THE TOXICOLOGY AND METABOLISM OF
NICKEL COMPOUNDS

Progress Report
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Abstract of Progress Report

The toxicology and metabolism of nickel compounds [NiCl_2 , Ni_3S_2 , NiS , Ni powder and $\text{Ni}(\text{CO})_4$] were investigated in rats. The new knowledge includes:

- (1) Demonstration that exposure of pregnant rats to inhalation of nickel carbonyl on the eighth day of gestation induces fetal anomalies (i.e. microphthalmia and anophthalmia).
- (2) Development of a furildioxime extraction technique for measurements of nickel in body fluids by electrothermal atomic absorption, and demonstration that the technique provides a practical method for monitoring occupational exposures to nickel compounds.
- (3) Demonstration that sodium diethyldithiocarbamate is more effective than d-penicillamine and triethylenetetramine as an immediate antidote for nickel carbonyl poisoning in rats, and observation that d-penicillamine is more effective than diethyldithiocarbamate at 6 hours after exposure to nickel carbonyl.
- (4) Elucidation of the sequence of chemical reactions whereby nickel subsulfide becomes dissolved in body fluids *in vitro*.
- (5) Demonstration that intratesticular injection of nickel subsulfide in rats is an effective method for induction of testicular sarcomas, including rhabdomyosarcomas.
- (6) Identification of morphological and biochemical responses to nickel subsulfide in tissue culture cells. These observations may lead to development of *in vitro* systems for nickel carcinogenesis.
- (7) Demonstration of increased concentrations of nickel in lungs from victims of "Legionnaires' Disease".
- (8) Acquisition of new information regarding pathophysiological mechanisms of nickel-induced erythrocytosis and hyperglycemia in rats.

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Scientific Accomplishments During the Past Year:

A. Studies of the Embryotoxicity, Fetal Toxicity, Teratogenicity, Mutagenicity and Transplacental Carcinogenicity of Nickel Compounds in Rats.

We have completed a study of the embryotoxicity and fetal toxicity of nickel chloride (NiCl_2) and nickel subsulfide (Ni_3S_2) in rats. Injection of NiCl_2 (16 mg Ni/kg, *im*) on day 8 of gestation reduced the mean number of live pups/dam and resulted in diminished body weights of fetuses on day 20 of gestation and of weanlings at 4 to 8 weeks after birth. Injection of Ni_3S_2 (80 mg Ni/kg, *im*) on day 6 of gestation reduced the mean number of live pups/dam. No congenital anomalies were found in fetuses from any Ni-treated dams, including dams that received 10 *im* injections of NiCl_2 (2 mg Ni/kg, twice daily, on days 6-10 of gestation). $^{63}\text{NiCl}_2$ (12 mg Ni/kg, *im*) was administered to a group of non-pregnant female rats and to two groups of pregnant rats on days 8 or 18 of gestation. After 24 hours, the relative concentrations of ^{63}Ni in tissues were: kidney>serum>adrenal=lung>ovary>spleen=heart=liver>skeletal muscle. Pituitary ^{63}Ni concentrations were much higher in pregnant rats than in non-pregnant

females. ^{63}Ni concentrations in products of conception (fetuses and fetal membranes) on day 9 and in placentas on day 19 were equivalent to ^{63}Ni concentrations in adrenal, lung, and ovary tissues of the dams. Autoradiography of fetuses and placentas on day 19 of gestation showed ^{63}Ni localization in fetal urinary bladders and in the basal laminae and yolk sacs of the placentas. These studies show (a) that *in* injection of NiCl_2 and Ni_3S_2 during early gestation causes death of embryos at dosages that do not cause maternal mortality, and (b) that $^{63}\text{Ni}(\text{II})$ can cross the feto-maternal barriers and enter the embryos and fetuses during early and late gestation. A paper that describes these findings was presented at the annual meeting of the *Society of Toxicology* (abstract¹ submitted herewith). The completed manuscript² (submitted herewith) has been accepted for publication in *Toxicology and Applied Pharmacology*.

As the second phase of this avenue of our research, we have investigated the embryotoxicity of nickel carbonyl, $\text{Ni}(\text{CO})_4$, in rats. Rats were placed in an exposure chamber and exposed to inhalation of specified concentrations of $\text{Ni}(\text{CO})_4$ for 15 min. The concentrations of $\text{Ni}(\text{CO})_4$ in the chamber were monitored by gas chromatography. Based upon a pilot study of 88 non-pregnant adult Fischer rats, the LD_{50} of $\text{Ni}(\text{CO})_4$ is 0.58 (S.E. ± 0.09) mg $\text{Ni}(\text{CO})_4$ /liter/15 min, and the LD_5 is 0.12 mg $\text{Ni}(\text{CO})_4$ /liter/15 min. Pregnant rats were exposed on day 8 of gestation to inhalation of $\text{Ni}(\text{CO})_4$ in dosages of 0.12 and 0.06 mg $\text{Ni}(\text{CO})_4$ /liter/15 min (equivalent to 120 and 60 ppb). The results are summarized in Table 1. Twenty-four of 87 live fetuses from 13 litters have had ophthalmic malformations. In comparison, no malformations have been found in 100 live fetuses from 12 control litters. The ophthalmic abnormalities have included unilateral microphthalmia (15/87), bilateral microphthalmia (6/87), and unilateral anophthalmia (3/87). These findings indicate that $\text{Ni}(\text{CO})_4$ is

TABLE 1
EMBRYOTOXICITY OF Ni(CO)₄ IN FISCHER RATS

On day 8 of gestation, dams were exposed to inhalation of Ni(CO)₄ in atmospheric concentration of 0.06 or 0.12 mg Ni(CO)₄/liter for 15 min. Control dams were exposed to inhalation of ambient air in an identical chamber.

<u>Observations</u>	<u>Control Dams</u>	<u>Ni(CO)₄-Treated Dams</u>	
		<u>(0.06 mg/liter 15 min)</u>	<u>(0.12 mg/liter 15 min)</u>
Number of Dams:	12	6	7
Corpora Lutea/Dam:	9.5 ± 1.9	7.5 ± 3.0	11.1 ± 0.7
Live Fetuses/Dam:	9.2 ± 2.1	7.2 ± 3.1	6.3 ± 4.7
Ratio of Dead Fetuses/ Conceptuses:	4/114 (3.5%)	2/45 (4.4%)	15/59 (25.4%)
Body Wt of Live Fetuses(g):	3.36 ± 0.19	3.01 ± 0.38	2.79 ± 0.31
Ratio of Malformed Fetuses/ Live Fetuses:	0/111 (0%)	12/43 (28%) ^a	12/44 (27%) ^b

^a Unilateral microphthalmia (8); bilateral microphthalmia (3) and unilateral anophthalmia (1).

^b Unilateral microphthalmia (7); bilateral microphthalmia (3) and unilateral anophthalmia (2).

teratogenic in rats. Further investigation of the teratogenicity of $\text{Ni}(\text{CO})_4$ is a major focus of our current research. Experiments are in progress to elucidate the dose-response and time-response relationships for embryotoxicity and teratogenicity of $\text{Ni}(\text{CO})_4$ in rats.

As the third phase of this avenue of research, we have initiated studies of the transplacental carcinogenicity of Ni_3S_2 in rats. Eight pregnant rats were given an *im* injection of 20 mg of Ni_3S_2 on day 6 of gestation, and 8 pregnant control rats were given a similar injection of the vehicle. The pregnancies were allowed to proceed to parturition. Fifty-eight (24 σ and 34 η) pups from the 8 litters of Ni_3S_2 -treated dams, and 62 pups (33 σ and 29 η) from the 8 litters of control dams are being observed for 2 years for possible tumor development. This study is in progress and will be completed in approximately 1.5 years.

B. Chelation Therapy of Acute Nickel Carbonyl Poisoning in Rats. The therapeutic efficacies of sodium diethyldithiocarbamate (DDC), d-penicillamine (d-PEN), and triethylenetetramine (TETA) as antidotes for acute $\text{Ni}(\text{CO})_4$ poisoning have been tested in rats. The chelating drugs were administered *im* to rats (a) at 10 min prior to inhalation of $\text{Ni}(\text{CO})_4$ in an atmospheric concentration sufficient to produce immediate death (4.2 mg $\text{Ni}(\text{CO})_4$ /liter/15 min); (b) at 10 min after inhalation of $\text{Ni}(\text{CO})_4$ in an atmospheric concentration sufficient to produce delayed death within 3 to 5 days (1.4 mg $\text{Ni}(\text{CO})_4$ /liter/15 min); and (c) at 6 hours after inhalation of $\text{Ni}(\text{CO})_4$ in the same concentration of 1.4 mg ($\text{Ni}(\text{CO})_4$ /liter/15 min. The results of this study are summarized in Table 2. Sodium diethyldithiocarbamate was found to be the most effective antidote for acute $\text{Ni}(\text{CO})_4$ poisoning when it was administered immediately prior or after the exposure. On the other hand, d-penicillamine was the best antidote when administration of the chelating drug was delayed

TABLE 2

ANTIDOTAL EFFICACY OF CHELATING DRUGS IN ACUTE $\text{Ni}(\text{CO})_4$ POISONING IN RATS

Group	$\text{Ni}(\text{CO})_4$ Exposure (mg $\text{Ni}(\text{CO})_4$ /liter/15 min)	Drug	Interval Between Administration of Drug and Exposure to $\text{Ni}(\text{CO})_4$	Dosage of Drug ^a (mM/kg)	Mortality Ratio	Time of Death (days)
A	4.2	Controls	-10 min	0	33/33	0(0-2)
B	4.2	DDC	-10 min	4.1	1/28 ^c	6
C	4.2	d-PEN	-10 min	13.1	16/28 ^c	2(0-6)
D	4.2	TETA	-10 min	1.36	22/26	3(0-5)
E	1.4	Controls	+10 min	0	19/26	3(2-5)
F	1.4	DDC	+10 min	4.1	2/26 ^c	3(2-5)
G	1.4	d-PEN	+10 min	13.1	4/26 ^c	3(1-5)
H	1.4	TETA	+10 min	1.36	6/26 ^c	4(3-5)
I	1.4	Controls	+6 hr	0	17/26	4(1-6)
J	1.4	DDC	+6 hr	4.1	18/25	2.5(1-6)
K	1.4	d-PEN	+6 hr	13.1	6/25 ^b	3.5(2-6)
L	1.4	TETA	+6 hr	1.36	12/25	4(3-7)

^a Each drug was injected *im* at dosage equivalent to 0.6 times its respective LD_{50} values.

^b $P < 0.01$ vs corresponding control rats, by χ^2 test.

^c $P < 0.001$ vs corresponding control rats, by χ^2 test.

for 6 hours after exposure to $\text{Ni}(\text{CO})_4$. This study is nearing completion, and a manuscript describing our findings will be drafted within the next three months.

C. Atomic Absorption Spectrometry of Nickel in Body Fluids. A furildioxime extraction method has been developed for analysis of nickel in body fluids by atomic absorption spectrometry. After the sample of serum or urine (1 ml) has been subjected to acid digestion, $\text{Ni}(\text{II})$ is extracted into methylisobutylketone as a furildioximate complex, and is measured by electrothermal atomic absorption spectrometry. The method is more sensitive and more convenient than previous methods, and is less subject to nickel contamination, since the digestion and extraction steps are performed in a single tube. Recovery of $\text{Ni}(\text{II})$ (10 or 20 $\mu\text{g}/\text{liter}$) added to 20 specimens of serum or urine averaged 101% (range = 94 to 107). The following values (mean $\pm$ SD) were obtained for nickel concentrations in specimens of serum and urine from 19 healthy adults living in Central Connecticut: serum = 3.1 ± 1.6 $\mu\text{g}/\text{liter}$; urine = 2.7 ± 1.6 $\mu\text{g}/\text{liter}$. The furildioxime method provides a practical means for monitoring occupational exposures of workers to nickel compounds. A paper that describes this method was presented at an *International Symposium on Metal Toxicology* (abstract³ submitted herewith). The completed manuscript⁴ (submitted herewith) has been published in *Clinical Chemistry*.

The furildioxime method has been used to measure nickel concentrations in urine from workers who are exposed to nickel compounds in several industrial processes, including (a) nickel refinery workers, (b) nickel-plating workers, (c) fabricators of Ni-Cd batteries, and (d) workers in the ERDA pilot plant for coal gasification (Bruceton, PA) which employs Raney nickel as a hydrogenation catalyst. The results (Table 3) indicate that urinary excretion

TABLE 3
URINE NICKEL AS AN INDEX OF OCCUPATIONAL EXPOSURES

<u>Subjects</u>	<u>Number</u>	<u>Urine Ni Concentration^a</u>	
		<u>µg/liter</u>	<u>µg/g creatinine</u>
Non-Exposed Hospital Workers	19	2.7±1.6 (0.4-5.1)	2.5±1.3 (0.7-5.7)
Non-Exposed Industrial Workers	14	2.8±1.3 (0.4-4.2)	2.5±1.1 (0.6-3.6)
Workers in a Coal Gasification Plant	9	4.2±2.4 (0.4-7.9)	3.2±1.6 (0.1-5.8)
Ni-Cd Battery Workers	6	11.7±7.7 (3.4-26)	10.2±6.4 (7.2-23)
Ni Plating Workers	21	28±21 (5.3-65)	19±15 (2.4-62)
Ni Refinery Workers	12	140±138 (4.0-390)	85±84 (3.1-281)

^a Mean ± S.D.; range in parentheses

of nickel is increased in the nickel-plating workers, in the Ni-Cd battery workers and in the nickel refinery workers. Nickel concentrations have also been measured in serum samples from nickel refinery workers (Table 4).

TABLE 4
SERUM NICKEL AS AN INDEX OF OCCUPATIONAL EXPOSURES

<u>Subjects</u>	<u>Number</u>	<u>Serum Ni Conc.^a</u>
		<u>µg/liter</u>
Non-Exposed Hospital Workers	19	3.1±1.6 (0.6-5.3)
Non-Exposed Industrial Workers	6	2.3±2.0 (0.3-5.0)
Ni Refinery Workers	8	15.1±13.2 (1.6-34)

^a Mean ± S.D.; range in parentheses

Simultaneous measurements of nickel in urine and serum have been performed in 14 subjects (6 non-exposed industrial workers and 8 nickel refinery workers). As shown in Figure 1, there was excellent correlation between the concentrations of nickel in serum and urine. The equation of the regression line in Figure 1 is: $Y = 11.83 X - 17.5$, and the correlation coefficient is 0.972. Since the concentrations of nickel are much higher in urine of exposed workers than in serum, and since it is more practical to obtain samples of urine than samples of serum from workers, we advocate measurements of urine nickel concentrations as a biological index of occupational exposures to nickel. A paper that describes these findings has been presented at the 1977 Spring Meeting of the *Association of Clinical Scientists* (abstract⁵ submitted herewith). A manuscript that describes these analyses and additional studies which are in progress will be drafted within the next 6 months.

The International Union of Pure and Applied Chemistry has appointed the Principal Investigator to serve as Chairman of a Subcommittee on Environmental and Occupational Toxicology of Nickel. The IUPAC Nickel Subcommittee includes 16 toxicologists from 9 countries. Under the auspices of the IUPAC Nickel Subcommittee, two interlaboratory comparisons have been performed of nickel analyses by electrothermal atomic absorption spectrometry. Samples of urine and plasma were distributed to 11 laboratories in 7 countries, and the analytical results were returned to Dr. Sunderman for statistical analysis. These comparisons identified serious weaknesses in methods that were employed in various laboratories for nickel analyses in body fluids. A preliminary report of these findings has been presented at an *International Symposium on Metal Toxicology* (abstract⁶ submitted herewith). An additional survey will be performed during October, 1977, and a manuscript describing these interlaboratory

Figure 1: Correlation between nickel concentrations in serum and urine of non-exposed industrial workers (solid circles) and nickel refinery workers (open circles).

surveys will be drafted for publication within the next year. Financial support for these interlaboratory surveys has been derived in part from this ERDA contract. These interlaboratory comparisons have contributed to decisions regarding the proposed *IUPAC International Reference Method for Nickel Analyses in Body Fluids*. It seems likely that a modification of the furil-dioxime extraction method⁴ for electrothermal atomic absorption of nickel in body fluids will be accepted as the International Reference Method at the meeting of the IUPAC Nickel Subcommittee which is scheduled to be held in Kristiansand, Norway, in May, 1978.

D. Mechanisms of Solubilization of Ni_3S_2 in Body Fluids.

To elucidate the initial steps of carcinogenesis by nickel subsulfide, the mechanisms of dissolution of $\alpha\text{Ni}_3\text{S}_2$ were studied by incubating $\alpha\text{Ni}_3\text{S}_2$ dust in (a) water, (b) rat serum, and (c) rat serum ultrafiltrate. The sediments that remained following incubation of $\alpha\text{Ni}_3\text{S}_2$ in the 3 media for 2 weeks were examined by X-ray diffractometry. The rates of solubilization of ^{63}Ni from $\alpha^{63}\text{Ni}_3\text{S}_2$ during incubation in the 3 media were measured by liquid scintillation counting. These studies showed that solubilization of Ni(II) from $\alpha^{63}\text{Ni}_3\text{S}_2$ in rat serum requires the presence of O_2 and involves three reactions: [I] $2\alpha\text{Ni}_3\text{S}_2 + \text{O}_2 + 2\text{H}_2\text{O} \rightarrow 4\text{NiS} + 2\text{Ni(OH)}_2$; [II] $6\text{NiS} + 2\text{O}_2 + \text{Ni}^{2+} + \text{SO}_4^{2-}$; and [III] $\text{Ni}^{2+} + \text{Ni(OH)}_2 + \text{serum ligands [albumin \& amino acids]} \rightarrow \text{soluble Ni(II)-complexes}$. The rate of solubilization of ^{63}Ni from $\alpha^{63}\text{Ni}_3\text{S}_2$ was initially more rapid in rat serum than in serum ultrafiltrate and much more rapid than in water. After 2 days of incubation, the rates of dissolution of $\alpha^{63}\text{Ni}_3\text{S}_2$ in the 3 media became progressively retarded, probably owing to deposition of insoluble surface coatings of $\beta^{63}\text{NiS}$ and $\beta^{63}\text{Ni(OH)}_2$ on the $\alpha^{63}\text{Ni}_3\text{S}_2$ particles. A manuscript⁷ (submitted herewith) that describes these findings has been published in *Research Communications in Chemical Pathology and*

Pharmacology.

E. Nickel Carcinogenesis in Experimental Animals. In an endeavor to expand the variety of experimental models for study of nickel carcinogenesis, nickel subsulfide (Ni_3S_2) was administered to rodents by several previously untested routes. In two groups of Syrian hamsters, sarcomas developed at the sites of single *im* injections (5 or 10 mg of Ni_3S_2). In contrast, Ni_3S_2 did not induce any malignant tumors of the cheek pouches, oral cavities or gastrointestinal tracts of hamsters, despite multiple local applications of Ni_3S_2 to the cheek pouches in total dosages as large as 1.1 g of Ni_3S_2 . In a group of Fischer rats, single intratesticular injections of Ni_3S_2 (10 mg) induced many testicular sarcomas. In contrast, no malignant tumors developed in two groups of rats that received single injections into the submaxillary gland (2.5 mg of Ni_3S_2) or into the liver (5 mg of Ni_3S_2 *via* the portal venous system). A paper that describes these findings was presented at an *International Symposium on Inorganic and Nutritional Aspects of Cancer* (manuscript⁸ submitted herewith).

Intratesticular injection of Ni_3S_2 in rats has been found to be an efficient method for induction of sarcomas of the testis, including rhabdomyosarcomas. The occurrence of testicular rhabdomyosarcomas was unexpected, since the testis does not normally contain striated muscle cells. Intratesticular injection has three practical advantages over *im* injection as an experimental system to investigate nickel carcinogenesis. *First*, the injection site is localized and encapsulated, and it can be excised completely at any time during the latent period. *Second*, the testis is easily palpated and tiny tumors can be detected more readily in the testis than in the leg musculature. *Third*, study of the differentiation of rhabdomyocytes from

undifferentiated mesenchymal cells is more readily accomplished in testis than in muscle, where rhabdomyoblasts and rhabdomyocytes are components of the normal populations of cells. A paper⁹ (abstract submitted herewith) that describes testicular sarcomas induced in rats by intratesticular injection of Ni_3S_2 was presented at the meeting of the *American Association for Cancer Research*. The completed manuscript¹⁰ (submitted herewith) has been submitted for publication in *Cancer Research*.

F. Effects of Nickel Compounds in Tissue Culture Cells. As an initial phase of our attempts to study Ni_3S_2 carcinogenesis in tissue culture, we have investigated the effect of *in vitro* additions of Ni_3S_2 , NiS , NiCl_2 and Ni_3Se_2 upon the growth, morphology and biochemical parameters of Chinese hamster ovary (CHO) cells. Ni_3S_2 caused rounded CHO cells to elongate. NiS , NiCl_2 and a variety of other metal compounds that were tested had no similar effects on CHO morphology over a wide range of toxic and non-toxic concentrations. CHO cells were elongated following exposure to Ni_3S_2 at concentrations which did not affect cell proliferation and the elongation was not presumed to be related to toxicity. CHO cells also became elongated following addition of agents that elevate cellular cAMP levels. CHO cells treated with either dibutyryl-cAMP or Ni_3S_2 had very similar fibroblastic morphology. Upon removal of media containing dibutyryl-cAMP, elongated CHO cells reverted to control morphology within 4 hours. In contrast, removal of Ni_3S_2 from the cultures did not result in reversion of elongated cells to normal fibroblastic morphology. The Ni_3S_2 treated cells, however, eventually reverted to normal morphology after a few weeks in culture. Nickel subsulfide activated cAMP-dependent protein kinase within 3 hours after its addition to cultures while addition of NiS or NiCl_2 did not significantly

activate protein kinase. Dibutyryl-cAMP addition to CHO cultures completely activated protein kinase. High doses of actinomycin D, or cycloheximide did not affect the dibutyryl-cAMP-induced elongation of CHO cells while addition of colcemid did inhibit the elongation. All three of these agents inhibited Ni_3S_2 -induced elongation of CHO cells. These data demonstrate that Ni_3S_2 causes specific morphological changes in CHO cells which may be mediated by a cAMP-dependent mechanism. A manuscript¹⁰ (submitted herewith) that describes these experiments has been submitted for publication in *Toxicology and Applied Pharmacology*.

As the second phase of this avenue of study, we have attempted to produce *in vitro* neoplastic transformation of primary cultures of fetal cells from Syrian hamsters. Preliminary studies showed that addition of Ni_3S_2 (1 $\mu\text{g}/\text{ml}$) to a primary cell culture for 5 days, followed by incubation for 7 days in fresh medium without added nickel, results in the growth of piled-up colonies, as illustrated in Figure 2A. In contrast, no such proliferative growth of hamster fetal cells was observed in control cultures (Figure 2B that were grown without exposure to Ni_3S_2). In Table 5 are summarized the results that were obtained when hamster fetal cells were exposed to Ni_3Se_2 , NiS , NiCl_2 , Ni dust and benzo(a)pyrene (positive control). Each of these compounds was added to the cultures for 5 days, and the cells were removed from the monolayer and plated in fresh media to form colonies for 7 days. Similar numbers of cells were inoculated into the plates that were exposed to each of the compounds, and the plates that were examined all had similar numbers of colonies. It may be seen from Table 5 that the proliferative responses to Ni_3S_2 and benzo(a)pyrene were most intense, and the proliferative responses to the other nickel compounds were less marked. Additional experimentation

Figure 2A: (Top) A colony of Syrian hamster fetal cells treated with Ni_3S_2 .

Figure 2B: (Bottom) Control colony of untreated Syrian hamster fetal cells.

TABLE 5
TRANSFORMATION OF SYRIAN HAMSTER FETAL CELLS BY
NICKEL DUST, NICKEL COMPOUNDS AND BENZO(A)PYRENE

<u>Metal or</u> <u>Compound</u>	<u>Concentration</u>		<u>Plates with "Piled up"</u> <u>Colonies or Foci</u>	<u>No. of "Piled up"</u> <u>Colonies or Foci</u> <u>in each plate</u>	<u>Average No. of</u> <u>"Piled up" Colon-</u> <u>ies or Foci/Plate</u>
	<u>ug/ml</u>	<u>ng-atom</u> <u>of metal/ml</u>			
NiS (Amorphous)	0.1	1.1	1/6	2,0,0,0,0,0	0.3
	1	12	3/6	3,2,1,0,0,0	1.0
α Ni ₃ S ₂	0.1	1.2	5/6	6,5,3,3,2,0	3.2
	1	12	6/6	14,9,8,3,3,2	6.5
α Ni ₃ Se ₂	0.1	1	3/6	7,4,6,0,0,0	2.8
	1	10	5/6	7,7,5,2,1,0	3.8
NiCl ₂	0.1	0.8	2/6	7,1,0,0,0,0	1.3
	1	8	1/6	2,0,0,0,0,0	0.3
Benzo(a)pyrene	0.1	-	5/6	8,5,4,2,1,0,0	3.3
	1	-	4/4	20,14,12,4	12.5
Nickel Dust	0.1	1.6	3/6	7,6,3,0,0,0	2.7
	1	16	3/6	10,7,2,0,0,0	3.2
Control	-	-	0/5	0,0,0,0,0	0.0

needs to be undertaken before the significance of these changes in colonial morphology can be assessed. To date, we have not succeeded in attempts to induce tumors in Syrian hamsters following inoculation of hamster fetal cells that were treated *in vitro* with Ni_3S_2 .

G. Investigations of Ni_3S_2 -Induced Erythrocytosis. Attempts to collaborate with Dr. Albert Gordon of New York University in an investigation of mechanisms of Ni_3S_2 -induced erythrocytosis were not fruitful, and hence we have pursued this study independently. Comparisons have been made of the erythrocytic responses in Fischer rats following intrarenal injections of various nickel compounds (Table 6). Intrarenal injection of nickel as nickel

TABLE 6
BLOOD HEMATOCRIT VALUES IN RATS AT 2 MONTHS AFTER
INTRARENAL INJECTION OF NICKEL COMPOUNDS

Group	Compounds	Dosage		Hematocrit (%) Mean $\pm$ SD (N)
		mg/rat	$\mu\text{g-atoms}^a$	
A	NaCl sol'n	-	-	47 $\pm$ 1 (24)
B	Glycerol	-	-	47 $\pm$ 1 (18)
C	Fe	7.0	125	47 $\pm$ 1 (18)
D	Ni	7.3	125	67 $\pm$ 8 ^b (18)
E	$\alpha\text{Ni}_3\text{S}_2$	10.0	125	73 $\pm$ 3 ^b (24)
F	$\alpha\text{Ni}_3\text{Se}_2$	13.9	125	67 $\pm$ 7 ^b (24)
G	αNiS	11.3	125	71 $\pm$ 7 ^b (15)
H	NiS(amorphous)	11.3	125	48 $\pm$ 1 (15)

^a Dosage of metal, as $\mu\text{g-atoms/rat}$.

^b $P < 0.001$ vs corresponding values in groups A and B (control rats) that received intrarenal injections of the vehicles (NaCl solution and glycerol).

subulfide (Ni_3S_2) produced marked erythrocytosis in Fischer rats. Intrarenal injection of Ni dust, Ni_3Se_2 , or αNiS produced moderate erythrocytosis. Intrarenal injection of amorphous NiS or Fe dust had no significant effects upon mean hematocrits of comparable groups of rats. As shown in Table 7, injection *ir* of Mn dust plus Ni_3S_2 delayed the development of erythrocytosis, indicating that Mn dust is antagonistic to the effect of Ni_3S_2 . ^{59}Fe

TABLE 7
MANGANESE ANTAGONISM OF Ni_3S_2 -INDUCTION
OF ERYTHROCYTOSIS IN RATS

Group	$\alpha\text{Ni}_3\text{S}_2$ (ng/rat)	Mn (ng/rat)	Hematocrit (%) ^a
A	0	6.9	47 ± 1 (24)
B	10.0	0	73 ± 3^b (24)
C	10.0	6.9	$56 \pm 6^{b,c}$ (24)
A'	0	4.0	48 ± 1 (12)
B'	1.3	0	70 ± 3^b (12)
C'	1.3	4.0	$53 \pm 5^{b,c}$ (12)

^a Hematocrit at 2 months after *ir* injection; Mean $\pm$ SD (N)

^b $P < 0.001$ vs groups A or A'

^c $P < 0.001$ vs groups B or B'

incorporation into erythrocytes was measured in Fischer rats at intervals from 1 to 8 weeks after *ir* injection of Ni_3S_2 (Table 8). Increases in RBC uptake of ^{59}Fe occurred at 4 weeks after injection of Ni_3S_2 (24 hr uptake = 99 ± 7 percent of the ^{59}Fe dose in Ni_3S_2 -treated rats vs 28 ± 9 percent in vehicle-treated control rats ($P < 0.001$). This study shows that induction of erythrocytosis by *ir* injection is not specific for Ni_3S_2 but also occurs

after *ir* injection of some other nickel powders. This study also demonstrates that the mechanism of nickel-induced erythrocytosis involves a greatly accelerated rate of RBC production. A paper that describes these findings was presented at the Spring Meeting of the *Association of Clinical Scientists* (abstract¹² submitted herewith). This research will be continued during the next year and further data will be obtained before a manuscript will be submitted for publication.

TABLE 8
ERYTHROCYTE ⁵⁹Fe UPTAKE AT INTERVALS AFTER INTRARENAL
INJECTION OF αNi₃S₂ (5 mg/rat)

Interval after <i>ir</i> injection (weeks)	⁵⁹ Fe Uptake into RBC (% of dose at 24 hr)	
	Control Rats	Ni ₃ S ₂ -treated rats
2	24 ± 6 (6)	71 ± 10 ^a (4)
4	28 ± 9 (6)	99 ± 7 ^a (6)
6	31 ± 6 (6)	71 ± 9 ^a (6)
8	27 ± 4 (6)	52 ± 13 ^a (6)

^a $P < 0.001$ vs corresponding control rats

H. Measurements of Nickel in Tissues of Victims of "Legionnaires' Disease".

Upon the request of the Department of Health of the Commonwealth of Pennsylvania, our laboratory performed analyses of nickel in specimens of tissue and urine from victims of "Legionnaires' Disease", as well as analyses of nickel in water samples from the Bellvue-Stratford Hotel in Philadelphia. The analyses were performed by electrothermal atomic absorption spectrometry. Increased concentrations of nickel were found in five of six lung specimens from "Legionnaires' Disease" victims, suggesting that these persons may have

inhaled a nickel compound, (e.g. $\text{Ni}(\text{CO})_4$). The mean Ni concentration in lungs from the six victims was 68 ± 50 (range = 12 to 162) $\mu\text{g}/100 \text{ g}$ (dry weight) vs a mean Ni concentration of 7.4 ± 5.3 (range = 1.4 to 14.6) $\mu\text{g}/100 \text{ g}$ (dry weight) in lung samples from six control subjects who died of unrelated causes. Analyses of liver, kidney, brain and urine samples from victims of "Legionnaires' Disease" did not reveal any increased concentrations of nickel. Our findings were not conclusive evidence of nickel poisoning, since it was not possible to exclude contamination from exogenous nickel. Our results were reported to the Commonwealth of Pennsylvania (report¹³ submitted herewith) and we testified on the possible role of nickel in the etiology of "Legionnaires' Disease" at the Congressional investigation (statement¹⁴ submitted herewith). A paper that describes our analyses was presented at the *Annual Meeting of the American Academy of Forensic Sciences* (abstract¹⁵ submitted herewith). The costs of our study of "Legionnaires' Disease" were supported almost exclusively by the ERDA Contract. Independent confirmation of our finding of increased nickel concentrations in lungs of victims of "Legionnaires' Disease" has recently been published in *Science* by Chen *et al*¹⁶.

I. Studies of Nickel-Induced Hyperglycemia. Two previous studies^{18,19} in our laboratory have been focused upon the mechanisms of hyperglycemia that is induced in rats by parenteral injection of Ni(II). In our previous study¹⁹, we observed marked hyperglucagonemia in rats immediately following an injection of NiCl_2 , and we speculated that Ni(II)-induction of glucagon release might be responsible for the hyperglycemia. As the next phase of this investigation, we have studied the effect of somatostatin upon Ni(II)-induced hyperglucagonemia and hyperglycemia. The experimental animals were 54 female Fischer rats (weight = $162 \pm 7 \text{ g}$; range = 150 to 178 g). The rats were fasted

overnight prior to administration of somatostatin by *iv* infusion into a tail vein. Somatostatin (500 μ g dissolved in 0.5 ml of NaCl solution, 154 mmole/liter) was administered by slow infusion, commencing 15 min prior to *ip* injection of Ni(II) and continuing for 25 min after the injection of Ni(II). NiCl₂ was injected *ip* in a dosage of 68 μ mole/kg body weight. Control rats received a sham *ip* injection and/or an *iv* infusion of NaCl solution. The rats were exsanguinated by cardiac puncture at 30 min after the *ip* injection of Ni(II) or saline. Measurements of plasma glucose, glucagon and insulin were performed as previously described^{18,19}. The results of this study are summarized in Table 9. Infusion of somatostatin decreased the basal levels of plasma glucagon in rats that did not receive Ni(II). However, infusion of somatostatin had no effect upon Ni(II)-induced hyperglycemia, hyperglucagonemia or hyperinsulinemia. This result is unexpected, since somatostatin usually suppresses pancreatic release of glucagon.

As the next step in this avenue of research, we have investigated the possibility that Ni(II) might induce hyperglycemia by blocking cellular uptake of glucose. As the experimental system to test this possibility, we have measured *in vitro* uptake of ¹⁴C-glucose into erythrocytes that were collected from rats at intervals of 0.5, 1.2 and 3 hours after *ip* injection of Ni(II) (68 μ mole/kg, body weight). The data in Table 10 demonstrate that administration of Ni(II) did not block RBC uptake of ¹⁴C-glucose but on the contrary had a slight stimulatory effect. This effect did not parallel the increases in concentrations of plasma glucose.

Our previous studies of the effects of nickel on plasma glucose were concerned with soluble salts of Ni(II). A test was performed to ascertain whether inhalation of Ni(CO)₄ also produced acute hyperglycemia in Fischer

TABLE 9

EFFECTS OF SOMATOSTATIN UPON Ni(II) -INDUCED HYPERGLYCEMIA, HYPERGLUCAGONEMIA AND HYPERTENSULINEMIA

Group	No. of Rats	Treatments NiCl_2 Injection ^a	Somatostatin Infusion ^b	Plasma Glucose mg/100 ml	Plasma Glucagon $\mu\text{g}/100 \text{ ml}$	Plasma Insulin $\mu\text{U}/\text{ml}$
A	12	-	-	135 $\pm$ 19 (100 - 168)	14.8 $\pm$ 3.8 (9.1 - 21.0)	23.2 $\pm$ 3.2 (19.7 - 32.5)
B	10	-	+	134 $\pm$ 13 (116 - 165)	10.8 $\pm$ 1.4 ^e (8.3 - 13.5)	23.8 $\pm$ 4.6 (17.5 - 33.3)
C	15	+	-	192 $\pm$ 36 (145 - 274)	19.5 $\pm$ 8.9 ^d (10.5 - 37.0)	36.7 $\pm$ 14.4 ^d (14.6 - 72.2)
D	16	+	+	197 $\pm$ 52 ^e (140 - 295)	20.5 $\pm$ 8.4 ^e (9.0 - 39.0)	37.4 $\pm$ 20 ^c (13.9 - 94.1)

^a NiCl_2 , 68 $\mu\text{mole}/\text{kg}$, was administered ip at 15 min after the start of the somatostatin infusion. The rats were killed at 45 min after the start of the somatostatin infusion (i.e. at 30 min) after the ip injection of NiCl_2 . Control rats received injections and/or infusions of the vehicle.

^b Somatostatin, 500 μg , slowly infused iv in a volume of 0.5 ml during 35 min.

^c $P < 0.05$ vs Group A by "t" test.

^d $P < 0.01$ vs Group A by "t" test.

^e $P < 0.001$ vs Group A by "t" test.

TABLE 10
IN VITRO UPTAKE OF ^{14}C -GLUCOSE BY ERYTHROCYTES FROM
FISCHER RATS FOLLOWING IP INJECTION OF Ni(II) ^a

<u>Interval</u> <u>following</u> <u>ip Ni(II)</u> <u>(hr)</u>	<u>Plasma</u> <u>Glucose</u> <u>Conc.^b</u> <u>(mg/100 ml)</u>	<u>RBC Uptake</u> <u>of ^{14}C-Glucose^{b,c}</u> <u>(umole/liter</u> <u>of RBC/min)</u>
0	97 ± 13 (88 - 124)	23.4 ± 3.1 (18.1 - 26.2)
0.5	212 ± 33 ^e (181 - 268)	23.1 ± 4.5 (15.9 - 31.4)
1	219 ± 46 ^e (165 - 288)	26.9 ± 23 ^d (21.8 - 29.4)
2	228 ± 46 ^e (132 - 313)	26.1 ± 3.6 (20.2 - 31.3)
3	220 ± 32 ^e (173 - 283)	31.1 ± 5.0 ^e (22.0 - 37.9)

^a Injection of NiCl_2 ip in dosage of 68 $\mu\text{mole/kg}$ body weight to 12 female Fischer rats. Blood (50 μl) was collected from the tail vein of each rat at each of the specified intervals.

^b Mean ± S.D. (and range).

^c Measurements by the method of Rabinow *et al* (*Clin. Chem.* 22: 2010-2017, 1976).

^d $P < 0.01$ vs corresponding value at 0 hr, computed by "t" test.

^e $P < 0.001$ vs corresponding value at 0 hr, computed by "t" test.

rats. As shown in Table 11, exposure to $\text{Ni}(\text{CO})_4$ did induce hyperglycemia, with a response that was essentially the same as that observed with $\text{Ni}(\text{II})$.

TABLE 11
MEASUREMENTS OF PLASMA GLUCOSE IN RATS FOLLOWING INHALATION OF $\text{Ni}(\text{CO})_4$

Group	No. of Rats	$\text{Ni}(\text{CO})_4$ Exposure (mg $\text{Ni}(\text{CO})_4$ /liter/15 min)	Plasma Glucose Concentration (mg/100 ml, mean $\pm$ SD) at Intervals following start of Exposure to $\text{Ni}(\text{CO})_4$				
			0 hr	0.5 hr	1 hr	2 hr	3 hr
A	20	0	123 $\pm$ 13	111 $\pm$ 15	110 $\pm$ 13	104 $\pm$ 4	107 $\pm$ 10
B	19	0.2	122 $\pm$ 15	142 $\pm$ 19 ^a	130 $\pm$ 16 ^a	108 $\pm$ 15	98 $\pm$ 14
C	20	0.6	119 $\pm$ 11	171 $\pm$ 28 ^a	153 $\pm$ 32 ^a	109 $\pm$ 21	95 $\pm$ 20
D	20	1.1	126 $\pm$ 7	212 $\pm$ 22 ^a	189 $\pm$ 25 ^a	126 $\pm$ 17	102 $\pm$ 14

^a $P < 0.001$ vs Group A, computed by "t" test.

Inhalation of $\text{Ni}(\text{CO})_4$ did not cause any significant change in RBC uptake of ^{14}C -glucose at 0.5 or 1 hr after the start of the 15 min period (Table 12). These studies of Ni-induced hyperglycemia will be included in a manuscript to be drafted within the next year.

J. Studies of 5'-Nucleotidase Activity. During the past two years our laboratory has investigated the possible use of analyses of serum 5'-nucleotidase activity as a biological indicator of nickel toxicity, since it has been known for many years that $\text{Ni}(\text{II})$ inhibits 5'-nucleotidase activity *in vitro*. We have developed a new assay for serum 5'-nucleotidase activity based upon the difference between the enzymatic hydrolysis of adenosine-5'-monophosphate in the presense and absence of concanavalin-A. The concanavalin-A inhibition method for serum 5'-nucleotidase is more specific than previous methods, and is convenient for routine use in clinical laboratories for the diagnosis of

TABLE 12
IN VITRO UPTAKE OF ^{14}C -GLUCOSE BY ERYTHROCYTES FROM
FISCHER RATS FOLLOWING INHALATION OF $\text{Ni}(\text{CO})_4$ ^a

Interval Following Inhalation of $\text{Ni}(\text{CO})_4$ (hr)	Plasma Glucose Concentration ^b (mg/100 ml)		RBC Uptake of ^{14}C -Glucose ^b ($\mu\text{mole/liter RBC/min}$)	
	Control	$\text{Ni}(\text{CO})_4$	Control	$\text{Ni}(\text{CO})_4$
	Rats	Treated Rats	Rats	Treated Rats
0.5	119 $\pm$ 8 (107 - 131)	239 $\pm$ 11 ^c (220 - 249)	25.7 $\pm$ 3.8 (20.4 - 30.7)	24.8 $\pm$ 2.4 (20.3 - 26.6)
1	128 $\pm$ 7 (116 - 138)	196 $\pm$ 30 ^c (173 - 249)	24.9 $\pm$ 2.4 (20.7 - 28.1)	24.2 $\pm$ 2.0 (20.0 - 26.3)

^a Eight rats were exposed to inhalation of $\text{Ni}(\text{CO})_4$ in concentration of 1.1 mg $\text{Ni}(\text{CO})_4$ /liter of air for 15 min. Eight control rats were placed in an exposure chamber for 15 min without $\text{Ni}(\text{CO})_4$.

^b Mean $\pm$ S.D. (and range).

^c $P < 0.001$ vs corresponding value in control rats, computed by "t" test.

hepatobiliary diseases. However, measurements of serum 5'-nucleotidase have not proven to be useful as an indicator of nickel exposure in rats. A paper describing our new assay for serum 5'-nucleotidase was presented at the meeting of the *American Association for Clinical Chemistry* (abstract¹⁷ submitted herewith). The completed manuscript¹⁸ (submitted herewith) has been submitted for publication in *Clinical Chemistry*.

K. Miscellaneous Accomplishments. A review paper (abstract¹⁹ submitted herewith) on the metabolism and toxicology of nickel was presented at an *International Symposium on Metal Toxicology*. This manuscript²⁰ (submitted herewith) has been accepted for publication in *Annals of Clinical and Laboratory Science*.

A review paper on metal carcinogenesis was presented at a *Symposium on Environmental Carcinogens* that was sponsored by the American Society of Pharmacology and Experimental Therapeutics during the FASEB meetings, and the manuscript²¹ (submitted herewith) is in press in *Federation Proceedings*.

The principal investigator served as a member of the NIOSH review panel for the Criteria Document on Occupational Exposures to Inorganic Nickel²². This document recommends lowering the permissible occupational exposure to nickel in air from 1 mg per m³ to 15 µg per m³.

The principal investigator collaborated in a study of lead concentrations in semen from healthy men. A paper (abstract²³ submitted herewith) describing this research was presented at an *International Symposium on Metal Toxicology*. The completed manuscript²⁴ (submitted herewith) has been accepted for publication in *Annals of Clinical and Laboratory Science*. This paper describes the first reported analyses of lead in human semen, and provides baseline data for further studies of lead concentrations in semen from exposed workers.

Assistance was given to Dr. Max Costa for an investigation of thermal sensitivities of cyclic AMP-dependent protein kinases. Dr. Costa's manuscript²⁵ (submitted herewith) has been accepted for publication in the *Journal of Cyclic Nucleotide Research*.

L. Compliance with Contract Requirements. As specified in last year's renewal proposal, the specific goals of this year's research were:

- (1) *Chelation Therapy of Acute Nickel Carbonyl Poisoning in Rats.*

This work has been accomplished (see section B).

- (2) *Studies of Fetal Toxicity, Teratogenicity, Mutagenicity and Transplacental Carcinogenicity of Nickel Compounds in Rats.* Most of the

proposed research has been accomplished and the remainder is in progress (see section A).

(3) *Studies of Differences in Solubilities, Metabolism and Toxicity of Carcinogenic and Non-Carcinogenic Nickel Compounds.* Substantial work in this area has been accomplished (see section D), although this study is not yet completed.

(4) *Development of International Reference Method for Nickel Analysis in Biological Materials.* Major steps towards this goal have been accomplished (see section C).

(5) *Investigation of Mechanisms of Ni_3S_2 -Induced Erythrocytosis.* The experimental design has been modified, but important progress in this project has been achieved (see section G).

(6) *Induction of Ni-Deficiency in Rats.* No work was done in this area, in compliance with priorities recommended by our ERDA scientific advisors in Germantown, Maryland during the principal investigator's visit to review this contract during September, 1976.

In addition to the work towards these specified goals, valuable contributions have been made to knowledge of nickel toxicology in the areas of: (7) nickel carcinogenesis in experimental animals (see section E); (8) effects of nickel compounds on cells in tissue cultures (see section F); (9) investigation of "Legionnaires' Disease" (see section H); and (10) study of the mechanisms of Ni-induced hyperglycemia (see section I).

M. Effort of the Principal Investigator. The principal investigator is devoting 25% of his time and effort to this project during the period from December 1, 1976 to November 30, 1977.

N. Significance of the Research and its Relevance to National Priorities.

There is very serious national and international concern regarding environmental

pollution and industrial toxicity from nickel and its compounds, as indicated (a) by the report on nickel of the *Committee on Medical and Biological Effects of Environmental Pollutants* of the *National Academy of Sciences*²⁶; (b) by the monograph on *Carcinogenic Risk to Man from Exposure to Nickel and Inorganic Nickel Compounds* which has been issued by the *International Agency for Cancer Research*²⁷; (c) by the *Criteria Document on Occupational Exposures to Inorganic Nickel* which has been issued by the *National Institute of Occupational Safety and Health*²²; and (d) by the creation of the *Subcommittee on Environmental and Occupational Toxicology of Nickel* of the *International Union of Pure and Applied Chemistry*. The *World Health Organisation* is drafting an *Environmental Criteria Document for Nickel* which will focus additional attention upon the human hazards from exposure to nickel compounds. Insofar as the principal investigator can ascertain, this research program at the University of Connecticut is the only research program in the United States which is specifically focused upon the toxicology and metabolism of nickel compounds. The relevance of this research to national priorities has been enhanced by the introduction of nickel catalysts in several industrial processes for gasification of coal. Formation and release of volatile nickel compounds (e.g. $\text{Ni}(\text{CO})_4$) and of nickel sulfides (e.g. Ni_3S_2) may represent serious hazards to industrial workers and even to the general population. The data on toxicity, carcinogenicity, teratogenicity and mutagenicity of nickel compounds, and the biochemical indices and analytical methods for detection of nickel exposures that are being derived from this research program will become especially important to national priorities if nickel catalyzed gasification technologies for coal achieve widespread adoption.

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