	4
25	9.7	22	
30	11.7	18	6
40	15.6	10	5
50	19.5	9	6
60	23.3	4	2
70	27.2	7	4
80	31.1	4	1
100	38.9	3	1

3. Black NiO

µg/mL	µg Ni/mL	% Survival average	std
0.0	0.0	100	0
2.5	1.9	103	
5.0	3.9	98	
7.5	5.8	93	
10.0	7.8	86	10
15.0	11.7	73	
20.0	15.5	61	1
25.0	19.4	43	
30.0	23.3	28	6
40.0	31.1	21	12
50.0	38.9	11	5
60.0	46.6	9	5
70.0	54.4	7	7
80.0	62.2	3	2

4. Green NiO

µg/mL	µg Ni/mL	% Survival average	std
0	0.0	100	0
50	39.3	95	5
75	59.0	94	9
100	78.6	81	12
125	98.3	69	25
150	117.9	57	23
175	137.6	51	26
200	157.2	39	27
250	196.5	29	16
300	235.8	21	16
500	393.0	10	5

5. $Li_2Ni_8O_{10}$

µg/mL	µg Ni/mL	% Survival average	std
0	0.0	100	0
10	7.2	92	2
25	18.1	90	12
50	36.2	81	13
75	54.3	72	20
100	72.4	52	15
125	90.5	43	12
150	108.6	29	14
175	126.7	21	8
200	144.8	17	14
250	181.0	11	8

6. NiS, amorphous

µg/mL	µg Ni/mL	% Survival average	std
0.0	0.0	100	0
1.0	0.4	103	9
2.0	0.8	97	11
3.0	1.2	93	16
4.0	1.6	92	15
5.0	2.1	86	20
6.0	2.5	72	25
8.0	3.3	60	31
10.0	4.1	51	22
15.0	6.2	28	14
20.0	8.2	16	12
25.0	10.3	6	3

7. Ni_7S_6

µg/mL	µg Ni/mL	% Survival average	std
0.0	0.0	100	0
0.5	0.3	101	7
1.0	0.7	94	13
2.5	1.7	89	18
5.0	3.4	79	17
7.5	5.1	69	14
10.0	6.8	58	21
12.5	8.5	51	23
15.0	10.2	36	22
20.0	13.6	26	17
30.0	20.4	16	11
40.0	27.1	11	11
50.0	33.9	7	5

8. Ni_3S_2

µg/mL	µg Ni/mL	% Survival average	std
0.0	0.0	100	0
0.5	0.4	102	
1.0	0.7	90	1
1.5	1.1	87	
2.0	1.5	82	5
2.5	1.8	79	
3.0	2.2	66	
4.0	2.9	61	6
5.0	3.7	55	15
6.0	4.4	46	1
8.0	5.9	37	9
10.0	7.3	27	5
15.0	11.0	12	5
20.0	14.7	8	3
25.0	18.4	5	2

continued next page

Table 5: Toxicity Testing of Nickel Compounds in AS52 Cells (continued)[a]

9. $NiCl_2$ (5 h)

		% Survival	
mM	ug Ni/mL	average	std
0.00	0.0	100	0
0.50	29.4	94	10
0.75	44.0	90	15
1.00	58.7	86	12
1.50	88.1	78	15
2.00	117.4	64	26
2.50	146.8	49	17
3.00	176.1	44	24
3.50	205.5	24	8
4.00	234.8	32	27
5.00	293.5	20	16
7.50	440.3	5	

10. $NiSO_4$ (5 h)

		% Survival	
mM	ug Ni/mL	average	std
0.00	0.0	100	0
0.50	29.4	89	8
0.75	44.0	94	4
1.00	58.7	80	15
1.50	88.1	60	22
2.00	117.4	59	31
2.50	146.8	45	27
3.00	176.1	44	32
3.50	205.5	19	22
4.00	234.8	35	32
5.00	293.5	28	19
7.50	440.3	9	

11. Ni(CH3COO)2 (5 h)

		% Survival	
mM	ug Ni/mL	average	std
0.00	0.0	100	0
0.50	29.4	95	8
0.75	44.0	98	13
1.00	58.7	87	14
1.25	73.4	93	
1.50	88.1	61	23
2.00	117.4	57	28
2.50	146.8	23	
3.00	176.1	33	28
3.50	205.5	7	
4.00	234.8	26	38
5.00	293.5	11	16
7.50	440.3	2	

#12-14 (24 h)

		% Survival		
		#12	#13	#14
mM	ug Ni/mL	$NiCl_2$	$NiSO_4$	$Ni(CH_3COO)_2$
0.00	0.0	100	100	100
0.05	2.9	85	102	81
0.10	5.9	83	104	90
0.20	11.7	71	94	78
0.30	17.6		80	79
0.40	23.5	83	81	70
0.50	29.4	71	79	66
0.60	35.2	74	63	61
0.80	47.0	59	61	48
1.00	58.7	50	47	41
2.00	117.4	0	1	0
3.00	176.1	0	1	1

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[a]AS52 cells were seeded in F12/10% FBS medium at 500 cells per 60 mm diameter dish 2 d before treatment. The cells were exposed to the range of nickel compounds in serum free medium at the concentrations indicated for 24 h (or 5 h 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 (7-9 d). The % survival was determined by comparing the number of colonies counted to the number obtained in control (non-exposed) dishes. Averages are based on 3-5 replicate experiments (3 dishes per experiment per test condition) for each compound. Since all experiments may not have covered exactly the same range of nickel concentrations, the absence of a standard deviation indicates the that the corresponding dose was evaluated in a single experiment.

Figure 4: Nickel Toxicity Curves in AS52 Cells

AS52 cells were seeded in F12/10% FBS medium at 500 cells per 60 mm diameter dish 2 d before treatment. The cells were exposed to the range of nickel compounds in serum free medium at the concentrations indicated for 24 h (or 5 h 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 (7-9 d). The % survival was determined by comparing the number of colonies counted to the number obtained in control (non-exposed) dishes. Averages are based on 3-5 replicate experiments (3 dishes per experiment per test condition) for each compound. These figures have been plotted from the data compiled in Table 5.

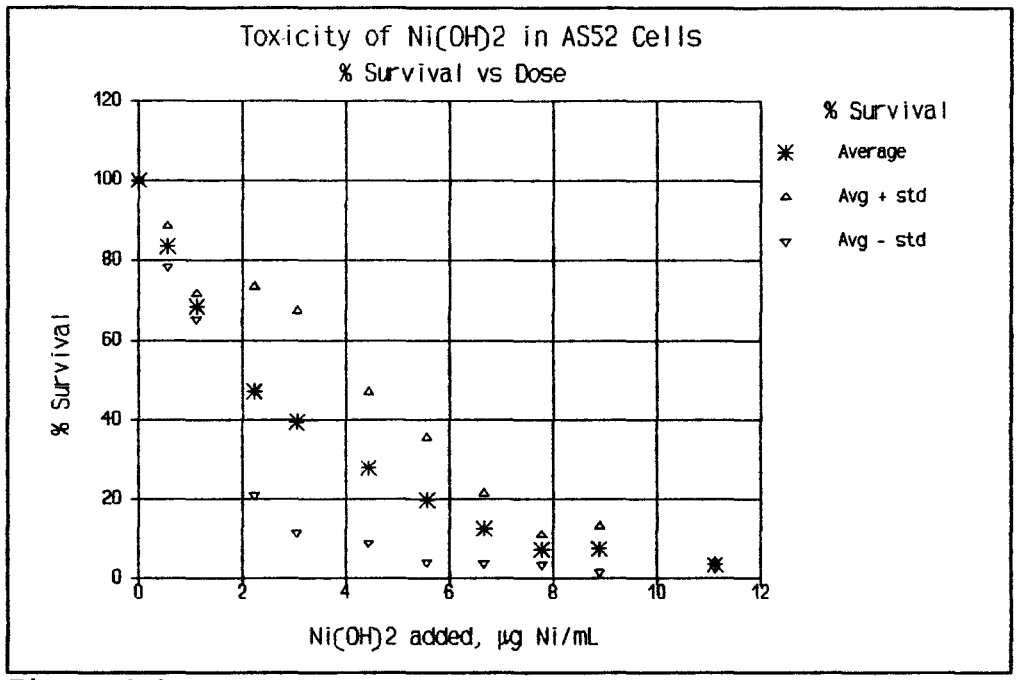

Figure 4-1

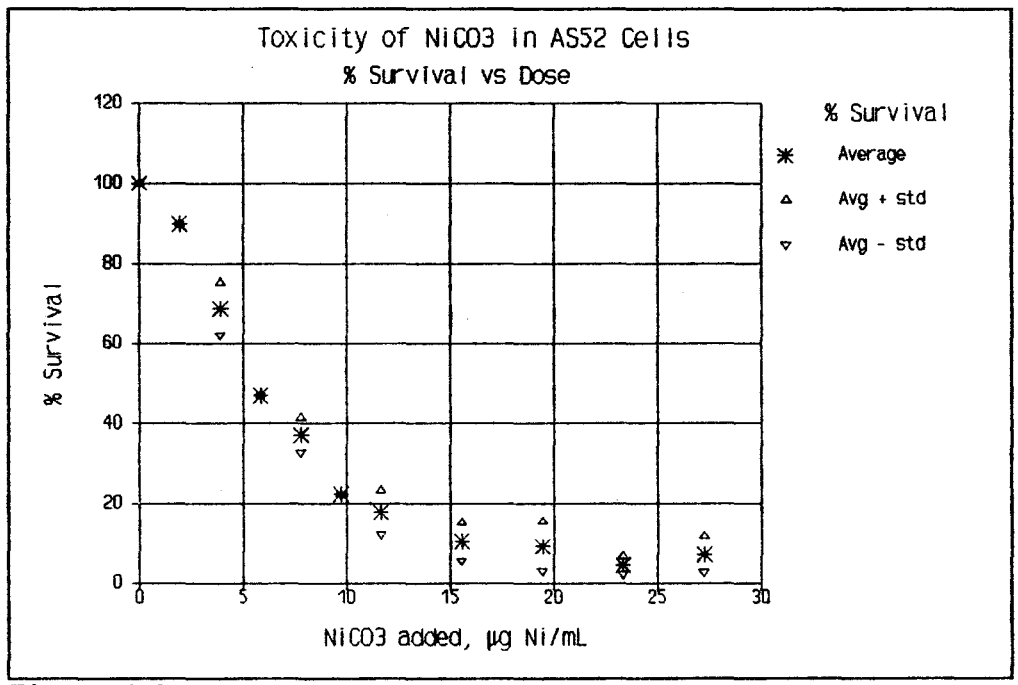

Figure 4-2

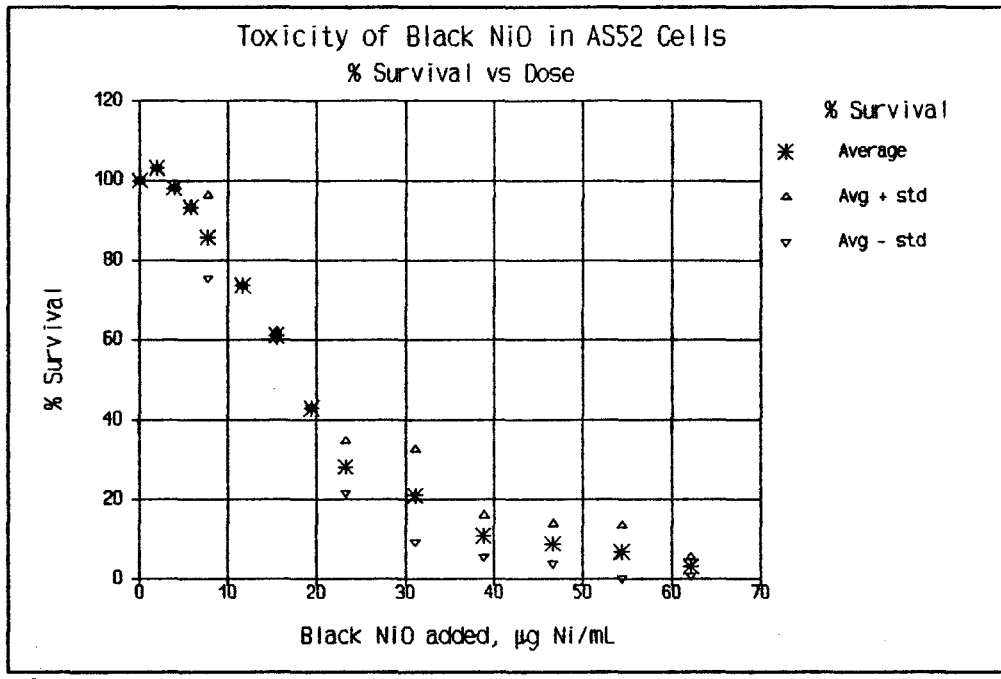

Figure 4-3

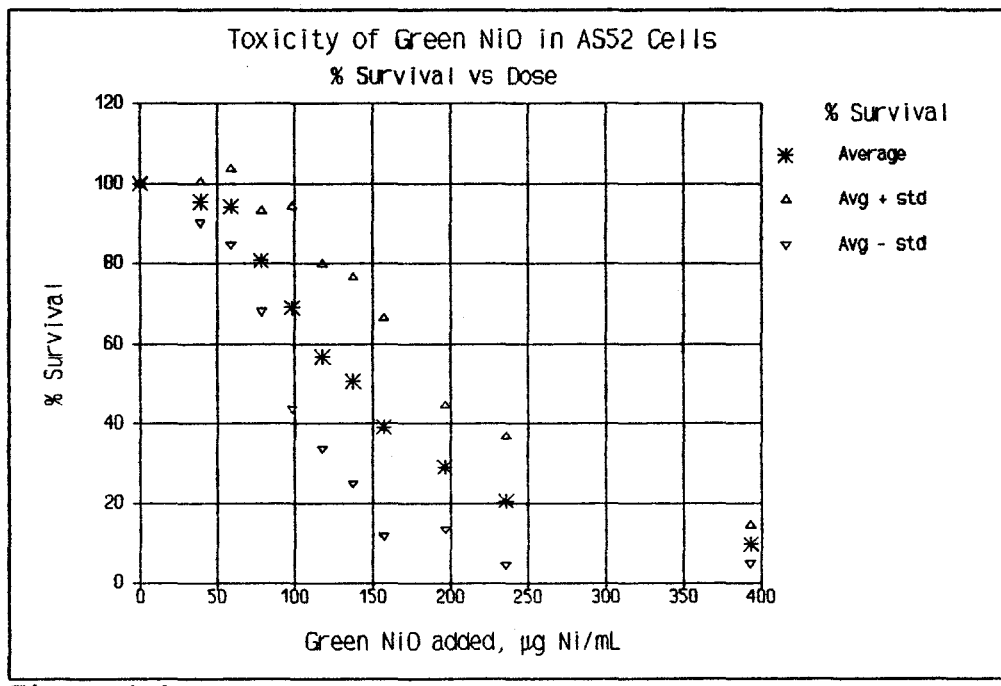

Figure 4-4

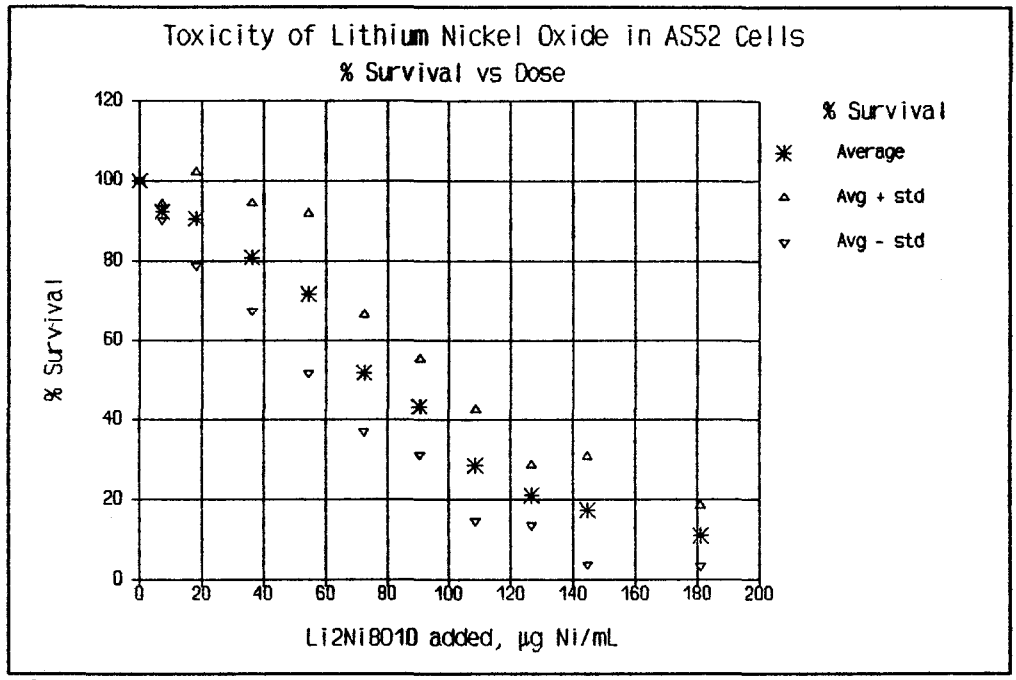

Figure 4-5

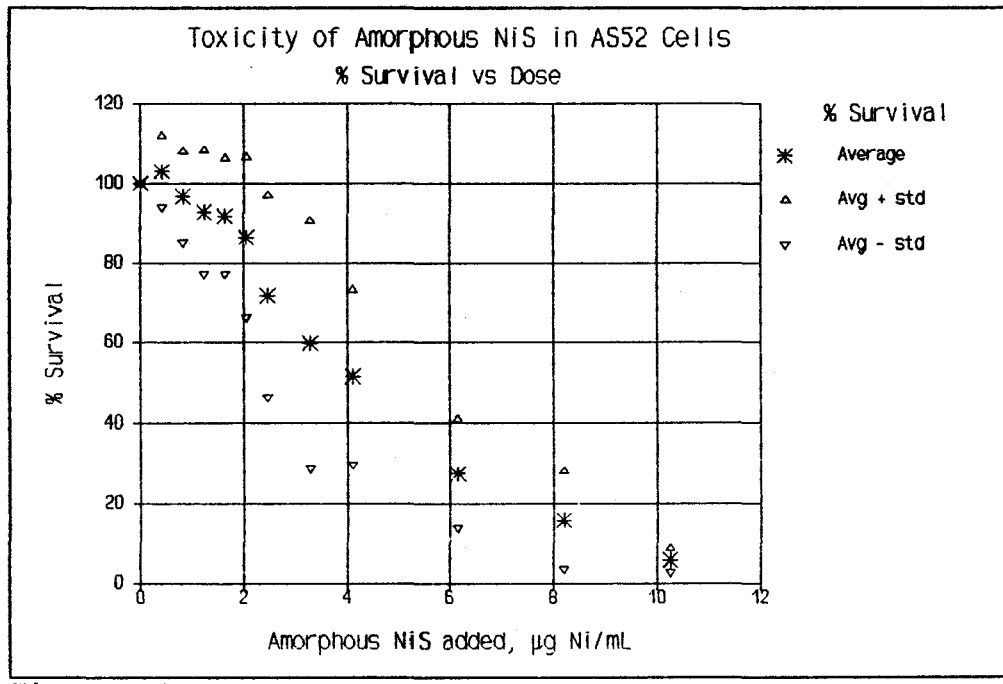

Figure 4-6

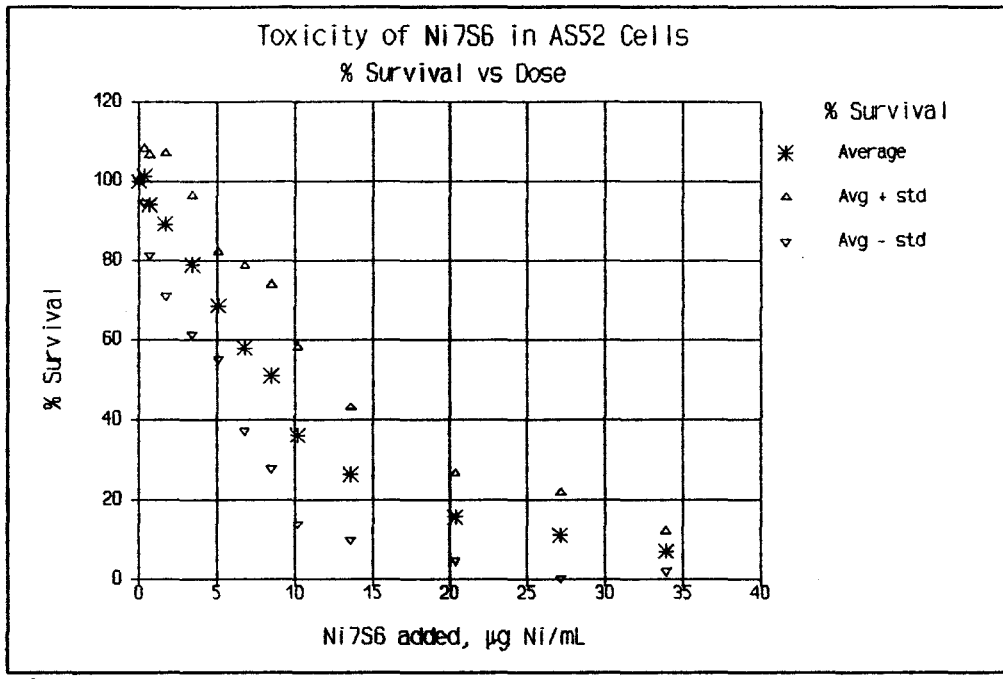

Figure 4-7

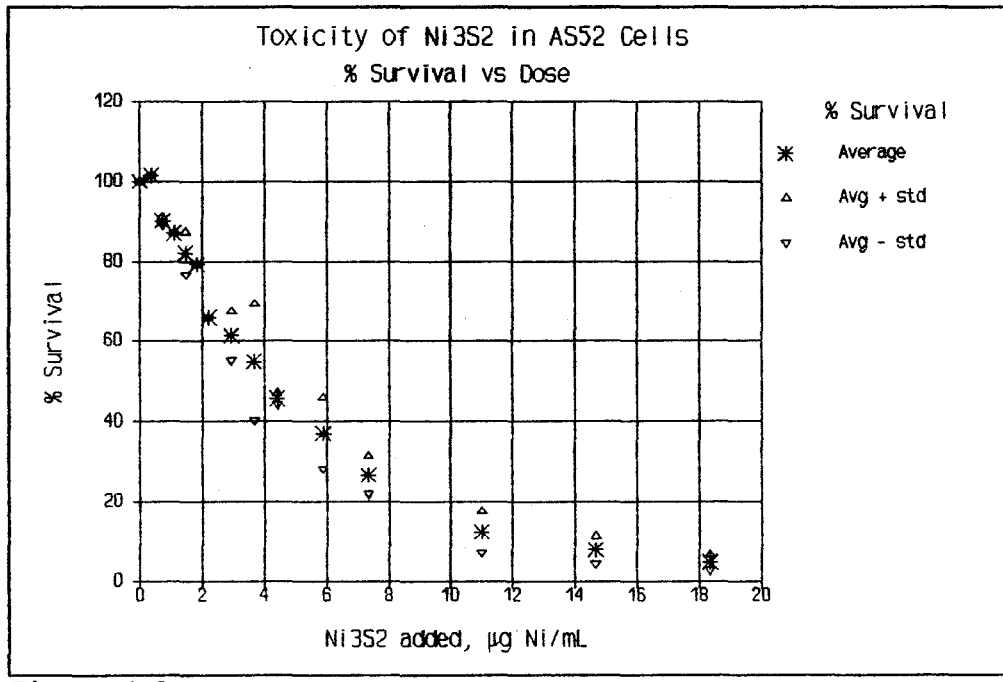

Figure 4-8

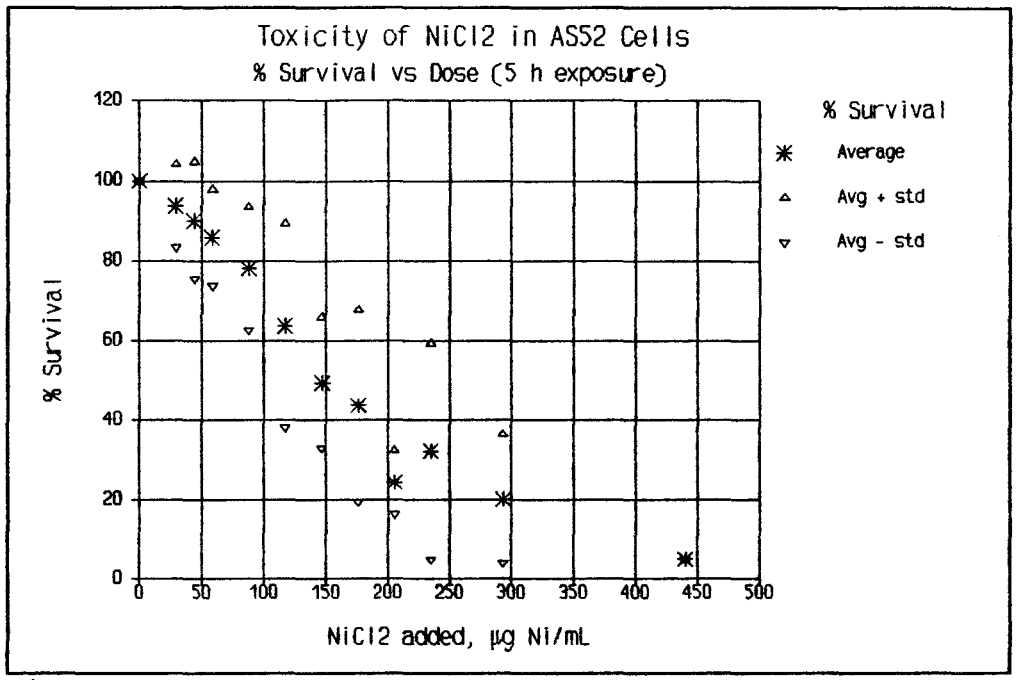

Figure 4-9

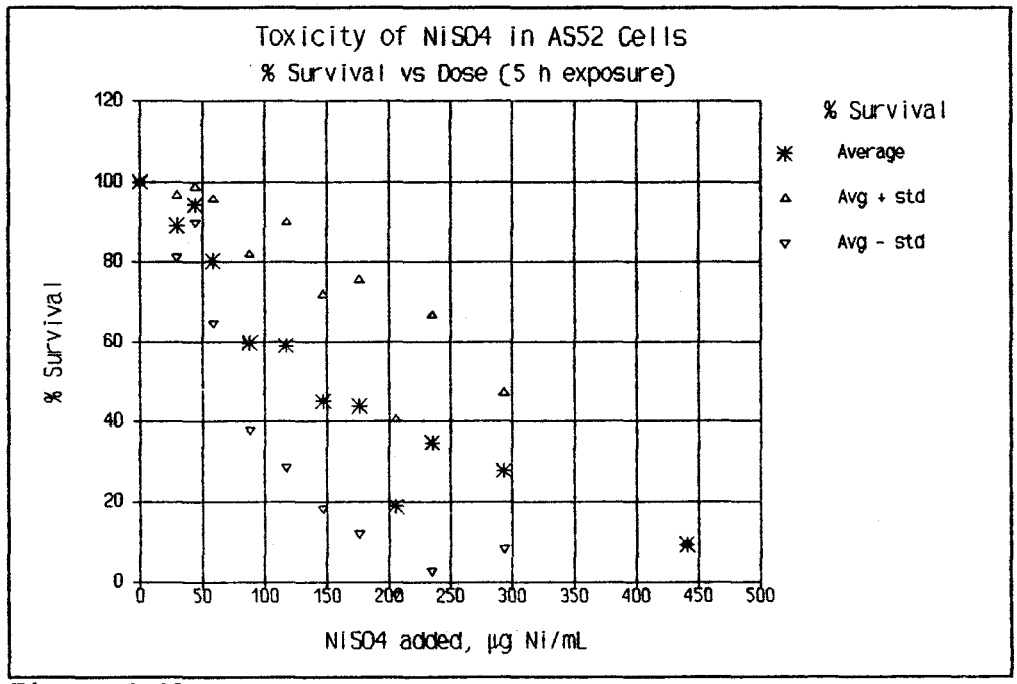

Figure 4-10

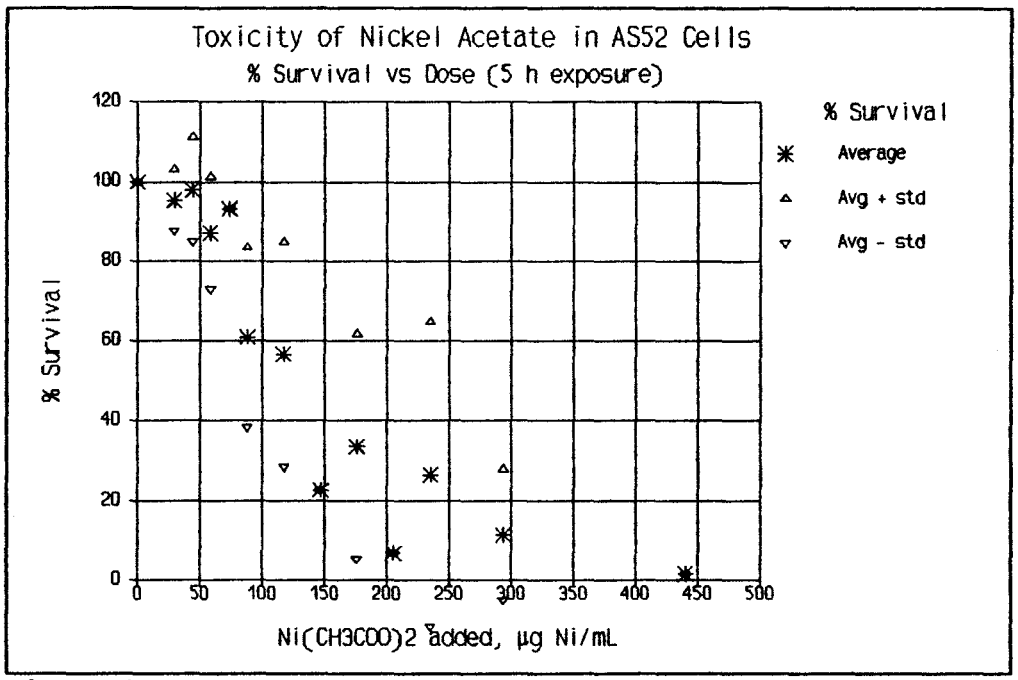

Figure 4-11

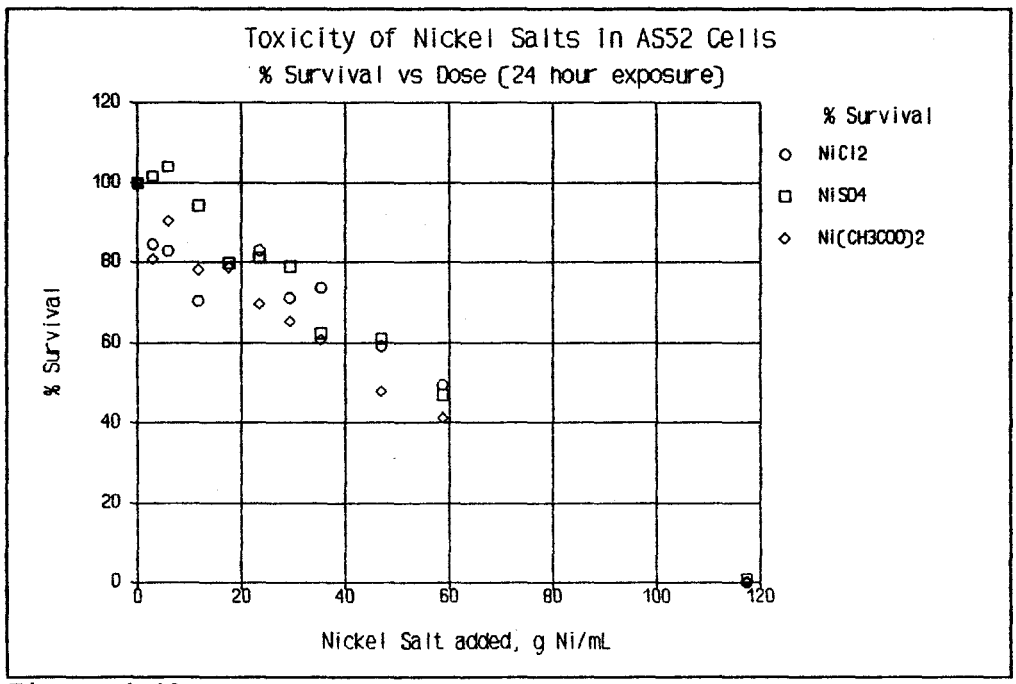

Figure 4-12

Table 6: Mutagenicity of Nickel Compounds in AS52 Cells, Set 1[g]

Compound	Label	Compound Added µg/mL	µg Ni/mL	Mutation Frequency	Confidence Interval 95% level[a]	Comparison with controls by t test[b] t		Dose-response correlation coefficient[a] r	
$Ni(OH)_2$	1 A	1.9	1.1	42	31 - 53	1.715	c	0.270	
	1 B	3.1	1.7	54	34 - 74	3.287	f		
	1 C	4.3	2.4	38	27 - 48	0.937			
	1 D	6.2	3.4						
	1 E	9.9	5.5	42					
$NiCO_3$	2 A	6.4	2.5	57	40 - 74	3.942	f	0.347	
	2 B	11.2	4.4	93	82 - 104	10.762	f		
	2 C	16.0	6.2	58	41 - 76	4.114	f		
	2 D	21.1	8.2	72	0 - 151	4.020	f		
	2 E	28.8	11.2	51	35 - 67	2.675	e		
NiO, black	3 A	12.2	9.5	22	5 - 40	-1.614		0.755	
	3 B	18.0	14.0	55	42 - 69	3.954	f		
	3 C	23.3	18.1	31	7 - 56	-0.172			
	3 D	28.5	22.2	30	19 - 42	-0.352			
	3 E	40.9	31.8	93	56 - 130	7.472	f		
NiO, green	4 A	108.6	85.3	66	43 - 89	4.817	f	-0.879	
	4 C	183.0	143.8	34	20 - 49	0.320			
	4 D	229.2	180.2	42	35 - 50	1.810	c		
	4 E	358.3	281.6	23	16 - 29	-1.829			
$Li_2Ni_8O_{10}$	5 A	52.2	37.8	98	65 - 132	7.839	f	0.532	
	5 B	83.3	60.3	28	15 - 41	-0.790			
	5 C	104.9	76.0	53	34 - 71	3.184	f		
	5 D	139.0	100.6	19	11 - 27	-2.505			
	5 E	201.4	145.8	42	10 - 74	1.281			
NiS, amorphous	6 A	4.4	1.8	22	2 - 43	-1.543		0.152	
	6 B	6.9	2.8	73	45 - 101	5.306	f		
	6 C	9.5	3.9	81	63 - 99	7.646	f		
	6 D	12.7	5.2	35	13 - 57	0.382			
	6 E	17.4	7.1		-				
Ni_7S_6	7 A	4.7	3.2	49	14 - 85	1.935	c	0.716	
	7 B	8.3	5.7	44	19 - 70	1.631			
	7 C	12.7	8.6	28	4 - 52	-0.603			
	7 D	15.5	10.5	25	15 - 35	-1.334			
	7 E	28.4	19.3	29	0 - 174	-0.434			
Ni_3S_2	8 A	2.1	1.6	38	23 - 54	1.002		0.580	
	8 B	4.1	3.0	74	42 - 106	5.072	f		
	8 C	5.9	4.3	46	32 - 59	2.264	d		
	8 D	8.5	6.2	61	7 - 115	3.440	f		
	8 E	12.4	9.1	73	59 - 86	5.810	f		
positive controls	EMS A	50		66	54 - 78	5.452	f	0.880	c
	EMS B	100		117	79 - 156	9.262	f		
	EMS C	200		288	79 - 498	26.909	f		
	EMS D	300		213	0 - 664	14.046	f		
	EMS E	400		345	0 - 2060	8.217	f		
negative controls (no addition)	0 A			31	22 - 40	-0.247			
	0 B			28	15 - 41	-0.716			
	0 C			37	21 - 54	0.802			
	0A-0E			32	26 - 39	0.000			

[a] two-tailed significance test
[b] one-tailed significance test
[c] significant at the .05 probability level
[d] significant at the .025 probability level
[e] significant at the .01 probability level
[f] significant at the .005 probability level
[g] See Table 7 for description of experiment.

Table 7: Mutagenicity of Nickel Compounds in AS52 Cells, Set 2[g]

Compound	Label	Compound Added µg/mL	µg Ni/mL	Mutation Frequency	Confidence Interval 95% level[a]	Comparison with controls by t test[b] t		Dose-response correlation coefficient[a] r	
$Ni(OH)_2$	1 A	1.9	1.1	23	10 - 37	-1.126		0.974	e
	1 B	3.1	1.7	50	41 - 60	3.203	f		
	1 C	4.3	2.4	42	26 - 57	1.689			
	1 D	6.2	3.4	71	52 - 90	6.184	f		
	1 E	9.9	5.5	121	94 - 148	12.566	f		
$NiCO_3$	2 A	6.4	2.5	34	25 - 44	0.516		0.626	
	2 B	11.2	4.4	20	7 - 33	-1.831			
	2 C	16.0	6.2	27	12 - 43	-0.634			
	2 D	21.1	8.2	86	56 - 117	7.346	f		
	2 E	28.8	11.2	57	33 - 81	3.736	f		
NiO, black	3 A	12.2	9.5	48	38 - 57	2.799	f	0.695	
	3 B	18.0	14.0	30	8 - 52	-0.185			
	3 C	23.3	18.1	44	34 - 54	2.176	d		
	3 D	28.5	22.2	28	17 - 39	-0.609			
	3 E	40.9	31.8	99	76 - 122	9.928	f		
NiO, green	4 A	108.6	85.3	41	28 - 55	1.643		0.826	
	4 C	183.0	143.8	50	25 - 74	2.647	e		
	4 D	229.2	180.2	44	28 - 59	1.960	c		
	4 E	358.3	281.6	55	26 - 84	3.198	f		
$Li_2Ni_8O_{10}$	5 A	52.2	37.8	33	22 - 43	0.211		0.822	
	5 B	83.3	60.3	51	42 - 59	3.323	f		
	5 C	104.9	76.0	31	17 - 45	-0.030			
	5 D	139.0	100.6	44	23 - 64	1.868	c		
	5 E	201.4	145.8	81	72 - 91	8.540	f		
NiS, amorphous	6 A	4.4	1.8	21	12 - 31	-1.724		0.953	c
	6 B	6.9	2.8	39	27 - 51	1.239			
	6 C	9.5	3.9	33	29 - 38	0.371			
	6 D	12.7	5.2	72	48 - 96	5.903	f		
	6 E	17.4	7.1	127	80 - 173	10.068	f		
Ni_7S_6	7 A	4.7	3.2	53	37 - 69	3.523	f	0.623	
	7 B	8.3	5.7	18	5 - 31	-2.238			
	7 C	12.7	8.6	41	24 - 59	1.542			
	7 D	15.5	10.5	39	5 - 73	0.983			
	7 E	28.4	19.3	70	60 - 81	6.621	f		
Ni_3S_2	8 A	2.1	1.6	40	23 - 56	1.317		0.932	c
	8 B	4.1	3.0	37	21 - 52	0.894			
	8 C	5.9	4.3	51	30 - 72	2.984	f		
	8 D	8.5	6.2	64	49 - 79	5.288	f		
	8 E	12.4	9.1	67	48 - 87	5.555	f		
positive controls	EMS A	50		178	150 - 205	20.329	f	0.969	e
	EMS B	100		325	305 - 345	44.998	f		
	EMS C	200		449	417 - 480	54.410	f		
	EMS D	300		966	931 - 1000	116.356	f		
	EMS E	400		1035	945 - 1125	73.416	f		
negative controls (no addition)	0 A			31	18 - 44	-0.077			
	0 B			33	14 - 51	0.222			
	0 C			38	27 - 49	1.154			
	0 D			37	25 - 49	0.955			
	0 E			18	12 - 24	-2.358			
average	0A-0E			31	26 - 36	0.000			

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Table 7: Mutagenicity of Nickel Compounds in AS52 Cells, Set 2 (continued)[g]

Compound	Label	Compound Added mM	Compound Added µg Ni/mL	Mutation Frequency	Confidence Interval 95% level[a]	Comparison with controls by t test[b] t	Dose-response correlation coefficient[a] r
$NiCl_2$ 5 hours	9 A	1.0	58.7	38	29 - 47	1.157	0.130
	9 B	2.0	117.4	90	61 - 119	7.977 [f]	
	9 C	3.0	176.1	100	74 - 125	9.773 [f]	
	9 D	4.0	234.8	16	6 - 26	-2.645	
	9 E	5.0	293.5	61	43 - 79	4.621 [f]	
$NiSO_4$ 5 hours	10 A	1.0	58.7	62	51 - 73	5.180 [f]	0.570
	10 B	2.0	117.4	34	24 - 44	0.427	
	10 C	3.0	176.1	74	51 - 98	6.270 [f]	
	10 D	4.0	234.8	65	49 - 81	5.408 [f]	
	10 E	5.0	293.5	78	60 - 95	7.321 [f]	
$Ni(CH_3COO)_2$ 5 hours	11 A	1.0	58.7	13	4 - 22	-3.125	0.632
	11 B	2.0	117.4	33	15 - 51	0.282	
	11 C	3.0	176.1	73	56 - 90	6.587 [f]	
	11 D	4.0	234.8	22	11 - 34	-1.534	
	11 E	5.0	293.5	78	67 - 89	7.848 [f]	
$NiCl_2$ 24 hours	12 A	0.25	14.7	90	70 - 109	8.948 [f]	0.246
	12 B	0.50	29.4	24	13 - 36	-1.182	
	12 C	0.75	44.0	38	18 - 57	0.951	
	12 D	1.00	58.7	19	13 - 25	-2.202	
	12 E	1.25	73.4	69	62 - 76	6.479 [f]	
$NiSO_4$ 24 hours	13 A	0.25	14.7	50	36 - 65	3.110 [f]	0.886 [c]
	13 B	0.50	29.4		-		
	13 C	0.75	44.0	56	39 - 72	3.862 [f]	
	13 D	1.00	58.7	59	27 - 90	3.573 [f]	
	13 E	1.25	73.4	57	25 - 89	3.295 [f]	
$Ni(CH_3COO)_2$ 24 hours	14 A	0.25	14.7	28	17 - 39	-0.558	0.637
	14 B	0.50	29.4	40	25 - 54	1.348	
	14 C	0.75	44.0	30	19 - 40	-0.254	
	14 D	1.00	58.7	76	54 - 98	6.659 [f]	
	14 E	1.25	73.4	49	35 - 63	2.916 [f]	

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[a] two-tailed significance test
[b] one-tailed significance test
[c] significant at the .05 probability level
[d] significant at the .025 probability level
[e] significant at the .01 probability level
[f] significant at the .005 probability level

[g] AS52 cells grown in MPA medium to eliminate spontaneous mutations and/or loss of the *gpt* gene were plated in F12/10% FBS 2 d ($\approx 5*10^5$ cells/100 mm dish) prior to treatment. Cells were exposed to the test compounds for 24 h (or 5 h for nickel salts as indicated) in serum free medium, then rinsed, trypsinized, and replated in F12/10% FBS. The cells were subcultured ≈every 3 d 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 10-15 d, dishes were rinsed with PBS and colonies stained and counted. The mutation frequency is expressed as the number of mutant colonies per million surviving cells (based on the number of cells plated corrected for plating efficiency at the time of selection and refers to a plating efficiency of 50%). Results are reported in this table for a single experiment based on 5-6 dishes per treatment condition with further replicates for negative controls as indicated. The previous table (Table 6) shows results from a separate but identical experiment.

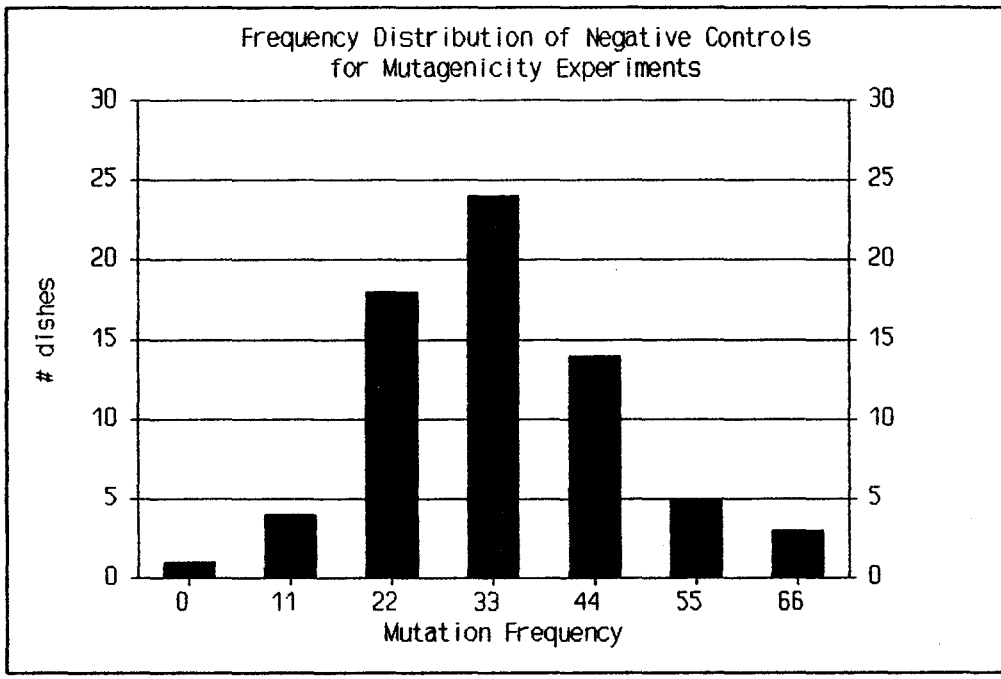

Figure 5

Figure 5: Frequency Distribution of Negative Controls for Mutagenicity Experiments

Mutation frequencies at the *gpt* locus in AS52 cells per 10^6 cells were determined for negative control cultures. The distribution of results for 3 experiments is shown.

C. Mutagenicity of Nickel Compounds

Mutagenicity results involving selection at the *gpt* locus in AS52 cells are given in Tables 6 and 7 for two separate experiments. The mutation frequency represents the number of mutations observed per million cells plated, with a correction for the plating efficiency (PE) of the cells in non-selective medium at the time of mutant selection. Since the maximum plating efficiency was approximately 50%, all results were normalized to this level [(mutation frequency) = (observed mutation frequency)*50/PE]. The statistical significance of the increase in mutation frequency after nickel exposure was assessed by comparison to the negative control data (no nickel added). As indicated in Figure 5, the mutation frequency of non-exposed cells appears to adhere to a normal statistical distribution. Since the mutation frequency reported is based on the average of 5-6 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 Tables 6 and 7. A comparison of the mean mutation frequency with that of non-exposed (negative) control cultures using the one-tailed Student 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 concentration and the induced mutation rate. For this comparison, the correlation coefficient (r) was calculated and the significance determined using standard two-tailed statistical tables.

D. Cellular, Cytosolic, and Nuclear Nickel Content of Cells

Initial analytical experiments involved measurements of the total cell-associated nickel content of CHO cells after exposure to $NiCl_2$ or Ni_3S_2. Using HNO_3 to digest $NiCl_2$ exposed cells and to release the nickel, the effect of the cell number used in the collection and digestion procedure was investigated; results are given in Table 8. At the lowest cell number (10^5 cells), it appeared recovery was incomplete, while using $4x10^5$ to $3x10^6$ cells seemed appropriate and was confirmed in further experiments. In another experiment (Table 9), the levels of nickel in the growth medium to which the nickel had been added, two saline rinses of the cells adhering to the plate, the trypsin/medium mixture after removing the cells from the plate and pelleting them, and the cells themselves were all analyzed. The sharp decrease in nickel with progressive steps indicate that the two rinses were sufficient to remove extraneous $NiCl_2$ not taken up by the cells.

In Table 10 is shown the cellular nickel content for several doses of $NiCl_2$ and Ni_3S_2. The average and standard deviation have been calculated based on 3 or more experiments for each compound. Note that for Ni_3S_2 there were some particles associated with the exterior of the cells that could not be removed in this procedure, as well as phagocytized material. These nickel sources make a large contribution to the analyzed nickel levels.

Results for one experiment involving nuclear isolation in CHO cells after Ni_3S_2 exposure are presented in Table 11. The cytosolic nickel content was determined, with the cytosol defined as the fraction not

pelleted after membrane disruption and release of nuclei from the cells. This fraction would represent dissolved Ni_3S_2 in the cytosol, since any particulates would pellet down with the nuclei. The pellet, consisting of nuclei plus Ni_3S_2 particles was also analyzed. Results from analysis of nuclei after attempting to separate the particles from the nuclei using a sucrose pad are also given. Due to difficulty in recovering the nuclei and some sucrose interference with the nickel analysis, not too much weighting should be placed on these data. Nevertheless, a rough mass balance is evident.

At this point in time, AS52 cells were received, and all subsequent fractionation and analysis were done using these cells. Due to low recovery of nuclei, the procedure was revised and the yield and purity of the nuclei investigated. Observation using both light and fluorescence microscopy indicated a high yield of pure nuclei, free from cytoplasmic contamination. Under the fluorescent microscope, acridine orange stained nuclei were initially seen as bright orange spheres against a dark background. Upon standing, the nuclei started to fluoresce green instead of orange. Cytoplasmic contamination, expected to exhibit red fluorescence, was vary sparse, appearing on only one nucleus in the field and for a few very small specks between nuclei. Rinsing of the nuclei after isolation and before staining eliminated all red cytoplasmic fluorescence. The procedure was therefore deemed to produce nuclei of sufficient purity for nickel analysis.

A comparison is shown in Table 12 of nickel contents at various stages of the cell fractionation procedure, both in controls (no exposure)

and in samples exposed to $NiCl_2$ or Ni_3S_2 at levels giving approximately 50% survival. Nickel in the exposure medium (containing added nickel), 3 rinses of cells attached to the culture dishes, intact cell suspension (trypsin + cells), rinses of the cells, cell cytosol, nuclear pellet (includes particles for Ni_3S_2 exposure) and nuclei free of Ni_3S_2 particles. As noted for the previously reported experiment with CHO cells, it was not possible to obtain a clear separation of nuclei and Ni_3S_2 particles with satisfactory levels of nuclear recovery. Further modifications were thus required at this stage in the procedure.

It was decided that separation of nuclei and particles was not possible at this point. Since we were concerned with the free or available nickel levels, and it has been shown that no nickel particles are contained in the nuclei, it was decided to change the approach and measure only the dissolved nickel content of the nuclei. A solution of SDS plus EDTA in a HEPES buffered solution, as used in chromosomal isolation (F Rossetto, personal communication), was used to disrupt nuclei and release dissolved nickel from the chromosomes/proteins. Particles were pelleted by centrifugation and supernatant analyzed for nickel. Concern about the possibility of EDTA affecting dissolution of particles still present led to consideration of other methods of nuclear disruption and nickel release. Use of DNAase I, followed by addition of SDS plus Proteinase K to disrupt membranes and proteins was thus decided on. A comparison of these methods is given in Table 13, in which green NiO or Ni_3S_2 particles were treated under the conditions used in the two disruption methods. The EDTA procedure was found to cause significantly

more dissolution of the particulates, while short incubations with DNAase I plus Proteinase K conditions gave much lower dissolution. This second method was thus chosen for nuclear nickel determinations. Table 14 summarizes cytosolic (average of 4 experiments) and nuclear (average of 2 experiments) nickel levels (present as Ni^{2+}) employing the optimum isolation and disruption procedures.

E. Correlation of Toxicity, Mutagenicity, and Nickel Content

Combined data from the experiments discussed above are presented in Figures 6-1 to 6-14. Toxicity (% survival), mutation frequency, and cytosolic and nuclear nickel levels are all plotted as a function of the concentration of nickel added to the culture medium in which the cells were growing. From the toxicity curves, the LC_{50} values (exposure concentration lethal to 50% of the cells plated, ie giving 50% survival) have been determined for each compound and are tabulated in Table 15. To allow comparison, the cytosolic and nuclear nickel levels corresponding to the LC_{50} values have been evaluated from the data in Figures 6-1 to 6-14 and are included in Table 15.

Table 8: Effect of Cell Number on Analysis of Total Cell-Associated Nickel[a]

$[NiCl_2]$ (µg/mL)	µg Ni/mL	Cells in sample	ng Ni/10^6 cells
25	11.3	1x105	14.5
"	11.3	4x105	22.9
"	11.3	7x105	22.7
"	11.3	1x106	30.6
"	11.3	2x106	28.3
"	11.3	3x106	26.8

[a]CHO cells exposed to $NiCl_2$ for 24 h were rinsed, trypsinized, and counted. The number of cells indicated was treated with ≈ 1N HNO_3 to release the nickel, followed by AA analysis.

Table 9: Investigation of Nickel Levels in Cell Rinses[a]

		[Ni] measured (µg Ni/L)				
$[NiCl_2]$ (µg/mL)	µg Ni/mL	Growth Medium	Rinse 1 (HBS)	Rinse 2 (HBS)	medium + trypsin	ng Ni/ 10^6 cells
0	0.0	2172	0.4	2.0	3.4	10
25	11.3	8647	6.7	9.2	0.0	71
50	22.6	14886	9.0	21.0	5.1	176
75	34.0	24994	19.1	28.6	0.0	239
100	45.3	32733	138.8	41.0	0.0	359
no cells				0.0	2.1	

[a]CHO were exposed to $NiCl_2$ for 24 h. The exposure medium and 2 saline rinses of the cells were collected for analysis. After trypsinization the cells were counted and then pelleted by centrifugation. The supernatant (medium + trypsin) and the cell pellet were analyzed for nickel content.

Table 10: Total Cell-Associated Nickel for CHO Cells Exposed to $NiCl_2$ or Ni_3S_2 for 24 h[a]

[$NiCl_2$] (µg/mL)	µg Ni/mL	µg Ni/10^6 cells Average[b]	Std	Range
0	0.0	0.003	0.004	(0.000 - 0.010)
10	4.5	0.015	0.001	(0.014 - 0.015)
20	9.1	0.039	0.016	(0.028 - 0.051)
30	13.6	0.048	0.044	(0.017 - 0.079)
40	18.1	0.045	0.042	(0.015 - 0.075)
50	22.6	0.111	0.075	(0.050 - 0.209)
60	27.2	0.163	0.168	(0.044 - 0.282)
70	31.7	0.253	0.134	(0.158 - 0.347)
80	36.2	0.268		
90	40.8	0.590		
100	45.3	0.325	0.284	(0.101 - 0.713)
120	54.3	1.148		

[Ni_3S_2] (µg/mL)	µg Ni/mL	µg Ni/10^6 cells Average[c]	Std	Range
0.0	0.0	0.00	0.00	(0.00 - 0.01)
0.1	0.1	0.10	0.10	(0.03 - 0.17)
0.5	0.4	0.26	0.35	(0.05 - 0.67)
1.0	0.7	0.97	0.71	(0.47 - 1.47)
1.5	1.1	0.87	0.92	(0.22 - 1.52)
2.0	1.5	1.70		
2.5	1.8	2.32	1.46	(1.29 - 3.36)
3.0	2.2	1.76	1.92	(0.40 - 3.11)
4.0	2.9	5.01		
5.0	3.7	4.78	3.95	(1.98 - 7.58)
6.0	4.4	3.84	4.54	(0.63 - 7.05)
8.0	5.9	10.47		
10.0	7.3	9.83	3.38	(7.44 - 12.23)
15.0	11.0	22.41		
20.0	14.7	29.97		

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[a]CHO cells were exposed to $NiCl_2$ or Ni_3S_2 for 24 h, rinsed, collected, and treated with HNO_3 to disrupt cells and release the cell associated nickel. After centrifugation the nickel content of the supernatant was determined by AA.

[b]Correlation coefficient, $NiCl_2$ added versus nickel measured, is $r = .837$, $p < 0.01$

[c]Correlation coefficient, Ni_3S_2 added versus nickel measured, is $r = .981$, $p < 0.01$

Table 11: Investigation of Nickel Content of CHO Cell Fractions[a]

[Ni_3S_2] (μg/mL)	μg Ni/mL	μg Ni/10^6 cells or nuclei			
		Whole Cells	Cytosol	Pellet	Nuclei
0	0.00	0.007	0.001	0.002	0.002
1	0.73	0.348	0.008	0.229	0.085
10	7.34	3.810	0.203	2.828	0.890

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[a]CHO cells exposed to Ni_3S_2 for 24 h were rinsed, collected, and counted. Cells were suspended in hypotonic buffer and lysed with a Triton X-100/Triton X-114 solution. After centrifugation the supernatant (cytosol) and the pellet (nuclei + Ni_3S_2 particles) were analyzed for nickel content. Separation of the nuclei and particulates was attempted by centrifugation at 50000g for 15 min on a 97% sucrose pad. The nickel content of the resultant supernatant, referred to as nuclei in the table, was determined.

Table 12: Distribution of $NiCl_2$ and Ni_3S_2 in Media, Rinses, Cells, and Nuclei[b]

		Control (no nickel)		$NiCl_2$, 5 h (1.5 mM)		$NiCl_2$, 5 hours, then 19 h incubation (1.5 mM)		Ni_3S_2, 24 h (5 µg/mL)	
	Fraction[a]	total nickel (µg)	ng Ni/ 10^6 cells or nuclei	total nickel (µg)	ng Ni/ 10^6 cells or nuclei	total nickel (µg)	ng Ni/ 10^6 cells or nuclei	total nickel (µg)	ng Ni/ 10^6 cells or nuclei
1	Exposure medium	0.167		6170.46		5622.37		842.38	
2	F12 Rinse #1	0.071		296.42		4.96		435.89	
3	F12 Rinse #2	0.071		12.83		14.71		0.46	
4	PBS Rinse	0.445		1.68		0.00		0.25	
5	Trypsin + cells	0.095	4.0	0.68	53.6	0.47	32.4	17.34	996.5
6	Trypsin supernatant	0.128		0.42		0.00		0.70	
7	Supernatant of cell pellet	0.092		0.20		0.16		0.91	
8	cell cytosol	0.012	0.6	1.17	103.1	0.60	42.1	3.20	213.2
9	Rinse of nuclear pellet	0.008		0.07		0.03		0.24	
10	Nuclei	0.009	0.4	0.09	8.3	0.05	3.6	19.14	1272.3
11	Nuclei after Sucrose	0.127	6.1					1.77	120.3

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[a]1. Residual nickel in the exposure medium after exposure
2-4. Nickel in cell rinses
5. Total cell nickel (cell-associated nickel plus nickel in trypsin/medium)
6. Nickel in trypsin/medium after removal of cells
7. Nickel in rinse of intact cell pellet
8. Nickel in lysing solution after cell disruption (non-particulate nickel in cytoplasm)
9. Nickel in rinse of intact nuclear pellet (pellet = nuclei + particulates of nickel compounds)
10. Nickel in nuclear pellet (nuclei + particulates of nickel compounds)
11. Nickel in nuclei (after removal of particulates of nickel compounds)

[b]AS52 cells were exposed to $NiCl_2$ or Ni_3S_2 for the time indicated. The above rinses and fractions were collected and the nickel content determined.

Table 13: Particle Dissolution During DNAse I or EDTA Treatment[a]

		Ni dissolved in suspension (mg/L)			
		Green NiO		Ni_3S_2	
		Avg	std	Avg	std
Add DNase I buffer, incubate, add SDS/HEPES					
A	no incubation	0.024	0.004	1.70	0.07
B	30 min @ RT then 60 min @ RT	0.031	0.002	1.06	0.12
C	10 min @ 37°C then 20 min @ 60°C	0.047	0.009	1.52	0.06
D	30 min @ 37°C then 60 min @ 60°C	0.069	0.014	4.14	1.65
E	60 min @ 37°C then 120 min @ 60°C	0.052	0.018	10.71	1.35
Add SDS/HEPES/EDTA					
F	60 min @ 60°C	0.141	0.045	30.12	7.20
G	180 min @ 60°C	0.150	0.070	34.79	9.60

[a]200-300 µL aliquots of green NiO (≈ 1.8 mg/tube) or Ni_3S_2 (≈ 0.9 mg/tube) stock solution in F12/5% FBS were placed into replicate test tubes for each of the above conditions. Two mL of hypotonic buffer was added, the suspension mixed by vortexing, and then the particles were immediately pelleted by centrifugation. For conditions A-E, 1 mL of hypotonic buffer was added, the particles vortexed, and the suspension incubated at RT (room temperature) or 37°C for the first time period indicated. An additional 1 mL of 10 mM HEPES/0.8% SDS solution was added and the tubes were vortexed and incubated for the second time period at RT or 60°C. For conditions F and G, 2 mL of lysis buffer consisting of 10 mM HEPES/40 mM EDTA/0.4% SDS was added and then tubes were vortexed and incubated at 60°C. At the end of the incubation period the tubes were centrifuged (2000g for 10 minutes) and the supernatant analyzed for dissolved nickel.

Table 14: Nickel Content in the Cytosol and Nuclei of AS52 Cells[c]

Compound	Label	Compound Added µg/mL	Compound Added µg Ni/mL	Cytosol µg Ni/10^6 cells Average	Cytosol Std	Cytosol Range	Nuclei µg Ni/10^6 cells Average	Nuclei Std	Nuclei Range	Correlation Coefficient (R) Cytosol	Nuclei
$Ni(OH)_2$	1 A	1.9	1.1	0.088	0.035	(0.059 - 0.135)	0.113	0.065	(0.067 - 0.159)	0.999[b]	0.891[a]
	1 B	3.1	1.7	0.136	0.043	(0.099 - 0.181)	0.085	0.065	(0.039 - 0.131)		
	1 C	4.3	2.4	0.202	0.059	(0.149 - 0.280)	0.084	0.028	(0.064 - 0.104)		
	1 D	6.2	3.4	0.274	0.079	(0.191 - 0.355)	0.133	0.060	(0.091 - 0.175)		
	1 E	9.9	5.5	0.421	0.105	(0.361 - 0.578)	0.292	0.118	(0.208 - 0.375)		
$NiCO_3$	2 A	6.4	2.5	0.260	0.198	(0.091 - 0.478)	0.022	0.000	(0.022 - 0.022)	0.966[b]	0.916[a]
	2 B	11.2	4.4	0.536	0.163	(0.402 - 0.718)	0.051	0.001	(0.050 - 0.051)		
	2 C	16.0	6.2	0.854	0.281	(0.554 - 1.112)	0.067	0.026	(0.049 - 0.086)		
	2 D	21.1	8.2	1.014	0.371	(0.585 - 1.230)	0.104	0.018	(0.092 - 0.117)		
	2 E	28.8	11.2	1.161	0.011	(1.153 - 1.170)	0.097	0.044	(0.066 - 0.128)		
$NiCO_3$ crystalline (Sample 2B)	2 cry A	2.5	1.0	0.077	0.022	(0.061 - 0.093)	0.020	0.006	(0.015 - 0.024)	0.988[b]	0.882[a]
	2 cry B	5.0	1.9	0.146	0.006	(0.142 - 0.150)	0.026	0.001	(0.025 - 0.027)		
	2 cry C	7.5	2.9	0.204	0.007	(0.198 - 0.209)	0.045	0.038	(0.018 - 0.071)		
	2 cry D	10.0	3.8	0.246	0.042	(0.217 - 0.276)	0.064	0.047	(0.031 - 0.097)		
	2 cry E	15.0	5.7	0.444	0.045	(0.412 - 0.475)	0.059	0.045	(0.027 - 0.090)		
	2 cry F	20.0	7.6	0.444	0.148	(0.340 - 0.549)	0.099	0.042	(0.069 - 0.129)		
	2 cry G	30.0	11.4	0.764	0.146	(0.661 - 0.867)	0.285	0.222	(0.128 - 0.442)		
	2 cry H	40.0	15.2	0.640	0.299	(0.428 - 0.852)	0.262	0.253	(0.082 - 0.441)		
NiO, black	3 A	12.2	9.5	0.076	0.038	(0.044 - 0.131)	0.039	0.015	(0.028 - 0.050)	0.970[b]	0.858
	3 B	18.0	14.0	0.098	0.034	(0.064 - 0.132)	0.042	0.014	(0.032 - 0.052)		
	3 C	23.3	18.1	0.094	0.020	(0.072 - 0.120)	0.046	0.003	(0.044 - 0.049)		
	3 D	28.5	22.2	0.129	0.038	(0.099 - 0.181)	0.041	0.034	(0.017 - 0.065)		
	3 E	40.9	31.8	0.181	0.054	(0.121 - 0.251)	0.057	0.039	(0.029 - 0.085)		
NiO, green	4 A	108.6	85.3	0.029	0.012	(0.018 - 0.047)	0.007	0.004	(0.004 - 0.010)	0.973[b]	0.939[a]
	4 C	183.0	143.8	0.035	0.009	(0.027 - 0.043)	0.005	0.000	(0.005 - 0.006)		
	4 D	229.2	180.2	0.062	0.029	(0.028 - 0.098)	0.017	0.006	(0.013 - 0.022)		
	4 E	358.3	281.6	0.094	0.071	(0.046 - 0.175)	0.045				
$Li_2Ni_8O_{10}$	5 A	52.2	37.8	0.024	0.014	(0.015 - 0.040)	0.009	0.006	(0.005 - 0.013)	0.943[a]	0.951[a]
	5 B	83.3	60.3	0.060	0.028	(0.040 - 0.080)	0.010				
	5 C	104.9	76.0	0.061	0.022	(0.037 - 0.080)	0.017	0.009	(0.011 - 0.023)		
	5 D	139.0	100.6	0.062	0.010	(0.055 - 0.069)	0.027				
	5 E	201.4	145.8	0.116	0.049	(0.082 - 0.151)	0.031				
NiS amorphous	6 A	4.4	1.8	0.108	0.044	(0.056 - 0.162)	0.072	0.003	(0.069 - 0.074)	0.983[b]	0.539
	6 B	6.9	2.8	0.145	0.089	(0.075 - 0.263)	0.121	0.077	(0.066 - 0.175)		
	6 C	9.5	3.9	0.267	0.091	(0.182 - 0.346)	0.070	0.012	(0.061 - 0.079)		
	6 D	12.7	5.2	0.290	0.106	(0.178 - 0.428)	0.086	0.008	(0.080 - 0.092)		
	6 E	17.4	7.1	0.420	0.136	(0.260 - 0.569)	0.133	0.025	(0.115 - 0.150)		
Ni_7S_6	7 A	4.7	3.2	0.043	0.016	(0.026 - 0.061)	0.027	0.009	(0.021 - 0.033)	0.994[b]	0.336
	7 B	8.3	5.7	0.068	0.024	(0.046 - 0.099)	0.040	0.001	(0.039 - 0.041)		
	7 C	12.7	8.6	0.082	0.015	(0.072 - 0.093)	0.072	0.056	(0.032 - 0.111)		
	7 D	15.5	10.5	0.099	0.008	(0.093 - 0.105)	0.045				
	7 E	28.4	19.3	0.189	0.001	(0.188 - 0.189)	0.016				
Ni_3S_2	8 A	2.1	1.6	0.076	0.023	(0.058 - 0.108)	0.048	0.004	(0.046 - 0.051)	0.985[b]	0.912[a]
	8 B	4.1	3.0	0.141	0.044	(0.104 - 0.200)	0.098	0.059	(0.057 - 0.139)		
	8 C	5.9	4.3	0.195	0.032	(0.165 - 0.234)	0.076	0.013	(0.067 - 0.085)		
	8 D	8.5	6.2	0.235	0.159	(0.093 - 0.406)	0.127	0.045	(0.095 - 0.159)		
	8 E	12.4	9.1	0.421	0.194	(0.216 - 0.638)	0.145	0.047	(0.111 - 0.178)		

continued next page

Table 14: Nickel Content in the Cytosol and Nuclei of AS52 Cells (continued)[c]

Compound	Label	Compound Added mM	Compound Added µg Ni/mL	Cytosol µg Ni/10⁶ cells Average	Cytosol Std	Cytosol Range	Nuclei µg Ni/10⁶ cells Average	Nuclei Std	Nuclei Range	Correlation Coefficient R Cytosol	Correlation Coefficient R Nuclei
$NiCl_2$ 5 h	9 A	1.0	58.7	0.079	0.014	(0.060 - 0.091)	0.003	0.002	(0.002 - 0.005)	0.970[b]	0.930[a]
	9 B	2.0	117.4	0.182	0.064	(0.123 - 0.270)	0.006	0.002	(0.005 - 0.008)		
	9 C	3.0	176.1	0.250	0.115	(0.127 - 0.404)	0.005	0.001	(0.005 - 0.005)		
	9 D	4.0	234.8	0.269	0.101	(0.169 - 0.400)	0.013	0.005	(0.009 - 0.016)		
	9 E	5.0	293.5	0.324	0.099	(0.211 - 0.438)	0.016	0.003	(0.014 - 0.018)		
$NiSO_4$ 5 h	10 A	1.0	58.7	0.077	0.025	(0.060 - 0.114)	0.006	0.004	(0.003 - 0.009)	0.935[a]	0.723
	10 B	2.0	117.4	0.155	0.080	(0.093 - 0.272)	0.007	0.001	(0.006 - 0.007)		
	10 C	3.0	176.1	0.260	0.119	(0.169 - 0.435)	0.012	0.008	(0.006 - 0.017)		
	10 D	4.0	234.8	0.267	0.103	(0.186 - 0.407)	0.008	0.000	(0.008 - 0.008)		
	10 E	5.0	293.5	0.288	0.061	(0.235 - 0.374)	0.011	0.003	(0.009 - 0.013)		
$Ni(CH_3COO)_2$ 5 h	11 A	1.0	58.7	0.090	0.022	(0.068 - 0.110)	0.005	0.001	(0.005 - 0.006)	0.997[b]	0.991[b]
	11 B	2.0	117.4	0.158	0.083	(0.064 - 0.266)	0.008	0.003	(0.006 - 0.010)		
	11 C	3.0	176.1	0.253	0.104	(0.154 - 0.399)	0.011	0.004	(0.008 - 0.014)		
	11 D	4.0	234.8	0.312	0.158	(0.138 - 0.515)	0.016	0.009	(0.010 - 0.022)		
	11 E	5.0	293.5	0.374	0.147	(0.182 - 0.495)	0.020	0.007	(0.015 - 0.025)		
$NiCl_2$ 24 h	12 A	0.25	14.7	0.053						0.961[b]	
	12 B	0.50	29.4	0.077							
	12 C	0.75	44.0	0.180							
	12 D	1.00	58.7	0.184							
	12 E	1.25	73.4	0.231							
$NiSO_4$ 24 h	13 A	0.25	14.7	0.048						0.958[a]	
	13 B	0.50	29.4	0.095							
	13 C	0.75	44.0	0.154							
	13 D	1.00	58.7	0.146							
	13 E	1.25	73.4	0.238							
$Ni(CH_3COO)_2$ 24 h	14 A	0.25	14.7	0.048						0.798	
	14 B	0.50	29.4	0.160							
	14 C	0.75	44.0	0.111							
	14 D	1.00	58.7	0.287							
	14 E	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]significant at the .05 probability level
[b]significant at the .01 probability level

[c]AS52 cells were seeded in F12/10% FBS medium at ≈ 0.5-1.0*10^6 cells per 100 mm diameter dish 1-2 d before treatment. The cells were exposed to the range of nickel compounds in serum free medium at the concentrations indicated for 24 h (or 5 h 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 were obtained based on 4 replicate experiments for the cytosolic nickel and 2 experiments for the nuclear nickel content.

Figure 6: Toxicity, Mutagenicity, and Uptake of Nickel Compounds in AS52 Cells

In Figures 6-1 to 6-14, results of the toxicity, mutagenicity, and uptake experiments are summarized for each of the nickel compounds at equivalent levels of cell survival. Toxicity data was determined from the detailed survival curves (Figures 4-1 to 4-12), while uptake data was read from Table 14. An average of the results of the two mutagenicity experiments reported in Tables 6 and 7 was used.

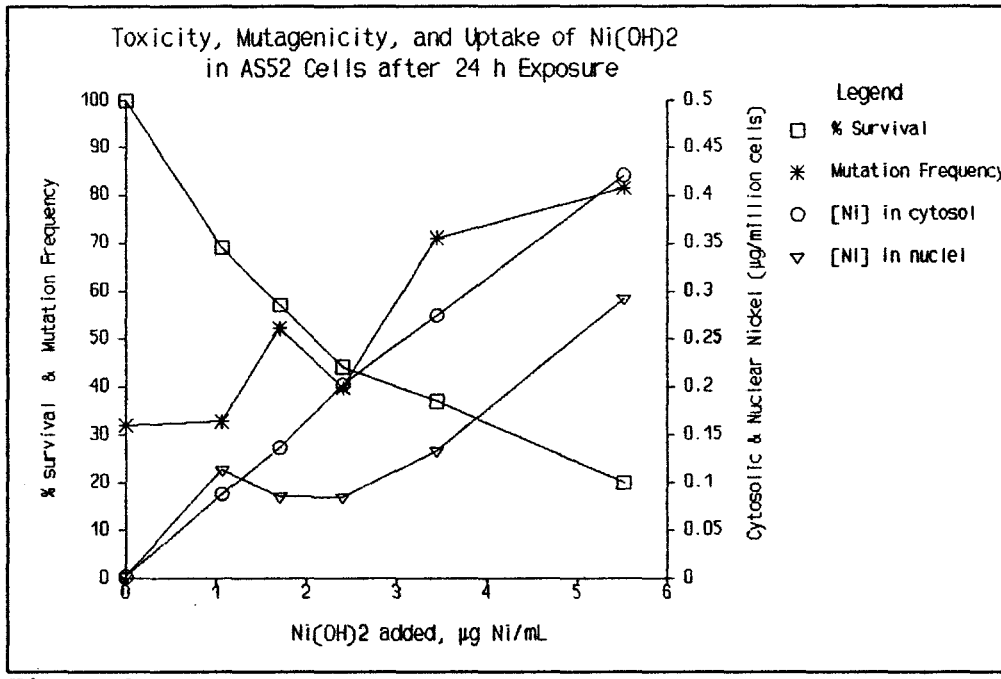

Figure 6-1

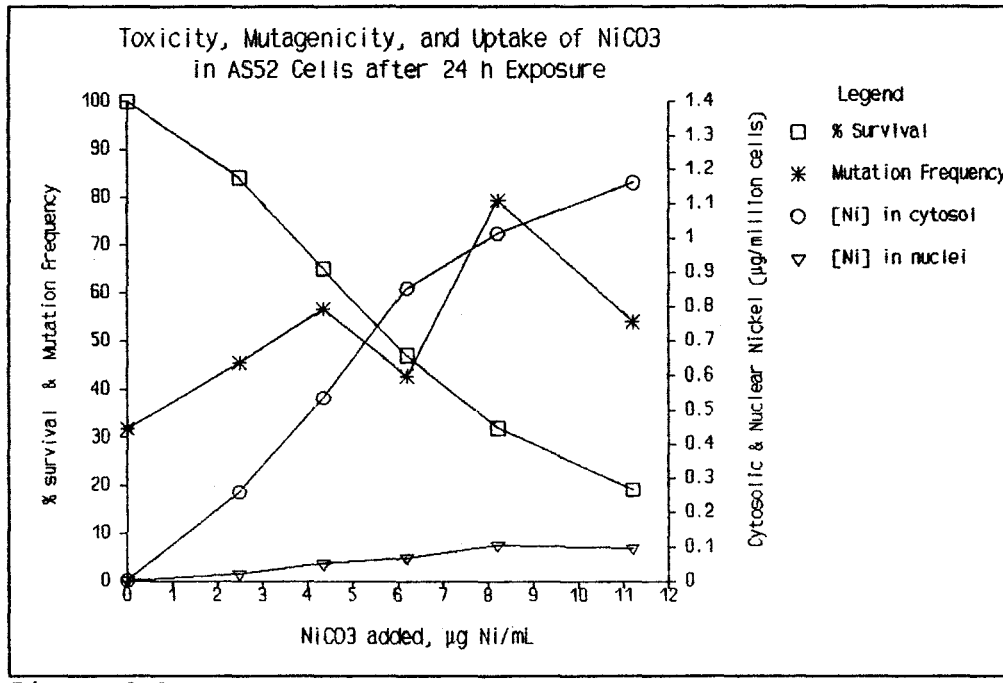

Figure 6-2

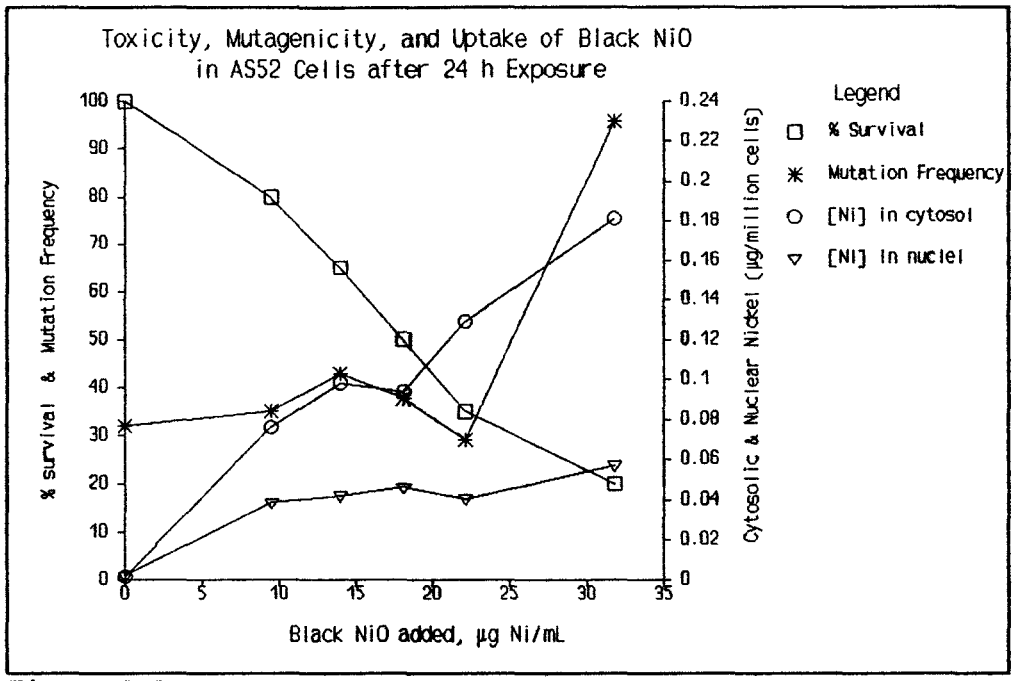

Figure 6-3

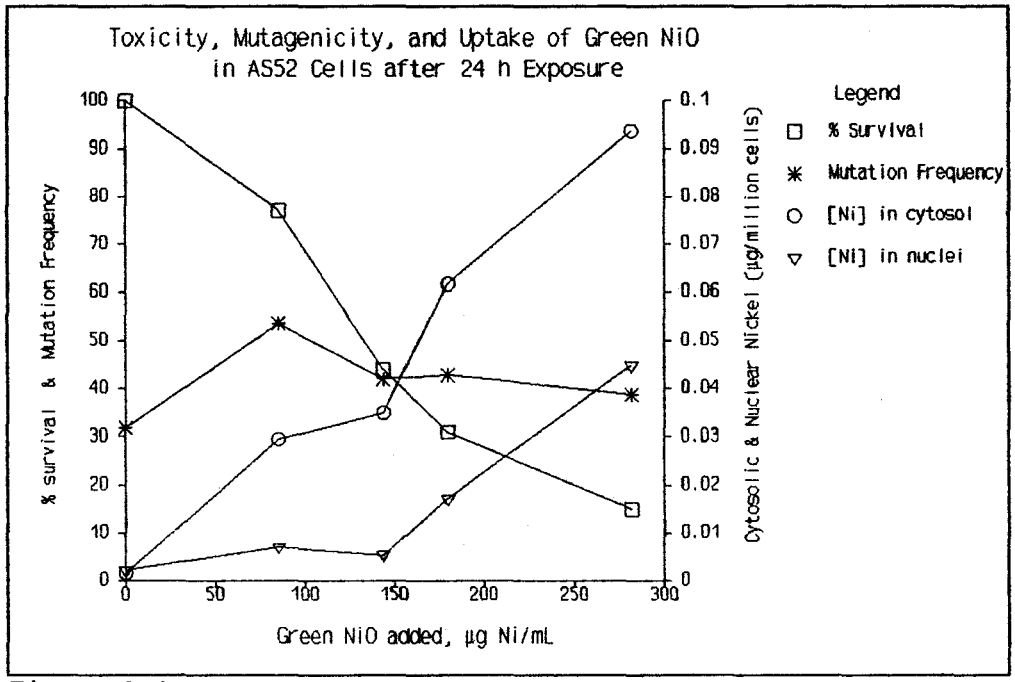

Figure 6-4

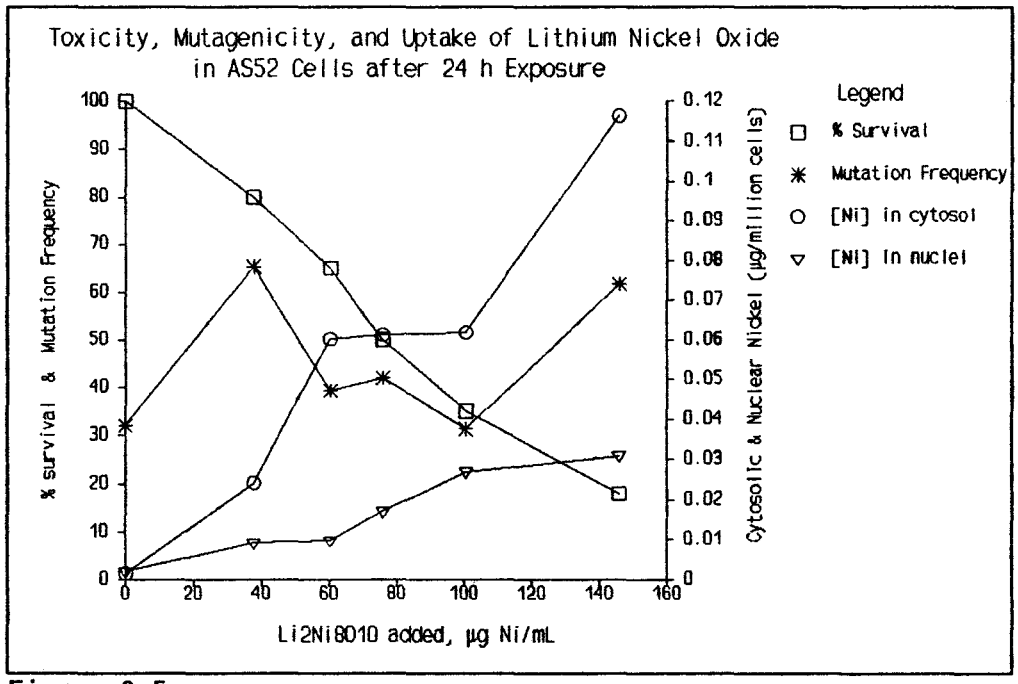

Figure 6-5

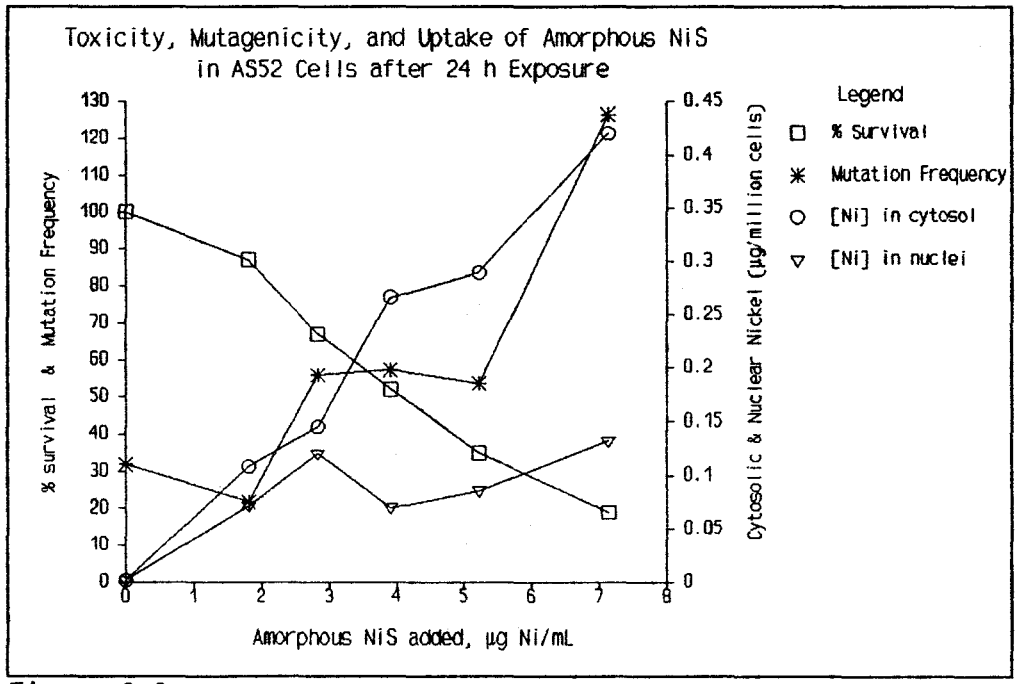

Figure 6-6

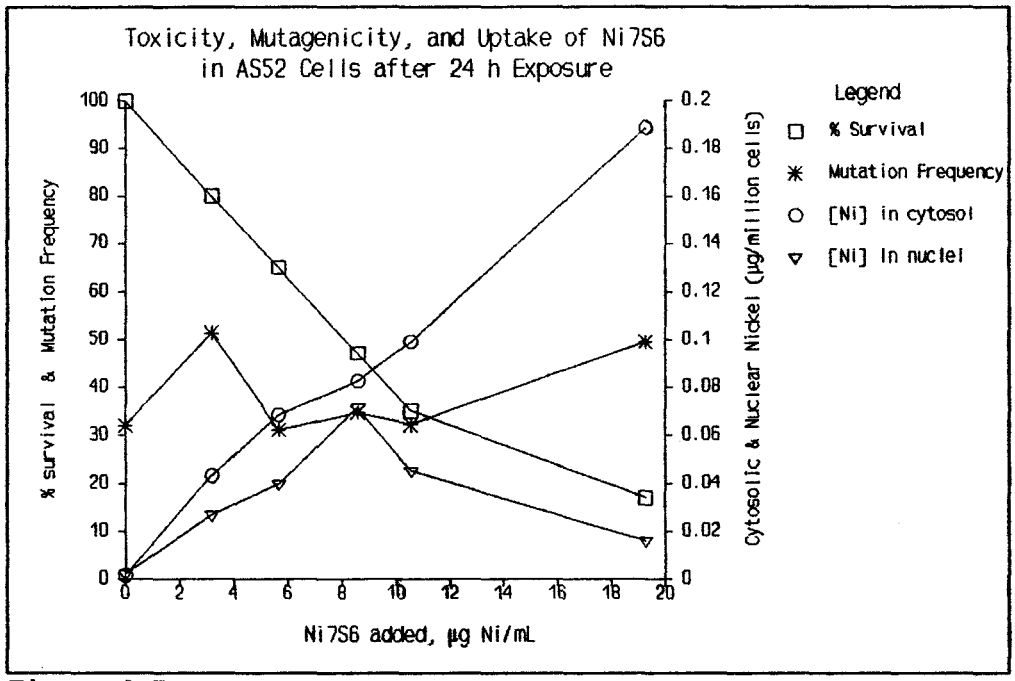

Figure 6-7

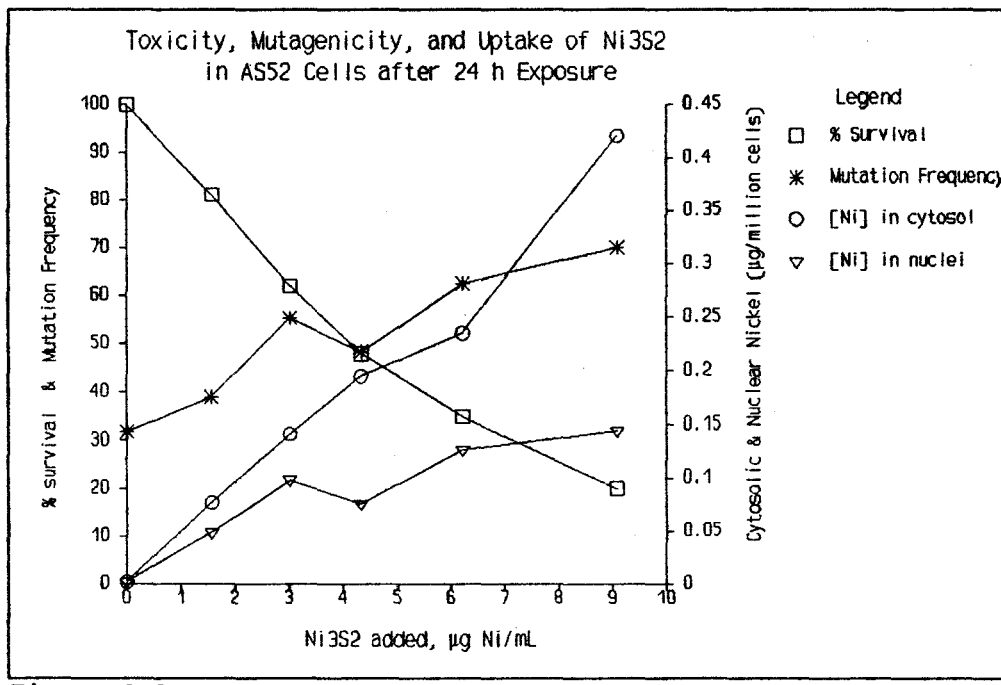

Figure 6-8

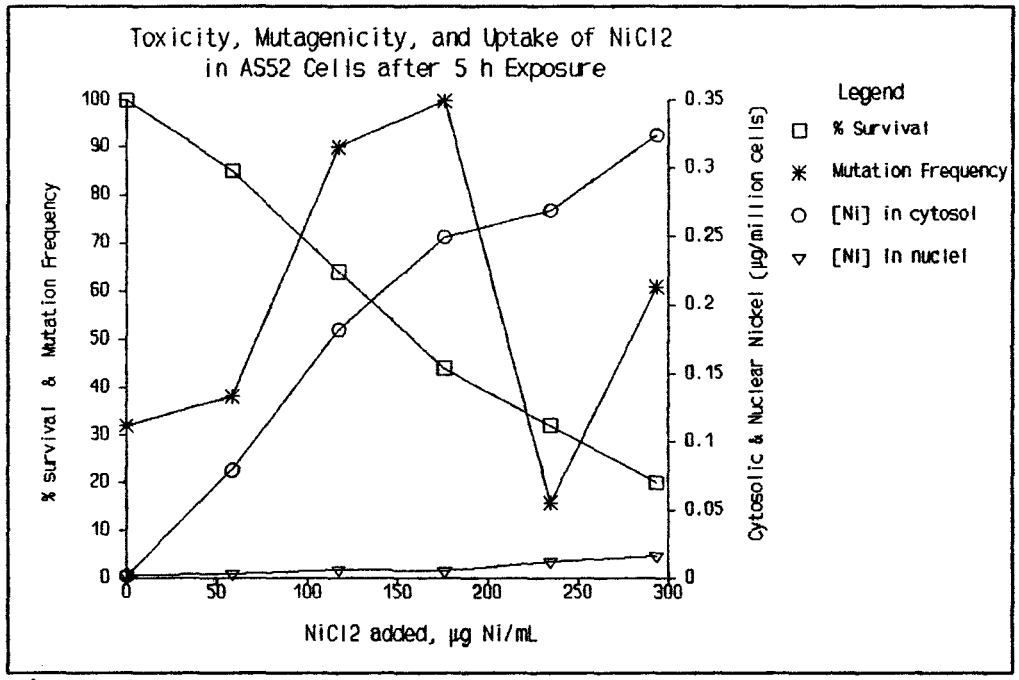

Figure 6-9

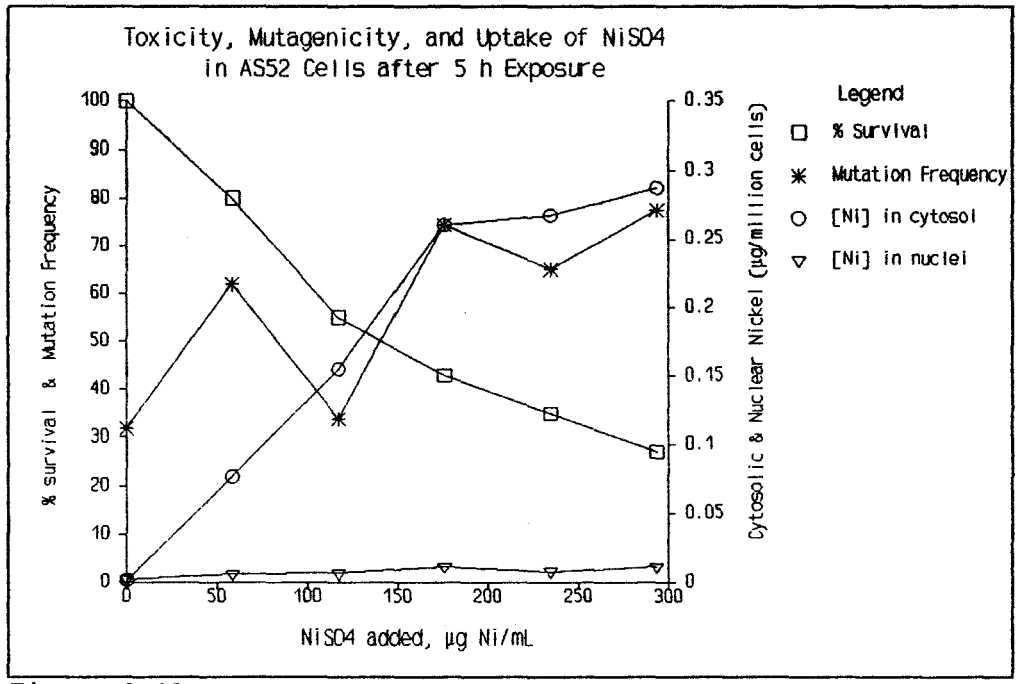

Figure 6-10

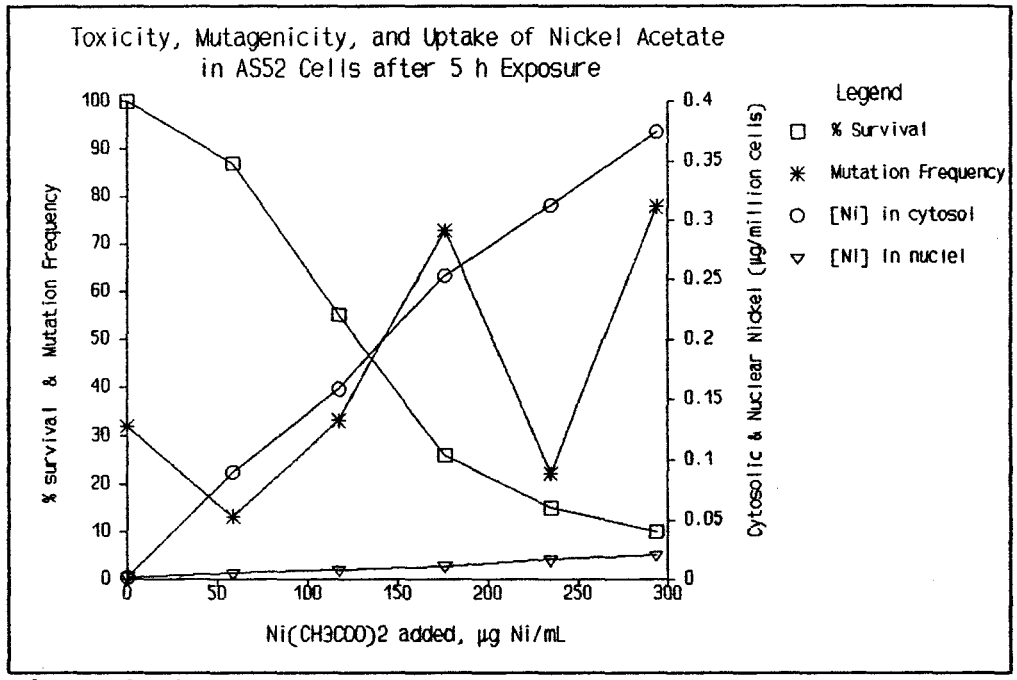

Figure 6-11

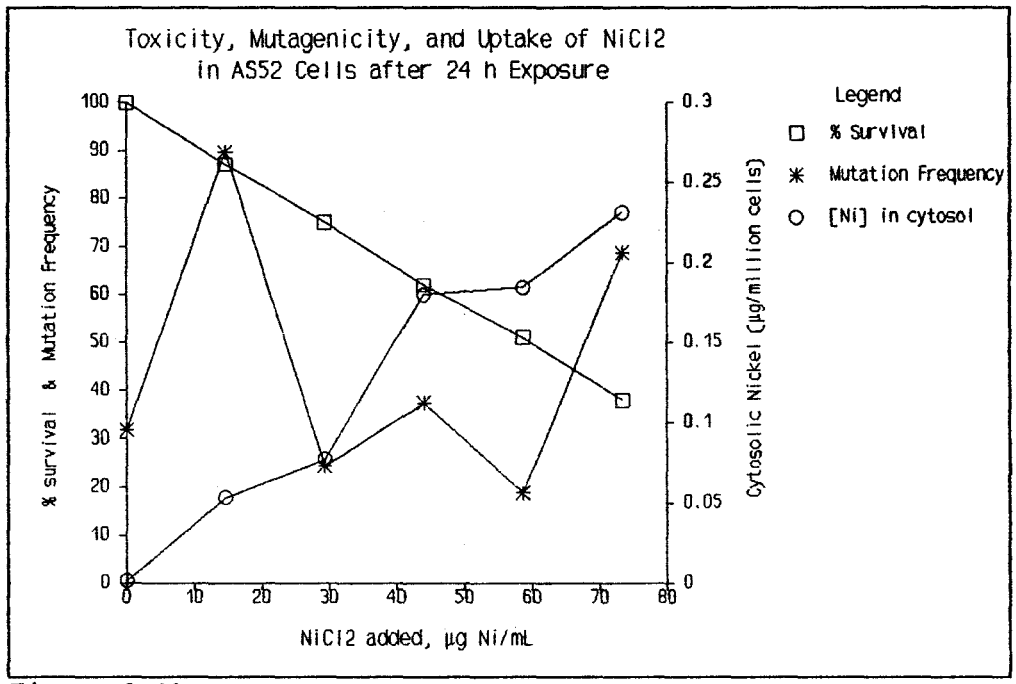

Figure 6-12

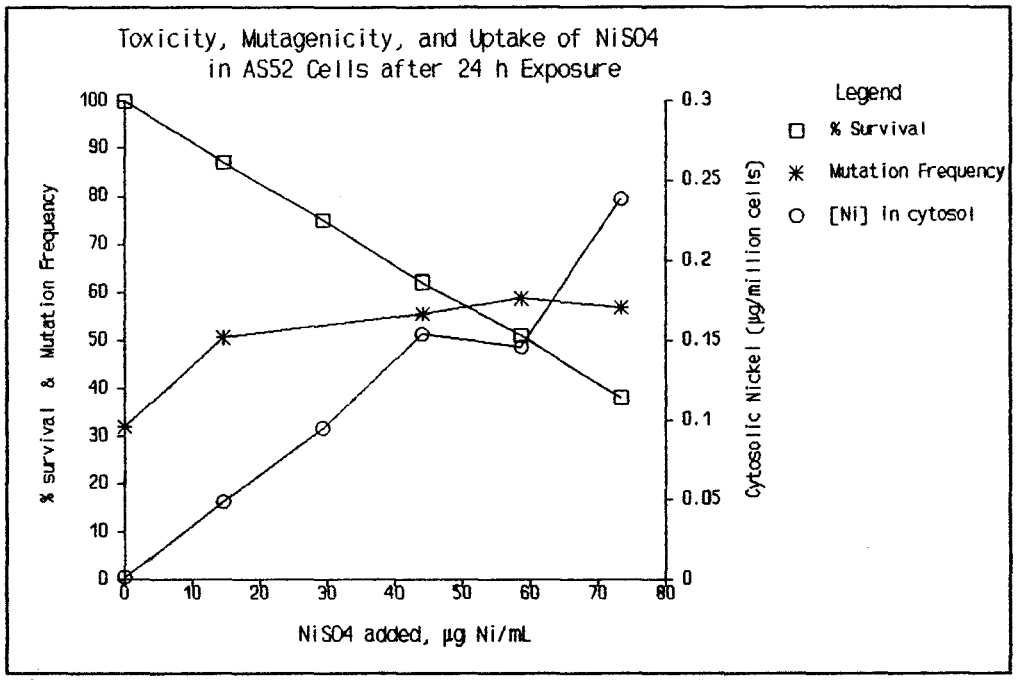

Figure 6-13

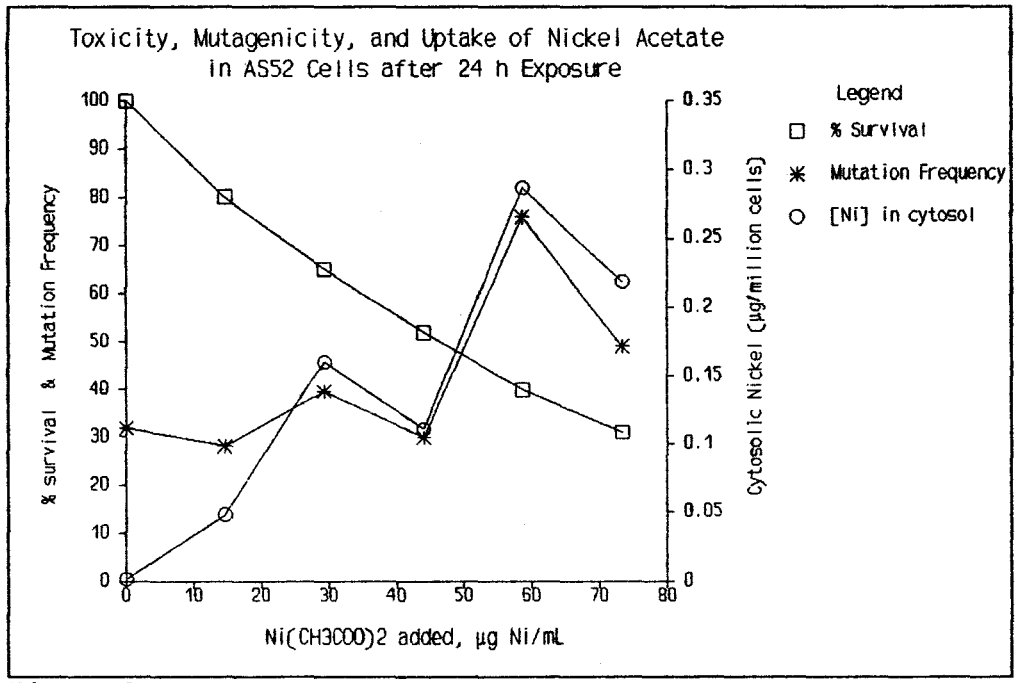

Figure 6-14

Table 15: LC_{50} Values for Nickel Compounds in AS52 and CHO Cells and Corresponding Cytosolic and Nuclear Nickel Levels

	Compound	LC_{50}[a] (µg/mL or mM)	LC_{50}[a] µg Ni/mL	Nickel (µg/10^6 cells) Cytosol[b,f]	Nuclei[b,g]	Dissolution Half Time (T_{50})[c] (Rat/Human Serum)
AS52 Cells						
1	$Ni(OH)_2$	3.6 µg/mL	2.0	0.16	0.08	< 1 d
2	$NiCO_3$	14.9	5.8	0.80	0.07	
3	black NiO	23.3	18.1	0.11	0.045	0.8 y
4	green NiO	165.4	130.0	0.035	0.007	> 11 y
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 d[d], <96 d[e]
7	Ni_7S_6	12.1	8.2	0.08	.04-.07	
8	Ni_3S_2	5.6	4.1	0.19	0.08	28 d
9	$NiCl_2$ (5 h)	2.6 mM	150	0.22	0.006	
10	$NiSO_4$ (5 h)	2.1	125	0.17	0.007	
11	$Ni(CH_3COO)_2$ (5 h)	2.0	120	0.16	0.008	
12	$NiCl_2$ (24 h)	1.0	60	0.18		
13	$NiSO_4$ (24 h)	1.0	60	0.17		
14	$Ni(CH_3COO)_2$ (24 h)	0.8	45	0.15		
CHO Cells						
	Ni_3S_2		4.0			
	$NiCl_2$ (24 hours)		37			

---

[a] LC_{50} is the concentration at which the survival of exposed cells is 50% of that of non-exposed (control) cells as determined in colony forming assays. Values were determined from Figures 3-1 and 3-2 for CHO cells and Figures 4-1 to 4-12 for AS52 cells

[b] Dissolved cytosolic and nuclear nickel levels determined from Figures 5-1 to 5-14 based on the LC_{50} values recorded above.

[c] Values taken from Table 16

[d] Value for amorphous NiS

[e] Value for NiS_2 determined in water

[f] For LC_{50} versus cytosolic nickel, $LC_{50} = 75 - 87\ Ni_{cytosol}$, $r = -0.29$, $p > 0.05$, N=14

[g] For LC_{50} versus nuclear nickel, $LC_{50} = 129 - 1810\ Ni_{nuclei}$, $r = -0.93$, $p < 0.01$, N=11

IV. DISCUSSION

A. Characterization and Selection of Nickel Compounds

It has been observed that certain nickel compounds, though closely related in chemical composition, exhibit significant differences in biological activity (Sunderman et al., 1987). For example, in a study of 6 different nickel oxides prepared by subjecting black nickel oxide (prepared at < 650°C) to calcination for 1 h at 735, 800, 850, 918, or 1045°C (yields green nickel oxide), only NiO (bunsenite) was identified in x-ray diffraction patterns but differences in solubility, phagocytosis by C3H-10T½ cells, SHE cell transformation, and erythropoietic response were observed. Various forms of nickel sulphides have also been shown to exhibit differing biological responses. A recent study by VJ Zatka (unpublished report to NiPERA), in which 21 basic nickel carbonates, 5 nickel sulphides, and 14 nickel oxides labelled as containing Ni_2O_3 were purchased from a number of suppliers and chemically analyzed, found that the suppliers' labels contained a number of errors, omissions, and misrepresentations. Because of uncertainties in compound identification and significant variation between different forms of the same compound, chemical and x-ray diffraction analysis of the compounds tested was deemed crucial to this present study.

The nickel hydroxide synthesized was found by chemical analysis to be $Ni(OH)_2 \cdot 0.6H_2O$; essentially pure nickel hydroxide with a small moisture content or hydration. The X-ray pattern indicated a crystalline

structure, though the broad peaks may be evidence of incomplete crystallinity. During preparation the sample had been heated and dried at 80°C in order to promote development of crystal formation from the initially colloidal product (Kasprzak et al., 1983).

Nickel carbonate samples commercially available or prepared by traditional methods are in the form of basic nickel carbonate (nickel hydroxycarbonate); generally containing a $NiCO_3$ to $Ni(OH)_2$ ratio ranging from 1:2 to 2:1. In an attempt to avoid testing a mixture of compounds, or an unknown composition, $NiCO_3$ was prepared by a procedure stated to give nickel carbonate essentially free of nickel hydroxide. A typical analysis by this procedure was reported to be $NiCO_3 \cdot 0.005Ni(OH)_2 \cdot 5.5H_2O$ (VJ Zatka and JS Warner, personal communication), although the method of analysis was not noted. Diffraction patterns of the nickel carbonates tested with varying amounts of nickel hydroxide were not reported in Zatka's report. Since great care was taken to keep the solutions chilled (temperatures were kept at 0°C, further evidenced by the formation of a few ice crystals before mixing the solutions) and the reaction mixture saturated with gaseous CO_2, only $NiCO_3 \cdot 6H_2O$ would be expected to form. The crystals formed were a distinct blue-green (aquamarine) colour as expected, and were dried only at room temperature in all stages of preparation, since exposures from 70-140°C or greater have been reported to cause partial conversion to nickel hydroxide. Sample 2D which was prepared by grinding these crystals (final diameter 10-25 μm) exhibited an X-ray diffraction pattern indicative of $NiCO_3 \cdot 6H_2O$, while Sample 2A which was obtained from particles < 10 μm diameter without grinding

exhibited no diffraction peaks but considerable biological activity. It may be possible that some degree of micro-crystallinity was still present though the crystals were too fine to give a sharp diffraction pattern. Chemical analysis at INCO's J Roy Gordon Research Laboratory indicated these compounds contain a considerable amount of nickel not accounted for by the amount of carbonate determined from the evolution of CO_2 by gasometry. It was thought that $Ni(OH)_2$ would be the most feasible additional species to be present. Since the background intensity in the X-ray pattern for Sample 2D is approximately 10% of that of the maximum peak, it is possible that amorphous $Ni(OH)_2$ is present. Samples 2A and 2D, though varying in the degree of crystallinity, had a very similar chemical analysis, with estimated formulas of $NiCO_3 \cdot 0.55Ni(OH)_2 \cdot 3.7H_2O$ and $NiCO_3 \cdot 0.58Ni(OH)_2 \cdot 4.2H_2O$ respectively. Since there is no indication that the reaction conditions or drying/storage temperatures used would cause formation of nickel hydroxide, it is hypothesized that this species must have formed during the particle sizing preparation or storage. If storage conditions are indeed critical, this might account for some of the labelling discrepancies noted in Dr Zatka's report (Zatka, unpublished report to NiPERA). Whatever the cause, the large differences in activity and uptake of the nickel hydroxide (Sample 1) and the nickel carbonate (Sample 2A) tested (to be discussed later) would indicate that distinct compounds are responsible for the biological activities noted.

As was noted in the Results section, the black and green nickel oxides tested match their expected diffraction patterns and chemical analysis (NiO). In a study of nickel oxides (Sunderman et al., 1987), it

was found that INCO black nickel oxide (also tested in this present investigation, Sample 3) had a substantially higher surface area than green or intermediate nickel oxides, contained appreciable Ni(III) (.81%), and produced significantly greater *in vivo* and *in vitro* effects. The sample of black nickel oxide (Sample 3B) analyzed prior to shipment to us was reported to contain this same level of Ni(III), as well as a small amount of undecomposed basic nickel carbonate used in the NiO preparation (2.0% $(OH)^-$ and .15% C). Green nickel oxides are produced at higher temperature than the black NiO forms, either directly or by heat treatment of the black nickel oxide form (eg 1045°C for 1 h produced Sample 4B from the black NiO Sample 3B). These green nickel oxides are less variable in composition since the additional heat treatment destroys any residual starting material, eliminates any Ni(III), and may give a more well defined crystal structure. In the accompanying x-ray diffraction patterns, it is seen that the peaks for the green NiO (Samples 4A and B) are a bit narrower and the background slightly lower than for the black NiO samples (3A and B).

The $Li_2Ni_8O_{10}$ included in this study is a nickel oxide in which the added lithium (2.39%) stabilizes nickel in the Ni(III) state (19.2%). It was hoped to observe whether or not nickel added in the Ni(III) form as opposed to Ni(II) as in the other compounds possessed any additional biological activity. Although the x-ray diffraction pattern of the $Li_2Ni_8O_{10}$ sample tested was slightly shifted relative to the NiO samples, attributes of both the NiO and $Li_2Ni_8O_{10}$ standard spectra were present. It is possible that a closely related species with slightly different

lithium content is the actual compound tested. Chemical analysis indicated 2.39% Li and 72.4% Ni (balance assumed O), giving a formula of $Li_{2.23}Ni_8O_{10.22}$.

The nickel-sulphur system exhibits a complex phase diagram, with species including NiS, NiS_2, Ni_3S_2, Ni_3S_4, and Ni_7S_6 (sometimes called Ni_6S_5 or Ni_9S_8) depending on the Ni/S ratio and temperature (Kullerud & Yund, 1962; Jellinek, 1968). Different crystal structures may also exist for some of these compounds, such as the low-temperature (millerite, βNiS) and high-temperature (NiAs-type, αNiS) forms of NiS (Grice & Ferguson, 1974). Clear identification of any nickel sulphides studied is therefore clearly desirable. Amorphous nickel sulphide was to have been synthesized and tested for *in vitro* activity. The x-ray diffraction pattern of the compound made and tested, however, indicated the presence of NiS_2 and $NiSO_4 \cdot 6H_2O$. Chemical analysis suggested an identity of NiS_2 + $NiSO_4 \cdot 6H_2O$ + NiS in a mole ratio of 1:2.25:4.7. The NiS, present in an amorphous form, would be expected from the preparation procedure and is consistent with the x-ray diffraction pattern, accounting for the relatively high background observed (≈ 20% of the maximum peak intensity). It has been reported that nickel sulphides prepared by precipitation from aqueous solutions are often unstable, oxidizing in air to give products which may include NiS_2, hydroxides, or sulphates (Jellinek, 1968). The primary precipitate is said to be an almost amorphous $Ni(SH,OH)_2$, with the OH content depending on the pH of the supernatant solution. Therefore the identified products in the prepared sample are as to be expected from the procedure followed.

Sample 7, identified when shipped by INCO as NiS, was found by x-ray diffraction pattern to be mainly Ni_7S_6 with possible inclusion of small amounts of other nickel sulphides (NiS, $NiSO_4$, Ni_3S_2). Chemical analysis agreed with the identification of Ni_7S_6 as the major species, with ≈ 5% NiS and ≈ 1% $NiSO_4$ (anhydrous). Crystalline NiS was therefore not tested in the present study. As noted before, Sample 8 was identified by both x-ray diffraction and chemical analysis as being crystalline Ni_3S_2.

B. Toxicity Studies

A comparison of toxicity results obtained in the CHO cells (Figures 3-1 and 3-2, also Table 4) with those in AS52 cells (Figures 4-8 and 4-12) indicates a similar response in these two related cell lines. For Ni_3S_2, the toxicity curves were almost identical, with LC_{50} values of 4.0 and 4.1 μg Ni/mL obtained, respectively, in CHO and AS52 cells. Lower standard deviations in the AS52 cells may have been obtained due to refinements in the toxicity testing methods, including sonication of the particulate stock solutions to break apart aggregates formed during the drying process. Results of $NiCl_2$ testing were also similar in the two cell types, though with larger variation than the Ni_3S_2. Individual experiments with nickel salts in the same cells also showed larger variation than did nickel subsulphide experiments. The majority of $NiCl_2$ testing used a 24-h exposure in CHO cells, which was changed to 5 h in the AS52 cells. Due to the limited number of experiments using 24-h nickel-

salt exposure in AS52 cells, performed mainly to allow comparison of the cell lines, the results presented may not be entirely representative. Nevertheless, it is concluded that, as expected, there is no significant difference in the response of the CHO cells or the derived AS52 cells. Subsequent experimental work was therefore performed using the AS52 cells.

As experimental work shifted from the CHO to the AS52 cells and the number of compounds to be tested increased, it was necessary to resolve the question of what exposure time to use. Although very crystalline compounds such as Ni_3S_2 have be found to reach a maximum uptake within a few hours (80% of cells contained particles after 6 h), amorphous compounds enter the cells much more slowly (Costa & Mollenhauer, 1980). To obtain sufficient uptake, an exposure period of 24 h was therefore chosen for particulate compounds. At the concentrations of nickel salts required to produce toxicity, severe though reversible growth inhibition occurs (Conway et al., 1986). They reported that after a 6 h exposure to 1.0-2.5 μM $NiCl_2$, DNA synthesis had been decreased to 30-40% of the control level; 48 h after exposure DNA synthesis and cell growth were still severely repressed, with the degree depending on the exposure level (cells treated with 2.5 mM $NiCl_2$ had recovered to ≈ 50% of the controls); by 72 h recovery was complete. In the present study, this effect was also noted in toxicity experiments. CHO cells exposed to nickel salts for 24 h exhibited very much slower growth and significantly smaller colonies compared to control cells. Two to three additional days of growth were required to obtain colonies of sufficient size for staining and counting. In the early stages of investigation this severe growth inhibition, which

was not present in positive (4-nitroquinoline oxide) or negative controls, hindered interpretation of the toxicity results. It might be expected that the longer 24-h exposure would cause more severe and prolonged growth inhibition than the 6-h exposure reported by Conway et al. In order to assess mutations in cell culture, a period of active cell replication is required following the exposure period and prior to mutant selection. This may account, at least in part, for the inability to detect $NiCl_2$ induced mutations in this study and by others after a 20-24 h exposure. Using a shorter $NiCl_2$ exposure time of 5 h, Hartwig & Beyersmann (1989) found a significant increase in mutations at the HPRT locus in Chinese hamster V79 cells. After considering the above factors, it was decided to perform all nickel salt testing using a 5 h exposure period.

As illustrated by the AS52 toxicity curves of Figures 4-1 to 4-12 (data compiled in Table 5), and the LC_{50} data in Table 15, there are significant differences in the toxicity of the various nickel compounds tested. The LC_{50} values range from 2.0 µg Ni/mL for $Ni(OH)_2$ to 130 µg Ni/mL for the green NiO and 120-150 µg Ni/mL for the water-soluble salts. The nickel hydroxide, carbonate, and sulphides show similar toxicities, while the nickel oxides are shown to be less toxic, with the potency depending on the compound tested. The black NiO gave a LC_{50} of 18.1 µg Ni/mL compared to values of 75 and 130 µg Ni/mL for the lithium nickel oxide and green nickel oxide, respectively. Clearly, the form of the nickel oxide is critical to its toxicity and biological activity. The green nickel oxide, which usually is quite inert in biological tests (e.g. Sunderman et al., 1987), is also much less toxic to the AS52 cells tested.

It should be noted that although the lithium nickel oxide is more toxic than the green nickel oxide, it is still much less toxic than the black nickel oxide and the other particulate compounds tested. The fact that the compound contains a high proportion of Ni(III) does not appear to be a major determinant of toxicity. Comparison of the three nickel salts (nickel chloride, nickel sulphate, and nickel acetate) indicates similar toxicity for all of these compounds. The slight differences in the LC_{50} values reported in Table 15 might be due to the uncertainty involved in interpolation from the respective toxicity curves. Toxicity testing using a 5 h exposure appeared more prone to inter-experimental variation (and thus larger standard deviations on the survival curves) than using a 24 h nickel exposure. It was noted that with shorter exposure, control of cell treatment and incubation conditions were much more critical (e.g. opening the incubator during a 5 h exposure appeared to affect the resultant cell survival).

C. Mutagenicity Testing

Although nickel compounds have been shown to be only weak mutagens (Amacher & Paillet, 1980; Miyaki et al., 1979; Costa et al., 1980), the results for two separate experiments presented in Tables 6 and 7 indicate a significant increase in mutation rate for all the nickel compounds. There is considerable variability in the mutation rates obtained for the different doses of a compound tested. The mutation frequency exhibits a dose-dependent manner only in the cases of $Ni(OH)_2$, amorphous NiS, and

Ni_3S_2 reported in Table 7. In other reports of metal mutation testing this general lack of dose-response has also been noted (Hsie et al., 1979). A possible explanation is that due to the low rate of mutation induction observed, the likelihood of non-representative sampling and selection of cells (normal versus mutated) at each stage of replating is greatly increased. Since the background mutation frequency is reproducible for replicate platings from several separate dishes, the average control mutation frequency may be accepted with considerable confidence (see Figure 5). Consequently, statistically significant increases ($p < 0.05$ or $p < 0.01$) in mutation frequency may be interpreted as a positive result.

D. Cellular/Nuclear Nickel Levels

As illustrated in Table 8 and verified in other experiments, significant cell numbers need to be present to permit proper pellet formation and circumvent significant cell loses during the collection, rinsing, and digestion stages. Therefore, whenever possible $\approx 10^6$ or more cells were harvested for analysis. Table 9 shows the levels of nickel detected in the exposure medium and cell rinses before trypsinization, as well as in the medium/trypsin mixture after removal of the cells by centrifugation. The results indicate that the majority of nickel is removed with the exposure medium, and that two rinses are adequate to remove any residual nickel not taken up by the cells.

It can be seen from the data in Table 10 that there is a consistent

association between the administered dose and the measured nickel content ($r = 0.837$, $p < 0.01$ for $NiCl_2$; $r = 0.981$, $p < 0.01$ for Ni_3S_2). As the nickel content increases, the variance also increases, but the trends are still very clear. Comparison of the Ni_3S_2- and $NiCl_2$-treated cells shows a 200-fold difference in nickel levels at the same administered dose. This is due in large part to the presence of undissolved nickel subsulphide particles. For nickel chloride exposure, the cell associated nickel must correspond to intracellular nickel and that attached externally to the cell, while for nickel subsulphide it would represent the nickel content of the cells (dissolved + phagocytized particulates) plus any externally attached particles not removed in the rinsing steps.

Attempts to separate nuclei from nickel particulates using a sucrose pad (denser particulates should pellet while nuclei remain at the top of the sucrose) were only partially successful (Tables 11 and 12). In fact, the nuclei/particulate separation was deemed unsatisfactory and the reported nuclear nickel in these two tables is not expected to be nuclear nickel free from extraneous particulate contamination. Comparison of cytosolic nickel and total cell associated nickel (whole cells) indicates that $\approx$ 2-6% of the total nickel measured is in the cytosol fraction for the experiment summarized in Table 11. References to cytosolic nickel throughout this report refer to nickel in the supernatant of disrupted cells after low speed centrifugation (10 min at 1000g) to pellet nuclei and any cell-associated particulate nickel (phagocytized and externally attached). Smaller organelles and membrane fragments (though not normally included in the definition of cytosol) would not pellet under these

conditions and would thus be included with the cytosolic fraction. The nickel in the cytosolic fraction is interpreted to represent nickel in its ionic form Ni^{2+} (free or bound to cytosolic components). Comparison of nickel levels in $NiCl_2$-treated cells immediately after exposure with levels in cells incubated for a further 19 hours indicates a loss of cellular and nuclear nickel of about 60% during this period (Table 12). A similar decay pattern was reported by Patierno et al. (1987) for CHO cells (see Section 1G). The apparent discrepancy between the nickel levels in the cytosol and total cell nickel in Table 12 appears to be within the normal fluctuations observed (see for example the inter-experimental ranges in Table 10). As already indicated, the rinsing protocol is successful in preventing carry over of the administered dose. It should also be noted that the levels of nickel in the cellular and nuclear rinses are small.

To improve on the protocol employed in the experiment summarized in Table 12, nuclei were initially lysed using a buffered SDS mixture containing EDTA to aid the denaturing of DNA and thus circumvent pelleting of the DNA and bound nickel upon centrifugation. Concern over the possibility of EDTA adding to the Ni^{2+} content by enhancing dissolution of residual nickel particles led to a further experimental modification. Comparison of these methods and the effect of EDTA on nickel compound dissolution is shown in Table 13. In the controls (vortexing, but no incubation period), nickel levels of .024 mg/L and 1.70 mg/L were measured for the green NiO and Ni_3S_2, respectively. Using the DNAase method a maximum level of .069 mg/L with the green NiO was reached (< 3 times the control), while dissolution of the Ni_3S_2 showed a much stronger time

dependence. Incubation of Ni_3S_2 for 10 min at 37°C in the DNAse buffer, followed by addition of SDS/HEPES (Proteinase K buffer) and incubation for 20 min at 60°C, produced a nickel level (1.52 mg/L) comparable to the control. Increasing the two incubation times to 30 + 60 min, respectively, gave 4.14 mg/L, while times of 60 + 120 min gave 10.71 mg Ni/L (6.3 times the control level). By comparison, the EDTA method gave nickel levels of .141 mg/L for the green NiO (6 times control) and 30.12 mg/L for the nickel subsulphide (18 times control). Therefore the DNAse method was selected for nuclear nickel determinations, choosing incubation times to maximize the DNAase I and Proteinase K activity while minimizing particulate nickel release (DNAse I digestion for 15 minutes at 37°C; Proteinase K/SDS incubation for 45 minutes at 60°C).

E. Toxicity, Mutagenicity, and Cytosolic and Nuclear Nickel Levels

As illustrated by several of the compounds (see Figures 6-1, 6-2, 6-3, 6-6, 6-8, 6-10, 6-13, 6-14), there is a general trend towards increasing mutation frequency with increasing added nickel concentrations, increasing toxicity (decreasing survival) and increasing nickel uptake. All of the compounds tested exhibit an increase in cytosolic and nuclear nickel as the administered dose increases (see Table 14). An anomaly is the Ni_7S_6 which exhibits a decrease in nuclear nickel levels at the 2 highest doses. Due to loss of dishes in one of the experiments, this observation could not be confirmed and the validity of these points is suspect. For most of the particulate nickel compounds, the nuclear nickel

was ≈ ¼ to ½ the cytosolic nickel level. By comparison, the nickel salts exhibited a nuclear nickel content that was ≈ 1/20 of the cytosolic nickel level. The nickel carbonate appears to be intermediate in behaviour between the particulate and water-soluble compounds. The nickel carbonate (Sample 2A) employed in the biological testing produced a nuclear nickel content ≈ 1/12 the cytosolic level, while the more crystalline nickel carbonate (Sample 2B) gave a nuclear nickel content equal to ¼ the cytosolic concentration. Interestingly, the difference in cytosolic content appears to account for this discrepancy.

Comparing the cytosolic and nuclear nickel contents of the compounds at 50% survival (Table 15) indicates less variability in these parameters than in the administered dose. Thus at LC_{50}, the exposure concentrations ranged from 2.0-150 μg Ni/mL while the cytosolic nickel levels were in the range 35-800 ng/10^6 cells (35-250 excluding the carbonate) and the nuclear levels covered the range of 6-80 ng/10^6 cells. This corresponds to a 75-fold difference in administered dose compared to a 23-fold (7-fold excluding the $NiCO_3$) and 13-fold differences in cytoplasmic and nuclear nickel levels, respectively. Interestingly, the compounds with the lowest toxicity (green NiO and lithium nickel oxide), and therefore necessitating the highest extracellular nickel concentrations to give measurable cell toxicity, also showed the lowest intracellular nickel levels. The lower cellular nickel level for the green NiO may be due to a much slower dissolution rate for this compound even after cellular uptake (see Table 16). There appears to be a reasonable correspondence between the dissolution half times (T_{50}'s) of the compounds tested and the cytosolic

Table 16: Solubility of Nickel Compounds

Compound	Reference	Dissolution Half Time (T_{50}) Water	Rat serum	Rat Renal Cytosol
NiO (green)	A[a]	> 11 y	> 11 y	> 11 y
NiS_2	A	96 d		
NiS (millerite)	A	3.3 y	2.6 y	1.4 y
NiS, amorphous	A	34 d	24 d	19 d
Ni_3S_2	A	10.4 y	34 d	21 d
Black NiO	B[b]	> 11 y	0.8 y	1.1 y
Green NiO	B	> 11 y	> 11 y	> 11 y
Ni_3S_2	B	7.2 y	23 d	26 d
			Human Blood Serum	Artificial Lung Fluid
$Ni(OH)_2$, colloidal	C[c]		< 5 min[d]	2-4 min
$Ni(OH)_2$, intermediate	C		12 h[d]	15 d
$Ni(OH)_2$, crystalline	C		> 24 h[d]	78 d
Ni_3S_2	C		56 d	178 d

[a,b]An amount of compound containing 4 mg nickel + 2 mL dissolution medium were placed in Pyrex tubes and agitated, then incubated at 37 °C with agitation. At time 0 and regular intervals thereafter the tubes were centrifuged (1200g for 10 min) and the supernatant analyzed for nickel content by atomic absorption.

[c]10 mg of the compound was shaken with 20 mL of dissolution medium, which was then changed every 24 h. Dissolved nickel was determined by the dimethylgloxime-bromine colorimetric method.

[d]Time for complete dissolution

A. Kuehn & Sunderman, 1982
B. Sunderman et al., 1987
C. Kasprzak et al., 1983

nickel levels at a given toxicity level (Tables 15 and 16). In a study of the solubility of various forms of $Ni(OH)_2$, Kasprzak et al. (1983) found that the colloidal form was very soluble (dissolved in human serum in < 5 min); an air-dried preparation with partial crystallinity was slightly less soluble (dissolved in 12 h), while a crystalline preparation was the least soluble (not totally dissolved in 24 h). From the x-ray diffraction patterns, it would appear that the $Ni(OH)_2$ tested in the present study was most similar to the crystalline form of Kasprzak et al.. Similarly, the solubility of the $NiCO_3$ samples would be expected to depend on the degree of crystallinity. It was found that the less crystalline sample (2A) produced the highest cytosolic nickel levels of all the compounds, while the more crystalline sample (2B) produced levels more similar to the other compounds tested. The compounds most soluble in rat (or human) blood serum ($NiCO_3$, $Ni(OH)_2$, amorphous NiS, Ni_3S_2) yielded the highest cytosolic nickel levels. These levels (with the exception of the $NiCO_3$) were similar to those produced by the nickel salts. Black NiO, known to be more soluble and more reactive than the green NiO, produced considerably higher cellular nickel levels, though lower than the previously mentioned compounds. The lithium nickel oxide has an intermediate LC_{50} and corresponding cytosolic and nuclear nickel levels.

Though the more soluble compounds, including the water soluble salts, produced relatively high cytosolic nickel levels at the LC_{50} values, the nuclear nickel levels showed much less dependence on solubility. It appears that the compounds could be divided into three classes; these being water-soluble salts producing very low nuclear levels and high

cytosolic levels, inert nickel oxides (green NiO and lithium nickel oxide) with relatively low nuclear and cytosolic nickel levels, and the remaining compounds (the major class) with relatively high cytosolic and nuclear nickel levels.

Nickel compounds are known to exhibit differential surface activities which, for example, result in quite different abilities to induce haemolysis in human erythrocytes (Nieboer et al., 1984). Consequently, physicochemical surface/cell interactions probably contribute to cell injury. Such an effect may be especially important when large doses are required, such as for the green and lithium nickel oxides. Compared to the other water-soluble and particulate compounds in Table 15, these compounds have the lowest intracellular nickel levels at the same toxicity, which lends evidence to this perspective.

Comparison of these results with reported carcinogenicity in rats (Sunderman, 1984; EPA, 1986) or induction of morphological transformation rates (Costa & Heck, 1982; Sunderman et al., 1987) indicates that, roughly speaking, animal carcinogenicity (based primarily on injection studies) correlates with the LC_{50} values. Crystalline Ni_3S_2 and NiS have the greatest potency for tumour induction (100% induction rate) and transformation. The evidence for NiO (green) is equivocal, being quite carcinogenic when injected intramuscularly but non-carcinogenic when administered intrarenally. The study of Sunderman et al. (1987) shows that green NiO can be both refractory or active in erythropoiesis tests, depending on its source. Thus the relative inactivity of green NiO in the current work is consistent with previous studies. As reported previously,

black oxides are known to have a range of biological activities (Sunderman et al., 1987). Nickel salts have been reported to have very weak or no carcinogenic activity but possess moderate transformation activity (35% of the Ni_3S_2 level). These compounds were found in the present study to have low relative toxicity and result in low nuclear nickel levels at the LC_{50}, though cytosolic nickel levels were comparable to the active particulate compounds tested. Amorphous NiS is reported to have low but significant tumorigenicity (12% induction rate in rats, $p < 0.01$ vs controls), though insignificant transformation potential. Our results indicate the amorphous NiS to behave in a manner similar to the $Ni(OH)_2$ and Ni_3S_2 with respect to LC_{50} and cellular nickel levels. The mixed nature of the compound tested, especially the presence of NiS_2, may account for some of this discrepancy. Interestingly, in Sunderman's intramuscular rat study, NiS_2 was only slightly less potent than Ni_3S_2. $Ni(OH)_2$ was found to be the most toxic compound tested and generated cellular nickel levels similar to those of nickel subsulphide. This compound was reported by Kasprzak et al. (1983) to have a tumour induction rate of $\approx$ 15% (compared to 80% for Ni_3S_2). It is obvious that the carcinogenicity of nickel compounds based on animal bioassays is a complex phenomena that is not well understood. Biological activity appears to depend on the physical properties of the nickel compounds. The result of the present study suggests that the degree of uptake and intracellular bioavailability of Ni^{2+} appears to be important.

Overall, the data suggests that the nickel-ion hypothesis appears to hold in terms of cytotoxicity, at least as a first approximation. The

substantially lower variation between the cytosolic and nuclear nickel levels compared to that for the LC_{50} values is accepted as corroborating evidence. When removing the $NiCO_3$ anomaly and taking cognizance of possible surface property contributions to the toxicity of nickel oxides, the cytosolic nickel concentrations at the LC_{50} are of comparable magnitude. Even though the nuclear nickel levels exhibit a narrower range than the LC_{50} values, there is nevertheless a significant inverse correlation ($r = -0.93$, $p < 0.01$) between these 2 variables. This relationship suggests that the nickel compounds with the highest potency (lowest administered dose at the LC_{50}) generate the highest nuclear nickel levels. Presumably, this reflects the ability of the phagocytotic process to promote nuclear loading of the nickel ion.

The dependence of mutagenicity on the nickel concentration in the 2 intracellular compartments is less clear. However, based upon the data in Table 7, the compounds with the strongest overall mutagenic responses are those with the lowest LC_{50} values and substantial nuclear and cytosolic nickel concentrations (namely $Ni(OH)_2$, amorphous NiS, and Ni_3S_2). These compounds show both enhanced mutation frequency and a dose response ($p < 0.05$). Although the dose-response is not exhibited by the data in Table 6, it is our judgement that the quality of the data is strongest in Table 7, (i.e., the observed reproducibility of both samples and positive controls is better and the responses of the positive controls are stronger, including the dose-response pattern).

Nickel-induced mutagenicity 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. Low nickel levels relative to those found in the cytosolic fraction are observed in the nuclei of cells exposed to the chloride, sulphate, or acetate salts (and also the carbonate, which appears to be the most soluble of the particulate compounds). By contrast, the cytosolic concentrations are substantially elevated for these compounds. It is reasonable to conclude that the measured cytosolic nickel represents different compartments for the salts and the particulate nickel compounds. It is known that phagocytized particulates of Ni_3S_2 are often present in cytoplasmic vacuoles (Costa & Mollenhauer, 1980; Costa et al., 1981a). The lysing step employed in releasing cytosolic nickel presumably makes available dissolved nickel from such compartments. On the other hand, the nickel(II) ions taken up from salt solutions appear, after uptake, to bind to intracellular proteins and low-molecular-mass ligands such as amino acids (Nieboer et al., 1988b). Greater complexation of nickel salts with the proteins and other cytosolic components and lack of intracellular transport could result in a proportionally lower amount of nickel being available to enter the nucleus and interact with the genetic material. Of course, some fraction of nickel delivered to the cell as particulates might also be expected to be present in this more general compartment. The concept of compartmentalization opens up the door for interesting mechanistic interpretations.

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 primarily 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 (Nieboer et al., 1988a, 1988b, 1989). 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 chromosomes and inducing chromosomal conformational changes that can result in permanent changes in gene expression, i.e., mutations (Nieboer et al., 1988a; Costa et al., 1981b). These interpretations receive strong support from the work of Sen & Costa (1986a), who demonstrated that the pathway of delivery of nickel to Chinese hamster ovary cells determined the type of interaction with chromosomes. Nickel(II) salts, like crystalline NiS, induced chromosomal aberrations (gaps, breaks, and exchanges), but only the latter 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 has therefore illustrated by direct measurement of nickel, that the nuclear concentration of nickel might well determine such differences in genotoxic responses.

The response to $NiCO_3$ warrants an additional comment. Crystallinity has generally been accepted as a prerequisite for the uptake of nickel compounds by phagocytosis. Although poorly crystalline, the $NiCO_3$ used in our studies was considerably more efficient in delivering nickel to the cells than the water-soluble salts. Like the salts, the nuclear fraction

of nickel was found to be small compared to the cytosolic levels observed, though still comparable to the other particulate compounds. This suggests that the $NiCO_3$ may cause cellular effects in a manner similar to the salts. Perhaps the rapid dissolution after uptake results in a lower degree of vacuolation than for the other solids.

In conclusion, this work has demonstrated that intracellular nickel(II) concentration, irrespective of the parent compound, constitutes an important determinant in the AS52 cell survival and mutagenic response to nickel compounds. This finding is consistent with the Nickel-Ion Hypothesis. However, this hypothesis must be augmented to allow for compartmentalization of dissolved nickel within the cell that is dependent on the nickel-uptake pathway. The uptake of particulates by phagocytosis elevates both the dissolved nickel levels in the cytoplasm and the nucleus, while nickel from salts accumulates preferentially in the cytoplasm. Our work strongly suggests that cytoplasmic and nuclear nickel accumulations appear to produce mutations by different mechanisms.

V. APPENDIX: X-Ray Diffraction d-Spacings and Intensities

Tables A-1 to A-5 are compilations of the standard and observed crystal lattice d-spacings and the corresponding relative intensities obtained from the X-ray powder diffraction data. The experimental diffraction patterns were obtained using CuK_{α} radiation at 1.5405 Å. For each compound tested, the d-values and relative intensities for the most closely matching JCPDS standard diffraction spectra are compiled next to the test compound data, with the corresponding d-values aligned. Standard spectra are from the compilations by the former Joint Committee on Powder Diffraction Standards (JCPDS--International Centre for Diffraction Data, 1988). Peaks of relative intensity of less than 5, in most cases, have not been detected and are therefore omitted from the standard spectra listings. Similarly, angles corresponding to d-values less than 1.3-1.4 Å were not scanned, and therefore several of the standard spectra lines below these values are not listed in the following tables.

This data has also been presented in Figures 2-1 to 2-8 in the form of plots of relative intensity (I/I_{max} or I/I_1, i.e. intensity of the peak compared to that of the strongest peak observed) versus the diffraction angle 2θ.

Table A-1: X-Ray Diffraction Analysis of $NiCO_3$ Samples

Standard JCPDS #12-276 $NiCO_3 \cdot 6H_2O$		Samples 2B		2C		2D	
d (A)	I/I^1	d(A)	I/I^1	d(A)	I/I^1	d(A)	I/I^1
9.4	100						
6.06	100	6.04	100	6.05	100	6.01	100
5.38	20	5.38	20	5.38	57	5.36	20
5.17	20	5.16	20			5.14	14
4.99	10	4.98	5	4.97	8	4.98	5
4.81	6					4.80	4
4.66	6						
3.76	20	3.75	10	3.77	12	3.75	13
3.65	70	3.65	47	3.65	57	3.64	56
3.40	60	3.40	33	3.41	19	3.39	32
3.21	16	3.21	8	3.23	16	3.21	8
3.11	40	3.11	21	3.11	23	3.10	21
2.98	10					2.99	4
2.78	40	2.78	16	2.78	19	2.78	14
2.69	10	2.69	5			2.67	2
2.62	20					2.63	7
2.57	10					2.57	4
2.38	50	2.37	17	2.38	21	2.37	21
2.31	40	2.31	12			2.31	11
2.26	6	2.26	7			2.25	4
2.21	6						
2.17	30	2.17	10			2.17	8
2.11	16					2.11	6
2.01	10					2.01	2
1.986	6						
1.965	16					1.962	7
1.949	16					1.946	4
1.824	35	1.82	8			1.824	8
1.790	16					1.786	4
1.724	30						
1.660	10						
1.634	6						
1.604	10						
1.553	20						
1.521	6						
1.499	20						

Table A2: X-Ray Diffraction Analysis of Various Nickel Oxide Samples

Standard #22-1189 NiO		Samples 3A		3B		4A		4B		5		Standard #23-362 Li2Ni8O10	
d (A)	I/I^1	d(A)	I/I^1	d(A)	I/I^1	d(A)	I/I^1	d(A)	I/I^1	d(A)	I/I^1	d(A)	I/I^1
												4.73	16
												2.465	6
2.412	60	2.417	64	2.410	64	2.408	58	2.412	69	2.389	53	2.376	20
2.088	100	2.089	100	2.088	100	2.086	100	2.090	100	2.069	100	2.058	100
1.477	35	1.476	39	1.477	42	1.476	36	1.477	33	1.463	42	1.455	35
1.476	35											1.240	10
1.260	18											1.188	10
1.259	12											1.029	6
1.206	16											0.944	6
1.045	8											0.920	10

Table A3: X-Ray Diffraction Analysis of "Amorphous NiS" Samples

Standard JCPDS #33-955 $NiSO_4 \cdot 6H_2O$		Standard JCPDS #11-99 NiS2		Samples 6A		6B	
d(Å)	I/I^1	d(Å)	I/I^1	d(Å)	I/I^1	d(Å)	I/I^1
5.98	5						
5.824	20			5.821	22	5.823	21
5.538	6						
5.424	21			5.421	18	5.429	20
5.061	21			5.062	20	5.058	26
4.900	51			4.892	23	4.896	20
4.782	24			4.781	9	4.789	13
						4.583	10
4.519	6					4.526	7
4.367	100			4.364	100	4.367	100
4.314	21						
4.096	22			4.084	14	4.089	15
4.003	60			4.001	54	4.001	65
3.865	4			3.874	6		
3.625	14			3.616	14	3.623	18
3.576	9						
3.544	20			3.545	9		
3.432	8						
3.340	8						
		3.27	20	3.300	6	3.276	12
3.162	7					3.166	12
3.001	6						
2.992	11			2.993	6	2.980	14
2.979	17						
2.916	26			2.911	28		
2.890	38			2.893	29	2.891	41
		2.83	100	2.840	50	2.834	65
2.818	8						
2.774	9						
2.690	5			2.701	10		
2.681	5					2.681	18
2.660	5						
2.570	9						
		2.54	40	2.540	12	2.544	16
				2.518	13		
2.471	12						
		2.32	40			2.321	13
2.306	5						
2.272	13						
2.246	5						
2.209	5						
1.995	7						
		2.00	50	2.002	13	1.999	11
1.990	10						
1.981	12			1.978	6		
1.954	5					1.951	5
1.909	5						
1.890	7	1.892	5				
1.853	14			1.851	6		
		1.707	80	1.708	11	1.708	28
		1.634	20				
		1.570	30				
		1.514	30				

Table A4: X-Ray Diffraction Analysis of Crystalline Nickel Sulphide

JCPDS Standard Patterns									
#24-1021		12-41		13-435		#30-863			
Ni7S6		NiS, millerite		NiSO4		Ni3S2		Sample 7	
d(A)	I/I[1]	d(A)	I/I[1]	d(A)	I/I[1]	d(A)	I/I[1]	d(A)	I/I[1]
5.67	10							5.673	27
								4.962	11
		4.81	60					4.807	12
4.67	25							4.657	8
4.50	10								
				4.314	40				
4.11	25							4.199	10
						4.081	69	4.070	38
3.93	10			3.921	30			3.915	11
								3.637	21
				3.564	80			3.569	8
3.43	15							3.404	15
3.32	10			3.334	50				
3.14	5							3.126	9
		2.946	40					2.938	22
2.920	50							2.910	44
						2.873	100	2.871	28
2.841	15							2.830	27
2.792	30	2.777	100					2.800	37
2.734	30								
2.713	90							2.703	91
2.630	10								
				2.579	45				
				2.553	100			2.553	13
		2.513	65					2.514	13
2.465	55							2.460	26
2.438	65	2.406	12			2.378	36	2.433	51
2.333	15			2.331	50	2.349	12	2.329	9
								2.259	7
2.246	25	2.228	55					2.241	25
2.146	5								
2.113	10							2.119	10
								2.102	17
				2.037	6	2.040	37	2.039	9
				2.001	25				
1.969	30			1.959	16			1.959	27
1.930	10							1.902	9
1.875	100	1.863	95	1.879	8			1.863	100
				1.860	8				
1.846	10							1.848	22
1.830	25					1.832	52	1.825	24
1.812	50	1.818	45			1.819	45	1.812	40
1.783	5			1.781	25				
1.760	35							1.759	19
1.737	10	1.737	49						
1.715	45							1.704	10
1.698	25					1.679	18	1.689	19
				1.679	8				
				1.667	16	1.664	59	1.659	4
1.637	50	1.634	18					1.635	48
1.626	25			1.629	9			1.623	18
		1.604	35						
		1.547	25						

Table A5: X-Ray Diffraction Analysis of Ni_3S_2

Standard JCPDS #30-863 Ni3S2		Sample 8	
d(A)	I/I^1	d(A)	I/I^1
4.0810	69	4.0825	59
2.8734	100	2.8721	100
2.3781	36	2.3793	33
2.3493	12	2.3497	12
		2.0681	2
2.0404	37	2.0410	32
1.8323	52	1.8326	49
1.8186	45	1.8192	40
1.6789	18	1.6797	13
1.6638	59	1.6638	47
1.3718	2		
1.3607	2		
1.3552	3	1.3547	3
1.2944	8		
1.2256	7		

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