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In Vitro Toxicity and Transformation Potency of Nickel Compounds

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An *in vitro* bioassay utilizing BHK-21 cells in culture is used to determine the relative transformation potential of a number of nickel compounds including, as relatively insoluble particulates a known carcinogen (Ni_3S_2) and several oxides either of commercial interest or found in the working environment in the metal industry (e.g., NiO), and a soluble salt [$Ni(CH_3COO)_2$]. Although a wide range of transformation potency is found as a function of the dose of Ni per area of culture, all substances produce the same number of transformed colonies at the same degree of toxicity (e.g., 50% survival). If toxicity is a direct measure of intracellular concentration, then apparently nickel *per se* is the ultimate transforming agent independent of source or uptake mechanism.

Introduction

Nickel compounds exhibit a wide range of *in vivo* toxicity and carcinogenicity, and show a similarly large variation of solubility in water and biologically relevant fluids *in vitro* and *in vivo* (*1 – 7*); they are also active in short-term bioassays utilizing mammalian cells in culture, e.g., cell transformation (*8 – 10*) and sister chromatid exchange (SCE) (*11*). It has been of considerable interest to determine which properties of the individual substances or which rate-limiting steps of the *in vitro* or *in vivo* bioassay determine the extent of these variations. In recent attempts to identify the relevant mechanism, Niebuhr et al. (*11*) have shown that SCE in human lymphocytes is dependent only on the strongly pH-dependent dissolved molar nickel concentration regardless of source material, and Costa et al. (*8*) have demonstrated that the transformation potential of a number of nickel compounds at equal doses is determined by the degree of phagocytotic activity induced. Although the data do not permit a quantitative comparison of compounds of high and low transforming potency, an independent analysis of the results suggests that, once nickel compounds have entered the cell, their transformation potential may depend only on the intracellular nickel content and be independent of the nature of original material.

In order to determine whether or not this is the case in general, a series of semiquantitative experiments using a BHK-21 cell transformation assay has been undertaken. If it can be assumed that toxicity is a direct measure of the intracellular concentration of nickel, regardless of source, then transformation experiments performed at equivalent toxic levels, e.g., 50% survival rate, should uniquely permit a direct comparison of the specific (molar) transformation potential of a variety of nickel compounds.

Materials and Methods

BHK-21 cells (baby hamster kidney cell line) were cultured in small plastic flasks in Dulbecco's modified Eagle's medium supplemented with 20% newborn calf serum. When the cell monolayer was 50-80% confluent, a transformation test was performed as follows: the growth medium was renewed (4 mL), then the test solution or suspension added in the appropriate concentration, and the culture flasks incubated at 37°C in 5% CO_2 for either 6 or 24 hr. After this treatment, the test solutions were discarded, and the cell monolayers rinsed with PBS (phosphate buffered saline); the cells were then trypsinized and converted to a single cell suspension by gentle pipetting. Approximately 5.0×10^5 cells from each culture flask were transferred to 8 mL of culture medium, of which 2×20 μL was plated in Petri dishes for a determination of the toxic effect of the test compound. Melted agar was added to the rest of the cell suspension to a final concentration of 0.3% and quickly poured into precooled Petri dishes. All dishes were then incubated in 5% CO_2 at 37°C; pH 7.3 is maintained constant throughout the test.

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To determine toxicity, the appropriate dishes were stained with crystal violet after 5 days of incubation. After 3 weeks of incubation in soft agar, the transformed colonies were counted. Nontransformed cells divide only a limited number of times in soft agar and hence form colonies smaller than 100 μm diameter. Only transformed cells grow into large colonies of 300-600 μm diameter, which can easily be identified and counted. All dishes were scored under a stereomicroscope at 8 × magnification. This test procedure is a modification of that originally proposed by Styles (*12*).

Two nickel oxides—Ni_2O_3 (Merck) and NiO (reagent grade, B.D.H. Laboratory Reagents)—nickel subsulfide α-Ni_3S_2 and corundum Al_2O_3 (Merck) (as a negative control) were ground in an agate mortar until 90-95% of the particles were smaller than 4 μm and none of them larger than 10 μm, as measured optically. This treatment is necessary since BHK cells are only able to phagocytize particles smaller than 4-5 μm (*8*).

A fume produced by metal inert-gas welding (MIG) with a pure Ni wire was included in the study as an example of a nickel containing aerosol found in the working environment. This fume which has a mass median diameter of approximately 1 μm (but which contains some much larger particles) is composed of 56.3% nickel of which 0.13% is water-soluble, distributed as Ni:NiO in a ratio of approximately 1:10. The fume also contains 0.04% Cr and 19% Fe and has been described elsewhere (*11, 13*). It has a carcinogenic potency in rats similar to that of NiS (*14*). The $Ni_2O_3 \cdot 6\ H_2O$ (a commercial catalyst for organic reactions) is a mixture of Ni(II) and Ni(IV) oxides.

The nickel acetate was dissolved, and the ground particles and welding fume were suspended in sterile distilled water. A preliminary toxicity test was performed for all compounds to ensure that the 50% toxic level was included in each transformation test sequence. The cells were exposed to the range of nickel compounds indicated in Figures 1 and 2, for 6 and 24 hr in two separate runs of three compounds each.

Results and Discussion

The effect of the nickel compounds on the BHK cells is shown in Figure 1 [for Run One ($Ni_2O_3 \cdot 6\ H_2O$,

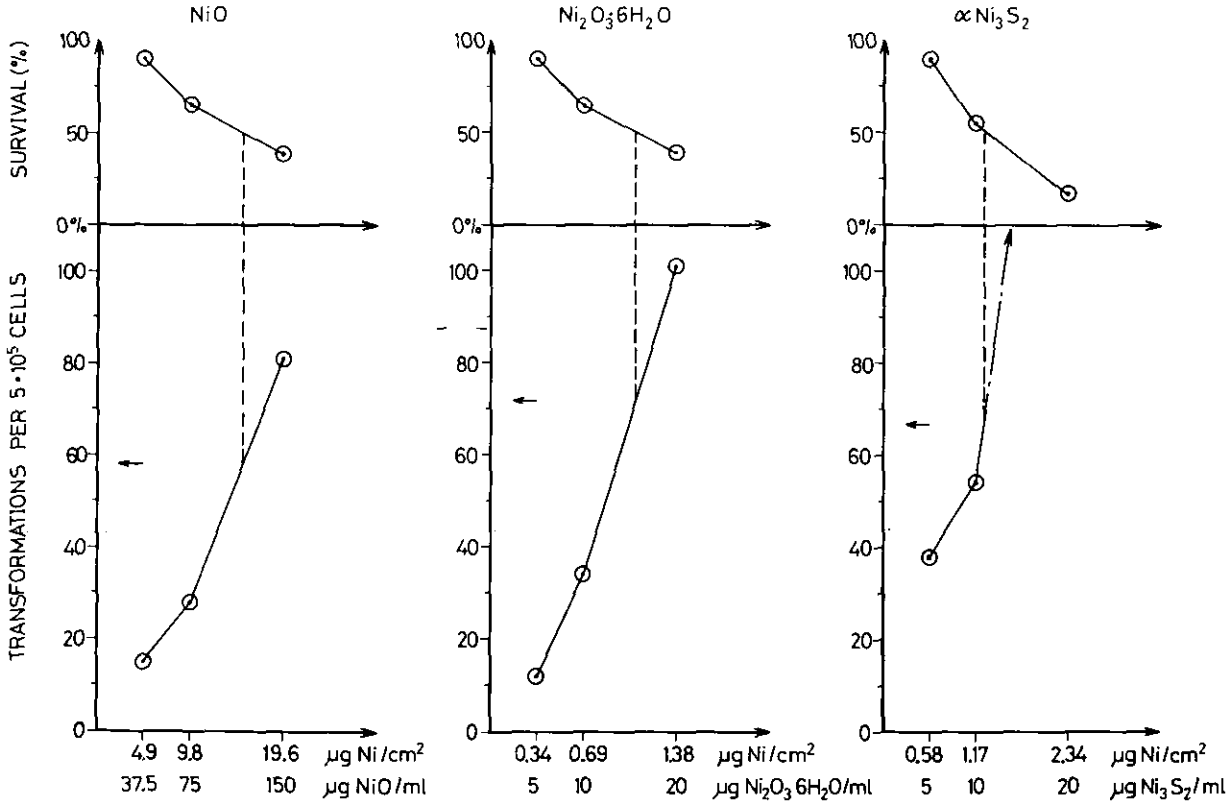

FIGURE 1. Run 1. Survival rate as a function of dose, and number of transformed colonies as a function of dose, for insoluble particulates of NiO, Ni_3S_2 and $Ni_2O_3 \cdot 6\ H_2O$. Note that the dose is given both as concentration of test compound and as dose of Ni per unit area of cells. Exposure for 6 hr and 24 hr results in identical values of toxicity and transformation rates. Transformation frequencies at 50% survival are identical.

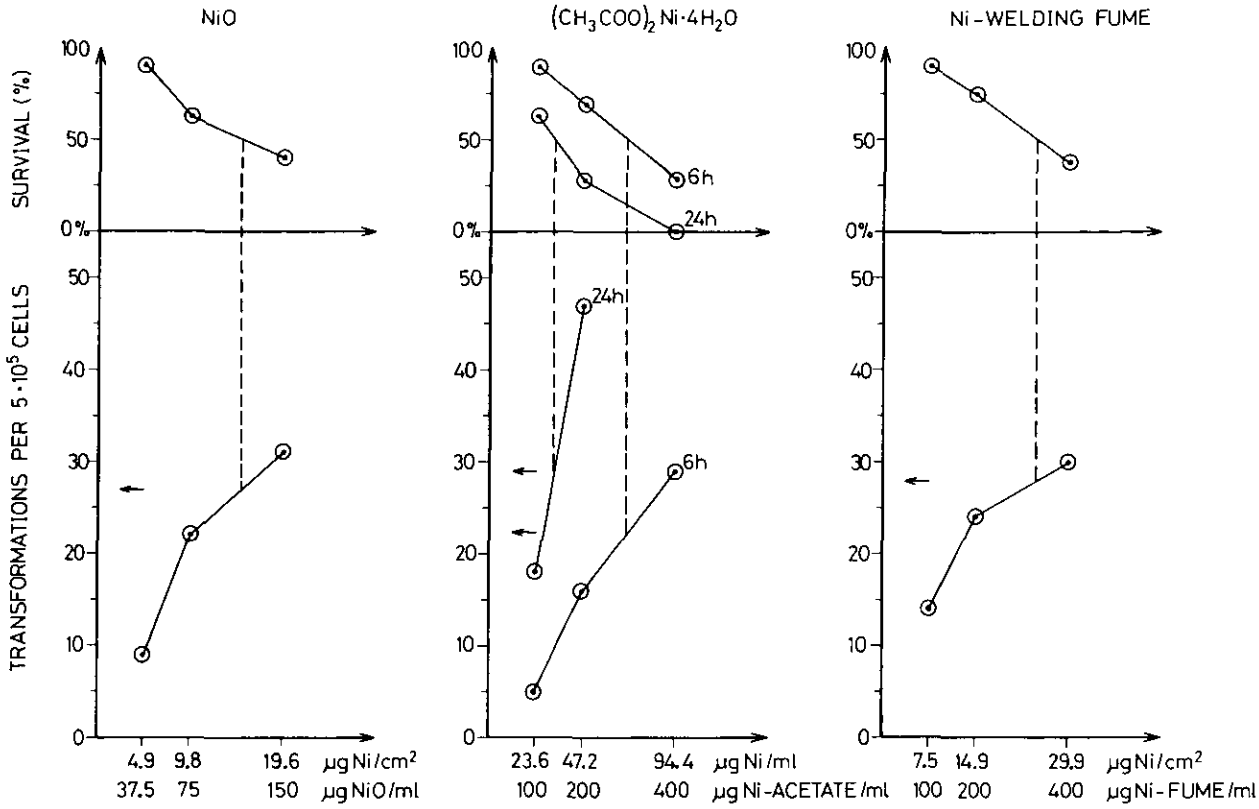

FIGURE 2. Run 2. Survival rate as a function of dose, and number of transformed colonies as a function of dose, for NiO, Ni:NiO MIG welding fume, and $Ni(CH_3COO)_2$. Transformation frequencies and toxicities for particulates are independent of exposure time: those for nickel acetate are proportional to exposure time as indicated. Transformation frequencies at 50% survival are identical. To compare with results of run 1, normalize values of NiO common to both. Nickel acetate dose given only as volume concentration: (4 mL medium for each exposure).

NiO and Ni_3S_2)] and Figure 2 [for Run Two ($Ni(CH_3COO)_2 \cdot 4\ H_2O$, Ni:NiO MIG fume, NiO)]. No difference in either toxicity or transformation frequency was observed after 6 hr exposure compared to 24 hr exposure for the particulates; hence only one of the results is shown in each case. For nickel acetate the transformation rate and toxicity are proportional to exposure time, and the results for both exposures are presented.

The test procedure as described above ensures a constant and low transformation frequency of 3.9 ± 2.3 (SD, n = 22). Corundum was nontoxic and nontransforming at a concentration of 32 μg/cm² of cell monolayer.

The actual number of transformed colonies for a given dose of Ni varies from run to run; however, the response ratio for different substances at equal toxicity (or at equal dose) remains constant for all runs. The absolute response of the system to any given substance can therefore be determined by normalizing the responses of different runs with respect to that of substances common between them, e.g., NiO for runs 1 and 2.

Examination of Figures 1 and 2 shows that at the 50% toxicity level all compounds tested have essentially the same transforming activity compared to NiO. This suggests that the transformation effect depends only on the amount of nickel picked up by the cells. It should be noted, however, that the 50% toxic dose level is 10 times lower for Ni_3S_2 and $Ni_2O_3 \cdot 6\ H_2O$ as compared to that for NiO and $Ni(CH_3COO)_2$ (or conversely, at equal Ni dose levels the transformation rate for Ni_3S_2 is approximately 10 times that for NiO); it is also noteworthy that the commercial catalyst $Ni_2O_3 \cdot 6\ H_2O$ has the same transforming effect as the carcinogen Ni_3S_2.

Nickel metal (Bie and Berntsen A/S) was also assayed in the cell transformation test and was found to be neither toxic nor transforming at a maximum concentration of 48 μg/cm². Since more than 50% of the particles were larger than 5 μm, and some were larger than 20 μm, this result is misleading, as little of the material is available for phagocytosis, and the effective dose is probably at least a factor of 10 below that used. Ni metal ultimately can be made transforming at sufficiently high doses. This result empha-

sizes the fact that insoluble compounds are only picked up by the cells through phagocytosis.

Because no rise in toxicity was observed after 24 hr of exposure compared to 6 hr of exposure for any of the relatively insoluble particulates, apparently all available nickel had been picked up by the cells or was at least firmly attached to the cell membrane and thereby carried over to the incubation step after the PBS rinse by the end of the 6 hr exposure. On the other hand, the nickel acetate solution was more toxic after 24 hr than after 6 hr, indicating a difference in the rates associated with different uptake mechanisms for particulates and dissolved material.

If the initial assumption is correct that toxicity is a direct measure of intracellular nickel concentration, then these observations of equal transformation rates at equal survival levels for all compounds tested can be interpreted as supporting a model whereby nickel is the ultimate intracellular biologically active material, independent of source. This implies that the only property which determines potency of various nickel compounds is the bioavailability of nickel critical to a given bioassay, *in vitro* and presumably *in vivo* as well.

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