Selective Phagocytosis of Crystalline Metal Sulfide Particles and DNA Strand Breaks as a Mechanism for the Induction of Cellular Transformation

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ABSTRACT

Crystalline NiS, CuS, CoS₂, and CdS particles were actively phagocytosed by cells and potently induced morphological transformation of Syrian hamster embryo cells in a concentration-dependent fashion. In contrast, the respective amorphous metal sulfide particles (amorphous NiS, CuS, CoS₂, and CdS) were not as actively phagocytosed by cultured cells and, in comparison to the crystalline form of these compounds, induced considerably less morphological transformation at both cytotoxic and noncytotoxic exposure levels. Chemical reduction of positively charged amorphous NiS with LiAlH₄ resulted in active phagocytosis of these particles which was also associated with enhancement of cellular transformation. Crystalline but not amorphous NiS caused considerable strand breaks in the DNA of Chinese hamster ovary cells following 2 to 3 hr exposure at 10 μg/ml as determined by alkaline sucrose gradient techniques with subsequent determination of DNA molecular weight. Phagocytized inert particles such as latex beads did not induce transformation or DNA damage, suggesting that genotoxic dissolution products such as Ni²⁺ rather than the phagocytized particles are responsible for the observed DNA damage and cellular transformation. NiCl₂ was about one-third to one-half as potent in inducing cellular transformation compared to crystalline NiS on a weight basis. These results correlate the selective phagocytosis of crystalline metal sulfides to their more potent activity in the induction of cellular transformation.

INTRODUCTION

Water-insoluble crystalline nickel sulfide compounds have been reported to induce a consistently high incidence of cancers in experimental animals at numerous sites of administration (10, 13, 16, 23). In contrast, similar treatment with amorphous NiS does not induce cancer at any of a variety of tested sites (23). Intrigued by these initial observations, the fact that certain nickel compounds have been implicated in human respiratory cancer (4, 14, 15), and the lack of tissue specificity in the induction of tumors displayed by these crystalline nickel sulfide particles, our laboratory has sought to understand the cellular basis for the differences in potency between crystalline and amorphous NiS compounds (1–3, 6–9, 17).

Using the SHE⁴ cell transformation assay, we demonstrated that crystalline NiS₂ (8) and other crystalline nickel sulfide compounds (9) induced neoplastic transformation while amorphous NiS was relatively inactive (8). Further studies showed that the relatively high transformation potential of the crystalline NiS particles in comparison to the low transforming activity of the amorphous NiS particles may be attributable to the selective phagocytosis of the former compounds by SHE cells in a manner similar to that which has been observed in various cultured fibroblasts (6, 7). We then demonstrated that, although crystalline and amorphous NiS particles have numerous chemical and physical similarities, crystalline NiS particles had a negative surface charge while amorphous NiS particles had positively charged surfaces (2) under physiological conditions. To further understand the relationship between phagocytosis, cell-transforming activity, and surface charge, we have investigated other crystalline and amorphous metal sulfide compounds. The present findings lend further support to and extend our previous hypothesis with the observation that the phagocytic uptake of a number of metal sulfide compounds is related to their in vitro cell-transforming potency and that such uptake is specific for particulate compounds with a crystalline configuration.

MATERIALS AND METHODS

Materials. The amorphous metal sulfides were prepared by precipitation of the sulfide salt from ultrapure solutions of NiCl₂, CdCl₂, CuCl₂, or CoCl₂ in H₂O using an excess of (NH₄)₂S. The respective crystalline metal sulfides were purchased from Alfa Products (Beverly, Mass.). The amorphous compounds were extensively washed with distilled water to remove any unreacted reagents. All particulate compounds were ground in an impact grinder (Spex Industries, Metuchen, N. J.). The small particles contained in the resulting ground material were purified by filtration through Nucleopore filters or by gravity sedimentation through a 2-m column of water containing 0.01% Triton X-100 to prevent particle flocculation and adsorption onto the column walls. Particle sizing was accomplished using a microscopic eyepiece micrometer and a Coulter particle size analyzer (Coulter Electronics, Hialeah, Fla.). Crystalline or amorphous structure was verified by powder X-ray diffraction.

Methods

Cell Transformation Assay and Phagocytosis of Metal Sulfide Particles in Cell Cultures. The SHE cell morphological transformation assay was performed as described previously (6–8). SHE cells isolated

⁴ The abbreviations used are: SHE, Syrian hamster embryo; CHO, Chinese hamster ovary.
on the 13th day of gestation were maintained in Dulbecco’s modification of Eagle’s minimal essential medium supplemented with 10% fetal bovine serum (Biolabs, Northbrook, Ill.). Second-passage cells were seeded at $2 \times 10^5$ cells/100-mm culture dish containing 10 ml of the growth medium. The metal particles were applied to cells maintained in complete growth medium as suspensions in 5 mM phosphate buffer, pH 7.2, containing 0.15 M NaCl in a series of 2 treatments 24 and 72 hr following cell plating. The cell monolayers were rinsed with this 0.15 M NaCl and given fresh growth medium before the second metal treatment. Forty-eight hr after this second treatment, the cells were washed, trypsinized, and replated as $3 \times 10^3$, $3 \times 10^4$, and $6 \times 10^4$ cells/plate in fresh growth medium. Following a 20-day growth period, surviving SHE cell colonies were fixed in 95% ethanol, stained with crystal violet, and scored for the disordered transformed colony morphology (11) by 3 independent observers who were unaware of the treatment condition.

CHO cells were used for uptake studies since this cell line forms a conspicuous cytoplasmic vacuole around phagocytized metal particles which facilitates differentiation between intracellular and surface-adSORBED particles (6, 7). It was considerably more difficult to quantitate actual uptake of the particles in SHE cells with light microscopy since confirmation of particle uptake by vacuolization in these cells required electron microscopy resolution (6, 7). CHO cells maintained in McCoy’s Medium 5A supplemented with 10% fetal bovine serum were treated with sonicated suspensions of crystalline or amorphous metal sulfides at concentrations ranging from 1 to 20 g/ml of medium (0.13 to 3.6 g/mg sq cm of surface growth area) for 24 hr, and phagocytosis of metal particles was quantitated following fixation of the cells with 95% ethanol and staining with crystal violet (6, 7). The percentage of cells with intracellular metal particles was determined by examination of at least 1000 cells/60-mm plate with the light microscope, as described previously (6, 7). Previous studies using both light and electron microscopy have shown a good correlation in the uptake of crystalline and amorphous NiS among SHE, CHO, and a number of other cell types (1, 2, 6, 7). Since the use of electron microscopy to confirm particle internalization in SHE cells is tedious and inefficient, light microscopic quantitation of particle endocytosis by the easily studied CHO cells was used.

Surface reduction of crystalline and amorphous NiS particles was performed in a nitrogen atmosphere by gently stirring for 1 hr 2 mg of each particle type in 2 ml of pyridine containing an excess of dissolved LiAlH4. Untreated particles were stirred in pyridine alone. The particles were then collected by centrifugation. Following 3 rinses in pyridine and then in ethyl ether, the particles were dried under N2 and suspended by sonication in sterile 0.15 M NaCl contained in capped, N2-purged vials.

**Assay of DNA Strand Breakage by Alkaline Sucrose Gradients.**

CHO cells were seeded (1 x 10^6 cells) into 60-mm tissue culture dishes in 5 ml of McCoy’s Medium 5A containing [H]thymidine (0.4 gCi/ml; specific activity, 55.2 Ci/mmol), and cells were labeled in this medium for 1 day, whereupon the radioactive medium was replaced with complete medium lacking [H]thymidine, and cells were allowed to reach confluence for an additional 2 days. The cells were then treated with the various nickel compounds for the time intervals specified in Chart 1. The cell monolayer was rinsed once with Puck’s Saline A and then scraped with a rubber policeman, and the cells were collected by centrifugation. The resulting cell pellet was then resuspended in sufficient Puck’s Saline A so that an aliquot of 100 ml contained approximately 8 x 10^6 cells. Cell number was determined with a Coulter Counter.

Alkaline sucrose gradients (5 to 20%, w/v) containing 0.3 M NaOH, 0.7 M NaCl, 10 mM EDTA, and 0.05 M Tris were formed over a 0.2-ml pad of 60% sucrose (w/v). The gradients were overlaid with 0.2 ml of a lysis solution consisting of 0.2% sodium dodecyl sulfate, 0.2 mM EDTA, and 0.05 M NaOH. An aliquot of cells was gently layered into the lysis solution and incubated for 18 hr at room temperature to ensure complete cell lysis. The gradients were then centrifuged in a Beckman SW 50.1 swinging bucket rotor at 40,000 rpm for 2 hr. The gradients were fractionated by collecting 10 drops per fraction onto Whatman No. 3MM filter paper strips. The strips were washed twice in 10% trichloroacetic acid and then washed in ethanol, dried, cut up, and counted in toluene-Omnifluor using a Packard liquid scintillation counter.

**DNA Molecular Weight Calculations from Alkaline Sucrose Gradients.**

To further analyze DNA strand breaks induced by nickel compounds, the molecular weight of DNA was calculated according to the following formulas:

$$M_0 = \frac{\Sigma w_i}{\Sigma (w_i/M_i)} \quad (A)$$

where $w_i$ is the weight of DNA in the $i$th fraction and is proportional to the amount of radioactivity and $M_i$ is the molecular weight in the $i$th fraction and is determined from the relationship

$$S_i = 0.0528M_i^{0.4} \quad (B)$$

where $S_i$ is the S value at the $i$th fraction and 0.0528 is a constant for alkaline sucrose containing sodium chloride. The S value was determined by using 53S gradient markers and by calculating from the relationship

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**Figure**

Chart 1. Representative effects of crystalline and amorphous NiS on DNA strand breaks in CHO cells. Molecular weight of DNA (see “Materials and Methods”) was calculated from these alkaline sucrose gradients ($x 10^{-7}$): untreated: 7.2; amorphous NiS (10 g/ml): 2.5 hr, 7.3; 4 hr, 7.2; 2 hr, 18 hr, 7.3; 24 hr, 7.6; crystalline NiS (10 g/ml): 0 hr, 6.8; 2 hr, 5.4; 3 hr, 5.2; 7 hr, 5.0; 24 hr, 4.9. Similar results have been observed in additional experiments.

SW 50.1 swinging bucket rotor at 40,000 rpm for 2 hr. The gradients were fractionated by collecting 10 drops per fraction onto Whatman No. 3MM filter paper strips. The strips were washed twice in 10% trichloroacetic acid and then washed in ethanol, dried, cut up, and counted in toluene-Omnifluor using a Packard liquid scintillation counter.
In this relationship, \( d \) is the distance the marker traveled, \( (\omega^2 t) \) is the rotor speed in rpm corrected for angular velocity, and \( t \) is the time of centrifugation in sec. The value for \( k \) is a rotor constant, and for our rotor it is \( 2.175 \times 10^{-2} \).

### RESULTS

**Importance of Surface Charge in Regulating the Phagocytosis and Subsequent Induction of Cellular Transformation.** Crystalline NiS particles were phagocytosed more than amorphous NiS particles and similarly resulted in a higher incidence of transformation (Table 1). Previous studies have regarded the surface properties of these compounds as an important determinant of their ability to be phagocytosed, and the high level of phagocytosis displayed by crystalline NiS was directly related to its negative surface charge \([-27 \text{ mV}, \xi \text{ potential (2)}]\); in contrast, the low level of phagocytosis displayed by amorphous NiS particles was related to a positive charge on the surface microenvironment \([+9 \text{ mV}, \xi \text{ potential (2)}]\). Table 1 confirms these findings by showing that treatment with a reducing agent (LiAIH4) resulted in significant enhancement of the phagocytic uptake of both crystalline and amorphous NiS concomitant with a proportional increase in the incidence of transformation. Previous studies have demonstrated that LiAIH4 treatment rendered the surface of crystalline and amorphous NiS particles more negative (2). Thus, positively charged, untreated amorphous NiS particles induced little transformation but, following treatment with LiAIH4, these particles exhibited a more negative surface charge and caused an incidence of transformation equaling that of untreated crystalline NiS. Solvent washing and chemical reduction of both amorphous and crystalline NiS also resulted in decreased colony survival. This was probably due to the enhancement of their uptake into cells (Table 1).

Fig. 1 demonstrates the appearance of a colony which was transformed by crystalline NiS particles while Fig. 2 shows the appearance of a normal colony of SHE cells. Fig. 4 highlights a section of a transformed colony at higher magnification revealing the disordered growth of the cells within that colony. The interaction and uptake of crystalline NiS particles with living cultured cells are shown in Fig. 3. Note the crystalline NiS particles which have been phagocytosed by the cells (arrow).

**Effect of Ionic Nickel and Latex Beads on the Incidence of Transformation.** NiCl₂ induced transformation in SHE cells, but its potency was less than that of crystalline NiS (Table 2). This result may be related to the higher cellular uptake of nickel in the form of crystalline NiS compared with the lower uptake of ionic nickel (9). Latex beads were actively phagocytosed by these cultured cells but induced little cell transformation (Table 2). Additionally, treatment of CHO cells with latex beads (5 µg/ml) resulted in no strand breaks in the DNA (i.e., number average molecular weight: untreated, \( 7.0 \times 10^{-7} \); latex beads, \( 6.9 \times 10^{-7} \)). These results indicated that phagocytosis of an inert particle does not lead to significant transformation or DNA strand breakage. Additionally, it should be noted that there were no transformations in untreated cultures (Table 2).

**Induction of Cellular Transformation by Crystalline and Amorphous NiS**

### Table 1

**Effect of various treatments on the induction of cell transformation by crystalline and amorphous NiS**

<table>
<thead>
<tr>
<th>Treatment compound</th>
<th>Particle size (µm)</th>
<th>µg/ml</th>
<th>µg/sq cm growth area (4.47)</th>
<th>Total no. of plates examined</th>
<th>Incidence of morphological transformation</th>
<th>% of phagocytosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated crystalline NiS</td>
<td>2.37</td>
<td>5</td>
<td>0.63</td>
<td>6</td>
<td>17/1155 (1.47)</td>
<td>ND</td>
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<td></td>
<td>10</td>
<td>1.27</td>
<td>6</td>
<td>26/972 (2.67)</td>
<td>29.2</td>
<td></td>
</tr>
<tr>
<td>Untreated amorphous NiS</td>
<td>1.0</td>
<td>1</td>
<td>0.13</td>
<td>7</td>
<td>2/510 (0.39)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.63</td>
<td>8</td>
<td>8/1949 (0.41)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.27</td>
<td>9</td>
<td>10/2054 (0.48)</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>Solvent-washed crystalline NiS</td>
<td>2.07</td>
<td>10</td>
<td>1.27</td>
<td>4</td>
<td>12/268 (4.47)</td>
<td>33.3</td>
</tr>
<tr>
<td>LIAIH₄-reduced crystalline NiS</td>
<td>3.76</td>
<td>10</td>
<td>1.27</td>
<td>7</td>
<td>40/543 (7.49)</td>
<td>51.0</td>
</tr>
<tr>
<td>Solvent-washed amorphous NiS</td>
<td>2.66</td>
<td>10</td>
<td>1.27</td>
<td>6</td>
<td>3/248 (1.21)</td>
<td>9.9</td>
</tr>
<tr>
<td>LIAIH₄-reduced amorphous NiS</td>
<td>2.26</td>
<td>10</td>
<td>1.27</td>
<td>5</td>
<td>6/183 (3.27)</td>
<td>27.7</td>
</tr>
</tbody>
</table>

* Number of morphologically transformed cells/total number of surviving colonies (SHE cells).
+ Number of cells with phagocytized NiS particles/total number of cells examined (CHO cells).
- Differs from amorphous NiS at corresponding exposure concentration; \( p < 0.001 \) (\( \chi^2 \) test).
\# Numbers in parentheses, percentage.
- ND, not determined.
- Differs from untreated crystalline or amorphous NiS particles; \( p < 0.05 \) (\( \chi^2 \) test).
\# Reduced crystalline NiS does not differ significantly from solvent-washed crystalline NiS; \( p = 0.11 \) (\( \chi^2 \) test).
\$ Reduced amorphous NiS differs from solvent-washed amorphous NiS; \( p < 0.03 \) (\( \chi^2 \) test).
Amorphous Metal Sulphides. At relatively noncytotoxic levels (see legend to Table 3), crystalline CoS$_2$ and CdS displayed greater cell-transforming activities than did the respective amorphous compounds (Table 3). At cytotoxic levels (10 µg/ml), crystalline CoS$_2$ and amorphous CoS induced 1.75 and 0.90% transformation, respectively, indicating some greater potency of the crystalline form. These differences in transformation were, however, not as large as the differences observed when less cytotoxic levels of the 2 compounds were compared (i.e., 1 and 5 µg/ml). At 10 µg/ml, crystalline CuS was more cytotoxic than was amorphous CuS, probably due to its greater uptake, and similarly induced more transformation. Crystalline CdS induced more transformation at both cytotoxic and noncytotoxic levels compared to amorphous CdS. The uptake of the crystalline compounds was generally greater than that of the amorphous structures (Table 3). This was quantitatively determined for amorphous CoS and CuS, while the uptake of amorphous CdS particles was difficult to observe quantitatively with light microscopy. However, qualitative assessment indicated considerably less uptake of amorphous CdS than of crystalline CdS. Detailed studies of surface charge have been conducted with crystalline NiS or CoS$_2$ and with amorphous NiS or CoS (2). The crystalline CoS$_2$ demonstrated a net negative surface charge, while the amorphous compound had a positive surface charge (2). These results are supportive of the findings shown in Table 1. Note also that cytotoxicity may result from the detachment of cells by particle interaction independent of their phagocytosis, since in our system all particles eventually settle on the monolayer of cells.

Effect of Crystalline and Amorphous NiS on DNA Strand Breaks. Alkaline sucrose gradient analysis of DNA from CHO cells treated with either crystalline NiS or amorphous NiS demonstrated that crystalline NiS but not amorphous NiS caused substantial strand breakage (Chart 1). Within 2 to 3 hr following treatment of cells with crystalline NiS (10 µg/ml),

Table 2

<table>
<thead>
<tr>
<th>Treatment identity</th>
<th>Particle size (µm)</th>
<th>µg/ml</th>
<th>µg/sq cm growth area</th>
<th>Total no. of plates examined</th>
<th>Incidence of morphological transformation $^a$</th>
<th>Number of transformed colonies/total number of surviving colonies $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NiCl$_2$</td>
<td>5</td>
<td>NA</td>
<td>10</td>
<td>6/2224 (0.35) $^{c,d}$</td>
<td>0.7</td>
<td>0.89/2224 (0.04) $^{c,d}$</td>
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<tr>
<td></td>
<td>10</td>
<td>NA</td>
<td>9</td>
<td>12/1101 (1.08)</td>
<td>0.9</td>
<td>0.89/1101 (1.08)</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>NA</td>
<td>8</td>
<td>13/1503 (0.86)</td>
<td></td>
<td>0.89/1101 (1.08)</td>
</tr>
<tr>
<td>Latex beads</td>
<td>1</td>
<td>0.13</td>
<td>9</td>
<td>1/549 (0.21)</td>
<td>0.5</td>
<td>0.00/549 (0.00)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.63</td>
<td>9</td>
<td>8/1806 (0.40)</td>
<td>0.5</td>
<td>0.00/549 (0.00)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.27</td>
<td>6</td>
<td>2/971 (0.21)</td>
<td>0.5</td>
<td>0.00/549 (0.00)</td>
</tr>
<tr>
<td>Latex beads</td>
<td>1</td>
<td>0.13</td>
<td>9</td>
<td>1/549 (0.21)</td>
<td>0.5</td>
<td>0.00/549 (0.00)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.63</td>
<td>9</td>
<td>8/1806 (0.40)</td>
<td>0.5</td>
<td>0.00/549 (0.00)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.27</td>
<td>6</td>
<td>2/971 (0.21)</td>
<td>0.5</td>
<td>0.00/549 (0.00)</td>
</tr>
<tr>
<td>Untreated</td>
<td>NA</td>
<td>NA</td>
<td>3</td>
<td>0/662 (0)</td>
<td></td>
<td>0.00/662 (0)</td>
</tr>
</tbody>
</table>

$^a$ Number of transformed colonies/total number of surviving colonies (SHE cells).

$^b$ Not statistically significant from untreated cultures ($\chi^2$ test).

$^c$ Numbers in parentheses, percentage.

$^d$ Not statistically significant versus amorphous metal sulfides at the same exposure level.

$^e$ Relatively nontoxic particle exposure concentration, colony survival comparable to untreated cells (compared by dividing total number of surviving colonies by total number of plates examined).

$^f$ Numbers in parentheses, percentage.

$^g$ Cytotoxic particle exposure conditions, colony survival <50% of untreated cells due to either cell detachment due to particle interaction with cells or to phagocytosis of particles.

$^h$ $p < 0.05$ versus amorphous metal sulfide particles ($\chi^2$ test).

$^i$ Not statistically significant versus amorphous metal sulfides at the same exposure level.

$^j$ Uptake of amorphous CdS was more difficult to determine by microscopy due to its yellow color.

Table 3

<table>
<thead>
<tr>
<th>Metal compound</th>
<th>Particle size (µm)</th>
<th>µg/ml</th>
<th>µg/sq cm growth area</th>
<th>Total no. of plates examined</th>
<th>Incidence of morphological transformation $^{a}$</th>
<th>% of phagocytosis $^{b}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amorphous CoS</td>
<td>2.0</td>
<td>1</td>
<td>0.13</td>
<td>6</td>
<td>1/1157 (0.10) $^{c,d}$</td>
<td>0.7</td>
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<tr>
<td></td>
<td>5</td>
<td>0.63</td>
<td>8</td>
<td>4/2434 (0.16)</td>
<td>0.9</td>
<td>0.89/2434 (0.16)</td>
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<tr>
<td></td>
<td>10</td>
<td>1.27</td>
<td>8</td>
<td>6/668 (0.90)</td>
<td>1.9</td>
<td>0.89/668 (0.90)</td>
</tr>
<tr>
<td>Crystalline CoS$_2$</td>
<td>1.25</td>
<td>1</td>
<td>0.13</td>
<td>9</td>
<td>13/2271 (0.57) $^{f}$</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.63</td>
<td>9</td>
<td>18/2017 (0.89)</td>
<td>28.1</td>
<td>18/2017 (0.89)</td>
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<tr>
<td></td>
<td>10</td>
<td>1.27</td>
<td>6</td>
<td>9/514 (1.75) $^{g}$</td>
<td>48.3</td>
<td>9/514 (1.75)</td>
</tr>
<tr>
<td>Amorphous CuS</td>
<td>0.98</td>
<td>1</td>
<td>0.13</td>
<td>6</td>
<td>2/1754 (0.11) $^{c}$</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.27</td>
<td>6</td>
<td>8/1183 (0.67)</td>
<td>6.0</td>
<td>8/1183 (0.67)</td>
</tr>
<tr>
<td>Crystalline CuS</td>
<td>0.76</td>
<td>5</td>
<td>0.63</td>
<td>8</td>
<td>8/326 (2.4) $^{e,f}$</td>
<td>21.4</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.27</td>
<td>3</td>
<td>9/357 (2.5) $^{e,f}$</td>
<td>43.8</td>
<td>9/357 (2.5)</td>
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<tr>
<td>Amorphous CdS</td>
<td>0.64</td>
<td>1</td>
<td>0.13</td>
<td>6</td>
<td>1/2568 (0.03) $^{c}$</td>
<td>&lt;1 $^{h}$</td>
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<tr>
<td></td>
<td>5</td>
<td>0.63</td>
<td>6</td>
<td>1/365 (0.27)</td>
<td>&lt;1 $^{h}$</td>
<td>1/365 (0.27)</td>
</tr>
<tr>
<td>Crystalline CdS</td>
<td>0.64</td>
<td>1</td>
<td>0.13</td>
<td>6</td>
<td>12/1068 (0.13) $^{e,f}$</td>
<td>8.3</td>
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<tr>
<td></td>
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<td>0.63</td>
<td>6</td>
<td>13/666 (1.90) $^{e,f}$</td>
<td>12.1</td>
<td>13/666 (1.90)</td>
</tr>
</tbody>
</table>

$^a$ Number of transformed colonies/total number of surviving colonies (SHE cells).

$^b$ Number of cells containing metal sulfide particles/total number of cells examined (CHO cells).

$^c$ Relatively nontoxic particle exposure concentration, colony survival comparable to untreated cells (compared by dividing total number of surviving colonies by total number of plates examined).

$^d$ Numbers in parentheses, percentage.

$^e$ Cytotoxic particle exposure conditions, colony survival <50% of untreated cells due to either cell detachment due to particle interaction with cells or to phagocytosis of particles.

$^f$ p < 0.05 versus amorphous metal sulfide particles ($\chi^2$ test).

$^g$ Not statistically significant versus amorphous metal sulfides at the same exposure level.

$^h$ Uptake of amorphous CdS was more difficult to determine by microscopy due to its yellow color.
strand breaks could be detected (Chart 1). Breakage is characterized by the change in sedimentation of the DNA toward the right in the graphs shown in Chart 1 (lower sucrose concentrations, upper portion of the gradient tube). Additionally, strand breaks were also quantitated by determining the number average molecular weight of DNA (see legend to Chart 1). The slower-sedimenting DNA under alkaline conditions represents smaller fragments of DNA. The line drawn at the 20% point of the gradient serves as a reference guide for observing the breakage of DNA caused by crystalline NiS and the low level of breakage following treatment with amorphous NiS (Chart 1).

In the amorphous NiS-treated culture, most of the DNA sedimented in the bottom 20% of the gradient while with crystalline NiS treatment, a substantial portion sedimented within the top 80% of the gradient. Note also that the average molecular weight of DNA in the amorphous NiS-treated cells ranged from 7.2 to 7.6 \times 10^{-7}, while in the crystalline NiS-treated cells the number average molecular weight ranged from 4.9 to 5.4 \times 10^{-7} (see legend to Chart 1).

DISCUSSION

Chart 2 shows the major sequence of events which are currently implicated in the induction of cell transformation by crystalline NiS. The interaction of particles with the cell membrane is the initial and perhaps the most critical event since the low transforming activity of a number of particulate metal compounds can be explained solely on the basis of their not entering the cell by phagocytosis (9). The interaction of crystalline NiS particles with the cell membrane leads to their phagocytosis, while amorphous NiS particles of similar size interact with the cell surface in a manner which does not trigger phagocytic uptake (Chart 2). The negative surface charge on the crystalline NiS particle appears to be an important property associated with its phagocytosis (3). In fact, if amorphous NiS particles are rendered more electronegative on their surface microenvironment through reduction with LiAlH4, they are then actively phagocytosed and induce a level of transformation equaling that of crystalline NiS (Table 1). Video intensification microscopy studies indicate that both amorphous and crystalline NiS particles bind to the cell membrane primarily in areas of active cell ruffling but that amorphous NiS particles tend to detach more often than do crystalline NiS particles (12). These results suggest that the negative charge on the particle surface allows for a characteristic binding such that the particle becomes endocytosed. Components in the culture media do not appear to influence the active phagocytosis of crystalline NiS and the low uptake of untreated amorphous NiS since similar uptake levels are observed in cells maintained with a simple salts-glucose medium (NaCl-KCl-glucose-buffer-Ca^{2+}). Calcium ions have been found to be essential for the uptake of crystalline NiS particles (3).

It should be noted that our model for selective phagocytosis of metal particles is related to the target cells for transformation and not terminally differentiated cells such as macrophages which cannot become transformed. Macrophages are likely to serve as a mechanism for the removal of metal particles capable of transforming other cells (facultative phagocytes) in vivo. However, it is interesting to note that preliminary data using mouse peritoneal macrophages indicated that crystalline NiS is phagocytosed more than is amorphous NiS. The active uptake of crystalline or amorphous NiS by macrophages may result in greater nickel dissolution in localized regions in vivo which could lead to transformation of other target cells or alternatively may lead to the production of reactive intermediates such as malonylaldehyde through the formation of oxygen radicals. These reactive intermediates could be released by macrophages after their death or could simply be released during the process of particle phagocytosis and thus become available to initiate oxidative attack upon neighboring cells less well equipped with antioxidant defenses. It is unlikely that differences in extracellular dissolution of particulate nickel compounds contribute significantly to their carcinogenic potency or to their ability to inhibit their own phagocytosis. This statement is supported by the observation that crystalline NiS, a potent carcinogen, has a dissolution rate in serum or other biological media similar to that of amorphous NiS. Should particle-derived Ni^{2+} play a significant role in inhibiting particle uptake, then it might be expected that compounds exhibiting similar solubilities would be taken up to a similar extent. The well-documented avid endocytosis of crystalline NiS and the lack of significant uptake of amorphous NiS indicate that particle solubility is not related to the propensity of these particles to be endocytosed.

Following phagocytosis, the particles appear to undergo dissolution either through interactions with lysosomes or by chelation of particle surface-dissolving nickel ions by intracellular binding agents having a high affinity for metal ions, creating a more favorable dissolution equilibrium. Video intensification microscopy studies indicate that lysosomes repeatedly interact with cytoplasmic particles (12). Other evidence implicates the involvement of lysosomes in the cytoplasmic dissolution of crystalline NiS particles (2). The cytoplasmic dissolution of crystalline NiS particles appears to be a prerequisite for the nuclear uptake of nickel since the nuclear membrane is impermeable to nickel in a particulate state (6, 7). Additionally, as shown in Chart 2, the generation of nickel ions in the cytoplasm results in a less restricted distribution of nickel in the cell, and thus ionic nickel can interact with oncogenically relevant binding sites.

\[\text{F. W. Sunderman, Jr., personal communication.}\]
In the upper portion of Chart 2, we have calculated the potential molar concentration of cellular nickel resulting from phagocytosis of a 1.5- or 4.0-μm particle of crystalline NiS. The details of this calculation have been described previously (9). Note the high cellular levels of nickel which may be achieved by phagocytosis of these particles compared to the levels attainable by extracellular exposure of cells to nickel (9).

It is interesting to note that nickel ions have been described as poor inducers of metallothionein in comparison to Cd2+ or Hg2+, but nickel ions are considerably less cytotoxic to cells. However, nickel compounds are at least equally if not more genotoxic than are the metal compounds which are potent inducers of metallothionein (19). Further studies in the area of structure-activity with respect to the induction and binding to metallothionein by metal compounds of defined cytotoxicity and genotoxicity may lead to a better understanding of the cellular role of this very interesting metal binding protein. However, the importance of metallothionein induction in protecting the cell from an initial insult with toxic metal ions is questionable since it appears to play a significant role in chelating metals only in metal-resistant cells or as a secondary defense mechanism.

Chart 2 indicates that nickel ions are believed to be the ultimate carcinogenic form of nickel compounds that enters the nucleus to interact with DNA. It is interesting to note that NiS particles have been shown to aggregate around the nucleus (12), as depicted in Chart 2, and this perinuclear aggregation may provide a constant source of ionic nickel to enter the nucleus or to interact with molecules leaving the nucleus, such as RNA. Nickel ions have a variety of effects on DNA structure and function including decreased fidelity of replication (19), induction of DNA strand breaks (17), and the induction of DNA-protein cross-links (5). These genotoxic effects may lead to mutations and sister chromatid exchanges, which have also been reported following Ni2S2 exposure (18), and ultimately to the induction of transformation and carcinogenesis (2, 6–9, 21, 22). Nickel ions are known to bind to both the base and phosphate moieties of DNA, and this interaction may be responsible for the observed induction of DNA strand breaks reported in the present and in previous studies (5, 17, 20). It should be noted that single-strand breaks in the DNA can be repaired more readily than can double-stranded breaks. However, the latter occurs less frequently than does the former, and thus the sensitivity is greater in analyzing for both single and double strand breaks under alkaline conditions. Preliminary studies have reported the induction of single-strand breaks in DNA following treatment of cells with nontoxic levels of NiCl2 and crystalline NiS, suggesting that nickel has very selective effects upon DNA (17). In the present study, DNA strand breaks were observed in cells following only 3 to 4 hr of exposure to crystalline NiS, indicating that nickel ions enter the nucleus rapidly following phagocytosis of the particles. Alkaline sucrose gradient analyses were initially performed on cells treated with nickel compounds 1 hr after removal of [3H]thymidine, which resulted in more pronounced fragmentation of DNA within the lower molecular weight range (17). Perhaps this represents the effects of nickel on nascent DNA. Subsequently, we have observed (Chart 1) that DNA isolated from cells treated with nickel compounds 48 hr after removal of [3H]thymidine caused a less conspicuous change in the molecular weight of DNA. The strand breaks described in the present report probably comprise the breakage of parental DNA by nickel.

The work of Sirover and Loeb (19) and other studies have indicated that Ni2+, Cu2+, Co2+, and various other heavy metals which are mutagenic or carcinogenic cause decreased fidelity of DNA synthesis. Thus, it seems that, if the metal ion can reach the nucleus in sufficient concentrations, genotoxic and carcinogenic effects are observed. Our studies (1–3, 6–9) are attempting to understand the cellular regulatory processes which deliver potentially carcinogenic particulate metal compounds to their site of genotoxic action so that the biological responses associated with cellular transformation may be better understood.

ACKNOWLEDGMENT

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Fig. 1. Colony of SHE cells transformed by crystalline NiS. Crystal violet, × 20.
Fig. 2. Normal colony of SHE cells. Crystal violet, × 20.
Fig. 3. Living CHO cells with phagocytized crystalline NiS particles. Arrow, phagocytized crystalline NiS particle. × 500.
Fig. 4. Edge of a crystalline NiS-transformed colony of SHE cells. Crystal violet, × 40.
Selective Phagocytosis of Crystalline Metal Sulfide Particles and DNA Strand Breaks as a Mechanism for the Induction of Cellular Transformation

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