is restricted to the chloroplasts of the bundle sheath cells.

To verify this conclusion, we sought to identify cellular and subcellular activities of the enzyme utilizing preparative gel electrophoresis. Two isozymes could be identified in the whole leaf protein extract (Fig. 1). The activity was approximately equal in the two isozyme fractions. Both isozymes were present in extracts of bundle sheath strands and bundle sheath chloroplasts (Fig. 1). The bundle sheath chloroplasts were enriched in the specific activity of the enzyme, which is consistent with our findings in Table 2. Further, both isozymes appeared in approximately the same ratio as observed in the whole leaf extract. Trace amounts of both isozymes were detected in the extract prepared from mesophyll protoplasts (Fig. 1), presumably because of a minor contamination of bundle sheath protoplasts in this preparation (compare with Table 1). In this experiment (Fig. 1) both protoplast types were isolated simultaneously from very young tissue.

We have found the activity of ATP sulfurylase to be largely in bundle sheath cells of a variety of C_4 plants. Within this cell type two isozymes exist, both of which are chloroplastic. It is not known whether the entire process of sulfur assimilation to the reduced level of cysteine is compartmentalized in leaves of C_4 plants. Recently, thiosulfonate reductase sulfite reductase activity was shown in both mesophyll and bundle sheath cells of *D. sanguinalis*, with the bundle sheath extracts containing two to three times more activity than did mesophyll cell extracts (*7*). While the consequences of cellular compartmentation during sulfur assimilation have yet to be examined fully, it is clear that sulfur activation has been modified in the leaves of C_4 plants, with the initial step of sulfate activation occurring primarily in one chloroplast type of the leaf. The cellular compartmentation of sulfur assimilation in leaves of C_4 plants is analogous to that of carbon and nitrogen assimilation; but it is distinct in that carbon and nitrogen assimilation are initiated in mesophyll cells while sulfur assimilation is initiated in bundle sheath cells.

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10. On a day-to-day basis we have found the isolation of bundle sheath protoplasts to be quite inconsistent. The data presented here on bundle sheath protoplasts were obtained only after numerous attempts at the isolation. Plant age, growth conditions, and type and amount of digestive enzymes seem to be the most important factors. With *P. miliaceum*, best results were obtained with 5- to 7-day-old greenhouse grown seedlings on which the first leaf was typically 2 to 3 cm long. However, when protoplasts are isolated, the results shown are reproducible.
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13. Supported in part by NSF grant PCM 770-8548 and the Mobil Foundation. Send correspondence to: Clanton C. Black, Jr., Department of Biochemistry, Boyd Graduate Studies Research Building, University of Georgia, Athens 30602.
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Carcinogenic Activity of Particulate Nickel Compounds Is Proportional to Their Cellular Uptake

Abstract. *Particles (≤5 micrometers) of the potent carcinogen crystalline nickel subsulfide were actively phagocytized by cultures of Syrian hamster embryo cells and Chinese hamster ovary cells. Cells did not take up significant quantities of similar-sized particles of the noncarcinogen amorphous nickel monosulfide. The carcinogenic activity of this and other metal compounds appears to be proportional to their cellular uptake.*

Lung, sinonasal, and laryngeal carcinomas in nickel refinery workers have been attributed to the inhalation of nickel compounds (*1*). Rats receiving a single intramuscular injection of crystalline Ni_3S_2 or crystalline Ni_3Se_2 have developed a 65 to 100 percent incidence of sarcomas (*2–4*). However, when amorphous NiS was administered under similar conditions, no cancers were observed to develop (*5*). The differences in carcinogenic activity between crystalline Ni_3S_2 and amorphous NiS have been documented in other experimental animals, including mice and Syrian hamsters, with various administration routes (that is, intrarenal and intratesticular injection) (*2–4, 6*). The inhalation of crystalline Ni_3S_2 has also been shown to induce lung cancer in experimental animals (*7*).

The striking difference in carcinogenic activity between amorphous NiS and crystalline Ni_3S_2 was also observed in a tissue culture cell transformation assay (*8–10*); Ni_3S_2 induced a concentration-dependent incidence of morphological transformation in cultured Syrian hamster embryo cells, whereas amorphous NiS produced little or no change (*10–12*). Colonies transformed by Ni_3S_2 were cloned and derived into immortal cell lines, which are capable of forming three-dimensional colonies in soft agar and of producing tumors when inoculated into nude athymic mice (*8*). Since both Ni_3S_2 and NiS particles have similar water solubility properties and do not readily dissolve in tissue culture media, we were able to study their cellular uptake with light and electron microscopy. Both compounds were ground separately and passed through a 5-μm screen.

Both compounds appeared to be of similar particle size under the light and electron microscopes (≤5 μm). When these compounds were added to cultured cells grown on plastic microscopic slides (Leighton tubes), the cultures actively phagocytized the Ni_3S_2 particles but did not readily phagocytize the NiS particles. Figure 1, A and B, shows light microscope photographs of Chinese hamster ovary (CHO) cells and Syrian hamster embryo (SHE) cells phagocytizing Ni_3S_2 particles. The phagocytized Ni_3S_2 particles were almost always contained in vacuoles. Figure 1C shows an electron microscope photograph of a CHO cell that has engulfed Ni_3S_2 particles. The particles are contained in a vacuole, and these nickel-containing vacuoles are primarily associated with the cytoplasmic compartment (*11*).

Within 30 minutes after the addition of Ni_3S_2 to the media of these cultures, Ni_3S_2 particles were seen entering the cells. The time course of the uptake of

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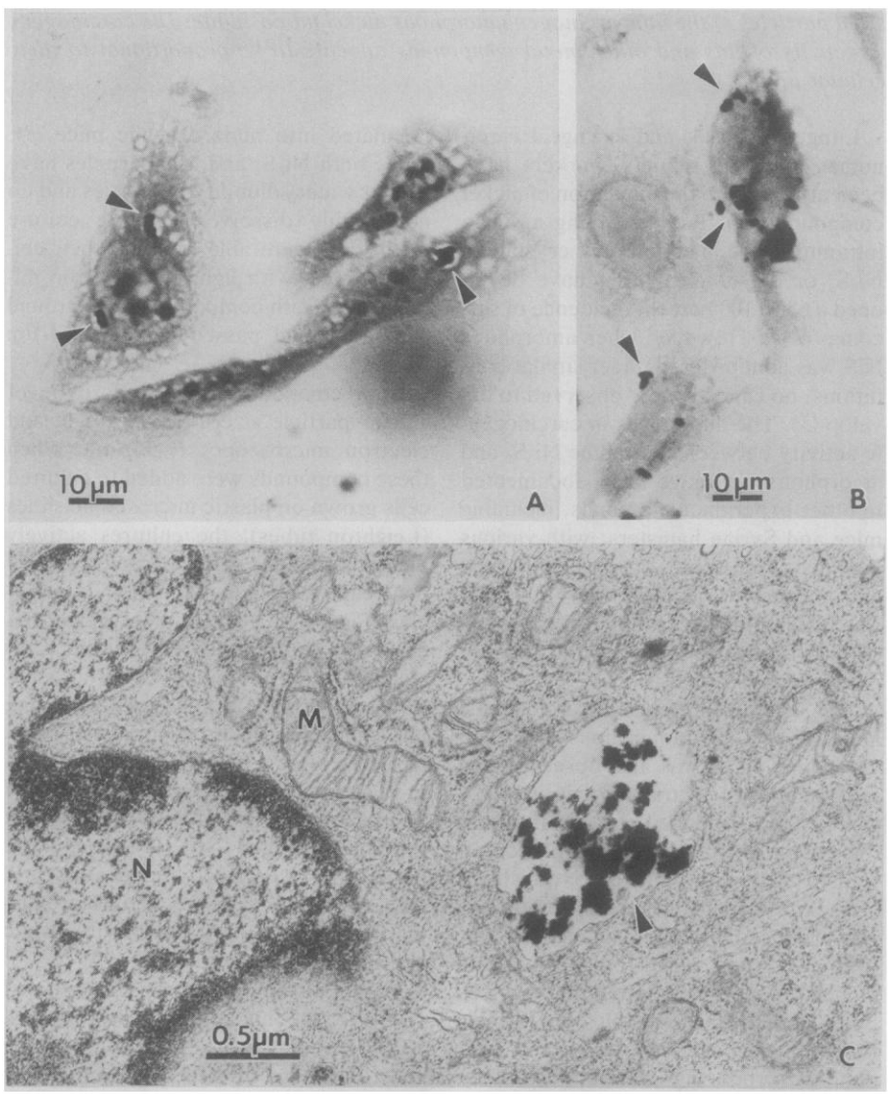
Table 1. Uptake and carcinogenic activity of particulate amorphous NiS and crystalline Ni_3S_2 in cultured cells. The SHE cells were exposed to amorphous NiS and crystalline Ni_3S_2 (three times for 2 days in each exposure). The free metal compounds were removed from contact with the cells by washing with normal saline, and the cells were removed from the monolayer by trypsinization. Cells (1000 to 5000) were placed in 100-mm tissue culture dishes to form colonies. After 12 days of incubation the colonies were fixed, stained, and evaluated for morphological transformation (*8*, *10*). The number of transformed colonies was expressed as a function of the number of surviving colonies for each plate (six plates were used for each condition described) [for further details of methodology, see (*8*)]. For the uptake studies, log-phase monolayer cultures grown on plastic microscopic slides were exposed to the nickel compounds (three exposures, 24 hours for each exposure). The cells were then washed twice with normal saline, fixed with 95 percent ethanol, and stained with a methanol-crystal violet solution (0.5 percent crystal violet). One thousand cells in each slide were examined with a light microscope for intracellular nickel particles as shown in Fig. 1. Each percentage shown is the mean of three slides representing a total of 3000 cells examined.

Concentration (μg/ml)	Transformed colonies/ total surviving colonies of SHE cells	Uptake of nickel particles (percent of 1000 cells scored)	
		SHE cells	CHO cells
	Crystalline Ni_3S_2		
0.1	2/268 (0.7 percent)	0.8	2.2
1.0	6/214 (2.8 percent)	6.7	9.8
5.0	12/138 (8.7 percent)	23.6	44.6
10.0	11/93 (11.8 percent)	42.9	79.3
	Amorphous NiS		
0.1	0/321 (0 percent)	0	0
1.0	0/222 (0 percent)	0.12	0.6
5.0	0/189 (0 percent)	0.35	2.4
10.0	0/166 (0 percent)	0.81	3.5



Ni_3S_2 particles was linear for the first several hours but then leveled off at 6 hours. We quantified the uptake of metal particles by counting a total of 1000 cells in each slide and determining with a light microscope the number of cells that contained nickel particles. Selected cells were also viewed with an electron microscope to study the uptake of smaller particles. In most instances the particles phagocytized by the cultured cells were visible with both the electron and light microscope.

Table 1 shows the striking differences in the cellular uptake of amorphous NiS and crystalline Ni_3S_2 particles in the two culture systems. The SHE system was also used to evaluate carcinogenic activity on the basis of changes in colonial morphology (*12*, *13*). The incidence of morphological transformation was dependent upon the dosage of crystalline Ni_3S_2 and also paralleled Ni_3S_2 uptake activity with the SHE cells. On the other hand, NiS did not induce transformation at all the concentrations tested and also was not significantly taken up by the two cultures examined (Table 1).

We have compared the uptake of Ni_3S_2 and NiS at a variety of time intervals, using several exposures at various concentrations. In all cases, Ni_3S_2 is actively taken up whereas NiS is not. It seems that the difference in uptake between crystalline Ni_3S_2 and amorphous NiS is more striking in the SHE cells than in the CHO cells. Carcinogenic activity, however, can only be assayed with the SHE cells and not with the CHO cells.

The striking difference in cellular uptake between crystalline Ni_3S_2 and amorphous NiS is not attributable to differences in particle size or solubility. Both compounds are relatively insoluble in tissue culture media, and both are of similar size. The NiS particles do not enter the cell at early time periods or after three separate exposures for 24 hours each to 10 μg/ml (a very high NiS concentration). The LC_{50} (concentration which killed 50 percent of the cells), determined by plating efficiency in CHO cells, was 0.5 μg/ml for Ni_3S_2 and about 2 μg/ml for NiS (*14*). Therefore, Ni_3S_2 is also more toxic to cells than amorphous NiS. This difference in toxicity is also explainable in terms of the differences in

Fig. 1. (A) Light microscope photograph of CHO cells that have phagocytized Ni_3S_2 particles (arrows) (×700). (B) Light microscope photograph of SHE cells phagocytizing Ni_3S_2 particles (arrows) (×700). (C) Electron microscope photograph of a CHO cell that has taken up Ni_3S_2 particles (arrow) (×25,000); *N*, nucleus and *M*, mitochondrion.

uptake activity between these two compounds. Another interesting relationship is the percentage of SHE cells that take up Ni_3S_2 relative to the incidence of morphological transformation. For 1.0, 5.0, and 10.0 μg/ml of Ni_3S_2 (SHE cells, Table 1) this ratio averages about 3. This suggests that one out of three cells that take up Ni_3S_2 particles undergoes morphological transformation. The uptake/carcinogenesis ratio may be governed by Ni_3S_2 toxicity or may be related to the occurrence of a critical mutation which initiates the carcinogenesis process. We have investigated the uptake and carcinogenic activity of other particulate nickel compounds and several other metals (*15*). There is a good correlation between the uptake of these metals and their respective carcinogenic activities; this result suggests that carcinogenic activity of other metal compounds is proportional to their uptake.

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11. Nickel particles probably enter the nucleus but apparently not in a physical form large enough to be easily detected with the electron microscope. Numerous nickel particles (2 to 5 μm) were seen pushing against the nuclear membrane. On the basis of current dogma and in vivo biochemical studies of nickel carcinogenesis, the nucleus is a primary site of nickel deposition during the course of malignant transformation. Our results suggest that nickel may enter the nucleus only as very small particles or as nickel ions broken down from larger Ni_3S_2 particles contained within cytoplasmic vacuoles. We are currently conducting studies in which measurements of total nickel by x-ray fluorescence will be correlated with particulate nickel visible in the light and electron microscopes.
12. The SHE cells were isolated from hamster embryos after 13 to 14 days of gestation. These cultures have all the properties of a normal cell (that is, orderly growth, defined life span in culture, inability to form three-dimensional colonies in soft agar, and lack of tumorigenicity when cells are administered to appropriate host). After exposure to a carcinogen such as Ni_3S_2, some of the normal cells undergo morphological transformation characterized by disordered growth. Cultures that exhibit this disordered growth pattern also have acquired the other properties of a cancer cell (growth in soft agar and tumor formation in nude mice). Therefore, on the basis of the incidence of morphological transformation, the carcinogenic activity of the substance may be accurately determined [see (*13*)].
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14. The LC_{50} was determined by exposing log-phase cultures of CHO cells to various concentrations of the metal compounds for 4 days, with fresh metal and media added every day. At the end of the exposure period, 100 to 1000 cells were plated into 100-mm tissue culture dishes to form colonies. The total numbers of surviving colonies in each dish were counted and expressed as a function of the number of cells plated to determine the plating efficiency. The control plating efficiency was 80 to 90 percent, and the concentration that reduced this 50 percent was the LC_{50}. The LC_{50} could also be measured in SHE cells, but the plating efficiency of these cells is lower (1 to 10 percent) and more variable than that of CHO cells.
15. The uptake of crystalline Ni_3Se_2 and crystalline NiS in SHE cells was approximately 70 to 80 percent that of Ni_3S_2 for similar exposure conditions. Both these compounds are potent carcinogens in experimental animals and in tissue culture transformation assays (*9*). Nickel metal was taken up about 10 percent more than amorphous NiS and has been shown to be weakly carcinogenic in experimental animals (*4*). Out of the CHO cells exposed to iron dust for 24 hours at 10 μg/ml, only 2.7 percent of the cells contained particulate material. Of CHO cells exposed to chromium and cobalt dust under similar conditions, 8.2 percent and 5.2 percent, respectively, contained particulate material. Iron dust is not carcinogenic, but chromium dust has been shown to be carcinogenic in experimental animals and has been implicated as a human carcinogen on the basis of epidemiological studies (*2*). Cobalt has induced tumors in experimental animals (*2*).
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Developmental Potential of Somatic Nuclei Transplanted into Meiotic Oocytes of *Rana pipiens*

Abstract. *Somatic nuclei, when transplanted into oocytes at the stage of first meiotic metaphase, were induced to undergo chromosome condensation and alignment on spindles. When the oocytes completed maturity in vitro and were activated, the somatic nuclei transformed into "fertilization" nuclei and promoted development through embryogenesis. Thus somatic nuclei can reversibly respond to cytoplasms directing either meiotic or mitotic events, and somatic nuclei from differentiated cells may be reversed by conditioning in oocytes.*

The genetic and developmental properties of somatic cell nuclei have been tested by transplantation of nuclei into mature eggs (*1*). Although somatic nuclei from young embryos promote development of amphibian eggs into normal larvae and adults, nuclei from well-defined cell types fail to promote normal development of the recipient eggs. These results suggest that genetic restrictions accompany cell specialization. However, nuclei from advanced cell types fail to integrate with the cytoplasmic division cycles of the egg and develop chromosomal abnormalities that lead to developmental restrictions of the nuclear transplants (*2*). Thus, conclusive proof of the theory of nuclear equivalence among specialized cell types is still lacking. One approach to distinguish between the above alternatives is to transplant somatic nuclei into oocytes where they may be first conditioned and then tested for developmental potential.

We report our initial studies on the developmental properties of somatic nuclei transplanted into maturing oocytes of *Rana pipiens*. The results demonstrate that blastula nuclei and endodermal nuclei of the tail-bud stage, when induced by meiotic cytoplasm to undergo chromosome condensation and alignment on spindles at the time of first meiotic metaphase, still retain the capacity to program for development. When the oocytes mature and are activated, the transplanted nuclei transform into "fertilization" nuclei and direct the formation of the various cell types required for the completion of embryogenesis. These results demonstrate that (i) somatic nuclei can reversibly respond to cytoplasms, directing either meiotic or mitotic events, and (ii) this system could be used for conditioning or reprogramming somatic nuclei from well-defined cell types that have so far exhibited genetic and developmental restrictions when transplanted into mature eggs (*1*).

Oocyte hosts utilized for nuclear transplantation were in the stage of first meiotic metaphase (first black dot). It is possible, by controlling the dose and time of pituitary and progesterone injections into frogs, to obtain maturing oocytes at first meiotic metaphase (*3*). These oocytes encased in jelly reach the uterus prematurely and can be stripped from the female, although they are not activatable or fertilizable at this time. About 24 hours later (18°C) when noninjected oocytes used for controls were

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