

# **Report on Carcinogens Background Document for**

# **Cobalt Sulfate**

**April 17, 2002**

Prepared for the:  
**U.S. Department of Health and Human Services  
Public Health Service  
National Toxicology Program  
Research Triangle Park, NC 27709**

Prepared by:  
**Technology Planning and Management Corporation  
Canterbury Hall, Suite 310  
4815 Emperor Blvd  
Durham, NC 27703  
Contract Number N01-ES-85421**



---

## FOREWORD

The Report on Carcinogens (RoC) is prepared in response to Section 301 of the Public Health Service Act as amended. The RoC contains a list of all substances (i) that either are known to be human carcinogens or may reasonably be anticipated to be human carcinogens; and (ii) to which a significant number of persons residing in the United States are exposed. The Secretary, Department of Health and Human Services (DHHS) has delegated responsibility for preparation of the RoC to the National Toxicology Program (NTP) who prepares the Report with assistance from other Federal health and regulatory agencies and non-government institutions.

Nominations for listing in or delisting from the RoC are reviewed by a formal process that includes a multi-phased, scientific peer review and multiple opportunities for public comment. The review groups evaluate each nomination according to specific RoC listing criteria. This Background Document was prepared to assist in the review of the nomination of Cobalt Sulfate. The scientific information in this document comes from publicly available, peer reviewed sources. Any interpretive conclusions, comments or statistical calculations, etc made by the authors of this document that are not contained in the original citation are identified in brackets [ ]. If any member(s) of the scientific peer review groups feel this Background Document does not adequately capture and present the relevant information they will be asked to write a commentary for this Background Document that will be included as an addendum to the document. In addition, a meeting summary that contains a brief discussion of the respective review group's review and recommendation for the nomination will be added to the Background Document, also as an addendum.

A detailed description of the RoC nomination review process and a list of all nominations under consideration for listing in or delisting from the RoC can be obtained by accessing the NTP Home Page at <http://ntp-server.niehs.nih.gov>. The most recent RoC, the 9<sup>th</sup> Edition, was published in May, 2000 and may be obtained by contacting the NIEHS Environmental Health Information Service (EHIS) at <http://ehis.niehs.nih.gov> (800-315-3010).

---

## CONTRIBUTORS

### NIEHS/NTP Staff

C.W. Jameson, Ph.D.	Head, Report on Carcinogens, Environmental Toxicology Program, NIEHS
Ruth M. Lunn, Dr. P.H.	Report on Carcinogens Group, Environmental Toxicology Program, NIEHS
Shawn Jeter, B.S.	Report on Carcinogens Group, Environmental Toxicology Program, NIEHS
AnnaLee Sabella	Report on Carcinogens Group, Environmental Toxicology Program, NIEHS

**Support to the National Toxicology Program for the preparation of this background document was provided by Technology Planning and Management Corporation through NIEHS Contract Number NO1-ES-85421**

Ronald Thomas, Ph.D., Principal Investigator

Sanford Garner, Ph.D., Co-Principal Investigator

Stanley Atwood, M.S., Senior Scientist

Susan Goldhaber, M.S., Senior Scientist

Greg Pazianos, B.S., Scientist

### *Support staff*

Angie Fralick, B.S.

Tracy Saunders, B.S.

### *Consultants*

Max Costa, Ph.D., Department of Environmental Medicine, NYU School of Medicine  
(General Reviewer)

Susan F. Dakin, Ph.D., Independent Consultant (Scientific Editing of Document)

## Criteria for Listing Agents, Substances or Mixtures in the Report on Carcinogens

### U.S. Department of Health and Human Services National Toxicology Program

#### Known to be Human Carcinogens:

There is sufficient evidence of carcinogenicity from studies in humans, which indicates a causal relationship between exposure to the agent, substance or mixture and human cancer.

#### Reasonably Anticipated to be Human Carcinogens:

There is limited evidence of carcinogenicity from studies in humans, which indicates that causal interpretation is credible but that alternative explanations such as chance, bias or confounding factors could not adequately be excluded; or

There is sufficient evidence of carcinogenicity from studies in experimental animals which indicates there is an increased incidence of malignant and/or a combination of malignant and benign tumors: (1) in multiple species, or at multiple tissue sites, or (2) by multiple routes of exposure, or (3) to an unusual degree with regard to incidence, site or type of tumor or age at onset; or

There is less than sufficient evidence of carcinogenicity in humans or laboratory animals, however; the agent, substance or mixture belongs to a well defined, structurally-related class of substances whose members are listed in a previous Report on Carcinogens as either a *known to be human carcinogen*, or *reasonably anticipated to be human carcinogen* or there is convincing relevant information that the agent acts through mechanisms indicating it would likely cause cancer in humans.

Conclusions regarding carcinogenicity in humans or experimental animals are based on scientific judgment, with consideration given to all relevant information. Relevant information includes, but is not limited to dose response, route of exposure, chemical structure, metabolism, pharmacokinetics, sensitive sub populations, genetic effects, or other data relating to mechanism of action or factors that may be unique to a given substance. For example, there may be substances for which there is evidence of carcinogenicity in laboratory animals but there are compelling data indicating that the agent acts through mechanisms which do not operate in humans and would therefore not reasonably be anticipated to cause cancer in humans.



---

## Executive Summary

### Introduction

Cobalt sulfate is an inorganic salt of divalent cobalt that is used in the electroplating and electrochemical industries and as a coloring and drying agent. Cobalt sulfate heptahydrate is the hydrated form of cobalt sulfate. The behavior of the anhydrous and hydrated forms in solution is indistinguishable, as dissolution of either form results in a system containing hydrated ions and water. Cobalt sulfate was nominated by the National Institute of Environmental Health Sciences for possible listing in the Report on Carcinogens based on a National Toxicology Program (NTP) two-year inhalation study of cobalt sulfate heptahydrate which concluded that there was clear evidence of carcinogenicity in female F344/N rats and male and female B6C3F<sub>1</sub> mice and some evidence of carcinogenicity in male F344/N rats. Cobalt and cobalt compounds have been categorized by the International Agency for Research on Cancer as possibly carcinogenic to humans (Group 2B), based on sufficient evidence of carcinogenicity in experimental animals.

### Human Exposure

*Use.* Cobalt sulfate is used in the electroplating and electrochemical industries; as a drier for lithographic inks, varnishes, paints, and linoleum; in storage batteries; and as a coloring agent in ceramics, enamels, glazes, and porcelain. In addition, cobalt sulfate has been used in animal feeds as a mineral supplement and used on pastures where the forage is cobalt deficient, to provide enough cobalt for ruminants to produce vitamin B<sub>12</sub>. Past uses include addition to beers to improve the stability of the foam, use in veterinary medication to prevent and treat cobalt deficiency in ruminants, and use in humans to improve hematocrit, hemoglobin, and erythrocyte levels.

*Production.* Cobalt sulfate is formed by the interaction of cobalt oxide, hydroxide, or carbonate with sulfuric acid. Production of cobalt sulfate in the United States in 1983 was estimated at 450,000 lb. Import of cobalt sulfate in 1986 was reported to be 79,700 lb. United States imports for consumption of cobalt sulfates were 1,360 metric tons in 1999 and 1,040 metric tons in 1998.

*Environmental exposure.* No information was found that specifically identified environmental exposure to cobalt sulfate. Exposure to cobalt occurs through inhalation of ambient air and ingestion of food and drinking water. Cobalt is an essential trace element in humans, because a cobalt atom is present in each molecule of vitamin B<sub>12</sub> (cobalamin). The National Health and Nutrition Examination Survey in 1999 reported that the geometric mean cobalt level in the urine of humans was 0.36 µg/L of urine (95% confidence interval = 0.32 to 0.40).

*Occupational exposure.* No information was found that specifically identified occupational exposure to cobalt sulfate. More than a million workers in the United States potentially are exposed to cobalt or cobalt compounds. Occupational exposure to cobalt occurs principally in refining processes, in production of alloys, and in the tungsten

carbide hard-metal industry. In addition, many workers are exposed to a limited degree when using cobalt-containing paint dryers. Occupational exposure is primarily dermal or through inhalation of cobalt metal dusts or fumes. A high degree of conformity between the concentration of cobalt in blood and urine and the average levels of cobalt in the air during a workweek has been reported for workers exposed to cobalt.

*Regulations.* No specific United States Environmental Protection Agency (EPA) regulations for cobalt sulfate were identified. Cobalt is regulated by the EPA, Food and Drug Administration, and Occupational Safety and Health Administration (OSHA). The current OSHA permissible exposure limit for cobalt metal, dust, and fume (as Co) is 0.1 mg/m<sup>3</sup> of air as an 8-hour time-weighted average (TWA) concentration. The National Institute for Occupational Safety and Health has established a recommended exposure limit for cobalt metal, dust, and fume of 0.05 mg/m<sup>3</sup> as a TWA for up to a 10-hour workday and a 40-hour workweek. The American Conference of Governmental Industrial Hygienists has assigned elemental cobalt and inorganic cobalt compounds (as Co) a threshold limit value of 0.02 mg/m<sup>3</sup> as a TWA for an 8-hour workday and a 40-hour workweek.

### **Human Cancer Studies**

Although no human studies are available in which exposure to cobalt sulfate is specifically evaluated, some human studies have investigated carcinogenicity of cobalt and cobalt compounds as a class. Several studies suggest that exposure to cobalt in hard-metal production is associated with an increased risk of lung cancer. However, because the exposure considered in these studies is to metallic cobalt and tungsten carbide together, the results are of uncertain relevance for the evaluation of cancer due to cobalt exposure alone. Exposure to cobalt without co-exposure to tungsten carbide was found to be associated with a twofold increase in risk of lung cancer in two studies; however, the most likely source of this exposure is cobalt metal. Only one study (at an electrochemical factory) specifically mentioned exposure to cobalt salts. The small study size and unstable risk estimates reflected in the discrepancy between the findings of the initial study and the updated study limit the usefulness of these results for evaluation of the carcinogenic effects of cobalt salts in humans. A biomarker study showed a strong association between esophageal cancer and cobalt present in nails but did not provide any information on exposure to specific cobalt compounds. The human studies thus provide limited information for the specific evaluation of the carcinogenicity of cobalt sulfate.

### **Studies in Experimental Animals**

Cobalt sulfate heptahydrate was found to be carcinogenic in B6C3F<sub>1</sub> mice and F344/N rats when administered by inhalation in a two-year study conducted by the NTP. There was clear evidence of carcinogenicity in male mice, female mice, and female rats, based on increased incidences of lung tumors. In addition, female rats had an increased incidence of pheochromocytoma of the adrenal medulla. Some evidence of carcinogenicity in male rats was reported, based on increased incidences of lung tumors at the highest exposure level.

**Genotoxicity**

The genotoxicity of cobalt compounds may depend on the ligand coordinated about the metal ion. Cobalt sulfate was mutagenic in *Salmonella typhimurium* strain TA100 but not in strains TA98 or TA1535. Cobalt sulfate induced cell transformation and micronuclei in Syrian hamster embryo cells and strongly induced p53 expression in mouse fibroblasts. In the presence of hydrogen peroxide, cobalt sulfate induced putative intrastrand cross-links in salmon sperm DNA and single-strand breaks in plasmid pBluescript K+ DNA. However, 8-hydroxy-2'-deoxyguanosine adducts were not induced in salmon sperm DNA. Sulfite in the presence of cobalt ions caused damage in DNA fragments derived from the human c-Ha-ras-1 protooncogene. Yields of DNA base products in human chromatin were increased by exposure to cobalt sulfate in the presence of hydrogen peroxide. Cobalt sulfate was not genotoxic to human lymphocytes.

**Other Relevant Data**

*Absorption and excretion.* Cobalt is absorbed from the gastrointestinal tract, lungs, and skin and is distributed throughout the body. The highest concentrations are found in the liver, kidney, and heart. It is excreted primarily in the urine, but fecal excretion also is important. There are two distinct elimination phases: the first is rapid and occurs within days of exposure, but the second phase may take several years.

*Toxicity.* Occupational exposure to cobalt has been associated with a severe and progressive respiratory disease known as hard-metal pneumoconiosis, as well as asthma and contact dermatitis. In the 1960s, several outbreaks of cardiomyopathy and polycythemia were reported in individuals who drank large quantities of beer containing added cobalt.

*Potential mechanisms of carcinogenicity.* Cobalt ions may mimic or replace other essential divalent metal ions (e.g., magnesium, calcium, iron, copper, or zinc), thus altering many important cellular reactions and functions. There is good evidence that cobalt ions can inhibit DNA repair processes or interact with hydrogen peroxide to form reactive oxygen species that can damage DNA. These mechanisms may contribute to the genotoxic and carcinogenic effects reported for cobalt sulfate and other cobalt compounds.



---

**Table of Contents**

Executive Summary .....	v
1 Introduction.....	1
1.1 Chemical identification.....	1
1.2 Physical-chemical properties.....	2
1.3 Role of cobalt in biological systems.....	3
2 Human Exposure.....	5
2.1 Use.....	5
2.2 Production.....	5
2.3 Analysis.....	7
2.4 Environmental occurrence.....	9
2.4.1 Air.....	9
2.4.2 Water.....	10
2.4.3 Soil.....	10
2.5 Environmental fate.....	10
2.5.1 Air.....	11
2.5.2 Water.....	11
2.5.3 Soil.....	11
2.6 Environmental exposure.....	11
2.7 Occupational exposure.....	12
2.8 Biological indices of exposure.....	12
2.9 Regulations.....	13
3 Human Cancer Studies.....	19
3.1 IARC assessment.....	19
3.2 Current human studies.....	20
3.2.1 Occupational studies.....	20
3.2.2 Biomarker study.....	21
3.3 Discussion and summary.....	22
4 Studies of Cancer in Experimental Animals.....	31
4.1 NTP carcinogenicity bioassay in mice.....	31
4.2 NTP carcinogenicity bioassay in rats.....	34
4.3 Summary.....	37
5 Genotoxicity.....	39
5.1 Prokaryotic systems.....	39
5.2 Mammalian systems.....	39
5.2.1 Rodent cells.....	39
5.2.2 Human cells.....	41
5.3 Summary.....	42
6 Other Relevant Data.....	43

6.1	Toxicity of cobalt sulfate .....	43
6.2	Mammalian absorption, distribution, metabolism, and excretion of cobalt .....	44
6.3	Syrian hamster embryo cell transformation assay .....	45
6.4	Possible mechanisms of cobalt-induced carcinogenesis.....	45
6.5	Cocarcinogenicity of cobalt and Rauscher leukemia virus.....	47
6.6	Summary .....	47
7	References.....	49
Appendix A: IARC (1991). Monographs on the Evaluation of Carcinogenic Risks to Humans. Chlorinated Drinking-Water: Chlorination By-products; Some Other Halogenated Compounds: Cobalt and Cobalt Compounds. V 52. PP A-1 – A-112.....		
		55
Appendix B: NTP (1998). Toxicology and Carcinogenesis Studies of Cobalt Sulfate Heptahydrate in F344/N Rats and B6C3F <sub>1</sub> Mice (Inhalation Studies). TR No. 471. PP B-1 – B-59.....		
		57

### List of Tables

Table 1-1.	Physical and chemical properties of cobalt sulfate.....	2
Table 2-1.	Cobalt production, consumption, import, and export.....	6
Table 2-2.	Patterns of cobalt consumption in the United States in 2001 .....	7
Table 2-3.	Analytical methods for determining cobalt in biological materials .....	8
Table 2-4.	Analytical methods for determining cobalt in environmental samples. ....	9
Table 2-5.	EPA regulations.....	14
Table 2-6.	FDA regulations .....	16
Table 2-7.	OSHA regulations .....	17
Table 3-1.	Current studies of human exposure to cobalt .....	24
Table 4-1.	Tumor incidence in B6C3F <sub>1</sub> mice following inhalation exposure to cobalt sulfate heptahydrate for 105 weeks.....	32
Table 4-2.	Incidences and severity of nonneoplastic lesions in B6C3F <sub>1</sub> mice following inhalation exposure to cobalt sulfate heptahydrate for 105 weeks .....	33
Table 4-3.	Tumor incidence in F344/N rats following inhalation exposure to cobalt sulfate heptahydrate for 105 weeks.....	35
Table 4-4.	Incidences and severity of nonneoplastic lesions in F344/N rats following inhalation exposure to cobalt sulfate heptahydrate for 105 weeks .....	36
Table 5-1.	Effects of cobalt sulfate hydrate on micronucleus formation in SHE cells.....	40
Table 5-2.	Genotoxic effects of cobalt chloride, cobalt nitrate hexahydrate, and cobalt sulfate heptahydrate in human lymphocytes.....	42

**List of Figures**

Figure 1-1. Structure of cobalt sulfate ..... 1



# 1 Introduction

Cobalt sulfate is an inorganic salt of divalent cobalt. It is the usual source of water-soluble cobalt, because it is more economical and has less tendency to dehydrate than cobalt chloride or cobalt nitrate (Budavari *et al.* 1996). Cobalt sulfate is used in the electroplating and electrochemical industries, as a coloring agent for ceramics, as a drying agent in inks, paints, varnishes, and linoleum, and as a mineral supplement additive to animal feed. Cobalt sulfate heptahydrate is the hydrated form of cobalt sulfate. The behavior of the anhydrous and hydrated forms in solution is indistinguishable, as dissolution of either form results in a system containing hydrated ions and water (Davis *et al.* 1999).

Cobalt sulfate was nominated by the National Institute of Environmental Health Sciences (NIEHS) for possible listing in the Report on Carcinogens based on a National Toxicology Program (NTP) two-year inhalation study of cobalt sulfate heptahydrate which concluded that there was clear evidence of carcinogenicity in female F344/N rats (alveolar/bronchiolar neoplasms and pheochromocytoma of the adrenal medulla) and male and female B6C3F<sub>1</sub> mice (alveolar/bronchiolar neoplasms) and some evidence of carcinogenicity in male F344/N rats (alveolar/bronchiolar neoplasms) (NTP 1998). Cobalt sulfate heptahydrate also has been found to be mutagenic in *Salmonella typhimurium* strain TA100 with and without liver S9 metabolic activation enzymes. Cobalt and cobalt compounds have been categorized by the International Agency for Research on Cancer (IARC) as Group 2B, possibly carcinogenic to humans, based on sufficient evidence of carcinogenicity in experimental animals. The majority of the cancers in animals reported for cobalt and cobalt compounds in the publications reviewed by IARC (1991) were local sarcomas at injection sites. Data specific for carcinogenicity of cobalt sulfate in animals were not available at the time of the IARC review.

## 1.1 Chemical identification

Cobalt sulfate (CoSO<sub>4</sub>, mol wt 155.0, CASRN 10124-43-3) occurs as red to lavender dimorphic, orthorhombic crystals. It also is known as cobalt monosulfate, cobaltous sulfate, and cobalt(II) sulfate. Its RTECS number is GG3100000. The structure of cobalt sulfate is illustrated in Figure 1-1.

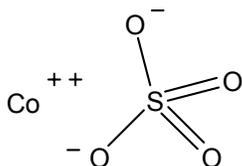


Figure 1-1. Structure of cobalt sulfate

In the majority of its compounds and complexes, cobalt exists in the +2 (cobaltous, cobalt[II]) and +3 (cobaltic, cobalt[III]) valence states. Evidence for cobalt(I) ( $\text{Co}^{+1}$ ) was first obtained from the electrolytic reduction of cyano-compounds. Cobalt(I) also may be found in coordination compounds of the organo-metallic class carbonyl, isonitriles, and unsaturated hydrocarbon derivatives. Cobalt(II) forms numerous salts, most of which are octahedral and tetrahedral. Cobalt(II) forms more tetrahedral complexes than any other transition-metal ion. The octahedral and tetrahedral complexes of cobalt(II) differ little in stability. Octahedral cobalt(II) salts typically are pink to reddish brown (as in the case of cobalt sulfate), whereas most tetrahedral cobalt(II) salts are blue (Kirk and Othmer 1999). Although the cobalt(III) ion exists, only a few simple cobalt(III) salts are known. Examples of cobalt(IV) ( $\text{Co}^{+4}$ ) compounds include cesium cobalt fluoride ( $\text{Cs}_2[\text{CoF}_6]$ ) and cobalt (IV) fluoride ( $\text{CoF}_4$ ) (Considine and Considine 1995, WebElements 2001).

## 1.2 Physical-chemical properties

Cobalt sulfate melts at 735°C. It is soluble in water (36.2 g/100 mL at 20°C), slightly soluble in methanol, and insoluble in ammonia. The physical and chemical properties of cobalt sulfate are summarized in Table 1-1.

**Table 1-1. Physical and chemical properties of cobalt sulfate**

Property	Information	Reference
Molecular weight	155.00	Budavari <i>et al.</i> 1996
Color	red to lavender	Budavari <i>et al.</i> 1996
Physical state	dimorphic, orthorhombic crystals	Budavari <i>et al.</i> 1996
Melting point (°C)	735	HSDB 2000
Decomposition point (°C)	> 708	Budavari <i>et al.</i> 1996
Density/specific gravity (at 25°C/4°C)	3.71	Budavari <i>et al.</i> 1996
Solubility:		
water (at 20°C)	36.2 g/100 mL	HSDB 2000
water (at 100°C)	84 g/100 mL	HSDB 2000

Cobalt salts are soluble to varying degrees (Lide 1999, Jensen and Tüchsen 1990). Those more soluble in water than cobalt sulfate include cobalt chloride (52.9 g/100 mL at 20°C), cobalt chloride hexahydrate (76.7 g/100 mL at 20°C), and cobalt nitrate hexahydrate (133.8 g/100 mL at 20°C). Cobalt acetate tetrahydrate also is considered soluble. Other salts are much less soluble than cobalt sulfate; cobalt formate is slightly soluble in water (5.03 g/100 mL at 20°C), and cobalt hydroxide is very slightly soluble in water. Salts insoluble in water include cobalt carbonate (1.1 g/100 mL at 15°C), cobalt linoleate, and cobalt oxalate.

### **1.3 Role of cobalt in biological systems**

Cobalt is considered an essential element for animals, including humans, because it is incorporated into the vitamin B<sub>12</sub> molecule. Green plants do not synthesize vitamin B<sub>12</sub>; microorganisms in ruminants (cud-chewing mammals with multi-chambered stomachs, such as cattle and sheep) are the only major producers of vitamin B<sub>12</sub> in the food chain. The normal sources of this vitamin for humans are milk, cheese, meat, and eggs (Considine and Considine 1995).

Vitamin B<sub>12</sub> contains about 4% cobalt by weight. Ruminants require 0.07 to 0.10 ppm cobalt in their feed, and lack of cobalt in the soil and feedstuffs prevents them from synthesizing enough B<sub>12</sub> for their needs. To prevent cobalt deficiency in cattle and sheep, cobalt sulfate may be added to feedstuffs, or cobalt may be added to the soil to increase its levels in plants. Areas of low cobalt content in the United States include Florida, the New England area, much of New York, western Iowa, southwestern Minnesota, and a small area of Illinois around Peoria (Considine and Considine 1995).

4/17/02

Draft RoC Background Document for Cobalt Sulfate  
Do not quote or cite

---

## 2 Human Exposure

### 2.1 Use

Cobalt sulfate is used in the electroplating and electrochemical industries, where it is added to nickel plating baths in order to improve the smoothness, brightness, hardness, and ductility of the deposits. It also is used as a drier for lithographic inks, varnishes, paints, and linoleum and in storage batteries. Cobalt sulfate is employed as a coloring agent in ceramics, enamels, and glazes to prevent discoloring and as a co-pigment for decorating porcelain. In addition, cobalt sulfate has been used in animal feeds as a mineral supplement (Budavari *et al.* 1996, Kirk and Othmer 1999).

Cobalt sulfate has been mixed, in small quantities, with fertilizers for use on pastures where the forage is cobalt deficient, to provide enough cobalt for ruminants to produce vitamin B<sub>12</sub>. In the United States in 1996, the total amount of fertilizer consumed containing cobalt sulfate was two tons. All of the fertilizer use was in Washington State, where the highest concentrations of cobalt in fertilizers were 44.8 to 222 mg/kg (dry weight) (EPA 1999, Washington State 1999).

In the 1960s, some breweries added cobalt sulfate to their beers to improve the stability of the foam by counteracting the antifoaming activity of detergent residues left on poorly rinsed glasses. Although only a small amount (1 ppm) was used in the beer, this practice was stopped after an epidemic of “beer drinker’s cardiomyopathy” was linked to the cobalt (NTP 1998).

Cobalt sulfate has also been used in veterinary medication to prevent and treat cobalt deficiency in ruminants, which causes reduction in feed intake and body weight, accompanied by emaciation, anemia, and debility. Cobalt sulfate had been used in the past to improve hematocrit, hemoglobin, and erythrocyte levels in human patients with refractory anemia, including sickle-cell disease, thalassemia, chronic infection or renal disease, anemia associated with neoplastic disease, and various other refractory anemias of unknown cause. In 1985, cobalt was used clinically only in the treatment of normochromic, normocytic anemia associated with severe renal failure (HSDB 2000, Hillman and Finch 1985). There is no listing for cobalt or cobalt sulfate in the current *Goodman & Gilman's Pharmacological Basis of Therapeutics* (Goodman and Gilman 2001).

### 2.2 Production

Cobalt sulfate is formed by the interaction of cobalt oxide, hydroxide, or carbonate with sulfuric acid. Production of cobalt sulfate in the United States in 1983 was estimated at 450,000 lb (NTP 1998). Current production levels are not available. There are currently 11 U.S. suppliers of cobalt sulfate (ChemFinder 2001).

The United States did not mine or refine cobalt in 2000, although negligible amounts of cobalt were produced as a byproduct of mining operations. The U.S. supply of cobalt in 2000 included imports, stock releases, and secondary materials. Stock releases originated from the U.S. government reserve (National Defense Stockpile) for military, industrial,

and civilian use during a national emergency. Sales of the National Defense Stockpile of cobalt began in March of 1993. Seven companies were known to be active in the production of cobalt compounds. It was estimated that 45% of U.S. cobalt usage was in superalloys, 9% in cemented carbides, 9% in magnetic alloys, and the remaining 37% in various other metallic and chemical uses (USGS 2001). Table 2-1 summarizes recent patterns of cobalt production, import, export, and consumption in the United States.

**Table 2-1. Cobalt production, consumption, import, and export**

Salient statistics	Metric tons of cobalt	
	1999	2000
<b>United States:</b>		
Production:		
Mine	NR	NR
Secondary	2,720	2,800
Consumption:		
Reported	8,420	8,400
Apparent	10,700	10,900
Imports for consumption	8,150	8,000
Exports	1,550	2,300
<b>World production:</b>		
Mine	29,900	32,300
Refinery	31,200	NR

Sources: Shedd 1999, USGS 2001.

NR = not reported.

Import of cobalt sulfate in 1986 was reported to be 79,700 lb (HSDB 2000). U.S. imports for consumption of cobalt sulfates were 1,360 metric tons in 1999 and 1,040 metric tons in 1998, valued at \$9,840,000 and \$10,400,000, respectively. Reported 1999 U.S. cobalt consumption was 2,530 metric tons for chemical and ceramic uses and 64 metric tons for miscellaneous and unspecified uses. Reported 1999 U.S. consumption of cobalt chemical compounds (organic and inorganic) was 1,910 metric tons. Imports of cobalt sulfates and other cobalt salts (acetates, carbonates, and chlorides) from 10 countries totaled \$12,400,000. Most imports were from Finland. The United States exported \$49,700,000 of cobalt and cobalt compounds in 1999. No specific information on cobalt sulfate exports was identified (USGS 2001). Table 2-2 summarizes U.S. cobalt consumption patterns in early 2001.

**Table 2-2. Patterns of cobalt consumption in the United States in 2001**

Consumption information	Date	Metric Tons	Compounds and uses
Reported consumption of cobalt materials	Jan–May	669	oxide and other chemical compounds
Reported consumption of cobalt by end use	Jan–May	883	chemical uses including catalysts, driers in paints, feed or nutritive additive, glass decolorizer, ground coat frit, pigments, other uses
Reported consumption of cobalt by end use	Jan–May	127	miscellaneous and unspecified uses
Imports by consumption	Jan–April	498	salts and compounds including acetates, carbonates, chlorides, and sulfates
Exports	Jan–April	74	salts and compounds

Source: USGS 2001.

Chem Sources identified 15 suppliers of cobalt(II) sulfate, four suppliers of cobalt(II) sulfate monohydrate, and 16 suppliers of cobalt(II) sulfate heptahydrate in the United States (Chem Sources 2001). The Hazardous Substances Data Bank listed seven manufacturers of cobaltous sulfate (HSDB 2000).

### 2.3 Analysis

Determination of cobalt, especially in biological samples containing low levels of cobalt, is accurate only if samples are not contaminated. Contamination from disposable syringes and technical-grade anticoagulants was responsible for erroneous reports in earlier literature of grossly high levels of cobalt in biological specimens. The common classical methods used for determining cobalt concentration in biological samples are polarographic and colorimetric methods. However, these older methods are unsuitable for determining low levels of cobalt in many biological samples, and samples must be chemically pretreated before quantification. The most common single-element instrumental techniques used are electrothermal atomic absorption spectrometry (AAS) and voltammetric techniques (ATSDR 1992). Analytical methods for determining cobalt in biological matrices are summarized in Table 2-3. The samples analyzed in the studies presented in this table were primarily from cobalt-exposed and non-exposed workers (Heinrich and Angerer 1984, Ichikawa *et al.* 1985, Alexandersson 1988). However, one study used samples from laboratory volunteers (Bouman *et al.* 1986), and another used hospital patients with knee or hip prostheses (Sunderman *et al.* 1989). IARC (1991) reported that serum cobalt concentrations in humans were in the range of 0.1 to 0.3 µg/L. As shown in Table 2-3, the level of detection for cobalt in serum by direct injection into electrothermal AAS with Zeeman background correction is 0.02 µg/L.

**Table 2-3. Analytical methods for determining cobalt in biological materials**

<b>Matrix</b>	<b>Analytical method</b>	<b>Detection limit</b>
Urine	electrothermal AAS with Zeeman background correction — direct injection	0.3 µg/L 0.1 µg/L
	electrothermal AAS with Zeeman background correction — chemical preparation	2.4 µg/L
	electrothermal AAS with deuterium background correction — chemical preparation	0.1 µg/L
	differential pulse cathodic stripping voltametry (DPCSV) — chemical preparation	0.2 µg/L
Whole blood	electrothermal AAS with deuterium background correction	2 µg/L
	DPCSV — chemical preparation	0.8 µg/L
	colorimetry — chemical preparation	0.15 µg/L
Serum	electrothermal AAS with Zeeman background correction — direct injection	0.02 µg/L
Blood	inductively coupled plasma-atomic emission spectrometry (ICP–AES) — chemical preparation	10 µg/kg
Tissue	ICP–AES — chemical preparation	200 µg/kg

Source: ATSDR 1992.

Because of its rapidity, accuracy, and low detection limit, electrothermal AAS with Zeeman background correction is the method most commonly used to quantify cobalt levels in environmental samples. To meet detection limits of some of the analytical methods, preconcentration may be necessary for some environmental samples (e.g., seawater). As with biological samples, contamination of environmental samples during collection, storage, and treatment are concerns (ATSDR 1992). Analytical methods for determining cobalt in environmental samples are detailed in Table 2-4.

**Table 2-4. Analytical methods for determining cobalt in environmental samples.**

<b>Matrix</b>	<b>Analytical method</b>	<b>Detection limit</b>	<b>Recovery<sup>a</sup></b>
Air (workroom)	$\tau$ -spectrometry with lithium-drifted germanium detector	0.17 $\mu\text{g}/\text{m}^3$	–
Air (occupational)	flame AAS with background correction	0.4 $\mu\text{g}/\text{m}^3$	98% with 12- to 96- $\mu\text{g}$ spiked filter
	ICP–AES	0.5 $\mu\text{g}/\text{m}^3$	95%–100% with 2.5- to 1,000- $\mu\text{g}$ spiked filter
Water (low ionic strength)	electrothermal AAS with Zeeman or deuterium background correction	< 0.5 $\mu\text{g}/\text{L}$	93%–113% at 8.5–30 $\mu\text{g}/\text{L}$
Lake water	ICP–AES	< 0.004 $\mu\text{g}/\text{L}$	–
Rainwater	photon-induced X-ray emission	0.08 $\mu\text{g}/\text{L}$	–
Seawater	electrothermal AAS with Zeeman background correction	0.0002 $\mu\text{g}/\text{L}$	90%
	DPCVS	0.0004 $\mu\text{g}/\text{L}$	103% at 0.02 $\mu\text{g}/\text{L}$
Water and waste water	flame AAS	0.05 $\text{mg}/\text{L}$	97%–98% at 0.2–5.0 $\text{mg}/\text{L}$
	electrothermal AAS with background correction	1 $\mu\text{g}/\text{L}$	–
Groundwater or leachate	flame AAS with background correction	0.05 $\text{mg}/\text{L}$	97%–98% at 0.2–5.0 $\text{mg}/\text{L}$
Groundwater or leachate	electrothermal AAS with background correction	1 $\mu\text{g}/\text{L}$	–
Food	electrothermal AAS with background correction	1.88 $\mu\text{g}/\text{L}$	100%–107% at 0.2–0.6 $\text{mg}/\text{kg}$ in leaves and liver

Source: ATSDR 1992.

<sup>a</sup> – = no data available.

## 2.4 Environmental occurrence

Very limited information is available on the environmental occurrence of cobalt sulfate or other soluble forms of cobalt. Thus, this section summarizes the information available on the environmental occurrence of nonspecific cobalt and cobalt compounds. Where information on cobalt sulfate or other cobalt compounds was available, the specific compound is cited.

### 2.4.1 Air

Sources of cobalt in the atmosphere are both natural and anthropogenic. Natural sources include wind-blown continental dust, seawater spray, volcanoes, forest fires, and continental and marine biogenic emissions. The worldwide emissions from natural

sources have been estimated to range from 13 to 15 million pounds per year. Cobalt in the atmosphere probably exists in particulate form (ATSDR 1992).

In the United States, the average ambient atmospheric concentration of cobalt was reported to be  $0.41 \text{ ng/m}^3$  (ATSDR 1992). Although the HSDB (2000) described the atmospheric concentration of cobalt in remote areas as being very low, the only value given was less than  $1 \text{ ng/m}^3$  for the Antarctic. The same source reported that the air concentration of cobalt can reach or exceed  $81 \text{ ng/m}^3$  in heavily industrialized cities. Near a beryllium-copper alloy facility, cobalt levels as high as  $610 \text{ ng/m}^3$  were observed (HSDB 2000, ATSDR 1992).

Atmospheric cobalt concentrations are much higher near cobalt manufacturing and production facilities. In the ambient air of a facility that manufactured cobalt salts, cobalt concentrations measured by personal sampling ranged from  $0.1$  to  $3.0 \text{ mg/m}^3$ , with a mean of  $0.2 \text{ mg/m}^3$ , and mean concentrations measured by stationary sampling were  $0.049$  and  $1.046 \text{ mg/m}^3$ . The cobalt concentration in the ambient air during painting of pottery with soluble cobalt salts ranged from  $0.07$  to  $8.61 \text{ mg/m}^3$  (HSDB 2000). More recent data on levels of cobalt in urban or rural areas were not located.

#### 2.4.2 Water

Concentrations of cobalt in uncontaminated freshwater have been reported to range from  $0.1$  to  $10 \text{ } \mu\text{g/L}$  (IARC 1991). The average concentration in seawater has been estimated at  $0.27 \text{ } \mu\text{g/L}$ . Concentrations in surface water and groundwater can be elevated over the natural background levels as a result of industrial activities. In polluted river water, the concentration may be  $27 \text{ } \mu\text{g/L}$ . Cobalt levels in suspended material in rivers typically range from  $7$  to  $94 \text{ mg/kg}$ , but approach  $500 \text{ mg/kg}$  in highly polluted rivers (ATSDR 1992).

The National Community Water Supply Study found that cobalt concentrations in drinking water in the United States ranged from nondetectable to  $19 \text{ } \mu\text{g/L}$ , with 62% of the water samples containing a concentration greater than  $1 \text{ } \mu\text{g/L}$ . The average cobalt concentration in drinking water was  $2.2 \text{ } \mu\text{g/L}$  (ATSDR 1992).

#### 2.4.3 Soil

The average concentration of cobalt is  $25 \text{ mg/kg}$  in the earth's crust,  $18 \text{ mg/kg}$  in igneous rocks, and  $7.2 \text{ mg/kg}$  in U.S. soils. Soils with cobalt concentrations less than  $3 \text{ mg/kg}$  are considered cobalt deficient, because plants that grow in these soils will not contain enough cobalt to meet the dietary needs of cattle and sheep ( $0.07$  to  $0.1 \text{ mg/kg}$ ). Soils near ore deposits, phosphate rocks, ore traffic sites, or industrial pollution sites have been reported to contain cobalt at concentrations of up to  $800 \text{ mg/kg}$  (ATSDR 1992).

### 2.5 Environmental fate

Very limited information is available on the environmental fate of cobalt sulfate or other soluble forms of cobalt. Thus, this section summarizes the information available on the environmental fate of nonspecific cobalt and cobalt compounds. Where information on cobalt sulfate or other cobalt compounds was available, the specific compound is cited.

### 2.5.1 Air

The Agency for Toxic Substances and Disease Registry (ATSDR 1992) proposed that cobalt originating from combustion sources would primarily be in the form of the oxide, whereas cobalt arsenide and sulfide could be released during ore extraction processes. Very few data, however, were available on the potential transformation of these forms to other chemical species, such as the sulfate. ATSDR speculated that chemical speciation of cobalt oxide in the air could lead to the formation of more-soluble cobalt sulfate, which would lead to a higher ratio of dissolved to particulate cobalt; however, no studies could be located on this subject in current literature.

### 2.5.2 Water

Many factors will affect the speciation and transport of cobalt in natural waters and sediments. Dissolved cobalt appears to be precipitated in the adsorbed state with oxides of iron and manganese and with crystalline sediments such as aluminosilicate and goethite. In addition, cobalt precipitates out as carbonate and hydroxide in water (ATSDR 1992). In freshwater, it is estimated that speciation may yield 76% free  $\text{Co}^{+2}$ , 19.4% carbonate or bicarbonate, 4% humic complexes, and 0.4% cobalt sulfate. Species of cobalt in seawater are  $\text{CoCl}^+$ , free  $\text{Co}^{+2}$ , carbonate, and humate. Seawater formation of cobalt sulfate is not estimated because of the high concentration of chloride ion. Organic waste concentration and pH play an important role in cobalt speciation (ATSDR 1992). Bioconcentration of cobalt in marine fish is expected to occur, with bottom-feeders accumulating high levels of cobalt (HSDB 2000, ATSDR 1992).

### 2.5.3 Soil

The speciation of cobalt is regulated primarily by pH, the concentration of chelating or complexing agents in the soil, and the redox potential of the soil. At low pH, cobalt is oxidized to trivalent cobalt and usually is associated with iron. In the process of weathering, cobalt is readily taken into solution. It also is adsorbed to a great extent by hydrolysate or oxidate sediments (HSDB 2000).

## 2.6 Environmental exposure

Very limited information is available on environmental exposure to cobalt sulfate or other soluble forms of cobalt. Thus, this section summarizes the information available on environmental exposure to nonspecific cobalt and cobalt compounds. Where information on cobalt sulfate or other cobalt compounds was available, the specific compound is cited.

Exposure to cobalt occurs through inhalation of ambient air and ingestion of food and drinking water (ATSDR 1992). The average intake of cobalt in foods by adults in the United States has been estimated at 300  $\mu\text{g}$  per day. Daily intake from water is estimated at 6  $\mu\text{g}$ , and intake from air is estimated at less than 0.1  $\mu\text{g}$ . The major source of cobalt is food, in the form of green leafy vegetables, which may contain as much as 0.5 mg/kg dry weight (HSDB 2000). Cobalt is an essential trace element in humans, because a cobalt atom is present in each molecule of vitamin  $\text{B}_{12}$  (cobalamin). Cobalt's presence in vitamin  $\text{B}_{12}$  is its only known essential function in humans (Anderson 2000). An adult human body contains approximately 1.1 mg of cobalt (NTP 1998).

The National Health and Nutrition Examination Survey in 1999 measured cobalt levels in the urine of 1,007 participants aged 6 years or older, to provide physicians with a reference range of cobalt in the urine of the U.S. population for use in determining whether individuals have been exposed to cobalt (CDC 2001). The geometric mean was 0.36 µg/L of urine (95% CI = 0.32 to 0.40). The geometric mean of the creatinine-adjusted levels was 0.33 µg/g of creatinine (95% CI = 0.29 to 0.36).

No information was found that specifically identified environmental exposure to cobalt sulfate.

## 2.7 Occupational exposure

Very limited information is available on occupational exposure to cobalt sulfate or other soluble forms of cobalt. Thus, this section summarizes the information available on occupational exposure to nonspecific cobalt and cobalt compounds. Where information on cobalt sulfate or other cobalt compounds was available, the specific compound is cited.

It has been estimated by Jensen and Tüchsen (1990) that more than a million workers in the United States potentially are exposed to cobalt or cobalt compounds, though for many, the degree of potential exposure is limited (HSDB 2000, NTP 1998). Occupational exposure to cobalt occurs principally in refining processes, in production of alloys, and in the tungsten carbide hard-metal industry (Kazantzis 1981). In addition, many workers are exposed to a limited degree when using cobalt-containing paint dryers. Occupational exposure is primarily dermal or through inhalation of cobalt metal dusts or fumes (HSDB 2000, NTP 1998).

Cobalt metal has been reported in the air of metal manufacturing, welding, and grinding factories at concentrations ranging from 1 to 300 µg/m<sup>3</sup> and in the dust of an electric welding factory at 4.2 µg/g (ATSDR 1992). Occupational exposure to cobalt also has been assessed from the concentrations of cobalt in workers' tissues and body fluids. Alexandersson (1988) reported a high degree of conformity between the concentration of cobalt in blood and urine and the average levels of cobalt in the air during a workweek. The cobalt levels in the urine of workers exposed to cobalt in the air at concentrations of 0.005 to 0.15 mg/m<sup>3</sup> were almost 700 times those of the control group. In workers exposed to high levels (0.09 mg/m<sup>3</sup>), cobalt concentrations in the blood were 20 times those of the control group, while in the low-exposure workers (0.01 mg/m<sup>3</sup>), the concentrations were only slightly higher than in the controls. Other studies have shown that lungs from occupationally exposed workers, such as coal miners and metal-industry workers, contained from 2.5 to 6 times as much cobalt as lungs from control groups (ATSDR 1992).

## 2.8 Biological indices of exposure

Cobalt sulfate, like other water-soluble metallic salts, dissolves directly into blood serum (362 g/L at 20°C) (Jensen and Tüchsen 1990). Cobalt can be detected in urine, blood, and tissues; however, there currently is no way to correlate cobalt sulfate exposure with cobalt levels observed in these matrices. Based on reports of accidental exposure to

radioactive cobalt ( $^{60}\text{Co}$ ) and intravenous or oral administration of  $^{60}\text{Co}$  to volunteer human subjects (Smith *et al.* 1972), approximately 90% of inhaled, injected, or ingested cobalt is eliminated within a few days; however, the remaining 10% has a half-life in the body of two years after parenteral administration or 5 to 15 years after inhalation. No biological use of cobalt is known other than its presence in vitamin B<sub>12</sub> (HSDB 2000).

## 2.9 Regulations

No specific U.S. Environmental Protection Agency (EPA) regulations for cobalt sulfate were identified. EPA regulates cobalt under the Clean Water Act (CWA), limiting effluent discharges of cobalt from facilities that produce cobalt from ore concentrate raw materials or process tungsten or tungsten carbide scrap raw materials. EPA also regulates cobalt and cobalt compounds under the Resource Conservation and Recovery Act (RCRA), establishing minimum criteria for all municipal solid waste landfills (MSWLFs). Under the Superfund Amendments and Reauthorization Act (SARA) of 1986, EPA mandates that all information regarding the release of toxic compounds, such as cobalt, be available to the public.

The Food and Drug Administration (FDA) regulates cobalt sulfate, barring its use in malted beverages as a foam stabilizer. Under the Federal Food, Drug, and Cosmetic Act (FFDCA), section 503A(a), all drugs containing cobalt or cobalt sulfate have been withdrawn because they were deemed unsafe or ineffective. Cobalt preparations intended for use in humans are regulated under section 301(p) of the FFDCA. They must go through the new drug application process outlined in sections 314 and 505. Warning and caution statements are required on all drugs containing cobalt or cobalt sulfate. The FDA recognizes that cobalt sulfate and other cobalt compounds are generally recognized as safe when added to animal feeds as nutritional dietary supplements.

The Occupational Safety and Health Administration (OSHA) regulates cobalt under Sections 4, 6, and 8 of the Occupational Safety and Health Act of 1970. The current OSHA permissible exposure limit (PEL) for cobalt metal, dust, and fume (as Co) is 0.1 mg/m<sup>3</sup> of air as an 8-hour time-weighted average (TWA) concentration. The regulation requirements are exactly the same for shipyard and construction workers. The National Institute for Occupational Safety and Health (NIOSH) has established a recommended exposure limit for cobalt metal, dust, and fume of 0.05 mg/m<sup>3</sup> as a TWA for up to a 10-hour workday and a 40-hour workweek. The American Conference of Governmental Industrial Hygienists (ACGIH) has assigned elemental cobalt and inorganic cobalt compounds (as Co) a threshold limit value of 0.02 mg/m<sup>3</sup> as a TWA for an 8-hour workday and a 40-hour workweek (OSHA 1998). The ACGIH has established a biological exposure index of 15 µg of cobalt per liter of urine; this index is used to “generally indicate a concentration below which nearly all workers should not experience adverse health effects” (CDC 2001).

EPA regulations are summarized in Table 2-5, FDA regulations in Table 2-6, and OSHA regulations in Table 2-7.

**Table 2-5. EPA regulations**

Regulatory action	Effect of regulation or other comments
40 CFR 60 – PART 60 – STANDARDS OF PERFORMANCE FOR NEW STATIONARY SOURCES. Promulgated: 36 FR 24877, 12/23/71. U.S. Codes: 42 U.S.C. 7401, 7411, 7413, 7414, 7416, 7601, and 7602.	The provisions of this part apply to the owner or operator of any stationary source that contains an affected facility, the construction or modification of which is commenced after the date of publication in this part of any standard applicable to that facility.
40 CFR 60.750ff. – Subpart WWW – Standards of Performance for Municipal Solid Waste Landfills. Promulgated: 61 FR 9919, 03/12/96.	This subpart describes methods that are applicable to the determination of cobalt emissions from stationary sources.
40 CFR 122 – PART 122 – EPA ADMINISTERED PERMIT PROGRAMS: THE NATIONAL POLLUTANT DISCHARGE ELIMINATION SYSTEM. Promulgated: 48 FR 14153, 04/01/83. U.S. Codes: 33 U.S.C. 1251 et seq., the CWA.	These regulations cover basic EPA permitting requirements for effluent discharges from point sources to waters of the United States. Appendix D lists pollutants that must be identified by dischargers if expected to be present. Cobalt is listed under Table IV — Conventional and nonconventional pollutants required to be tested by existing dischargers if expected to be present.
40 CFR 258 – PART 258 – CRITERIA FOR MUNICIPAL SOLID WASTE LANDFILLS. Promulgated: 56 FR 51016, 10/09/91. U.S. Codes: 33 U.S.C. 1345(d) and (e); 42 U.S.C. 6907(a)(3), 6912(a), 6944(a), and 6949a(c).	The provisions of this part establish minimum national criteria under RCRA, as amended, for all MSWLF units and under the CWA, as amended, for MSWLFs that are used to dispose of sewage sludge. The criteria ensure the protection of human health and the environment.
40 CFR 258 – APPENDIX II TO PART 258 – LIST OF HAZARDOUS AND ORGANIC CONSTITUENTS.	The practical quantitation limits (PQLs), which are the lowest concentrations of analytes in ground waters that can be reliably determined within specified limits of precision and accuracy by the indicated methods under routine laboratory operating conditions, for cobalt are 70 µg/L for method 6010, 500 µg/L for method 7200, and 10 µg/L for method 7201.
40 CFR 261 – PART 261 – IDENTIFICATION AND LISTING OF HAZARDOUS WASTES. Promulgated: 53 FR 13388, 04/22/88. U.S. Codes: 42 U.S.C. 6905, 6912(a), 6921, 6922, and 6938.	Cobalt is listed as a hazardous waste with a concentration limit of 4.6 mg/kg.
40 CFR 264.1200ff. – Subpart EE – Hazardous Waste Munitions and Explosives Storage. Promulgated: 62 FR 6652, 02/12/97.	The requirements of this subpart apply to owners or operators who store munitions and explosive hazardous wastes. The PQL for cobalt is 70 µg/L for method 6010, 500 µg/L for method 7200, and 10 µg/L for method 7201.
40 CFR 372 – PART 372 – TOXIC CHEMICAL RELEASE REPORTING: COMMUNITY RIGHT-TO-KNOW. Promulgated: 53 FR 4525, 02/16/88. U.S. Codes: 42 U.S.C. 11013 and 11028. The effective date of this regulation for cobalt is 1/1/87.	This part sets forth requirements for the submission of information relating to the release of toxic chemicals under section 313 of Title III of SARA (1986). Information collected under this part is intended to inform the general public and the communities surrounding covered facilities about releases of toxic chemicals, to assist research, and to aid in the development of regulations, guidelines, and standards.

Regulatory action	Effect of regulation or other comments
40 CFR 421 – PART 421 – NONFERROUS METALS MANUFACTURING POINT SOURCE CATEGORY. Promulgated: 49 FR 8790, 03/08/84. U.S. Codes: 33 U.S.C. 1311, 1314(b), (c), (e), and (g), 1316(b) and (c), 1317(b) and (c), 1318, and 1361.	The provisions of this part apply to facilities producing primary metals from ore concentrates and recovering secondary metals from recycled wastes which discharge pollutants to waters of the U.S. or which introduce or may introduce pollutants into a publicly owned treatment works.
40 CFR 421.230ff. – Subpart U – Primary Nickel and Cobalt Subcategory. Promulgated: 50 FR 38359, 09/20/85.	The provisions of this subpart are applicable to discharges resulting from the production of nickel or cobalt by primary nickel and cobalt facilities processing ore concentrate raw materials.
40 CFR 421.232 – Sec. 421-232. – Effluent limitations guidelines representing the degree of effluent reduction attainable by the application of the best practicable control technology currently available.	For raw material dust control, the cobalt maximum for any 1 day is 0.016, with a maximum monthly average of 0.007. For nickel wash water, the cobalt maximum for any 1 day is 0.007, with a maximum monthly average of 0.003. For nickel reduction decant, the cobalt maximum for any 1 day is 2.666, with a maximum monthly average of 1.143. For cobalt reduction recant, the cobalt maximum for any 1 day is 4.494, with a maximum monthly average of 1.926.
40 CFR 421.233 – Sec. 421-233. – Effluent limitations guidelines representing the degree of effluent reduction attainable by the application of the best available technology economically achievable.	For raw material dust control, the cobalt maximum for any 1 day is 0.011, with a maximum monthly average of 0.005. For nickel wash water, the cobalt maximum for any 1 day is 0.005, with a maximum monthly average of 0.002. For nickel reduction decant, the cobalt maximum for any 1 day is 1.777, with a maximum monthly average of 0.889. For cobalt reduction recant, the cobalt maximum for any 1 day is 2.996, with a maximum monthly average of 1.498.
40 CFR 421.234 – Sec. 421-234. – Standards of performance for new sources.	The requirements of this section are identical to those set forth in section 421-233.
40 CFR 421.236 – Sec. 421-236. – Pretreatment standards for new sources.	The requirements of this section are identical to those set forth in section 421-233.
40 CFR 421.310ff. – Subpart AC – Secondary Tungsten and Cobalt Subcategory. Promulgated: 50 FR 38386, 09/20/85.	The provisions of this subpart are applicable to discharges resulting from the production of tungsten or cobalt at secondary tungsten and cobalt facilities processing tungsten or tungsten carbide scrap raw materials.
40 CFR 421.312 – Sec. 421-312. – Effluent limitations guidelines representing the degree of effluent reduction attainable by the application of the best practicable control technology currently available.	For cobalt sludge leaching wet air pollution control, the cobalt maximum for any 1 day is 140.977, with a maximum monthly average of 61.901. For cobalt hydroxide filtrate, the cobalt maximum for any 1 day is 233.189, with a maximum monthly average of 97.999. For cobalt hydroxide filter cake wash, the cobalt maximum for any 1 day is 429.598, with a maximum monthly average of 188.631. Other maximum effluent limitations for various tungsten processes also are provided.

Regulatory action	Effect of regulation or other comments
40 CFR 421.313 – Sec. 421-313. – Effluent limitations guidelines representing the degree of effluent reduction attainable by the application of the best available technology economically achievable.	For cobalt sludge leaching wet air pollution control, the cobalt maximum for any 1 day is 98.756, with a maximum monthly average of 43.295. For cobalt hydroxide filtrate, the cobalt maximum for any 1 day is 156.346, with a maximum monthly average of 68.543. For cobalt hydroxide filter cake wash, the cobalt maximum for any 1 day is 300.094, with a maximum monthly average of 131.932. Other maximum effluent limitations for various tungsten processes also are provided.
40 CFR 421.314 – Sec. 421-314. – Standards of performance for new sources.	The requirements of this section are identical to those set forth in section 421-313.
40 CFR 421.316 Sec. 421-316 – Pretreatment standards for existing sources.	The requirements of this section are identical to those set forth in section 421-313.
40 CFR 716 – PART 716 – HEALTH AND SAFETY DATA REPORTING. Promulgated: 51 FR 32726, 09/15/86. U.S. Codes: 15 U.S.C. 2607(d). The effective date for cobalt is 06/01/87, and the sunset date is 06/01/97.	The provisions of this part require the submission of lists and copies of health and safety studies on chemical substances and mixtures selected for priority consideration for testing rules under section 4(a) of the Toxic Substances Control Act and on other chemicals for which EPA requires health and safety information in fulfilling the purposes of the Act.

Source: The regulations in this table have been updated through the Code of Federal Regulations 40 CFR, 1 July 2001.

**Table 2-6. FDA regulations**

Regulatory action	Effect of regulation or other comments
21 CFR 173 – PART 173 – SECONDARY DIRECT FOOD ADDITIVES PERMITTED IN FOOD FOR HUMAN CONSUMPTION. Promulgated: 42 FR 14526 03/15/77. U.S. Codes: 21 U.S.C. 321, 342, and 348.	Cobalt sulfate may be safely used as a catalyst in boiler water additives in the preparation of steam that will contact food.
21 CFR 189 – PART 189 – SUBSTANCES PROHIBITED FROM USE IN HUMAN FOOD. Promulgated: 42 FR 14659, 03/15/77. U.S. Codes: 21 U.S.C. 321, 342, 348, and 371.	Cobalt sulfate has been used in fermented malt beverages as a foam stabilizer and to prevent “gushing.” Food containing any added cobalt sulfate is deemed to be adulterated in violation of the act based upon an order published in the 31 FR 8788, 08/12/66.
21 CFR 216 – PART 216 – PHARMACY COMPOUNDING. Promulgated: 64 FR 10944, 03/08/99. U.S. Codes: 21 U.S.C. 351, 352, 353(a), 355, and 371.	All drug products containing cobalt salts, including cobalt sulfate (except radioactive forms of cobalt and its salts and cobalamin and its derivatives), were withdrawn or removed from the market because they were found to be unsafe or not effective.
21 CFR 310 – PART 310 – NEW DRUGS. Promulgated: 64 FR 401, 01/05/99. U.S. Codes: 21 U.S.C. 321, 331, 351, 352, 353, 355, 360(b)–360(f), 360(j), 361(a), 371, 374, 375, and 379(e); 42 U.S.C. 216, 241, 242(a), 262, and 263(b)–263(n).	Cobalt preparations intended for use by man have been determined by rulemaking procedures to be new drugs under the FFDCA. An approved new drug application under section 505 of the act and part 314 of this chapter is required for marketing.

Regulatory action	Effect of regulation or other comments
21 CFR 369 – PART 369 – INTERPRETATIVE STATEMENTS RE WARNINGS ON DRUGS AND DEVICES FOR OVER-THE-COUNTER SALE. Promulgated: 39 FR 11745, 03/29/74. U.S. Codes: 21 U.S.C. 321, 331, 351, 352, 353, 355, 356, 357, and 371.	Cobalt preparations must have the following warnings and caution statements: <b>Warning — Do not exceed the recommended dosage. Do not administer to children under 12 years of age unless directed by physician. Do not use for more than 2 months unless directed by physician.</b> This warning is not required on articles containing not more than 0.5 milligram of cobalt as a cobalt salt per dosage unit and which recommend administration of not more than 0.5 milligram per dose and not more than 2 milligrams per 24-hour period.
21 CFR 582 – PART 582 – SUBSTANCES GENERALLY RECOGNIZED AS SAFE. Promulgated: 41 FR 38657, 09/10/76. U.S. Codes: 21 U.S.C. 321, 342, 348, and 371.	Cobalt compounds, including cobalt sulfate, added to animal feeds as nutritional dietary supplements are generally recognized as safe when added at levels consistent with good feeding practice.

Source: The regulations in this table have been updated through the Code of Federal Regulations 21 CFR, 1 April 2001.

**Table 2-7. OSHA regulations**

Regulatory action	Effect of regulation or other comments
29 CFR 1910.1000 – TABLE Z-1 – Limits for Air Contaminants. Promulgated: 39 FR 23502, 06/27/74. U.S. Codes: 5 U.S.C. 553, 29 U.S.C. 653, 655, and 657.	Cobalt is identified as an air contaminant. The PEL for cobalt is 0.1 mg/m <sup>3</sup> as an 8-h TWA.
29 CFR 1915 – Subpart Z – Toxic and Hazardous Substances. Promulgated 58 FR 35514, 07/01/93.	The requirements applicable to shipyard employment under this section are identical to those set forth in section 1910.1000.
29 CFR 1926 – Subpart D – Occupational Health and Environmental Controls. Promulgated: 39 FR 22801, 06/24/74. U.S. Codes: 29 U.S.C. 653, 655, and 657.	The requirements applicable to construction employment under this section are identical to those set forth in section 1910.1000.

Source: The regulations in this table have been updated through the Code of Federal Regulations 29 CFR, 1 July 2001.

4/17/02

Draft RoC Background Document for Cobalt Sulfate  
Do not quote or cite

---

### 3 Human Cancer Studies

Although no human studies are available in which exposure to cobalt sulfate is specifically mentioned, some human studies have investigated carcinogenicity of cobalt and cobalt compounds as a class of chemicals. Most of these studies are cohort studies assessing occupational exposure to cobalt and cobalt compounds. They include studies of cobalt production workers, ceramics workers, hard-metal workers, and workers in nickel refineries. Studies on nickel refinery workers are not included in this discussion, because the main exposure is to nickel, which is a known human carcinogen.

#### 3.1 IARC assessment

IARC (1991) reviewed the carcinogenicity of cobalt and cobalt compounds in 1991 and classified them as possibly carcinogenic to humans (Group 2B). The IARC evaluation included occupational studies and studies of patients with implanted medical devices that may have contained cobalt. Most of the investigations concerning implanted medical devices were case reports, 10 of which described single cases of malignant neoplasia, primarily sarcoma, at the site of implants made of cobalt-containing alloys. The only cohort study of implant patients reported an increased risk of tumors of the lymphatic and hematopoietic system among hip-replacement patients; however, this study did not describe the composition of the hip prosthesis and thus is not informative for the evaluation of cobalt.

The IARC (1991) evaluation discussed four cohort studies of occupational exposure to cobalt, two of which were considered informative; two studies of nickel refinery workers were considered not informative for the evaluation of cobalt and cobalt compounds. Both studies evaluated by IARC, a cohort at a French electrochemical plant producing cobalt and a cohort of Swedish hard-metal workers, reported an excess of lung cancer. The French study (Mur *et al.* 1987) is discussed below (Section 3.2) because an update of this report was published after the IARC review. The Swedish cohort of Hogstedt and Alexandersson (1990) consisted of 3,163 male workers employed at three hard-metal manufacturing plants from 1940 to 1982, with at least one year of exposure to cobalt-containing hard-metal dust, and followed until 1951 to 1982. A standardized mortality ratio (SMR) for lung cancer of 1.34 (95% CI = 0.77 to 2.13, 17 cases) was observed for the cohort, and a higher value was reported for workers with more than 10 years of exposure and more than 20 years since first exposure (SMR = 2.78, 95% CI = 1.11 to 5.72, 7 cases). Smoking habits among the cohort did not differ from those of the male Swedish population. Workers in both studies also were exposed to known carcinogens, such as nickel and arsenic (in the French study) or tungsten carbide present in hard-metal dust (in the Swedish study). IARC concluded that there was inadequate evidence of carcinogenicity in humans for cobalt and cobalt compounds.

## 3.2 Current human studies

Current studies on human exposure to cobalt are summarized in Table 3-1.

### 3.2.1 Occupational studies

Mur *et al.* (1987) conducted a retrospective cohort study of 1,143 workers who had been employed for at least one year between 1950 and 1980 at an electrochemical plant in France that produced cobalt and sodium. Cobalt was produced by etching of roasted ore, followed by neutralization, filtration, and electrolysis of the cobalt chloride solution. The manufacturing process also included production of cobalt salts and oxides. Vital status was assessed in 1981 by Mur *et al.* (1987) and in 1988 by Moulin *et al.* (1993). In the first report (Mur *et al.* 1987), fewer deaths from all causes were observed in the entire cohort (213) than expected from the French male population (SMR 0.8, 95% CI = 0.7 to 0.9). An increased risk of lung cancer was observed only for cobalt production workers (adjusted SMR = 4.7, 95% CI = 1.5 to 10.6, 4 cases), and not for sodium production workers or maintenance and general service workers at the plant. However, seven years later, lung cancer risk was no longer elevated in cobalt production workers; the SMR (Cohort II) was 1.2 (95% CI = 0.2 to 3.4, 3 cases) (Moulin *et al.* 1993). The SMR in this later study was based on 3 lung cancer cases, rather than 4 (as in the earlier study). The discrepancy in the number of observed cases is due to differences in how the cause of death was ascertained; the 1987 study used only physicians' records, whereas the later study used death certificates for the years 1968 to 1988. The use of death certificates decreased the proportion of unknown causes of death from 20% to 11%, and no additional lung cancer cases were observed in the extended follow-up period (1981 to 1988). [The small number of exposed cases and high percentage of unknown causes of death limit the power of these studies to detect an effect of exposure to cobalt salts.] The authors stated that the negative finding of the updated study could not be considered a definite conclusion. [Other limitations of these studies include their inability to consider smoking status.]

Lasfargues *et al.* (1994) conducted a cohort mortality study of 709 men employed for at least one year between January 1956 and December 1989 at a French plant producing hard-metal tools. Exposure was categorized into four degrees of hard-metal exposure (none, low, medium, and high) based on job histories and periods of employment. Elevated SMRs in the entire cohort were observed for esophageal cancer (nonsignificant), leukemia (nonsignificant), and lung cancer (significant; SMR = 2.1, 95% CI = 1.0 to 3.9, 10 cases). Risk of lung cancer was highest in the highest exposure category but was not related to duration of employment or time since first exposure. Smoking status was ascertained for 81% of the cohort and 69% of the deceased population; the proportions of smokers were similar to the proportion in a sample of the French adult male population.

Moulin *et al.* (1998) conducted a multicenter study of a cohort consisting of all male (5,777) and female (1,682) workers employed for at least three months in any of ten French factories that produced hard metal. Causes of death (684) were ascertained from death certificates and medical records. In addition to production of hard metal, activities at these factories included power metallurgy processes. Exposure to cobalt and other agents was assessed and semiquantified from a job-exposure matrix, which was validated

by atmospheric measurements of cobalt. A case-control study of 61 cases of lung cancer and 180 controls was nested within the cohort of all workers employed in this industry. An increased risk of lung cancer was associated with “other” cobalt exposure, which was defined as exposure to cobalt alone or simultaneous exposure to cobalt and agents that did not include tungsten carbide (odds ratio [OR] = 2.2, 95% CI = 1.0 to 4.9). A later study of the largest production plant (2,860 workers) in the multicenter cohort (Wild *et al.* 2000) reported that other industrial processes related to cobalt exposure included production of magnets and stainless steel made with cobalt, production of cobalt powders by calcination, and reduction of cobalt hydroxide. Thus, the “other” cobalt exposure probably was to metallic cobalt, but may have included exposure to ionized cobalt generated during the production of metallic cobalt. Wild *et al.* (2000) also reported an increased risk of lung cancer for “other” cobalt exposure that did not include co-exposure with tungsten carbide (OR = 2.0, 95% CI = 1.1 to 3.2) and was assessed from the job-exposure matrix.

Both studies reported an association between simultaneous exposure to cobalt and tungsten carbide (hard-metal production) and lung cancer. The case-control study nested in the multicenter cohort found exposure-response relationships for duration of exposure (test for trend,  $P = 0.03$ ) and for the unweighted cumulative exposure to cobalt and tungsten carbide (test for trend,  $P = 0.01$ ). Unweighted measures of cumulative exposure treat occasional and full-time exposure equally, thus favoring peak exposure (Moulin *et al.* 1998). Wild *et al.* (2000) reported that lung cancer risk was associated with hard-metal production before sintering (SMR = 2.9) and that little risk was associated with hard-metal production after sintering (SMR = 1.1). Exposure to hard metals is higher before than after sintering. Risk associated with exposure to hard-metal dust (cobalt and tungsten carbide) remained elevated and significant after controlling for smoking (Moulin *et al.* 1998) and in a regression model that included smoking and exposure to any IARC carcinogen, including asbestos, polycyclic aromatic hydrocarbons (PAH), certain chromium compounds, certain nickel compounds, and silica (Wild *et al.* 2000).

Tüchsen *et al.* (1996) studied a cohort of Danish porcelain workers exposed to cobalt-aluminate spinel and/or cobalt silicate at two factories (382 women in Factory 1 and 492 women in Factory 2). A significantly increased risk of lung cancer was observed in the exposed women, compared with the Danish population (standardized incidence ratio [SIR] = 2.4, 8 cases); however, an increased risk of lung cancer also was observed in a reference group of non-exposed workers at one of the factories (520 women). The authors recommended a longer follow-up, because of the small number of exposed cases and the need to assess the effects of exposure to cobalt silicate dye, which replaced the cobalt-aluminate spinel dye in Factory 1 in 1972 and Factory 2 in 1989.

### 3.2.2 Biomarker study

Rogers *et al.* (1993) conducted a population-based case-control study on levels of certain elements (cobalt, calcium, iron, zinc, and chromium) in toenail clippings and cancer of the upper aerodigestive tract. Cases (661) were identified by the local Surveillance, Epidemiology and End Results (SEER) cancer registry, and controls (466), matched on sex and age, were identified by random-digit dialing. Cobalt was measured from toenail samples (507 cases and 434 controls) with neutron activation analysis. Significantly

increased risks for esophageal (OR = 9.0, 95% CI = 2.7 to 30.0) and oral cancer (OR = 1.9, 95% CI = 1.0 to 3.6) were observed for individuals with the highest nail cobalt levels (highest 25%; see Table 3-1), and an exposure-response relationship was observed for esophageal cancer (test for trend,  $P < 0.001$ ). These findings are in agreement with a small study conducted by Collecchi *et al.* (1986), which found higher plasma concentrations of cobalt in patients with laryngeal carcinoma (mean = 18.27 ng/mL, N = 11) than in healthy subjects (mean = 0.73 ng/mL, N = 15) (see Section 6). [Strengths of the study by Rogers *et al.* (1993) include its large population size, the use of a biomarker to measure cobalt-specific exposure, and adjustment for potential confounders. The study is limited because it measures recent exposure (perhaps after the development of cancer) rather than past exposure.] Cobalt is deposited in nails during matrix formation, which usually occurs from eight months to two years after exposure, depending on the age of the individual. No differences in risks were observed after stratification by time from diagnosis to interview or stage of disease. This study does not provide any information on the source or type of cobalt exposure. The authors speculated that the cobalt exposure was unlikely to come from vitamin B<sub>12</sub>, because the cancer patients tended to eat fewer animal products than controls, and there were no differences in the intake of vitamin B<sub>12</sub> supplements between cases and controls.

### 3.3 Discussion and summary

The studies discussed in this section are not specific for cobalt sulfate. Whether studies on exposure to cobalt as a class are relevant for evaluation of the carcinogenicity of cobalt sulfate probably depends on the mechanism(s) of carcinogenicity. As discussed in Section 6.4, the proposed mechanisms of cobalt-induced carcinogenesis are based on exposure to cobalt ions. Although several studies suggest that exposure to cobalt in hard-metal production is associated with an increased risk of lung cancer, these studies involve exposure to metallic cobalt and simultaneous exposure to tungsten carbide. Lung toxicity of hard-metal particles may result from a specific interaction between cobalt metal and carbide particles that produces reactive oxygen species (Moulin *et al.* 1998). Thus, these studies are of uncertain relevance for the evaluation of cancer due to cobalt exposure alone.

Other studies discussed in this section include studies on exposure to cobalt as a class of compounds. In most, the types of cobalt present are not specified. The exception is the study of porcelain workers exposed to cobalt-aluminate spinel and cobalt silicate dyes. The small numbers, the increased risk of lung cancer among the non-exposed reference group, and the uncertain relevance of these dyes to cobalt sulfate make this study difficult to interpret. Two of the hard-metal studies reported a twofold increase in risk of lung cancer for “other” cobalt exposure, where “other” was defined as exposure to cobalt without co-exposure to tungsten carbide or hard metal. The most likely source of this exposure is cobalt metal; however, ionic cobalt could have been released during the production of cobalt. Because the focus of these studies was hard-metal exposure, characterization of “other” cobalt exposure and analyses controlling for confounders (co-exposure to other carcinogens) was less detailed than for exposure to hard metal.

Only one study, of the French electrochemical factory, specifically mentioned exposure to cobalt salts. The small study size and unstable risk estimates, reflected in the

discrepancy between the findings of the initial study (Mur *et al.* 1987) and the updated study (Moulin *et al.* 1998), limits its usefulness for evaluation of the carcinogenic effects of cobalt salts in humans.

The biomarker study showed a strong association between esophageal cancer and cobalt present in nails but did not provide any information on specific cobalt compounds. Moreover, the study assessed recent cobalt exposure, whereas past exposure is more likely important for cancer development.

In conclusion, the human studies provide limited information for the specific evaluation of the carcinogenicity of cobalt sulfate.

**Table 3-1. Current studies of human exposure to cobalt**

Reference	Study design and population	Exposure	Effect: SMR, RR, or OR	Comments
Occupational studies				
Mur <i>et al.</i> 1987	Retrospective cohort study. <i>Cohort:</i> 1,143 workers employed for at least 1 year between 1950 and 1980 at an electrochemical factory producing cobalt, sodium, and other chemicals.  Vital status was assessed in 1981, and cause of death was ascertained from physicians' records.	Cobalt was produced by etching of the roasted ore, followed by neutralization, filtration, and electrolysis of the cobalt chloride solution. The manufacturing process included production of cobalt oxides and salts. Exposure was defined by the worker's occupation.	SMR (95% CI); number of cases <i>Entire cohort:</i> all causes 0.8 (0.7–0.9); 213 all cancer 0.8 (0.6–1.1); 44 lung cancer 0.9 (0.4–1.6); 9 <i>Cobalt production workers:</i> all causes 1.3 (0.9–1.9); 28 all cancers 1.7 (0.8–3.1); 8 lung cancer 4.7 (1.5–10.6); 4 oral cancer 3.4 (0.3–10.3); 2 <i>Sodium production workers:</i> all causes 0.8 (0.6–1.0); 62 all cancer 0.7 (0.4–1.2); 13 lung cancer 0.7 (0.1–2.2); 2 <i>Maintenance workers:</i> all causes 0.8 (0.6–1.1); 38 all cancer 1.0 (0.5–1.8); 8 lung cancer 0.5 (0–2.6); 1	<i>Confounders and limitations:</i>  (1) There was co-exposure to nickel and arsenic.  (2) Smoking was ascertained for only 30% of cohort and was not considered.  (3) 20% of deaths were due to unknown causes.  (4) Vital status assessment for foreign-born individuals was poor.  (5) The small number of exposed cases limited the study's power to detect an effect.

Reference	Study design and population	Exposure	Effect: SMR, RR, or OR	Comments
Moulin <i>et al.</i> 1993 France	<p>Retrospective cohort study, update of Mur <i>et al.</i> (1987) (reported in IARC 1991)</p> <p><i>Cohort I:</i> 1,143 workers employed at least 1 year between 1950 and 1980 at an electrochemical factory producing cobalt, sodium, and other chemicals.</p> <p>Vital status was assessed in 1988. The cause of death was ascertained from death certificates in the French national file for 1968 to 1988 and from physicians' records for 1950 to 1967.</p> <p>The cohort was divided into 2 subcohorts because of differences in overall mortality according to birthplace:</p> <p><i>Cohort I:</i> all members, but limited to age groups <math>\leq 74</math> for calculation of person-years for those born abroad.</p> <p><i>Cohort II:</i> limited to workers born in France.</p>	<p>Cobalt was produced by etching of the roasted ore, followed by neutralization, filtration, and electrolysis of the cobalt chloride solution. The manufacturing process included production of cobalt oxides and salts.</p> <p>Exposure was defined by the worker's occupation.</p>	<p>SMR (95% CI); number of cases</p> <p><i>Cohort I:</i> all causes 0.9 (0.8–1.0); 309 all cancer 0.8 (0.7–1.0); 84</p> <p><i>Cohort II:</i> all causes 1.0 (0.8–1.1); 247 all cancer 1.0 (0.8–1.3); 72</p> <p><i>Lung cancer in cobalt production workers:</i> cohort I 0.9 (0.2–2.5); 3 cohort II 1.2 (0.2–3.4); 3</p> <p><i>Duration/time since first exposure in cobalt production:</i></p> <p>Cobalt production workers: No trend of increased risk for increasing duration or time since first exposure; however, there were only 3 exposed cases.</p> <p>Maintenance workers: Risk increased with increasing time since first exposure. Risk was elevated (SMR &gt; 2 for cohort I and &gt; 3 for cohort II) and significant for longest duration (&gt; 30 years) and time since first exposure (&gt; 30 years) in both cohort I and II.</p>	<p><i>Confounders and limitations:</i></p> <p>(1) There was co-exposure to nickel and arsenic; maintenance workers may have been exposed to asbestos in sodium production areas.</p> <p>(2) Smoking was ascertained for only 30% of cohort and was not considered.</p> <p>(3) 11% of deaths were due to unknown causes.</p> <p>(4) Vital status assessment for foreign-born individuals was poor; the SMR for workers over 75 was low, so these age groups were excluded.</p> <p>(5) The small number of exposed cases limited the study's power to detect an effect; the small number of cases among exposed maintenance workers may have led to chance findings.</p>

Reference	Study design and population	Exposure	Effect: SMR, RR, or OR	Comments
Lasfargues <i>et al.</i> 1994 France	<p>Cohort mortality study</p> <p><i>Cohort:</i> all men (709) employed for at least 1 year between 1/1/1956 and 12/31/1989 at a French plant producing hard-metal tools. The plant consisted of two workshops (A and B). Workers in A had the highest exposure (powders mixing, pressing, and soft carbide machining); it opened in 1956, and preventive measures were taken between 1973 and 1976. Workers in B had lower exposure (maintenance, hard carbide machining); preventive measures were taken since its opening in 1974.</p> <p>Vital status was assessed on 1/1/1990, and cause of death was ascertained from physicians' records.</p>	<p>Exposure was defined by workers' job histories and periods of employment (to assess preventive measures); job histories before 1970 often were missing.</p> <p>Four degrees of cobalt exposure:</p> <p>(1) no exposure</p> <p>(2) low exposure: &lt; 10 µg/m<sup>3</sup> in 8 h</p> <p>(3) medium exposure: 15–40 µg/m<sup>3</sup> in 8 h</p> <p>(4) high exposure: &gt; 50 µg/m<sup>3</sup> in 8 h</p>	<p>SMR (95% CI); number of cases</p> <p><i>Entire cohort:</i></p> <p>all causes 1.1 (0.8–1.3); 75 all cancer 1.3 (0.8–1.8); 26 esophagus 1.9 (0.4–5.6); 3 leukemia 3.1 (0.4–11.1); 2 lung 2.1 (1.0–3.9); 10</p> <p><i>Degree of exposure:</i></p> <p>all cancers: increased risk with increasing exposure</p> <p>lung cancer:</p> <p>no 1.5 (0.0–8.5); 1 low 0.9 (0.0–5.2); 0 medium 1.4 (0.3–4.2); 3 high 5.0 (1.9–11.0); 6</p> <p><i>Duration of employment and time since first exposure:</i> no increase in SMR for lung cancer</p> <p><i>Smoking and exposure:</i> highest risk in smokers with medium (SMR = 9.2) and high exposure (SMR = 15.1); no risk for smokers with no or low exposure; comparison group was non-exposed individuals who had never smoked</p>	<p><i>Confounders and limitations:</i></p> <p>(1) Smoking was ascertained for 81% of the workers and 69% of the deceased; the proportion of smokers was similar to that in a sample of the French male adult population.</p> <p>(2) The expected number of deaths was calculated from national rates; local rates for lung cancer were available from 1971 to 1978 and were lower than national rates, so risks based on national rates are conservative.</p> <p>(3) Misclassification of exposure may have been most pronounced between medium and high exposure; some low exposure may have been classified as a higher exposure.</p> <p>(4) The small number of exposed cases limited the study's power to detect an effect.</p>

Reference	Study design and population	Exposure	Effect: SMR, RR, or OR	Comments
Moulin <i>et al.</i> 1998 France	<p>Historical (mortality) cohort and nested case-control study</p> <p><i>Cohort:</i> all male (5,777) and female (1,682) workers employed at least 3 months in any of 10 factories of the hard-metal industry from the time the factory opened until 12/31/1991. Other production activities at the factories included powder metallurgy processes.</p> <p>Mortality was followed from 1968 (or first date of employment) to 12/31/1991. Cause of death was ascertained from death certificates and medical records.</p> <p><i>Cases:</i> 61 cohort workers who died of lung cancer.</p> <p><i>Controls:</i> 180 living cohort members who were under follow-up on the date the case died and had completed 3 months of employment (3 per case).</p>	<p>Exposure to hard metal was assessed from a job-exposure matrix developed by a panel of experts. The matrix consisted of 320 job periods with assigned semiquantitative estimates of cobalt and tungsten carbide exposure. Exposure to other carcinogens (e.g., PAH, asbestos) was considered.</p> <p>Atmospheric concentrations of cobalt previously measured by plasma emission spectrometry were used to validate the job-exposure matrix.</p>	<p><i>Cohort study:</i> SMR (95% CI); number of cases all causes 0.9 (0.9–1.0); 684 all cancer 1.1 (0.9–1.2); 247 lung 1.3 (1.0–1.7); 63</p> <p><i>Case-control study – lung cancer:</i> OR (95% CI) for cobalt-related exposures other cobalt 2.2 (1.0–4.9) “Other cobalt” exposure refers to exposure to cobalt alone or simultaneously with agents other than tungsten carbide.</p> <p>Simultaneous cobalt and tungsten carbide exposure level: levels 0 to 1 1.0 (ref.) levels 2 to 9 1.9 (1.0–3.6)</p> <p><i>Exposure-response</i> (test for trend): duration: <math>P = 0.03</math> unweighted cumulative exposure: <math>P = 0.01</math> frequency-weighted cumulative exposure: <math>P = 0.08</math></p> <p>The unweighted cumulative exposure measure assigns the same value for occasional and full-time workers, thus favoring peak exposure, whereas the frequency-weighted measure reduces the effects of occasional exposures.</p>	<p><i>Confounders and limitations:</i></p> <p>(1) Healthy worker effect: there were fewer deaths than in the general population.</p> <p>(2) Adjusting for smoking (50 cases and 143 controls) increased the crude OR slightly and did not affect trend relationships; the sources of information on smoking were different for cases and controls.</p> <p>(3) Other carcinogens were present in the factories.</p> <p>(4) 1,131 subjects were lost to follow-up (875 born abroad), lowering the study’s power to detect an effect.</p> <p><i>Validation of exposure assessment:</i></p> <p>Linear relationship between cobalt levels assigned with job-exposure matrix and log-transformed atmospheric cobalt measurement: short-duration area samples (<math>P &lt; 0.0001</math>) long-duration area samples (<math>P = 0.015</math>) long-duration personal samples (<math>P = 0.015</math>).</p>

Reference	Study design and population	Exposure	Effect: SMR, RR, or OR	Comments
Wild <i>et al.</i> 2000 France	<p>Cohort study</p> <p><i>Cohort:</i> 2,860 subjects who had worked at a hard-metal production site (the largest site in the multicenter study of Moulin <i>et al.</i> 1998) for at least 3 months between 1/1/1950 and 6/30/1992, still alive on 1/1/1968, and with available work histories. 14 workshops at the plant, identified by type of production, were regrouped into the various stages related to hard-metal production (e.g. powder production, hard metal before sintering, hard metal after sintering, other alloy production, maintenance, and non-exposed workshops).</p> <p>Cause of death was ascertained from death certificates and physicians' records.</p>	<p>Exposure to cobalt, tungsten carbide, hard metal, and other carcinogens was assessed from an industry-specific job-exposure matrix (Moulin <i>et al.</i> 1998) implemented by a subgroup of the panel of experts. The matrix was validated by atmospheric measurements of cobalt.</p>	<p>SMR (95% CI), number of cases</p> <p><i>Entire cohort of women:</i> all causes 1.3 (1.0–1.6); 68 all cancers 1.3 (0.8–1.9); 22</p> <p><i>Entire cohort of men:</i> all causes 1.0 (0.9–1.1); 331 all cancers 1.1 (0.9–1.3); 118</p> <p><i>Lung cancer:</i> job exposure matrix: cobalt, not hard metal 2.0 (1.1–3.2); 15 smoking 2.3 (1.5–3.2); 29 any IARC carcinogen 2.1 (1.3–3.0); 26 hard metal 2.0 (1.3–3.0); 26</p> <p>workshops (only employed): non-exposed 1.0 (0.4–2.0); 7 hard metal/sintering before 2.9 (1.1–6.3); 6 after 1.1 (0.3–2.9); 3 powder production 1.4 (0.2–5.0); 2 maintenance 2.8 (1.3–5.4); 9</p> <p>Poisson regression* (RR): IARC carcinogen 1.5 (0.8–2.7) smoking 1.6 (0.7–3.6) unsintered dust 1.4 (1.0–2.0) sintered dust 0.8 (0.4–1.5)</p> <p>*Model included smoking and exposure to IARC carcinogens (asbestos, PAH, silica, nickel, and chromium compounds), unsintered hard-metal dust, and sintered hard-metal dust</p>	<p><i>Confounders and limitations:</i></p> <p>(1) Local death rates were used as the mortality reference.</p> <p>(2) Smoking was assessed from occupational records and co-workers.</p> <p>(3) Exposure to other carcinogens was not assessed in the same degree of detail as exposure to hard metal, which may have resulted in misclassification; however, job turnover was low, so the hard-metal exposure probably was not confounded by other industrial processes.</p> <p>(4) 21% of male subjects were lost to follow-up.</p>

Reference	Study design and population	Exposure	Effect: SMR, RR, or OR	Comments
Tüchsen <i>et al.</i> 1996 Denmark	Retrospective cohort study <i>Cohort:</i> all women employed at any time in the plate underglazing departments in two porcelain factories, Factory 1 (382 women from 1943) and Factory 2 (492 women from 1962), and a reference group from a cobalt-free department in Factory 1 (520 women); these workers decorated glazed porcelain with small amounts of dye in a dust-protected room. The cohort was followed until 1992; mortality was identified from the population register, and incident cancer cases (1943–1992) were identified from the cancer registry.	Cobalt silicate dye replaced cobalt-aluminate spinel dye in 1972 in Factory 1 and 1989 in Factory 2. Cobalt content in both dyes was 25%, and nickel content was less than 0.5%. Airborne cobalt exposure measured in 19 workers in 6/1981 exceeded hygienic standards by a factor of 1.3 to 172.	SIR (95% CI); number of cases <i>All cancers:</i> all exposed 1.2 (0.9–1.5); 67 referents 1.0 (NR); 60 <i>Lung cancer:</i> all exposed 2.4 (1.1–4.6); 8 factory 1: 1.6 (NR); 3 factory 2: 3.3 (NR*), 5 referents 2.0 (0.8–4.1); 7 Comparison between exposed and reference, RR = 1.2 (0.4–3.8) *lower limit of 95% CI was reported to be > 1.0. <i>Other cancers with elevated significant SIRs:</i> exposed: cervical cancer SIR = 2.3 (1.2–4.0); 12 reference: corpus uteri cancer SIR = 3.0 (1.4–5.7); 9	<i>Confounders and limitations:</i> (1) Smoking habits were available from two small surveys; Factory 1 may have had more smokers than the general population, but this was unlikely to explain the increased risk relative to the general population of women. (2) The small study population limited the study's power to detect an effect.

Reference	Study design and population	Exposure	Effect: SMR, RR or OR	Comments
Biomarker study				
Rogers <i>et al.</i> 1993 Washington State, USA	Population-based case-control study on cancer of the upper aerodigestive tract (1983–1987)  <i>Cases:</i> 507 cases with aerodigestive tract cancers (153 laryngeal, 73 esophageal, and 281 oral cancer) identified by the local SEER cancer registry, with available nail samples.  <i>Controls:</i> 434 controls identified by random-digit dialing and matched by gender and age, with available nail samples.	Cobalt exposure was determined from nail samples by neutron activation analysis; subjects were divided into strata: lowest 25% (< 0.05 ppm), mid 50% (0.05–0.17 ppm), and highest 25% (> 0.17 ppm).  Other elements to which exposure was assessed were iron, calcium, zinc, and chromium.	Adjusted OR for cobalt (ppm) and cancer (95% CI)  <i>Larynx:</i> < 0.05                    1.0 (ref.) 0.05–0.17                2.0 (1.0–3.8) > 0.17                     1.0 (0.4–2.6)  <i>Esophagus:</i> < 0.05                     1.0 (ref.) 0.05–0.17                2.4 (0.8–7.2) > 0.17                     9.0 (2.7–30.0)*  *test for trend, $P < 0.001$ .  <i>Oral cavity:</i> < 0.05                     1.0 (ref.) 0.05–0.17                1.5 (0.9–2.6) > 0.17                     1.9 (1.0–3.6)  <i>Significant associations between other element levels (highest dose) in nails and esophageal cancer:</i>  iron                        2.9 (1.1–7.5) calcium                    2.6 (1.0–7.1)	<i>Confounders and limitations:</i>  (1) ORs were adjusted for age, sex, cigarette use, alcohol use, energy intake, $\beta$ -carotene intake, and ascorbic acid intake.  (2) Exposure was assessed after diagnosis of disease, but no significant differences were observed in ORs by stage or time from diagnosis to interview.  (3) Elements (Co) are deposited in nails during formation of the nail matrix (8 months to 2 years depending on age), so element levels probably represent recent exposure in most cases.

<sup>a</sup>NR = not reported; RR = relative risk.

## 4 Studies of Cancer in Experimental Animals

In its evaluation of the carcinogenicity of cobalt and cobalt compounds, IARC (1991) found that several cobalt compounds induced sarcomas at injection sites in animals. The limitations of the animal studies available to IARC for review were that they all were either injection or implantation studies and did not adequately evaluate the potential carcinogenicity of cobalt and cobalt compounds by other routes of exposure. After publication of the IARC monograph, the NTP (1998) completed a two-year inhalation carcinogenicity study of cobalt sulfate heptahydrate with B6C3F<sub>1</sub> mice and F344/N rats. Results are reported separately below for mice (Section 4.1) and rats (Section 4.2).

### 4.1 NTP carcinogenicity bioassay in mice

Groups of six-week-old B6C3F<sub>1</sub> mice (50 of each sex) were administered cobalt sulfate heptahydrate aerosols by inhalation at target concentrations of 0, 0.3, 1.0, or 3.0 mg/m<sup>3</sup>, 6 h/day, 3 days/week, for 105 weeks (NTP 1998, Bucher *et al.* 1999). The corresponding concentrations expressed as elemental cobalt were 0, 0.063 mg/m<sup>3</sup>, 0.210 mg/m<sup>3</sup>, and 0.628 mg/m<sup>3</sup>. Exposure concentrations were based on previous subacute and subchronic studies (Bucher *et al.* 1990, NTP 1991). Cobalt sulfate heptahydrate was generated and delivered from an aqueous solution via a compressed-air-driven nebulizer, an aerosol charge neutralizer, and an aerosol distribution system. The aerosol was dried and mixed with humidified air before delivery to the inhalation chambers, thus allowing partial rehydration of the aerosol particles. The mass median aerosol particle diameter was 1 to 3 μm, and the aerosol consisted of 1 mole of cobalt, 1 mole of sulfate, and 5.9 moles of water per mole of aerosolized cobalt sulfate (Bucher *et al.* 1999). The overall chemical purity of the study material was reported to be 99%. Survival was not significantly affected by exposure (see Appendix B, pp. B-33 to B-34, Table 8 and Figure 3 in NTP 1998). Mean body weights were slightly higher in exposed females than in controls, and mean body weights were lower in the high-dose males than in controls from week 96 to the end of the study (see Appendix B, pp. B-35 to B-37, Figure 4 and Tables 9 and 10 in NTP 1998).

The incidences of alveolar/bronchiolar adenoma, alveolar/bronchiolar carcinoma, and alveolar/bronchiolar adenoma or carcinoma (combined) showed a positive exposure-response trend in all groups. The incidences of these neoplasms were significantly higher in all the high-dose groups than in the controls, as was the incidence of adenoma or carcinoma (combined) in mid-dose female mice (Table 4-1). The NTP (1998) concluded that there was clear evidence of carcinogenic activity in both male and female mice, based on increased incidences of lung tumors.

Although the incidence of hemangiosarcoma was significantly increased in male mice in the mid-dose group (Table 4-1), *Helicobacter hepaticus* infection was present in these mice, making interpretation of this finding difficult. Liver sections from several male mice were positive for bacteria, and the spectrum of liver lesions in these mice was consistent with *H. hepaticus* infection.

**Table 4-1. Tumor incidence in B6C3F<sub>1</sub> mice following inhalation exposure to cobalt sulfate heptahydrate for 105 weeks**

Sex	Exposure conc. (mg/m <sup>3</sup> )	Tumor incidence <sup>a</sup> (%) <sup>b</sup>			
		Alveolar/bronchiolar			Liver
		Adenoma	Carcinoma	Combined	Hemangiosarcoma
Male	0	9 (30.4%)	4 (13.2%)	11 (35.5%)	2 (9.1%)
	0.3	12 (30.9%)	5 (16.1%)	14 (36.5%)	4 (11.5%)
	1.0	13 (41.1%)	7 (25.3%)	19 (56.5%)	8 (23.5%)* <sup>d</sup>
	3.0	18 (54.6%)*	11 (43.7%)*	28 (78.8%)* <sup>***</sup>	7 (25.0%)
	Trend <sup>c</sup>	<i>P</i> = 0.018	<i>P</i> = 0.006	<i>P</i> < 0.001	<i>P</i> = 0.078
Female	0	3 (8.8%)	1 (2.9%)	4 (11.8%)	1 (2.9%)
	0.3	6 (15.0%)	1 (2.7%)	7 (17.5%)	0
	1.0	9 (25.2%)	4 (9.2%)	13 (32.6%)*	3 (7.3%)
	3.0	10 (32.8%)*	9 (25.3%)* <sup>**</sup>	18 (50.2%)* <sup>***</sup>	0 <sup>e</sup>
	Trend <sup>c</sup>	<i>P</i> = 0.024	<i>P</i> < 0.001	<i>P</i> < 0.001	<i>P</i> = 0.431N

Source: NTP 1998, Bucher *et al.* 1999.

\**P* ≤ 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001 (logistic regression test).

<sup>a</sup>The number of animals with the neoplasm, out of 50 animals per group unless otherwise noted.

<sup>b</sup>Kaplan-Meier estimated neoplasm incidence at the end of the study after adjustment for intercurrent mortality.

<sup>c</sup>Logistic regression test; lower incidence in an exposure group is indicated by N.

<sup>d</sup>Results were confounded by *H. hepaticus* infection.

<sup>e</sup>49 animals in the group.

In addition to the neoplastic lesions, exposure to cobalt sulfate induced a spectrum of inflammatory, fibrotic, and proliferative lesions in other portions of the respiratory tract that were consistent with results observed in the shorter-term studies (Table 4-2). These included hyperplasia of the olfactory epithelium (high-dose groups), squamous metaplasia of the larynx (all exposed groups), cytoplasmic vacuolization of the bronchi (all exposed groups), diffuse histiocytic cell infiltration (high-dose males), and focal histiocytic cell infiltration of the lung (high-dose females). Histiocytic infiltration was observed most often in lungs with alveolar/bronchiolar neoplasms and was attributed to the neoplasms, rather than to a direct effect of cobalt sulfate.

**Table 4-2. Incidences and severity of nonneoplastic lesions in B6C3F<sub>1</sub> mice following inhalation exposure to cobalt sulfate heptahydrate for 105 weeks**

Exposure concentration:	Incidence <sup>a</sup> (severity) <sup>b</sup>			
	Controls	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Male</b>				
Lung				
Diffuse histiocytic cellular infiltration	1 (3.0)	2 (3.0)	4 (2.3)	10** (1.5)
Focal histiocytic cellular infiltration	10 (2.7)	5 (2.6)	8 (3.0)	17 (2.7)
Bronchiolar cytoplasmic vacuolization	0	18** (1.0)	34** (1.0)	38** (1.0)
Larynx				
Squamous metaplasia	0 <sup>c</sup>	37** <sup>d</sup> (1.0)	48** <sup>c</sup> (1.0)	44** <sup>d</sup> (1.0)
Nose				
Atrophy of olfactory epithelium	0	0	29** <sup>c</sup> (1.2)	48** <sup>d</sup> (1.8)
Hyperplasia of olfactory epithelium	0	0	0 <sup>c</sup>	10** <sup>d</sup> (1.0)
Suppurative inflammation	0	1 (3.0)	0 <sup>c</sup>	6* <sup>d</sup> (2.2)
<b>Female</b>				
Lung				
Diffuse histiocytic cellular infiltration	0	0	0	4 (3.3)
Focal histiocytic cellular infiltration	2 (2.0)	5 (1.8)	7 (2.9)	10* (2.4)
Bronchiolar cytoplasmic vacuolization	0	6* (1.0)	31** (1.0)	43** (1.0)
Larynx				
Squamous metaplasia	0	45** <sup>d</sup> (1.0)	40** <sup>c</sup> (1.0)	50** (1.1)
Nose				
Atrophy of olfactory epithelium	0	2 (1.5)	12** <sup>d</sup> (1.0)	46** <sup>c</sup> (1.5)
Hyperplasia of olfactory epithelium	0	0	0 <sup>d</sup>	30** <sup>c</sup> (1.3)
Suppurative inflammation	0	1 (1.0)	5* <sup>d</sup> (1.6)	4 <sup>c</sup> (1.5)

Source: NTP 1998.

\* $P \leq 0.05$ , \*\* $P < 0.01$  (logistic regression test).<sup>a</sup>The number of animals with the lesion, out of 50 animals per group unless otherwise noted.<sup>b</sup>Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.<sup>c</sup>48 animals examined.<sup>d</sup>49 animals examined.<sup>e</sup>47 animals examined.

## 4.2 NTP carcinogenicity bioassay in rats

Groups of six-week-old F344/N rats (50 of each sex) were administered cobalt sulfate heptahydrate aerosols by inhalation at target concentrations of 0, 0.3, 1.0, or 3.0 mg/m<sup>3</sup>, 6 h/day, 5 days/week, for 105 weeks (NTP 1998, Bucher *et al.* 1999). Exposure concentrations were based on previous subacute and subchronic studies (Bucher *et al.* 1990, NTP 1991). Survival of exposed rats did not differ significantly from that of controls. Among males, survival was 34%, 30%, 42%, and 30% in the control, low-exposure, mid-exposure, and high-exposure groups, respectively. Overall, survival was higher in females than in males, at 56%, 51%, 52%, and 60% in the control, low-exposure, mid-exposure, and high-exposure groups, respectively (see Appendix B, pp. B-21 and B-22, Table 2, and Figure 1 in NTP 1998). Mean body weights in all exposed groups did not differ significantly from those of controls throughout the study (see Appendix B, pp. B-23 to B-25, Tables 3 and 4, and Figure 2 in NTP 1998).

The incidence of alveolar/bronchiolar adenoma or carcinoma (combined) showed a significant positive exposure-related trend in male rats and was significantly higher in the high-dose group than in the control group. A significant positive exposure-related trend for alveolar adenoma, carcinoma, and adenoma or carcinoma (combined) was observed in female rats, and incidences were significantly higher in the mid-dose and high-dose groups than in the controls (Table 4-3). In addition, squamous-cell carcinoma of the lung was observed in two female rats (one each in the mid-dose and high-dose groups). The incidence of benign adrenal pheochromocytoma was increased in high-dose females, and the incidence of benign, complex, or malignant pheochromocytoma (combined) was increased in mid-dose males and high-dose females (Table 4-3). The increased incidences in the high-dose females were considered to be exposure related. The NTP (1998) concluded that there was some evidence of carcinogenicity in male rats, based on increased incidences of alveolar/bronchiolar neoplasms. Marginal increases in adrenal medullary tumors in male rats may have been related to exposure to cobalt sulfate heptahydrate. There was clear evidence of carcinogenicity in female rats, based on increased incidences of alveolar/bronchiolar neoplasms and pheochromocytoma of the adrenal medulla.

**Table 4-3. Tumor incidence in F344/N rats following inhalation exposure to cobalt sulfate heptahydrate for 105 weeks**

Sex	Exposure conc. (mg/m <sup>3</sup> )	Tumor incidence <sup>a</sup> (%) <sup>b</sup>				
		Alveolar/bronchiolar			Adrenal medulla	
		Adenoma	Carcinoma	Combined	Benign <sup>c</sup>	Total
Male	0	1 (2.3%)	0	1 (2.3%)	14 (51.0%)	15 (52.1%)
	0.3	4 (17.7%)	0	4 (17.7%)	19 (70.0%)	19 (70.0%)
	1.0	1 <sup>c</sup> (2.4%)	3 <sup>c</sup> (11.3%)	4 <sup>c</sup> (13.4%)	23 <sup>f</sup> (71.9%)	25 <sup>f</sup> (74.1%)*
	3.0	6 (28.4%)	1 (6.7%)	7 (33.9%)*	20 (71.4%)	20 (71.4%)
	Trend <sup>d</sup>	<i>P</i> = 0.051	<i>P</i> = 0.360	<i>P</i> = 0.032	<i>P</i> = 0.172	<i>P</i> = 0.218
Female	0	0	0	0	2 <sup>e</sup> (5.1%)	2 <sup>e</sup> (5.1%)
	0.3	1 <sup>f</sup> (3.4%)	2 <sup>f</sup> (8.0%)	3 <sup>f</sup> (11.2%)	1 <sup>f</sup> (3.1%)	1 <sup>f</sup> (3.1%)
	1.0	10 (36.4%)*	6(20.2%)*	15 (50.6%)*	3 (9.3%)	4 (11.7%)
	3.0	9 (30%)*	6 (17.5%)*	15 (46.1%)*	8 <sup>e</sup> (26.4%)*	10 <sup>e</sup> (31.5%)*
	Trend <sup>d</sup>	<i>P</i> = 0.001	<i>P</i> = 0.023	<i>P</i> < 0.001	<i>P</i> = 0.004	<i>P</i> < 0.001

Source: NTP 1998, Bucher *et al.* 1999.

\**P* ≤ 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001 (logistic regression test).

<sup>a</sup>The number of animals with the neoplasm, out of 50 animals per group unless otherwise noted.

<sup>b</sup>Kaplan-Meier estimated neoplasm incidence at the end of the study after adjustment for intercurrent mortality.

<sup>c</sup>Pheochromocytoma.

<sup>d</sup>Logistic regression test.

<sup>e</sup>48 animals in the group.

<sup>f</sup>49 animals in the group.

Nonneoplastic lesions of the respiratory tract generally were more severe in rats than in mice (see Section 4.1). Significantly increased incidences of inflammatory, fibrotic, and proliferative lesions were observed in all dose groups in the lung (hyperplasia and metaplasia of the alveolar epithelium, granulomatous inflammation, interstitial fibrosis, and proteinosis), nose (lateral wall hyperplasia and olfactory epithelium atrophy), and larynx (squamous metaplasia of the epiglottis) (Table 4-4). The NTP characterized all fibroproliferative lesions as atypical hyperplasia. Several animals had malignant neoplasms with a very prominent fibrous component, some of which presumably had progressed from atypical hyperplasia. The NTP (1998) concluded that it was clear that all the morphologic variants of proliferative lesions represented a response to cobalt sulfate heptahydrate.

**Table 4-4. Incidences and severity of nonneoplastic lesions in F344/N rats following inhalation exposure to cobalt sulfate heptahydrate for 105 weeks**

Exposure concentration:	Incidence <sup>a</sup> (severity) <sup>b</sup>			
	Controls	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Male</b>				
Lung				
Alveolar epithelial hyperplasia	9 (1.8)	20* (2.0)	20* <sup>c</sup> (2.1)	23** (2.0)
Alveolar epithelial metaplasia	0	50** (1.9)	48** <sup>c</sup> (3.1)	49** (3.7)
Granulomatous inflammation	2 (1.0)	50** (1.9)	48** <sup>c</sup> (3.1)	50** (3.7)
Interstitial fibrosis	1 (1.0)	50** (1.9)	48** <sup>c</sup> (3.1)	49** (3.7)
Proteinosis	0	16** (1.4)	40** <sup>c</sup> (2.3)	47** (3.4)
Larynx				
Squamous metaplasia of epiglottis	0	10** <sup>d</sup> (1.3)	37** <sup>c</sup> (1.8)	50** (2.8)
Nose				
Hyperplasia of lateral wall	2 (1.5)	14** (1.4)	21** <sup>d</sup> (1.5)	20** (1.6)
Squamous metaplasia of lateral wall	1 (1.0)	3 (1.3)	5 <sup>d</sup> (1.4)	8* (2.0)
Atrophy of olfactory epithelium	8 (1.1)	24** (1.4)	42** <sup>d</sup> (1.5)	48** (2.5)
Metaplasia of olfactory epithelium	5 (1.2)	1 (3.0)	5 <sup>d</sup> (1.8)	30** (1.9)
<b>Female</b>				
Lung				
Alveolar epithelial hyperplasia	15 (1.4)	7 <sup>d</sup> (1.6)	20 (1.8)	33** (2.0)
Alveolar epithelial metaplasia	2 (1.0)	47** <sup>d</sup> (2.0)	50** (3.6)	49** (3.9)
Granulomatous inflammation	9 (1.0)	47** <sup>d</sup> (2.0)	50** (3.6)	49** (3.9)
Interstitial fibrosis	7 (1.0)	47** <sup>d</sup> (2.0)	50** (3.6)	49** (3.9)
Proteinosis	0	36** <sup>d</sup> (1.2)	49** (2.8)	49** (3.9)
Larynx				
Squamous metaplasia of epiglottis	1 (1.0)	22** <sup>d</sup> (1.1)	39** (1.4)	48** (2.6)
Nose				
Hyperplasia of lateral wall	1 (1.0)	8* <sup>d</sup> (1.3)	26** (1.4)	38** (1.7)
Squamous metaplasia of lateral wall	1 (1.0)	1 <sup>d</sup> (3.0)	4 (1.3)	10** (1.4)
Atrophy of olfactory epithelium	5 (1.4)	29** <sup>d</sup> (1.2)	46** (1.6)	47** (2.9)
Metaplasia of olfactory epithelium	2 (2.0)	2 <sup>d</sup> (1.5)	3 (1.7)	40** (2.3)

Source: NTP 1998.

\* $P \leq 0.05$ , \*\* $P < 0.01$  (logistic regression test).<sup>a</sup>The number of animals with the lesion, out of 50 animals per group unless otherwise noted.<sup>b</sup>Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.<sup>c</sup>48 animals examined.<sup>d</sup>49 animals examined.

### 4.3 Summary

IARC (1991) concluded that there was sufficient evidence for the carcinogenicity of cobalt metal powder and cobalt(II) oxide and limited evidence for the carcinogenicity of metal alloys containing cobalt, cobalt(II) sulfide, and cobalt(II) chloride in experimental animals when exposure was by injection or implantation. However, evidence for the carcinogenicity of cobalt and cobalt compounds in experimental animals by other routes of administration were not available at that time. In a subsequent study, cobalt sulfate heptahydrate was found to be carcinogenic in B6C3F<sub>1</sub> mice and F344/N rats when administered by inhalation. There was clear evidence of carcinogenicity in male mice, female mice, and female rats, based on increased incidences of lung tumors. In addition, female rats had an increased incidence of pheochromocytoma of the adrenal medulla. Some evidence of carcinogenicity in male rats was described, based on increased incidences of lung tumors at the highest exposure level.



## 5 Genotoxicity

IARC (1991) reviewed the genotoxicity of cobalt and cobalt compounds. Although many studies investigated the genotoxicity of soluble cobalt(II) salts (e.g., cobalt chloride, cobalt acetate, and cobalt nitrate), none of them specifically addressed cobalt sulfate. In general, cobalt(II) compounds were not genotoxic in bacteria but induced DNA damage, mutations, sister chromatid exchange (SCE), and aneuploidy in some *in vitro* tests with animal and human cells (see Appendix A, pp. A-78 to A-80, Table 21 in IARC 1991). In addition, chlorophyll mutations, chromosomal aberrations, and aneuploidy were induced in plant cells.

Léonard and Lauwerys (1990) reviewed the mutagenicity, carcinogenicity, and teratogenicity of cobalt metal and cobalt compounds and concluded that cobalt and cobalt compounds were only weakly mutagenic. In another review, Beyersmann and Hartwig (1992) noted that the cobalt(II) ion is relatively inactive in prokaryotic systems, as are other metallic ions. Factors potentially contributing to this inactivity include precipitation of phosphates in bacterial media, a low rate of uptake or indirect mechanisms of interaction with DNA, and trapping of metal ions by proteins present in exogenous metabolic activating systems. Nevertheless, these authors concluded the following: (1) cobalt(II) salts generally are nonmutagenic in prokaryotic assays and were antimutagenic in some studies, (2) cobalt chloride is mutagenic to mitochondrial genes but only weakly mutagenic or nonmutagenic to chromosomal genes in *Saccharomyces cerevisiae*, (3) cobalt(II) salts induce gene mutations and chromosomal aberrations in plants, (4) cobalt(II) compounds cause DNA strand breaks, SCE, and aneuploidy in mammalian cells *in vitro*, and (5) cobalt(II) salts are comutagenic with ultraviolet light but not with gamma rays in mammalian cells.

### 5.1 Prokaryotic systems

Zeiger *et al.* (1992) presented the results of *Salmonella typhimurium* mutagenicity tests for 311 chemicals tested within the NTP's mutagenicity testing program. *S. typhimurium* strains TA98, TA100, and TA1535 were used with and without rat or hamster S9 metabolic activation. Each trial included triplicate plates of concurrent positive and negative controls and five exposure levels (between 10 and 10,000 µg/plate) of a test chemical. A positive response was defined as a reproducible, dose-related increase in revertant colonies in any one strain/activation combination. Cobalt sulfate heptahydrate was mutagenic in strain TA100 without metabolic activation and with either 5% hamster or rat liver S9. It was not mutagenic in strains TA98 or TA1535 with or without metabolic activation (NTP 1998).

### 5.2 Mammalian systems

#### 5.2.1 Rodent cells

Kerckaert *et al.* (1996a) tested cobalt sulfate hydrate and other metal compounds in the Syrian hamster embryo (SHE) cell transformation assay. These authors noted that for heavy metals and heavy-metal compounds, the SHE transformation assay was a better predictor of rodent carcinogenicity than the *Salmonella* assay; concordance with the rodent bioassay was 92% for the SHE assay but only 33% for the *Salmonella* assay.

Cobalt sulfate hydrate caused a significant increase in SHE cell transformation at all five exposure levels tested (0.125 to 1 µg/mL) within 24 hours; however, no significant exposure-response trend was found. Nevertheless, the authors considered the results to be positive because significant cell transformation was observed for at least two exposure levels.

Gibson *et al.* (1997) tested 16 chemicals, including cobalt sulfate hydrate, in the SHE micronucleus assay. Cobalt sulfate hydrate was tested at 1.0, 2.0, and 4.0 µg/mL. All exposure levels significantly increased the percentage of micronucleated binucleated cells (MNBC) (Table 5-1).

**Table 5-1. Effects of cobalt sulfate hydrate on micronucleus formation in SHE cells**

Exposure level (µg/mL)	Relative cell number	Binucleated cells (%)	MNBC (%)	Fisher's exact P value
Control	100	42	25/1000 (2.5)	–
1.0	190	45	43/1000 (4.3)	0.0176
2.0	219	38	47/1000 (4.7)	0.0056
4.0	159	34	63/1000 (6.3)	< 0.001

Adapted from Gibson *et al.* 1997.

Cellular levels of the tumor-suppressor protein p53 increase following DNA damage. Therefore, Duerksen-Hughes *et al.* (1999) developed and tested a mammalian *in vitro* assay for genotoxicity based on p53 induction. NCTC 929 cells derived from mouse fibroblasts were exposed to 25 test chemicals being tested by the NTP for carcinogenicity in rodents. Cultured cells were exposed to cobalt sulfate heptahydrate at a concentration of 1, 10, 20, 50, or 100 µg/mL. Control plates were exposed to the vehicle alone (culture medium or dimethylsulfoxide [DMSO]). Cells were incubated at 37°C and harvested at 6 hours (first series) or 17 hours (second series) post-treatment. Cobalt sulfate heptahydrate strongly induced p53 in NCTC 929 cells exposed at 50 or 100 µg/mL for 6 hours or at 20 or 50 µg/mL for 17 hours. A concentration of 100 µg/mL was cytotoxic to cells exposed for 17 hours.

Lloyd *et al.* (1997, 1998) investigated the generation of putative intrastrand cross-links, formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG), and single- and double-strand breaks in DNA by Fenton-type reactions. In both studies, DNA was exposed to hydrogen peroxide at a concentration of 50 mM and to various transition-metal ions, including cobalt sulfate. In the first report (Lloyd *et al.* 1997), salmon sperm DNA exposed to cobalt sulfate developed putative intrastrand cross-links in a dose-dependent manner at concentrations of up to 1 mM cobalt. Six radioactive spots detected by thin-layer chromatography were thought to be hydroxyl radical-mediated oxidative DNA lesions; however, no DNA strand breaks were detected. The authors tentatively identified two of the adducts as products of a reaction between the metal ion and the purine dimers 2'-deoxyadenylyl-(3'-5')-2'-deoxyadenosine and 2'-deoxyadenylyl-(3'-5')-2'-deoxyguanosine,

which they interpreted as consistent with the formation of intrastrand cross-links. In the latter study (Lloyd *et al.* 1998), a more sensitive method for detecting DNA strand breaks was used. Double-stranded plasmid pBluescript K+ DNA was incubated with 1 mM hydrogen peroxide and each transition-metal ion for 15 minutes. No significant formation of 8-OHdG adducts was detected after incubation with cobalt sulfate; however, single-strand, but not double-strand, breaks were detected.

### 5.2.2 Human cells

Kawanishi *et al.* (1989) incubated <sup>32</sup>P-labeled DNA fragments obtained from human c-Ha-ras-1 protooncogene with 1 mM sodium sulfite and 20 μM cobalt(II) ion. Sulfite caused DNA damage in the presence of cobalt(II) and other metal ions; however, sulfite alone or metal ion alone did not cause damage. DNA damage was much greater in the presence of cobalt than with copper, manganese, or iron. Treatment with 3,5-dibromo-4-nitrobenzenesulfonate or primary or secondary alcohols inhibited DNA damage by sulfite plus cobalt(II), whereas treatment with superoxide dismutase, catalase, or tert-butyl alcohol did not. The authors noted that primary and secondary alcohols react readily with sulfate radicals but not sulfite radicals and that sulfate radicals react slowly with tert-butyl alcohol. They concluded that the DNA damage was caused by autooxidation of sulfite to the sulfate radical in the presence of cobalt(II).

Nackerdien *et al.* (1991) investigated the ability of mixtures of cobalt(II) and hydrogen peroxide to cause chemical changes in DNA bases in chromatin isolated from human K562 cells. Reaction mixtures consisted of chromatin (0.12 mg DNA/mL) alone, chromatin plus cobalt sulfate (25 μM), chromatin plus hydrogen peroxide (2.8 mM), and chromatin plus cobalt sulfate and hydrogen peroxide. In addition, the effects of adding ethylenediaminetetraacetic acid (EDTA) (120 μM), ascorbic acid (100 μM), glutathione (1 mM), mannitol (50 mM), DMSO (50 mM), or superoxide dismutase (200 units/mL) to the reaction mixture were measured. Yields of DNA base products were not increased in chromatin exposed to cobalt sulfate only or hydrogen peroxide only; however, yields of all base products were increased 2- to 18-fold in chromatin exposed to both cobalt sulfate and hydrogen peroxide for one hour. The major products included cytosine glycol, formamidopyrimidines, and 8-hydroxypurines. Addition of ascorbic acid had no effect, whereas addition of the hydroxyl radical scavengers mannitol and DMSO or chelation with EDTA inhibited product formation. Results for glutathione were mixed; yields of some products decreased moderately, while yields of others increased twofold. Superoxide dismutase increased product yields. The authors concluded that DNA damage in chromatin caused by cobalt ions in the presence of hydrogen peroxide might contribute to genotoxicity and carcinogenicity.

Olivero *et al.* (1995) compared the genotoxicity of cobalt chloride, cobalt sulfate heptahydrate, and cobalt nitrate hexahydrate in cultured human lymphocytes. The mitotic index, chromosomal aberrations, and micronuclei were measured in whole-blood samples obtained from a single healthy donor. Exposure to any of the three cobalt salts resulted in a dose-related decrease in the mitotic index; however, micronuclei increased significantly only in cells exposed to cobalt chloride. None of the cobalt salts increased the frequency of chromosomal aberrations. Results are summarized in Table 5-2.

**Table 5-2. Genotoxic effects of cobalt chloride, cobalt nitrate hexahydrate, and cobalt sulfate heptahydrate in human lymphocytes**

Compound	Conc. (µg/mL)	Conc. (mM)	Mitotic index	Micro-nucleated cells (%)	Aneuploidy (%)	Total structural aberrations (%)
Cobalt chloride	0	0	3.6	10	0	7
	0.0045	0.035	3.6	23*	0	11
	0.023	0.177	2.7	24*	1	12
	0.045	0.347	2.2	23*	2	12
	0.23	1.771	2.3	25**	2	14
	0.45	3.466	1.0	23*	0	8
Cobalt nitrate hexahydrate	0	0	3.5	5	0	16
	0.0045	0.015	3.4	4	0	10
	0.045	0.155	2.1	8	0	12
	0.45	1.546	1.2	8	0	16
Cobalt sulfate heptahydrate	0	0	3.5	5	1	8
	0.0045	0.016	2.4	1	2	8
	0.045	0.160	2.3	8	1	7
	0.45	1.601	1.3	7	1	9

Adapted from Olivero *et al.* 1995.\* $P < 0.05$ , \*\* $P \leq 0.01$  (chi-square test).

### 5.3 Summary

The genotoxicity of cobalt sulfate has been studied less extensively than that of other cobalt salts, especially cobalt chloride. There is evidence that the genotoxicity of cobalt compounds depends on the ligand coordinated about the metal ion. Overall, the data suggest that cobalt salts generally are not mutagenic in bacterial test systems. In one study, cobalt sulfate was mutagenic in *S. typhimurium* strain TA100 but not in strains TA98 or TA1535. Cobalt sulfate induced cell transformation and micronuclei in SHE cells and strongly induced p53 in mouse fibroblasts. In the presence of hydrogen peroxide, cobalt sulfate induced putative intrastrand cross-links in salmon sperm DNA and single-strand breaks in plasmid pBluescript K+ DNA. However, 8-OHdG adducts were not induced in salmon sperm DNA. Sulfite in the presence of cobalt ions caused damage in DNA fragments derived from the human *c-Ha-ras-1* protooncogene. Yields of DNA base products in human chromatin were increased by exposure to cobalt sulfate in the presence of hydrogen peroxide. In a study of three cobalt salts, cobalt sulfate was not genotoxic to human lymphocytes.

## 6 Other Relevant Data

IARC (1991) reviewed the carcinogenicity of cobalt and cobalt compounds. Although very little information specific to cobalt sulfate was presented in the IARC monograph, general information on cobalt(II) was considered relevant to the potential carcinogenicity of cobalt sulfate. IARC (1991) reached the following conclusions:

- There was sufficient evidence for the carcinogenicity of cobalt metal powder and cobalt(II) oxide in experimental animals.
- There was limited evidence for the carcinogenicity of metal alloys containing cobalt, chromium, and molybdenum and of cobalt(II) sulfide and cobalt(II) chloride in experimental animals.
- There was inadequate evidence for the carcinogenicity of cobalt-aluminium-chromium spinel, cobalt(II,III) oxide, cobalt naphthenate, and cobalt(III) acetate in experimental animals.
- There was inadequate evidence for the carcinogenicity of cobalt and cobalt compounds in humans.

This section summarizes the toxicity, toxicokinetics, and possible mechanisms of carcinogenesis of cobalt sulfate and similar cobalt compounds.

### 6.1 Toxicity of cobalt sulfate

As a component of vitamin B<sub>12</sub>, cobalt is an essential nutrient in humans. No other physiological function of cobalt has been identified. A daily intake of about 50 µg of cobalt, with about 80% (40 µg) as vitamin B<sub>12</sub>, is sufficient to meet the nutritional requirement (Léonard and Lauwerys 1990). However, excessive exposure to cobalt can result in many adverse effects. Cobalt can replace other essential divalent cations, such as magnesium and calcium ions; bind to sulfhydryl groups; inhibit heme synthesis; and reduce cytochrome P450 concentrations (Bucher *et al.* 1999).

The oral 50% lethal dose (LD<sub>50</sub>) of various inorganic cobalt(II) compounds in rats ranges from about 150 to 500 mg/kg body weight (b.w.) For cobalt sulfate, the oral LD<sub>50</sub> is 424 mg/kg b.w. in rats and 584 mg/kg b.w. in mice. Acute effects in animals include sedation, diarrhea, weight loss, tremor, and convulsions (IARC 1991, RTECS 2001). Rats and mice exposed to cobalt sulfate heptahydrate aerosols for 13 weeks at 0.3 to 30 mg/m<sup>3</sup> developed lesions in the respiratory tract, which included degeneration of the olfactory epithelium, squamous metaplasia of the respiratory epithelium, inflammation in the nose, epithelial hyperplasia in the alveoli, squamous metaplasia of the larynx, and other effects. In addition, polycythemia occurred in rats, and reproductive effects (e.g., abnormal sperm, decreased sperm motility, and decreased testis and epididymal weights) occurred in mice. Two of the 10 male mice exposed to the highest concentration died during the study (Bucher *et al.* 1990).

In humans, hard-metal pneumoconiosis and occupational asthma are considered the primary effects of occupational exposure to cobalt-containing dust. Hard-metal pneumoconiosis is a severe and progressive disease marked by interstitial fibrosis that may develop after a few months to several years of exposure to dust containing cobalt and other metals (e.g., titanium and tantalum) or tungsten carbide. Cobalt hypersensitivity has been associated with hard-metal asthma and allergic dermatitis in workers. In the 1960s, several outbreaks of cardiomyopathy, with mortality rates as high as 50%, were reported in individuals who drank large quantities of cobalt-fortified beer. At that time, cobalt sulfate, cobalt acetate, or cobalt chloride was added to some beers as a foaming agent. Polycythemia also was reported in some beer drinkers. Although the cobalt intake by the beer drinkers was a few milligrams per day, which is much higher than normal daily intakes, the exposure was much lower than the 25 to 300 mg/day once used to treat patients with anemia (IARC 1991). Therefore, the beer-drinkers' cardiomyopathy may have resulted from a synergistic effect with alcohol and poor nutrition (Lauwerys and Lison 1994).

## **6.2 Mammalian absorption, distribution, metabolism, and excretion of cobalt**

Cobalt is absorbed from the gastrointestinal tract, lungs, and skin. Normal levels in blood and urine in the general population are 0.2 to 2 µg/L, but concentrations greater than 200 µg/L have been reported in the urine of workers occupationally exposed to cobalt (IARC 1991, NTP 1998). Gastrointestinal tract absorption is highly variable depending on the compound, concentration, and other factors, but is estimated to range from 5% to 45% (Lauwerys and Lison 1994) and may be higher in females than in males (Christensen and Poulsen 1994). There is evidence that iron and cobalt share the same transport mechanism in the duodenum (Léonard and Lauwerys 1990). The degree of respiratory absorption in humans is unknown but varies with concentration. Some studies have shown a good correlation between concentrations in air and concentrations in urine of workers (Christensen and Poulsen 1994). Respiratory absorption of cobalt inhaled as cobalt oxide was about 30% (Lauwerys and Lison 1994). Scansetti *et al.* (1994) demonstrated substantial absorption of cobalt through the skin.

Once absorbed, cobalt is preferentially distributed to the liver, kidney, and heart (Léonard and Lauwerys 1990, Christensen and Poulsen 1994). Without occupational exposure, the cobalt content in the adult human body is about 1 to 2 mg. The cobalt content of bone and muscle account for 14% and 13%, respectively, of the total body burden, with the rest occurring in soft tissues (Léonard and Lauwerys 1990, IARC 1991). The highest cobalt concentrations are in the liver, because vitamin B<sub>12</sub> is stored there; IARC (1991) reported that the cobalt concentration in the liver at autopsy ranged from 6 to 151 µg/kg, with a median value of 30 µg/kg. Patients dying of cardiomyopathy from excessive intake of cobalt-fortified beer had 10 times the normal amount of cobalt in the heart (IARC 1991).

Concentrations of arsenic and cobalt were evaluated in tissue and plasma of patients with laryngeal carcinoma (Collecchi *et al.* 1986). Plasma and histologically nonmalignant and malignant laryngeal tissues were obtained from each of 15 male patients with no known exposure to toxic amounts of cobalt. The cobalt concentrations in malignant laryngeal tissue ( $68.7 \pm 7.3$  ng/g dry weight, mean  $\pm$  SD) were significantly higher ( $P < 0.01$ ,

paired *t*-test and Wilcoxon's test) than those in nonmalignant laryngeal tissue ( $39.6 \pm 7.0$ ). The plasma cobalt concentrations were 25-fold higher in the 15 patients with laryngeal carcinoma than in 11 apparently normal male individuals ( $18.27 \pm 2.10$  and  $0.73 \pm 0.10$  ng/mL, respectively;  $P < 0.001$ , Student's *t*-test and Mann-Whitney U-test). Similar significant differences were reported for plasma and tissue arsenic levels. The authors reported that further studies were in progress to ascertain the clinical significance of the changes in tissue and plasma cobalt and arsenic concentrations; however, no additional publications on this subject were identified in a search of the literature since 1986.

Cobalt is excreted in the urine and, to a lesser degree, in the feces. In experimental animals, 70% or more is eliminated in the urine (IARC 1991). In humans, 28% to 56% of radiolabelled cobalt chloride was eliminated in the urine and 2% to 12% in the feces within eight days after parental administration. Between 9% and 16% of the administered dose was eliminated very slowly, with a biological half-life of about two years (Smith *et al.* 1972). Thus, cobalt excretion has two distinct phases: a rapid initial phase, with a half-life of a few days, followed by a slow second phase, with a half-life of a year or more (Léonard and Lauwerys 1990, Lauwerys and Lison 1994). Cobalt concentrations in the urine of workers in the Italian hard-metal industry were 10 to 100 µg/L at the beginning of the work shift, increasing to 16 to 210 µg/L at the end of the shift (Sabbioni *et al.* 1994). Clearance from the lungs has not been studied but is expected to be rapid for soluble cobalt salts (NTP 1998).

### 6.3 Syrian hamster embryo cell transformation assay

Kerckaert *et al.* (1996a, b) tested five heavy-metal compounds (cobalt sulfate hydrate, gallium arsenide, molybdenum trioxide, vanadium pentoxide, and nickel sulfate heptahydrate) in the SHE cell transformation assay. The cobalt compound induced morphological transformation in a 24-hour exposure at five concentrations from 0.125 to 1 µg/mL ( $P < 0.05$ , Fisher's exact test); the highest concentration caused 66% cytotoxicity. The exposure-response trend test was not significant ( $P = 0.0739$ , unstratified binomial exact permutation trend test); however, the authors concluded that the overall SHE assay results were positive, based on significant results for at least two concentrations.

Positive results ( $P < 0.05$ , Fisher's exact test) also were reported for nickel sulfate heptahydrate at concentrations of 20 to 50 µg/mL in a 24-hour exposure (Kerckaert *et al.* 1996a).

### 6.4 Possible mechanisms of cobalt-induced carcinogenesis

The mechanisms of cobalt-induced carcinogenesis are not well understood. IARC (1991) did not address the possible mechanism(s) for carcinogenicity of cobalt ions beyond proposing that cobalt(II) ions could decrease the fidelity of DNA polymerase and could damage DNA through generation of reactive oxygen species, to explain the genotoxicity of cobalt compounds.

Lison *et al.* (2001) published an updated review of the information on genotoxicity and carcinogenicity of cobalt compounds, including both ionic and metallic cobalt. They discussed several potential mechanisms for DNA damage specific to cobalt(II) ions, which fell into two general categories: direct mechanisms (induction of DNA breaks) and indirect mechanisms (inhibition of DNA repair systems). Several of the reviewed studies demonstrated that micromolar concentrations of cobalt(II) ions in the presence of hydrogen peroxide could damage isolated DNA through a Fenton-like mechanism with generation of hydroxyl radicals. In addition, cobalt ions were shown to substitute for zinc ions in protein-zinc-finger domains that control the transcription of specific genes, and it was suggested that this substitution could generate DNA-damaging free radicals close to the DNA molecule. Mechanisms proposed for the indirect genotoxic effects of cobalt(II) ions were (1) inhibition of binding of the mammalian damage-recognition protein xeroderma pigmentosum group A protein to DNA by inhibition of binding of magnesium ions to the enzyme or (2) binding of cobalt(II) ions to zinc finger domains of the repair proteins themselves. In addition, binding of p53 protein to DNA is a zinc-dependent process that can be modulated by cobalt (II) ions. Although few of the data on the effects of cobalt(II) ion on DNA damage or inhibition of DNA repair were from studies of cobalt sulfate, Lison (1996) concluded that “it seems reasonable to consider that all soluble cobalt(II) salts (chloride, sulphate, acetate) share this carcinogenic potential.”

Kawanishi *et al.* (1989) demonstrated that cobalt(II) ion catalyzed the autooxidation of sulfite to the sulfate radical that caused DNA damage. Several researchers have reported that the interaction of divalent cobalt and other metal ions with hydrogen peroxide may form oxygen radical species that react with DNA (Nackerdien *et al.* 1991, Beyersmann and Hartwig 1992, Kawanishi *et al.* 1994, Lloyd *et al.* 1998). Nackerdien *et al.* (1991) demonstrated that the DNA base products formed in isolated human chromatin exposed to cobalt sulfate and hydrogen peroxide were consistent with hydroxyl radical formation and concluded that the DNA base damage may contribute to the genotoxicity and carcinogenicity of the divalent cobalt ion. Although both hydroxyl and superoxide radicals were formed by the interaction of divalent cobalt ions and hydrogen peroxide, their role in causing DNA breaks in intact cells was not established (Beyersmann and Hartwig 1992).

Other possible mechanisms of carcinogenesis include effects on DNA synthesis, DNA repair inhibition, oxidative stress, and gene expression changes. Divalent cobalt ions may decrease the fidelity of DNA synthesis by replacing magnesium in DNA polymerases; however, it is not clear whether the high concentrations used *in vitro* are relevant *in vivo*. Cobalt may inhibit DNA repair by replacing magnesium in the polymerization step or by binding to the DNA template and interfering with the polymerase-DNA interaction. (Beyersmann and Hartwig 1992).

Both nickel and cobalt mimic the effects of hypoxia by inducing several genes that are under transcriptional control by hypoxia-inducible factor-1 (HIF-1). Following hypoxia, or exposure to transition metals, HIF-1 $\alpha$  protein is stabilized and accumulates in cells. If HIF-1 transcriptional activity is not induced under hypoxic conditions, tumor cells fail to grow and metastasize (Salnikow *et al.* 1999a, 2000).

Although cobalt exposure produces oxidative stress in cells, which can be substantial, as measured by dichlorofluorescein fluorescence (Salnikow *et al.* 2000), cobalt compounds are only weakly mutagenic (see Section 5 and Kitahara *et al.* 1996). Furthermore, human A549 lung cells exposed to cobalt chloride showed a time- and concentration-dependent increase in reactive oxygen species, which were much lower in A549 cells exposed to nickel chloride. Nevertheless, both cobalt chloride and nickel chloride equally increased upregulation of *Cap43*, an HIF-1-dependent gene (Salnikow *et al.* 2000). Another study showed that increased intracellular calcium levels were essential for *Cap43* upregulation in nickel-exposed cells (Salnikow *et al.* 1999b). The free-radical scavenger 2-mercaptoethanol did not block the increased expression of *Cap43* mRNA induced by cobalt chloride or nickel chloride, even though generation of reactive oxygen species was completely suppressed (Salnikow *et al.* 2000). Therefore, oxidative stress apparently is not involved in HIF-1 induction. These researchers suggested that the signaling cascade responsible for HIF-1 $\alpha$  stabilization and upregulation of *Cap43* could be activated if the iron in the oxygen sensor protein was replaced by cobalt or nickel (Salnikow *et al.* 2000). Carcinogenesis could be related to metal-induced hypoxia-like conditions with subsequent selection for increased HIF-1-dependent transcription (Salnikow *et al.* 1999a).

### 6.5 Cocarcinogenicity of cobalt and Rauscher leukemia virus

Gainer (1973) showed that cobalt sulfate may exert cocarcinogenic effects by activating an oncogenic virus. He studied the interaction between several metal salts and Rauscher leukemia virus (RLV) infection in mice. RLV disease was determined by the development of large spleens containing high titers of virus. Fifteen male CD-1 mice were given drinking water containing a 0.01 M solution of cobalt sulfate beginning at four weeks of age. A control group of 15 mice was not given cobalt sulfate. At six weeks of age, 10 mice in the treatment and control groups were inoculated with RLV. Treatment with cobalt sulfate induced RLV splenomegalies in male CD-1 mice. Spleen weights in the uninoculated mice exposed to cobalt sulfate were not significantly different from those in unexposed controls. Spleens from mice exposed to cobalt sulfate also contained high titers of virus, whereas spleens from virus-injected control mice did not contain virus. The authors speculated that exposure to cobalt sulfate might reduce interferon activity and permit easier replication of virus.

### 6.6 Summary

Cobalt is part of the vitamin B<sub>12</sub> complex. A daily intake of about 50  $\mu$ g is sufficient to meet the nutritional requirement. Occupational exposure to cobalt has been associated with a severe and progressive respiratory disease known as hard-metal pneumoconiosis, as well as asthma and contact dermatitis. In the 1960s, several outbreaks of cardiomyopathy and polycythemia were reported in individuals who drank large quantities of beer containing added cobalt.

Cobalt is absorbed from the gastrointestinal tract, lungs, and skin and is distributed throughout the body. The highest concentrations are found in the liver, kidney, and heart. It is excreted primarily in the urine, but fecal excretion also is important. There are two

distinct elimination phases: the first is rapid and occurs within days of exposure, but the second phase may take several years.

Cobalt ions may mimic or replace other essential divalent metal ions (e.g., magnesium, calcium, iron, copper, or zinc), thus altering many important cellular reactions and functions. There is good evidence that cobalt ions can inhibit DNA repair processes or interact with hydrogen peroxide to form reactive oxygen species that can damage DNA. These mechanisms may contribute to the genotoxic and carcinogenic effects reported for cobalt sulfate and other cobalt compounds.

## 7 References

1. Alexandersson, R. 1988. Blood and urinary concentrations as estimators of cobalt exposure. *Arch Environ Health* 43:299-303.
2. Anderson, J.J.B. 2000. Minerals. In Food, Nutrition, & Diet Therapy. Mahan, L.K. and S. Escott-Stump, eds. W. B. Saunders Company, Philadelphia. pp. 110-152.
3. ATSDR. 1992. Toxicological Profile for Cobalt. Agency for Toxic Substances and Disease Registry, U.S. Public Health Service. 140 pp.
4. Barberá, R. and R. Farré. 1988. Determination of cobalt in foods by flame and electrothermal atomization-atomic absorption spectrometry. A comparative study. *At Spectrosc* 9:6-8.
5. Beyersmann, D. and A. Hartwig. 1992. The genetic toxicology of cobalt. *Toxicol Appl Pharmacol* 115:137-145.
6. Bouman, A.A., A.J. Platenkamp, and F.D. Posma. 1986. Determination of cobalt in urine by flameless atomic absorption spectroscopy. Comparison of direct analysis using Zeeman background correction and indirect analysis using extraction in organic solution. *Ann Clin Biochem* 23:346-350.
7. Bucher, J.R., M.R. Elwell, M.B. Thompson, B.J. Chou, R. Renne, and H.A. Ragan. 1990. Inhalation toxicity studies of cobalt sulfate in F344/N rats and B6C3F1 mice. *Fundam Appl Toxicol* 15:357-372.
8. Bucher, J.R., J.R. Hailey, J.R. Roycroft, J.K. Haseman, R.C. Sills, S.L. Grumbein, P.W. Mellick, and B.J. Chou. 1999. Inhalation toxicity and carcinogenicity studies of cobalt sulfate. *Toxicol Sci* 49:56-67.
9. Budavari, S., M.J. O'Neil, A. Smith, P.E. Heckelman, and J.F. Kinneary. 1996. The Merck Index. Merck & Co., Inc., Whitehouse Station, NJ. pp. 414.
10. CDC. 2001. National Report on Human Exposure to Environmental Chemicals: Cobalt. Centers for Disease Control and Prevention, National Center for Environmental Health, Division of Laboratory Sciences. Available at (<http://www.cdc.gov/nceh/dls/report/results/cobalt.htm>).
11. Chem Sources. 2001. Chemical Sources International. Available at (<http://www.chemsources.com> and search 10124-43-3 or 10026-24-1).
12. ChemFinder. 2001. Cobalt sulfate. CambridgeSoft. Available at (<http://chemfinder.cambridgesoft.com> and search 10124-43-3).
13. Christensen, J.M. and O.M. Poulsen. 1994. A 1982-1992 surveillance programme on Danish pottery painters. Biological levels and health effects following exposure

- to soluble or insoluble cobalt compounds in cobalt blue dyes. *Sci Total Environ* 150:95-104.
14. Collecchi, P., M. Esposito, S. Brera, E. Mora, A. Mazzucotelli, and M. Oddone. 1986. The distribution of arsenic and cobalt in patients with laryngeal carcinoma. *J Appl Toxicol* 6:287-289.
  15. Considine, D.M. and G.D. Considine. 1995. Van Nostrand's Scientific Encyclopedia, Eighth Edition. Considine, D.M. and G.D. Considine eds. Van Nostrand Reinhold, New York, pp. 728-730.
  16. Davis, R.E., H.C. Metcalfe, J.E. Williams, and J.F. Castka. 1999. Modern Chemistry. Holt, Rinehart and Winston, Austin, TX. pp. 405
  17. Duerksen-Hughes, P.J., J. Yang, and O. Ozcan. 1999. p53 induction as a genotoxic test for twenty-five chemicals undergoing *in vivo* carcinogenicity testing. *Environ Health Perspect* 107:805-812.
  18. EPA. 1999. Background Report on Fertilizer Use, Contaminants and Regulations. Office of Pollution Prevention and Toxics, U.S. Environmental Protection Agency, Washington, DC. Available at ([www.epa.gov/opptintr/fertilizer.pdf](http://www.epa.gov/opptintr/fertilizer.pdf)).
  19. Gainer, J.H. 1973. Activation of the Rauscher leukemia virus by metals. *J Natl Cancer Inst* 51:609-613.
  20. Gibson, D.P., R. Brauninger, H.S. Shaffi, G.A. Kerckaert, R.A. LeBoeuf, R.J. Isfort, and M.J. Aardema. 1997. Induction of micronuclei in Syrian hamster embryo cells: comparison to results in the SHE cell transformation assay for National Toxicology Program test chemicals. *Mutat Res* 392:61-70.
  21. Goodman, L.S. and A.G. Gilman. 2001. Goodman & Gilman's The Pharmacological Basis of Therapeutics, Tenth Edition. Hardman, J.G., L.E. Limbird and A.G. Gilman eds. McGraw-Hill, New York.
  22. Hansson, H.C., P. Ekholm A-K, and H.B. Ross. 1988. Rainwater analysis: a comparison between proton-induced x-ray emission and graphite furnace atomic absorption spectroscopy. *Environ Sci Technol* 22:527-531.
  23. Heinrich, R. and J. Angerer. 1984. Determination of cobalt in biological materials by voltammetry and electrothermal atomic absorption spectrometry. *Int J Environ Anal Chem* 16:305-314.
  24. Hillman, R.S. and C.A. Finch. 1985. Drugs effective in iron-deficiency and other hypochromic anemias. In Goodman & Gilman's The Pharmacological Basis of Therapeutics, Seventh Edition. Gilman, A.G., L.S. Goodman, T.W. Rall and F. Murad, eds. Macmillan Publishing Company, New York. pp. 1308-1322.

25. Hogstedt, C. and R. Alexandersson. 1990. [Mortality among hard-metal workers]. *Arbete Hälsa* 21:1-26.
26. HSDB. 2000. Cobaltous Sulfate. Revised February 8, 2000. Last reviewed August 23, 1989. Hazardous Substances Data Bank, National Library of Medicine, Bethesda, MD. Available at (<http://toxnet.nlm.nih.gov/cgi-bin/sis/htm.gen?HSDB> and search 10124-43-3 and select Cobaltous Sulfate in search results).
27. IARC. 1991. Chlorinated Drinking-Water: Chlorination By-products; Some Other Halogenated Compounds: Cobalt and Cobalt Compounds, Vol. 52. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. World Health Organization, International Agency for Research on Cancer, Lyon, France. 363-472 pp.
28. Ichikawa, Y., Y. Kusaka, and S. Goto. 1985. Biological monitoring of cobalt exposure, based on cobalt concentrations in blood and urine. *Int Arch Occup Environ Health* 55:269-276.
29. Jensen, A.A. and F. Tüchsen. 1990. Cobalt exposure and cancer risk. *Crit Rev Toxicol* 20:427-437.
30. Kawanishi, S., K. Yamamoto, and S. Inoue. 1989. Site-specific DNA damage induced by sulfite in the presence of cobalt(II) ion. Role of sulfate radical. *Biochem Pharmacol* 38:3491-3496.
31. Kawanishi, S., S. Inoue, and K. Yamamoto. 1994. Active oxygen species in DNA damage induced by carcinogenic metal compounds. *Environ Health Perspect* 102 Suppl 3:17-20.
32. Kazantzis, G. 1981. Role of cobalt, iron, lead, manganese, mercury, platinum, selenium, and titanium in carcinogenesis. *Environ Health Perspect* 40:143-161.
33. Kerckaert, G.A., R.A. LeBoeuf, and R.J. Isfort. 1996a. Use of the Syrian hamster embryo cell transformation assay for determining the carcinogenic potential of heavy metal compounds. *Fundam Appl Toxicol* 34:67-72.
34. Kerckaert, G.A., R. Brauninger, R.A. LeBoeuf, and R.J. Isfort. 1996b. Use of the Syrian hamster embryo cell transformation assay for carcinogenicity prediction of chemicals currently being tested by the National Toxicology Program in rodent bioassays. *Environ Health Perspect* 104 Suppl 5:1075-1084.
35. Kirk, R.E. and D.F. Othmer. 1999. Kirk-Othmer Encyclopedia of Chemical Technology. Herman, F., J.J. McKetta, D.F. Othmer and A. Standen eds. Wiley, New York, pp. 482-484.

36. Kitahara, J., K. Yamanaka, K. Kato, Y.W. Lee, C.B. Klein, and M. Costa. 1996. Mutagenicity of cobalt and reactive oxygen producers. *Mutat Res* 370:133-140.
37. Lasfargues, G., P. Wild, J.J. Moulin, B. Hammon, B. Rosmorduc, C. Rondeau du Noyer, M. Lavandier, and J. Moline. 1994. Lung cancer mortality in a French cohort of hard-metal workers. *Am J Ind Med* 26:585-595.
38. Lauwerys, R. and D. Lison. 1994. Health risks associated with cobalt exposure--an overview. *Sci Total Environ* 150:1-6.
39. Léonard, A. and R. Lauwerys. 1990. Mutagenicity, carcinogenicity and teratogenicity of cobalt metal and cobalt compounds. *Mutat Res* 239:17-27.
40. Lide, D.R. 1999. CRC Handbook of Chemistry and Physics, 1999-2000. Lide, D.R. ed. CRC Press, Boca Raton.
41. Lison, D. 1996. Human toxicity of cobalt-containing dust and experimental studies on the mechanism of interstitial lung disease (hard metal disease). *Critical Reviews in Toxicology* 26:585-616.
42. Lison, D., M. De Boeck, V. Verougstraete, and M. Kirsch-Volders. 2001. Update on the genotoxicity and carcinogenicity of cobalt compounds. *Occup Environ Med* 58:619-625.
43. Lloyd, D.R., D.H. Phillips, and P.L. Carmichael. 1997. Generation of putative intrastrand cross-links and strand breaks in DNA by transition metal ion-mediated oxygen radical attack. *Chem Res Toxicol* 10:393-400.
44. Lloyd, D.R., P.L. Carmichael, and D.H. Phillips. 1998. Comparison of the formation of 8-hydroxy-2'-deoxyguanosine and single- and double-strand breaks in DNA mediated by Fenton reactions. *Chem Res Toxicol* 11:420-427.
45. Moulin, J.J., P. Wild, J.M. Mur, M. Fournier-Betz, and M. Mercier-Gallay. 1993. A mortality study of cobalt production workers: an extension of the follow-up. *Am J Ind Med* 23:281-288.
46. Moulin, J.J., P. Wild, S. Romazini, G. Lasfargues, A. Peltier, C. Bozec, P. Deguerry, F. Pellet, and A. Perdrix. 1998. Lung cancer risk in hard-metal workers. *Am J Epidemiol* 148:241-248.
47. Mur, J.M., J.J. Moulin, M.P. Charruyer-Seinerra, and J. Lafitte. 1987. A cohort mortality study among cobalt and sodium workers in an electrochemical plant. *Am J Ind Med* 11:75-81.

48. Nackerdien, Z., K.S. Kasprzak, G. Rao, B. Halliwell, and M. Dizdaroglu. 1991. Nickel(II)-and cobalt(II)-dependent damage by hydrogen peroxide to the DNA bases in isolated human chromatin. *Cancer Res* 51:5837-5842.
49. NTP. 1991. Toxicity Studies of Cobalt Sulfate Heptahydrate in F344/N Rats and B6C3F<sub>1</sub> Mice (Inhalation). National Toxicology Program, NTP Tox 5. 38 pp.
50. NTP. 1998. Toxicology and Carcinogenesis Studies of Cobalt Sulfate Heptahydrate in F344/N Rats and B6C3F<sub>1</sub> Mice (Inhalation Studies). National Toxicology Program TR No 471. 471 pp.
51. Olivero, S., P. Villani, and A. Botta. 1995. Genotoxic effects of cobalt chloride, sulfate and nitrate on cultured human lymphocytes. *Med Sci Res* 23:339-341.
52. OSHA. 1998. Cobalt, Metal, Dust & Fume (as Co). Revised November 19, 1998. Occupational Safety & Health Administration. U.S. Department of Labor. Available at ([http://www.osha-slc.gov/dts/chemicalsampling/data/CH\\_229100.html](http://www.osha-slc.gov/dts/chemicalsampling/data/CH_229100.html)).
53. Rogers, M.A., D.B. Thomas, S. Davis, T.L. Vaughan, and A.E. Nevissi. 1993. A case-control study of element levels and cancer of the upper aerodigestive tract. *Cancer Epidemiol Biomarkers Prev* 2:305-312.
54. RTECS. 2001. Cobalt(II) sulfate (1:1). Registry of Toxic Effects of Chemical Substances, National Institute for Occupational Safety and Health of the U.S. Department of Health and Human Services. Available at (<http://tomescps.com> and search Cobalt Sulfate, available to registered users only).
55. Sabbioni, E., C. Minoia, R. Pietra, G. Mosconi, A. Forni, and G. Scansetti. 1994. Metal determinations in biological specimens of diseased and non-diseased hard metal workers. *Sci Total Environ* 150:41-54.
56. Salnikow, K., W.G. An, G. Melillo, M.V. Blagosklonny, and M. Costa. 1999a. Nickel-induced transformation shifts the balance between HIF-1 and p53 transcription factors. *Carcinogenesis* 20:1819-1823.
57. Salnikow, K., T. Kluz, and M. Costa. 1999b. Role of Ca<sup>2+</sup> in the regulation of nickel-inducible *Cap43* gene expression. *Toxicol Appl Pharmacol* 160:127-132.
58. Salnikow, K., W. Su, M.V. Blagosklonny, and M. Costa. 2000. Carcinogenic metals induce hypoxia-inducible factor-stimulated transcription by reactive oxygen species-independent mechanism. *Cancer Res* 60:3375-3378.
59. Scansetti, G., G.C. Botta, P. Spinelli, L. Reviglione, and C. Ponzetti. 1994. Absorption and excretion of cobalt in the hard metal industry. *Sci Total Environ* 150:141-144.

- 
60. Shedd, K.B. 1999. Cobalt. Revised August 30, 2000. U.S. Geological Survey Minerals Yearbook. Available at (<http://minerals.usgs.gov/minerals/pubs/commodity/myb/> and select Cobalt).
  61. Smith, T., C.J. Edmonds, and C.F. Barnaby. 1972. Absorption and retention of cobalt in man by whole-body counting. *Health Phys* 22:359-367.
  62. Sunderman, F.W., Jr., S.M. Hopfer, T. Swift, W.N. Rezuze, L. Ziebka, P. Highman, B. Edwards, M. Folcik, and H.R. Gossling. 1989. Cobalt, chromium, and nickel concentrations in body fluids of patients with porous-coated knee or hip prostheses. *J Orthop Res* 7:307-315.
  63. Tüchsen, F., M.V. Jensen, E. Villadsen, and E. Lynge. 1996. Incidence of lung cancer among cobalt-exposed women. *Scand J Work Environ Health* 22:444-450.
  64. USGS. 2001. Mineral Industry Surveys. U.S. Department of the Interior. Available at (<http://minerals.usgs.gov/minerals/pubs/commodity/cobalt/21000501.pdf>).
  65. Washington State. 1999. Screening Survey for Metals and Dioxins in Fertilizer Products and Soils in Washington State. Washington State Department of Ecology. Available at ([www.ecy.wa.gov/pubs/99309.pdf](http://www.ecy.wa.gov/pubs/99309.pdf)).
  66. WebElements. 2001. Cobalt. The University of Sheffield and WebElements Ltd, UK. Available at (<http://www.webelements.com/webelements/elements/text/Co/comp.html>).
  67. Wild, P., A. Perdrix, S. Romazini, J.J. Moulin, and F. Pellet. 2000. Lung cancer mortality in a site producing hard metals. *Occup Environ Med* 57:568-573.
  68. Zeiger, E., B. Anderson, S. Haworth, T. Lawlor, and K. Mortelmans. 1992. Salmonella mutagenicity tests: V. Results from the testing of 311 chemicals. *Environ Mol Mutagen* 19 Suppl 21:2-141.

**Appendix A: IARC (1991). Monographs on the Evaluation of Carcinogenic Risks to Humans. Chlorinated Drinking-Water: Chlorination By-products; Some Other Halogenated Compounds: Cobalt and Cobalt Compounds. V 52. PP A-1 – A-112.**



**Appendix B: NTP (1998). Toxicology and Carcinogenesis Studies of Cobalt Sulfate Heptahydrate in F344/N Rats and B6C3F<sub>1</sub> Mice (Inhalation Studies). TR No. 471. PP B-1 – B-59.**



**NTP TECHNICAL REPORT**  
**ON THE**  
**TOXICOLOGY AND CARCINOGENESIS**  
**STUDIES OF**  
**COBALT SULFATE HEPTAHYDRATE**  
**(CAS NO. 10026-24-1)**  
**IN F344/N RATS AND B6C3F<sub>1</sub> MICE**  
**(INHALATION STUDIES)**

**NATIONAL TOXICOLOGY PROGRAM**  
**P.O. Box 12233**  
**Research Triangle Park, NC 27709**

**August 1998**

**NTP TR 471**

**NIH Publication No. 98-3961**

**U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES**  
**Public Health Service**  
**National Institutes of Health**

## FOREWORD

The National Toxicology Program (NTP) is made up of four charter agencies of the U.S. Department of Health and Human Services (DHHS): the National Cancer Institute (NCI), National Institutes of Health; the National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health; the National Center for Toxicological Research (NCTR), Food and Drug Administration; and the National Institute for Occupational Safety and Health (NIOSH), Centers for Disease Control. In July 1981, the Carcinogenesis Bioassay Testing Program, NCI, was transferred to the NIEHS. The NTP coordinates the relevant programs, staff, and resources from these Public Health Service agencies relating to basic and applied research and to biological assay development and validation.

The NTP develops, evaluates, and disseminates scientific information about potentially toxic and hazardous chemicals. This knowledge is used for protecting the health of the American people and for the primary prevention of disease.

The studies described in this Technical Report were performed under the direction of the NIEHS and were conducted in compliance with NTP laboratory health and safety requirements and must meet or exceed all applicable federal, state, and local health and safety regulations. Animal care and use were in accordance with the Public Health Service Policy on Humane Care and Use of Animals. The prechronic and chronic studies were conducted in compliance with Food and Drug Administration (FDA) Good Laboratory Practice Regulations, and all aspects of the chronic studies were subjected to retrospective quality assurance audits before being presented for public review.

These studies are designed and conducted to characterize and evaluate the toxicologic potential, including carcinogenic activity, of selected chemicals in laboratory animals (usually two species, rats and mice). Chemicals selected for NTP toxicology and carcinogenesis studies are chosen primarily on the bases of human exposure, level of production, and chemical structure. The interpretive conclusions presented in this Technical Report are based only on the results of these NTP studies. Extrapolation of these results to other species and quantitative risk analyses for humans require wider analyses beyond the purview of these studies. Selection *per se* is not an indicator of a chemical's carcinogenic potential.

Listings of all published NTP reports and ongoing studies are available from NTP Central Data Management, NIEHS, P.O. Box 12233, MD E1-02, Research Triangle Park, NC 27709 (919-541-3419). The Abstracts and other study information for 2-year studies are also available at the NTP's World Wide Web site: <http://ntp-server.niehs.nih.gov>.

**NTP TECHNICAL REPORT**  
**ON THE**  
**TOXICOLOGY AND CARCINOGENESIS**  
**STUDIES OF**  
**COBALT SULFATE HEPTAHYDRATE**  
**(CAS NO. 10026-24-1)**  
**IN F344/N RATS AND B6C3F<sub>1</sub> MICE**  
**(INHALATION STUDIES)**

**NATIONAL TOXICOLOGY PROGRAM**  
**P.O. Box 12233**  
**Research Triangle Park, NC 27709**

**August 1998**

**NTP TR 471**

**NIH Publication No. 98-3961**

**U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES**  
**Public Health Service**  
**National Institutes of Health**

## CONTRIBUTORS

### National Toxicology Program

*Evaluated and interpreted results and reported findings*

J.R. Bucher, Ph.D., Study Scientist  
 D.A. Bridge, B.S.  
 R.E. Chapin, Ph.D.  
 J.R. Hailey, D.V.M.  
 J.K. Haseman, Ph.D.  
 R.R. Maronpot, D.V.M.  
 G.N. Rao, D.V.M., Ph.D.  
 J.H. Roycroft, Ph.D.  
 C.S. Smith, Ph.D.  
 G.S. Travlos, D.V.M.  
 D.B. Walters, Ph.D.  
 K.L. Witt, M.S., Oak Ridge Associated Universities

### Battelle Pacific Northwest Laboratories

*Conducted studies, evaluated pathology findings*

B.J. Chou, D.V.M., Ph.D., Principal Investigator  
 J.A. Dill, Ph.D.  
 S.L. Grumbein, D.V.M., Ph.D.  
 P.W. Mellick, D.V.M., Ph.D.  
 S.E. Rowe, D.V.M., M.S.

### Experimental Pathology Laboratories, Inc.

*Provided pathology quality assurance*

J.F. Hardisty, D.V.M., Principal Investigator  
 M.R. Elwell, D.V.M., Ph.D.  
 C.C. Shackelford, D.V.M., M.S., Ph.D.

### Dynamac Corporation

*Prepared quality assurance audits*

S. Brecher, Ph.D., Principal Investigator

### NTP Pathology Working Group

*Evaluated slides, prepared pathology report on rats  
 (26 April 1996)*

M.P. Jokinen, D.V.M., Chairperson  
 Pathology Associates International  
 D. Dixon, D.V.M., Ph.D.  
 National Toxicology Program  
 J. Everitt, D.V.M.  
 Chemical Industry Institute of Toxicology  
 S.L. Grumbein, D.V.M., Ph.D.  
 Battelle Pacific Northwest Laboratories  
 F.F. Hahn, D.V.M., Ph.D.  
 IIT Research Institute  
 J.R. Hailey, D.V.M.  
 National Toxicology Program  
 R.A. Herbert, D.V.M., Ph.D.  
 National Toxicology Program  
 R.R. Maronpot, D.V.M.  
 National Toxicology Program  
 A. Nyska, D.V.M.  
 National Toxicology Program  
 C.C. Shackelford, D.V.M., M.S., Ph.D.  
 Experimental Pathology Laboratories, Inc.

*Evaluated slides, prepared pathology report on mice  
 (29 February 1996)*

M.P. Jokinen, D.V.M., Chairperson  
 Pathology Associates International  
 D. Dixon, D.V.M., Ph.D.  
 National Toxicology Program  
 M.R. Elwell, D.V.M., Ph.D.  
 Experimental Pathology Laboratories, Inc.  
 J. Everitt, D.V.M.  
 Chemical Industry Institute of Toxicology  
 J.R. Hailey, D.V.M.  
 National Toxicology Program  
 R.A. Herbert, D.V.M., Ph.D.  
 National Toxicology Program  
 R.R. Maronpot, D.V.M.  
 National Toxicology Program  
 A. Nyska, D.V.M.  
 National Toxicology Program  
 A. Radovsky, D.V.M., Ph.D.  
 National Toxicology Program

**Analytical Sciences, Inc.**

*Provided statistical analyses*

R.W. Morris, M.S., Principal Investigator

S.R. Lloyd, M.S.

N.G. Mintz, B.S.

**Biotechnical Services, Inc.**

*Prepared Technical Report*

S.R. Gunnels, M.A., Principal Investigator

L.M. Harper, B.S.

A.M. Macri-Hanson, M.A., M.F.A.

# CONTENTS

<b>ABSTRACT</b>		<b>5</b>
<b>EXPLANATION OF LEVELS OF EVIDENCE OF CARCINOGENIC ACTIVITY</b>		<b>10</b>
<b>TECHNICAL REPORTS REVIEW SUBCOMMITTEE</b>		<b>11</b>
<b>SUMMARY OF TECHNICAL REPORTS REVIEW SUBCOMMITTEE COMMENTS</b>		<b>12</b>
<b>INTRODUCTION</b>		<b>13</b>
<b>MATERIALS AND METHODS</b>		<b>21</b>
<b>RESULTS</b>		<b>29</b>
<b>DISCUSSION AND CONCLUSIONS</b>		<b>51</b>
<b>REFERENCES</b>		<b>55</b>
<b>APPENDIX A</b>	<b>Summary of Lesions in Male Rats in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate</b>	<b>63</b>
<b>APPENDIX B</b>	<b>Summary of Lesions in Female Rats in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate</b>	<b>105</b>
<b>APPENDIX C</b>	<b>Summary of Lesions in Male Mice in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate</b>	<b>139</b>
<b>APPENDIX D</b>	<b>Summary of Lesions in Female Mice in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate</b>	<b>173</b>
<b>APPENDIX E</b>	<b>Genetic Toxicology</b>	<b>209</b>
<b>APPENDIX F</b>	<b>Chemical Characterization and Generation of Chamber Concentrations</b>	<b>213</b>
<b>APPENDIX G</b>	<b>Ingredients, Nutrient Composition, and Contaminant Levels in NIH-07 Rat and Mouse Ration</b>	<b>225</b>
<b>APPENDIX H</b>	<b>Sentinel Animal Program</b>	<b>229</b>
<b>APPENDIX I</b>	<b>K-ras Mutation Frequency and Spectra in Lung Neoplasms from B6C3F<sub>1</sub> Mice Exposed to Cobalt Sulfate Heptahydrate for 2 Years</b>	<b>233</b>
<b>APPENDIX J</b>	<b>Impact of <i>Helicobacter Hepaticus</i> Infection in B6C3F<sub>1</sub> Mice from 12 NTP 2-Year Carcinogenesis Studies</b>	<b>241</b>

## ABSTRACT



### COBALT SULFATE HEPTAHYDRATE

CAS No. 10026-24-1

Molecular Weight: 281.13

**Synonyms:** Bieberite; cobalt(II) sulfate (1:1) heptahydrate; cobalt monosulfate heptahydrate; cobalt(II) sulphate heptahydrate; sulfuric acid, cobalt(2+) salt (1:1) heptahydrate

Cobalt sulfate is used in the electroplating and electrochemical industries. It is also used as a coloring agent for ceramics and as a drying agent in inks, paints, varnishes, and linoleum. Cobalt sulfate may be added to animal feed as a mineral supplement and has been used as a top dressing on pasture lands. Cobalt sulfate was nominated by the National Cancer Institute for study based on a lack of information on the toxicity of soluble salts. Male and female F344/N rats and B6C3F<sub>1</sub> mice were exposed to cobalt sulfate heptahydrate (approximately 99% pure) by inhalation for 2 years. Genetic toxicology studies were conducted in *Salmonella typhimurium*. The results of prechronic inhalation toxicity studies were reported previously (Bucher *et al.*, 1990; NTP, 1991).

#### 2-YEAR STUDY IN RATS

Groups of 50 male and 50 female rats were exposed to aerosols containing 0, 0.3, 1.0, or 3.0 mg/m<sup>3</sup> cobalt sulfate heptahydrate 6 hours per day, 5 days per week, for 105 weeks.

#### ***Survival and Body Weights***

Survival of exposed males and females was similar to that of the chamber controls. Mean body weights of exposed male and female rats were similar to those of the chamber controls throughout the study.

#### ***Pathology Findings***

The incidences and severities of proteinosis, alveolar epithelial metaplasia, granulomatous alveolar inflammation, and interstitial fibrosis were markedly greater in all exposed groups of male and female rats than in the chamber controls. The incidences of alveolar epithelial hyperplasia in all groups of exposed males and in females exposed to 3.0 mg/m<sup>3</sup> were significantly greater than those in the chamber control groups, as were the incidences of squamous metaplasia in 1.0 mg/m<sup>3</sup> females and atypical alveolar epithelial hyperplasia in 3.0 mg/m<sup>3</sup> females. In 3.0 mg/m<sup>3</sup> males, the combined incidence of alveolar/bronchiolar neoplasms (adenoma and/or carcinoma) was significantly greater than in the chamber controls. In female rats exposed to 1.0 or 3.0 mg/m<sup>3</sup>, the

incidences of alveolar/bronchiolar neoplasms were significantly greater than those in the chamber control group and exceeded the NTP historical control ranges. A squamous cell carcinoma was observed in one 1.0 mg/m<sup>3</sup> and one 3.0 mg/m<sup>3</sup> female.

The incidences of benign, complex, or malignant pheochromocytoma (combined) in 1.0 mg/m<sup>3</sup> males and in 3.0 mg/m<sup>3</sup> females were significantly greater than those in the chamber controls and exceeded the historical control ranges.

Hyperplasia of the lateral wall of the nose, atrophy of the olfactory epithelium, and squamous metaplasia of the epiglottis were observed in all exposed groups of males and females, and the severities of these lesions increased with increasing exposure concentration. The incidences of squamous metaplasia of the lateral wall of the nose and metaplasia of the olfactory epithelium were increased in 3.0 mg/m<sup>3</sup> males and females.

## 2-YEAR STUDY IN MICE

Groups of 50 male and 50 female mice were exposed to aerosols containing 0, 0.3, 1.0, or 3.0 mg/m<sup>3</sup> cobalt sulfate heptahydrate 6 hours per day, 5 days per week, for 105 weeks.

### ***Survival and Body Weights***

Survival of exposed males and females was similar to that of the chamber controls. Mean body weights of 3.0 mg/m<sup>3</sup> male mice were less than those of the chamber controls from week 96 until the end of the study. The mean body weights of all exposed groups of female mice were generally greater than those of the chamber controls from week 20 until the end of the study.

### ***Pathology Findings***

The incidences of diffuse histiocytic cell infiltration in 3.0 mg/m<sup>3</sup> males and of focal histiocytic cell infiltration in 3.0 mg/m<sup>3</sup> females were significantly greater than those in the chamber controls. The incidences of alveolar/bronchiolar neoplasms in 3.0 mg/m<sup>3</sup> males and females were significantly greater than those in the chamber control groups. The combined incidences

of alveolar/bronchiolar adenoma or carcinoma and the incidences of alveolar/bronchiolar carcinoma in 3.0 mg/m<sup>3</sup> males and females and the incidence of alveolar/bronchiolar adenoma in 3.0 mg/m<sup>3</sup> females exceeded the NTP historical control ranges for inhalation studies.

The incidences of atrophy of the olfactory epithelium in 1.0 and 3.0 mg/m<sup>3</sup> males and females and hyperplasia of the olfactory epithelium in 3.0 mg/m<sup>3</sup> males and females were significantly greater than in the chamber controls. Squamous metaplasia of the larynx was observed in all exposed groups of males and females.

Male mice had a pattern of nonneoplastic liver lesions along with silver-staining helical organisms within the liver, characteristic of an infection with *Helicobacter hepaticus*. In NTP studies with *H. hepaticus*-associated hepatitis, increased incidences of heman-giosarcoma were seen in the liver of male mice. In this study of cobalt sulfate heptahydrate, incidences of hemangiosarcoma were increased in exposed groups of male mice. Because of the above association, interpretation of the increased incidences of hemangiosarcoma in the livers of male mice was confounded. Incidences of lesions at other sites in this study of cobalt sulfate heptahydrate were not considered to have been significantly impacted by the infection with *H. hepaticus* or its associated hepatitis.

## GENETIC TOXICOLOGY

Cobalt sulfate heptahydrate was mutagenic in *S. typhimurium* strain TA100 with and without liver S9 metabolic activation enzymes; no mutagenic activity was detected in strain TA98 or TA1535, with or without S9.

## CONCLUSIONS

Under the conditions of these 2-year inhalation studies, there was *some evidence of carcinogenic activity\** of cobalt sulfate heptahydrate in male F344/N rats based on increased incidences of alveolar/bronchiolar neoplasms. Marginal increases in incidences of pheochromocytomas of the adrenal

medulla may have been related to exposure to cobalt sulfate heptahydrate. There was *clear evidence of carcinogenic activity* in female F344/N rats based on increased incidences of alveolar/bronchiolar neoplasms and pheochromocytomas of the adrenal medulla in groups exposed to cobalt sulfate heptahydrate. There was *clear evidence of carcinogenic activity* of cobalt sulfate heptahydrate in male and female

B6C3F<sub>1</sub> mice based on increased incidences of alveolar/bronchiolar neoplasms.

Exposure to cobalt sulfate heptahydrate caused a spectrum of inflammatory, fibrotic, and proliferative lesions in the respiratory tract of male and female rats and mice.

---

\* Explanation of Levels of Evidence of Carcinogenic Activity is on page 10. A summary of the Technical Reports Review Subcommittee comments and the public discussion on this Technical Report appears on page 12.

**Summary of the 2-Year Carcinogenesis and Genetic Toxicology Studies of Cobalt Sulfate Heptahydrate**

	<b>Male F344/N Rats</b>	<b>Female F344/N Rats</b>	<b>Male B6C3F<sub>1</sub> Mice</b>	<b>Female B6C3F<sub>1</sub> Mice</b>
<b>Concentrations</b>	Chamber control, 0.3, 1.0, or 3.0 mg/m <sup>3</sup>	Chamber control, 0.3, 1.0, or 3.0 mg/m <sup>3</sup>	Chamber control, 0.3, 1.0, or 3.0 mg/m <sup>3</sup>	Chamber control, 0.3, 1.0, or 3.0 mg/m <sup>3</sup>
<b>Body weights</b>	Exposed groups similar to chamber controls	Exposed groups similar to chamber controls	3.0 mg/m <sup>3</sup> group slightly less than chamber controls	Exposed groups slightly greater than chamber controls
<b>Survival rates</b>	17/50, 15/50, 21/50, 15/50	28/50, 25/49, 26/50, 30/50	22/50, 31/50, 24/50, 20/50	34/50, 37/50, 32/50, 28/50
<b>Nonneoplastic effects</b>	<p><u>Lung</u>: proteinosis (0/50, 16/50, 40/48, 47/50); alveolar epithelial metaplasia (0/50, 50/50, 48/48, 49/50); granulomatous alveolar inflammation (2/50, 50/50, 48/48, 50/50); interstitial fibrosis (1/50, 50/50, 48/48, 49/50); alveolar epithelial hyperplasia (9/50, 20/50, 20/48, 23/50)</p> <p><u>Nose</u>: lateral wall hyperplasia (2/50, 14/50, 21/49, 20/50); olfactory epithelial atrophy (8/50, 24/50, 42/49, 48/50); lateral wall squamous metaplasia (1/50, 3/50, 5/49, 8/50); olfactory epithelial metaplasia (5/50, 1/50, 5/49, 30/50)</p> <p><u>Larynx</u>: epiglottis squamous metaplasia (0/50, 10/49, 37/48, 50/50)</p>	<p><u>Lung</u>: proteinosis (0/50, 36/49, 49/50, 49/50); alveolar epithelial metaplasia (2/50, 47/49, 50/50, 49/50); granulomatous alveolar inflammation (9/50, 47/49, 50/50, 49/50); interstitial fibrosis (7/50, 47/49, 50/50, 49/50); alveolar epithelial hyperplasia (15/50, 7/49, 20/50, 33/50); squamous metaplasia (0/50, 1/49, 8/50, 3/50); atypical alveolar epithelial hyperplasia (0/50, 0/49, 3/50, 5/50)</p> <p><u>Nose</u>: lateral wall hyperplasia (1/50, 8/49, 26/50, 38/50); olfactory epithelial atrophy (5/50, 29/49, 46/50, 47/50); lateral wall squamous metaplasia (1/50, 1/49, 4/50, 10/50); olfactory epithelial metaplasia (2/50, 2/49, 3/50, 40/50)</p> <p><u>Larynx</u>: epiglottis squamous metaplasia (1/50, 22/49, 39/50, 48/50)</p>	<p><u>Lung</u>: diffuse histiocytic cell infiltrate (1/50, 2/50, 4/50, 10/50)</p> <p><u>Nose</u>: olfactory epithelial atrophy (0/50, 0/50, 29/48, 48/49); olfactory epithelial hyperplasia (0/50, 0/50, 0/48, 10/49)</p> <p><u>Larynx</u>: squamous metaplasia (0/48, 37/49, 48/48, 44/49)</p>	<p><u>Lung</u>: focal histiocytic cell infiltrate (2/50, 5/50, 7/50, 10/50)</p> <p><u>Nose</u>: olfactory epithelial atrophy (0/50, 2/50, 12/49, 46/48); olfactory epithelial hyperplasia (0/50, 0/50, 0/49, 30/48)</p> <p><u>Larynx</u>: squamous metaplasia (0/50, 45/49, 40/47, 50/50)</p>

---

**Summary of the 2-Year Carcinogenesis and Genetic Toxicology Studies of Cobalt Sulfate Heptahydrate**


---

	<b>Male F344/N Rats</b>	<b>Female F344/N Rats</b>	<b>Male B6C3F<sub>1</sub> Mice</b>	<b>Female B6C3F<sub>1</sub> Mice</b>
<b>Neoplastic effects</b>	<u>Lung</u> : alveolar/bronchiolar adenoma (1/50, 4/50, 1/48, 6/50); alveolar/bronchiolar carcinoma (0/50, 0/50, 3/48, 1/50); alveolar/bronchiolar adenoma or carcinoma (1/50, 4/50, 4/48, 7/50)	<u>Lung</u> : alveolar/bronchiolar adenoma (0/50, 1/49, 10/50, 9/50); alveolar/bronchiolar carcinoma (0/50, 2/49, 6/50, 6/50); alveolar/bronchiolar adenoma, alveolar/bronchiolar carcinoma, or squamous cell carcinoma (0/50, 3/49, 16/50, 16/50)  <u>Adrenal medulla</u> : benign, complex, or malignant pheochromocytoma (2/48, 1/49, 4/50, 10/48)	<u>Lung</u> : alveolar/bronchiolar adenoma (9/50, 12/50, 13/50, 18/50); alveolar/bronchiolar carcinoma (4/50, 5/50, 7/50, 11/50); alveolar/bronchiolar adenoma or carcinoma (11/50, 14/50, 19/50, 28/50)	<u>Lung</u> : alveolar/bronchiolar adenoma (3/50, 6/50, 9/50, 10/50); alveolar/bronchiolar carcinoma (1/50, 1/50, 4/50, 9/50); alveolar/bronchiolar adenoma or carcinoma (4/50, 7/50, 13/50, 18/50)
<b>Uncertain findings</b>	<u>Adrenal medulla</u> : benign, complex, or malignant pheochromocytoma (15/50, 19/50, 25/49, 20/50)	None	None	None
<b>Level of evidence of carcinogenic activity</b>	Some evidence	Clear evidence	Clear evidence	Clear evidence
<b>Genetic toxicology</b> <i>Salmonella typhimurium</i> gene mutations:			Positive in strain TA100 with and without S9 Negative in strains TA98 and TA1535 with and without S9	

---

## EXPLANATION OF LEVELS OF EVIDENCE OF CARCINOGENIC ACTIVITY

The National Toxicology Program describes the results of individual experiments on a chemical agent and notes the strength of the evidence for conclusions regarding each study. Negative results, in which the study animals do not have a greater incidence of neoplasia than control animals, do not necessarily mean that a chemical is not a carcinogen, inasmuch as the experiments are conducted under a limited set of conditions. Positive results demonstrate that a chemical is carcinogenic for laboratory animals under the conditions of the study and indicate that exposure to the chemical has the potential for hazard to humans. Other organizations, such as the International Agency for Research on Cancer, assign a strength of evidence for conclusions based on an examination of all available evidence, including animal studies such as those conducted by the NTP, epidemiologic studies, and estimates of exposure. Thus, the actual determination of risk to humans from chemicals found to be carcinogenic in laboratory animals requires a wider analysis that extends beyond the purview of these studies.

Five categories of evidence of carcinogenic activity are used in the Technical Report series to summarize the strength of the evidence observed in each experiment: two categories for positive results (**clear evidence** and **some evidence**); one category for uncertain findings (**equivocal evidence**); one category for no observable effects (**no evidence**); and one category for experiments that cannot be evaluated because of major flaws (**inadequate study**). These categories of interpretative conclusions were first adopted in June 1983 and then revised in March 1986 for use in the Technical Report series to incorporate more specifically the concept of actual weight of evidence of carcinogenic activity. For each separate experiment (male rats, female rats, male mice, female mice), one of the following five categories is selected to describe the findings. These categories refer to the strength of the experimental evidence and not to potency or mechanism.

- **Clear evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a dose-related (i) increase of malignant neoplasms, (ii) increase of a combination of malignant and benign neoplasms, or (iii) marked increase of benign neoplasms if there is an indication from this or other studies of the ability of such tumors to progress to malignancy.
- **Some evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a chemical-related increased incidence of neoplasms (malignant, benign, or combined) in which the strength of the response is less than that required for clear evidence.
- **Equivocal evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a marginal increase of neoplasms that may be chemical related.
- **No evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing no chemical-related increases in malignant or benign neoplasms.
- **Inadequate study** of carcinogenic activity is demonstrated by studies that, because of major qualitative or quantitative limitations, cannot be interpreted as valid for showing either the presence or absence of carcinogenic activity.

When a conclusion statement for a particular experiment is selected, consideration must be given to key factors that would extend the actual boundary of an individual category of evidence. Such consideration should allow for incorporation of scientific experience and current understanding of long-term carcinogenesis studies in laboratory animals, especially for those evaluations that may be on the borderline between two adjacent levels. These considerations should include:

- adequacy of the experimental design and conduct;
- occurrence of common versus uncommon neoplasia;
- progression (or lack thereof) from benign to malignant neoplasia as well as from preneoplastic to neoplastic lesions;
- some benign neoplasms have the capacity to regress but others (of the same morphologic type) progress. At present, it is impossible to identify the difference. Therefore, where progression is known to be a possibility, the most prudent course is to assume that benign neoplasms of those types have the potential to become malignant;
- combining benign and malignant tumor incidence known or thought to represent stages of progression in the same organ or tissue;
- latency in tumor induction;
- multiplicity in site-specific neoplasia;
- metastases;
- supporting information from proliferative lesions (hyperplasia) in the same site of neoplasia or in other experiments (same lesion in another sex or species);
- presence or absence of dose relationships;
- statistical significance of the observed tumor increase;
- concurrent control tumor incidence as well as the historical control rate and variability for a specific neoplasm;
- survival-adjusted analyses and false positive or false negative concerns;
- structure-activity correlations; and
- in some cases, genetic toxicology.

**NATIONAL TOXICOLOGY PROGRAM BOARD OF SCIENTIFIC COUNSELORS  
TECHNICAL REPORTS REVIEW SUBCOMMITTEE**

The members of the Technical Reports Review Subcommittee who evaluated the draft NTP Technical Report on cobalt sulfate heptahydrate on 11 December 1996 are listed below. Subcommittee members serve as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, subcommittee members have five major responsibilities in reviewing the NTP studies:

- to ascertain that all relevant literature data have been adequately cited and interpreted,
- to determine if the design and conditions of the NTP studies were appropriate,
- to ensure that the Technical Report presents the experimental results and conclusions fully and clearly,
- to judge the significance of the experimental results by scientific criteria, and
- to assess the evaluation of the evidence of carcinogenic activity and other observed toxic responses.

Gary P. Carlson, Ph.D., Chairperson  
School of Health Sciences  
Purdue University  
West Lafayette, IN

Irma Russo, M.D., Principal Reviewer  
Fox Chase Cancer Center  
Philadelphia, PA

Arnold L. Brown, M.D.  
University of Wisconsin Medical School  
Madison, WI

Louise Ryan, Ph.D.  
Division of Biostatistics  
Dana-Farber Cancer Institute  
Boston, MA

Thomas L. Goldsworthy, Ph.D.\*  
Department of Experimental Pathology and Toxicology  
Chemical Industry Institute of Toxicology  
Research Triangle Park, NC

Robert E. Taylor, M.D., Ph.D.  
Department of Pharmacology  
Howard University College of Medicine  
Washington, DC

Robert LeBoeuf, Ph.D.  
Corporate Professional and Regulatory Services  
Human Safety Department  
The Procter & Gamble Company  
Cincinnati, OH

Frederick L. Tyson, Ph.D., Principal Reviewer  
St. Mary's Hospital and Medical Center  
Cancer Research Institute  
Grand Junction, CO

Janardan K. Reddy, M.D.  
Department of Pathology  
Northwestern University Medical School  
Chicago, IL

Jerrold M. Ward, D.V.M., Ph.D., Principal Reviewer  
National Cancer Institute  
Frederick, MD

---

\* Did not attend

## SUMMARY OF TECHNICAL REPORTS REVIEW SUBCOMMITTEE COMMENTS

On 11 December 1996, the draft Technical Report on the toxicology and carcinogenesis studies of cobalt sulfate heptahydrate received public review by the National Toxicology Program's Board of Scientific Counselors' Technical Reports Review Subcommittee. The review meeting was held at the National Institute of Environmental Health Sciences, Research Triangle Park, NC.

Dr. J.R. Bucher, NIEHS, introduced the toxicology and carcinogenesis studies of cobalt sulfate heptahydrate by discussing the uses of the chemical and rationale for study, describing the experimental design, reporting on survival and body weight effects, and commenting on the chemical-related neoplastic and nonneoplastic lesions in male and female rats and mice. The proposed conclusions were *some evidence of carcinogenic activity* in male F344/N rats and *clear evidence of carcinogenic activity* in female F344/N rats and male and female B6C3F<sub>1</sub> mice.

Dr. Tyson, a principal reviewer, agreed with the proposed conclusions. Concerning the genetic mechanisms involved in murine lung tumorigenesis, he said that although a comprehensive study of *K-ras* activation was done in lung neoplasms, other molecular markers could have been assessed as well. Loss of heterozygosity or homozygous deletions on regions of chromosome 4, which are syntenic to regions of human chromosome 9p21 where frequent deletions are observed in human lung cancer, could have been

studied to determine if similar mechanisms are at work in both murine and human lung tumorigenesis via exposure to this chemical. Dr. R.C. Sills, NIEHS, reported that further studies were planned with the next step being to look at loss of heterozygosity not only on chromosome 4, but also to look at chromosomes 6 and 11, where the p53 genes are located.

Dr. Ward, the second principal reviewer, agreed with the proposed conclusions. He agreed with the rationale for the exposure concentrations chosen for the 2-year studies but because there was no concentration-related body weight gain depression, he thought that rats and mice could have tolerated higher concentrations. With regard to the extensive lesions in the nasal cavity and larynx, he stated that this was a classic case showing the association between toxic and regenerative/repairative lesions resulting in no neoplasms.

Dr. Russo, the third principal reviewer, agreed with the proposed conclusions.

Dr. Tyson moved that the Technical Report on cobalt sulfate heptahydrate be accepted with the revisions discussed and with the conclusions as written for male F344/N rats, *some evidence of carcinogenic activity* and for female F344/N rats and male and female B6C3F<sub>1</sub> mice, *clear evidence of carcinogenic activity*. Dr. Russo seconded the motion, which was accepted unanimously with eight votes.

## INTRODUCTION



### COBALT SULFATE HEPTAHYDRATE

CAS No. 10026-24-1

Molecular Weight: 281.13

**Synonyms:** Bieberite; cobalt(II) sulfate (1:1) heptahydrate; cobalt monosulfate heptahydrate; cobalt(II) sulphate heptahydrate; sulfuric acid, cobalt(2+) salt (1:1) heptahydrate

### CHEMICAL AND PHYSICAL PROPERTIES

Cobalt sulfate is a reddish, crystalline, water-soluble powder. It is usually produced as cobalt(II) sulfate but can also exist in the cobalt(III) sulfate form with a formula of  $\text{Co}_2(\text{SO}_4)_3 \cdot 18\text{H}_2\text{O}$ . The heptahydrate salt is reported to have a structure of  $[\text{Co}(\text{H}_2\text{O})_6] \cdot [\text{H}_2\text{SO}_5]$  (*Merck Index*, 1983). Cobalt(II) salts are stable to autoxidation in air or in solution (Smith and Carson, 1981).

### PRODUCTION, USE, AND HUMAN EXPOSURE

The production of cobalt sulfate in the United States in 1983 was estimated to be 450,000 pounds (204,000 kg) (J.V. Gandhi, Hall Chemical Co., personal communication); more recent production estimates are not available. Seven companies were listed as producing or handling cobalt sulfate at 10 facilities in the United States (USDHHS, 1992). Cobalt sulfate has been widely used in the electroplating and electrochemical industries. It is used as a coloring agent for ceramics and as a drying agent in inks, paints, varnishes, and linoleum. Cobalt sulfate

may be added to animal feed as a mineral supplement and has been used as a top dressing on pasture lands (De Bie and Doyen, 1962).

Cobalt is an essential trace element because it is an integral part of vitamin B<sub>12</sub>. The human body burden is approximately 1.1 mg, and the daily intake is about 0.3 mg, primarily via food (Hammond and Beliles, 1980). Cobalt is found in urban air (0.5 to 60 ng/m<sup>3</sup>) (Morgan *et al.*, 1970) and has been identified in trace amounts in natural waters; concentrations in excess of 10 µg/L are rare (NRC, 1977). Ocean water contains about 0.3 µg/L (Hamilton, 1994). Cobalt has been identified in chemical waste dumps (Barrett, 1983).

In the 1960s, several breweries added cobalt sulfate to beer at a level of about 1 ppm to counteract the antifoaming activity of detergent residues left on poorly rinsed glasses (Morin and Daniel, 1967). Soon after this, an epidemic of "beer-drinkers' cardiomyopathy" occurred, and cobalt was identified as the causative agent. The addition of cobalt salts to beer was discontinued, and the epidemic ceased. Doses of cobalt chloride of up to 200 to 300 mg per day were given orally to patients as treatment for various types of anemia in the 1950s (Finch, 1980). This practice

has largely stopped because of associated toxicity (gastrointestinal upset, goiter, cardiomyopathies) and the development of less hazardous therapies.

It has been estimated that over 1 million workers in the United States are exposed to cobalt or cobalt compounds (Jensen and Tüchsen, 1990). Occupational exposure to cobalt occurs principally in refining processes, in the production of alloys, and in the tungsten carbide hard metal industry (Kazantzis, 1981). Exposure under these conditions is primarily dermal or via inhalation of cobalt metal dusts or fumes, often in combination with other elements such as nickel, arsenic, or tungsten; adverse respiratory effects (such as pneumoconiosis) have been reported at cobalt concentrations between 0.1 and 2 mg/m<sup>3</sup> (Domingo, 1989). The threshold limit value-time weighted average for elemental cobalt is 0.02 mg/m<sup>3</sup> (ACGIH, 1996). Airborne levels of cobalt dust from spray painting in a Danish porcelain factory in 1981 were as high as 8.6 mg/m<sup>3</sup> (Jensen and Tüschchen, 1990).

## **ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION**

The absorption of cobalt salts after oral administration is variable and is influenced by the nature of the salt, the size of the dose, and the presence of food in the gastrointestinal tract (Murdock, 1959; Smith *et al.*, 1972). Clearance of inhaled soluble cobalt salts from the lung has not been studied but is expected to be rapid (Kerfoot *et al.*, 1975). Several processes could contribute to this effect. The water-soluble salts dissolve directly, and certain insoluble salts and cobalt metal powder appear to have an appreciable solubility in protein-containing fluids (Harding, 1950). Clearance by phagocytic alveolar macrophages may also occur (Kerfoot *et al.*, 1975). Cobalt is distributed to all tissues after administration by the oral or inhalation route or by injection (Smith and Carson, 1981). Tissue retention is not marked, but higher concentrations have been noted in the liver, kidney, spleen, and heart than in other organs (Domingo *et al.*, 1984a,b; Llobet *et al.*, 1986).

### ***Experimental Animals***

In an unspecified strain of rabbits administered cobalt sulfate at doses of 0.25 mg/kg per day orally or by injection for 2 months, some accumulation of cobalt

occurred in the liver, small intestine, lung, blood, kidney, and stomach (Kichina, 1974). Excretion is primarily via the urine and secondarily via the feces. The cobalt content of bile collected for 2 hours after intravenous administration of [<sup>57</sup>Co] cobalt chloride to Sprague-Dawley rats totaled about 2% to 5% of the dose over a thirty-fold dose range (0.03 mg/kg to 1 mg/kg of Co<sup>2+</sup>) (Gregus and Klaassen, 1986). Several studies have shown that a small portion of cobalt, given in several forms by parenteral or inhalation routes, is retained in tissues with a biological half-time of several years (IARC, 1991). The form of these materials has not been determined, but this could represent uptake into vitamin B<sub>12</sub> (Edel *et al.*, 1990).

### ***Humans***

A recent report has demonstrated significant dermal absorption of cobalt by humans exposed to mixed cobalt-tungsten carbide powders (Scansetti *et al.*, 1994). The concentration of cobalt in the blood and urine of nonoccupationally exposed humans is 0.2 to 2.0 µg/L (Hamilton, 1994). Cobalt concentrations in the urine of workers in the Italian hard metal industry were between 10 and 100 µg/L at the beginning of the work shift and increased to between 16 and 210 µg/L at the end of the work shift (Sabbioni *et al.*, 1994).

## **TOXICITY**

### ***Experimental Animals***

Exposure to cobalt results in a wide spectrum of toxicities in mammals. The ionic radius of cobalt is between that of Mg<sup>2+</sup> and Ca<sup>2+</sup>, so cobalt can replace or mimic these ions and also may influence reactions normally involving Fe<sup>2+</sup>, Zn<sup>2+</sup>, Cu<sup>2+</sup>, or Mn<sup>2+</sup> (Jennette, 1981). For example, cobalt can bind to Ca<sup>2+</sup>-binding proteins in or near microtubules (Phillips, 1980) and has been shown to block Ca<sup>2+</sup> channels in squid axons (Baker *et al.*, 1973). Cobalt promotes aberrant microtubule assembly (Buttlair *et al.*, 1980) and can alter the activity of metallo-enzymes such as carboxypeptidase (Jennette, 1981). Cobalt also inhibits the activity of DNA polymerase I from *Micrococcus luteus* (Korman *et al.*, 1978). Cobalt binds to sulfhydryl groups, including those of glutathione and cysteine, and through its binding to lipoic acid inhibits pyruvate dehydrogenase and α-ketoglutarate dehydrogenase, effectively stopping oxidative metabolism (Dingle *et al.*, 1962).

A 250  $\mu\text{mol/kg}$  (approximately 60 mg/kg) dose of cobalt chloride heptahydrate administered by subcutaneous injection to male Sprague-Dawley rats caused a rapid increase in biliary excretion of both reduced and oxidized glutathione, but total hepatic glutathione tended to increase after cobalt exposure (Stelzer and Klaassen, 1985).

A dose of 60 mg cobalt/kg body weight given to an unspecified strain of rats was found to inhibit heme synthesis in the liver (De Matteis and Gibbs, 1977). This apparently results from the formation of cobalt protoporphyrin by ferrochelatase and feedback inhibition of  $\delta$ -aminolevulinic acid synthetase activity by the abnormal protoporphyrin (Sinclair *et al.*, 1982). Cobalt also induces heme oxygenase (Maines and Kappas, 1976), and the combined effect of these actions is to rapidly decrease the cytochrome P<sub>450</sub> concentrations in the liver. Other cytochromes appear to be less affected (Tephly and Hibbeln, 1971).

In contrast to its actions on heme synthesis in the liver, cobalt administration promotes polycythemia. This effect is more pronounced in humans than in rodents (Smith and Carson, 1981) and is the basis for the use of cobalt chloride to treat anemia. The oral administration of 10 mg cobalt/kg body weight given as cobalt chloride to male rats of unspecified strain five times per week for 150 days resulted in an increase in the erythrocyte count, hematocrit value, and hemoglobin concentration of the blood; however, the mean cell volume and hemoglobin concentration per cell were unchanged, indicating a simple polycythemic effect (Murdock, 1959). This response is mediated by an increase in circulating erythropoietin, postulated to be a secondary response to a central nervous system effect of cobalt which results in respiratory alkalosis. Alkalosis increases the affinity of heme for oxygen, which is interpreted by tissue "sensors" as hypoxia (Miller *et al.*, 1974).

A second effect of cobalt administration on the blood is an increase in triglycerides, cholesterol, and free fatty acids (Taylor and Marks, 1978). This may be caused by inhibition of tissue lipoprotein lipase, resulting in failure to clear very low-density lipoprotein (Taylor and Marks, 1978), and perhaps by stimulation of lipoprotein synthesis in the liver (Eaton, 1972).

A single injection of 35 mg/kg cobalt chloride caused degranulation and disintegration of the  $\alpha$  cells of the pancreatic islets in rabbits (Telib, 1972). This was followed by degranulation of the  $\beta$  cells.

Although exposure to cobalt affects a wide variety of enzymatic processes, the acute toxicity of cobalt is not as great as might be expected. The oral LD<sub>50</sub> for anhydrous cobalt sulfate is 420 mg/kg in male and female Wistar rats (Speijers *et al.*, 1982).

Krasovskii and Fridlyand (1971) administered 0.5 or 2.5 mg/kg cobalt chloride by gavage to rats six times per week for 7 months. These investigators found polycythemia and a suppression of leukocyte function. Myocardial histologic changes were seen in 26 of 30 rats given 26 mg/kg cobalt sulfate by gavage once daily for 8 weeks (Grice *et al.*, 1969). This study is representative of a large number of animal studies designed to examine beer-drinkers' cardiomyopathy (cited in Smith and Carson, 1981, and USDHHS, 1992). Overall, these studies indicated that rather large doses of cobalt could mimic the cardiomyopathy caused by cobalt-treated beer, but that cobalt probably acted synergistically in humans with thiamine deficiency and an insufficient intake of sulfur-containing amino acids. Deficits in thyroid function have been shown in 1-day-old chicks and guinea pigs but not in young chicks, rats, mice, or rabbits given cobalt (Sederholm *et al.*, 1968).

A variety of cobalt dusts and aerosols have been administered to animals via inhalation. Results of these studies indicate that lung compliance is decreased and that electrical properties of the heart are affected as in beer-drinkers' cardiomyopathy (Kerfoot *et al.*, 1975; Smith, 1980). In general, similar toxicity has been elicited by cobalt whether administered orally or by inhalation. These effects have been seen after exposure of rats to atmospheres containing 0.05 or 0.5 mg/m<sup>3</sup> cobalt for 3 months (Popov, 1977). In addition, specific pulmonary effects in male rabbits exposed to 0.5 mg/m<sup>3</sup> cobalt (as cobalt chloride) by inhalation for 6 hours per day, 5 days per week, for 4 to 6 weeks included a change in the growth pattern of alveolar type II cells, resulting in clusters of cells projecting into the alveolar lumen, and changes in oxidative metabolism of lung macrophages (Johansson *et al.*, 1984, 1986).

Sixteen-day and 13-week inhalation studies with cobalt sulfate heptahydrate in F344/N rats and B6C3F<sub>1</sub> mice have been reported (Bucher *et al.*, 1990; NTP, 1991). In the 13-week studies, groups of 10 male and 10 female rats and mice were exposed to cobalt sulfate heptahydrate concentrations ranging from 0 to 30 mg/m<sup>3</sup>, 6 hours per day, 5 days per week. Two male mice exposed to 30 mg/m<sup>3</sup> died. All groups at this concentration initially lost weight, but then gained weight at rates similar to controls. At the end of the studies, lung weights were generally increased in rats and mice exposed to 1.0 mg/m<sup>3</sup> and higher, and polycythemia was observed in exposed rats but not in mice. Lesions observed in the respiratory tract of rats and mice included degeneration of the olfactory epithelium, squamous metaplasia of the respiratory epithelium, and inflammation in the nose; inflammation, necrosis, squamous metaplasia, ulcers (rats), and inflammatory polyps (rats) of the larynx; squamous metaplasia of the trachea (mice); and histiocytic infiltrates, bronchiolar regeneration, peribronchiolar and septal fibrosis, and epithelial hyperplasia in the alveoli of the lung. A no-observed-adverse-effect-level (NOAEL) was not reached in these studies as lesions, particularly in the larynx, were observed at the lowest exposure (0.3 mg/m<sup>3</sup>) used.

In other NTP studies (unpublished, available upon request), cobalt sulfate elicited contact hypersensitivity. Female Hartley guinea pigs received dermal applications of 100 µL of an aqueous 6% solution once per day for 14 days. A dose-related increase in contact hypersensitivity, as measured by retention of labeled inflammatory cells in the skin, was observed upon challenge application of solutions of 0.3%, 1%, or 3% aqueous cobalt sulfate to a site distant from the induction site 7 days after the last induction dose. Erythema and edema in the ears and paws of rats resulted from the administration of 5 mg cobalt sulfate by injection (Jasmin, 1974).

### **Humans**

Besides myocardial toxicity, as noted above, a second effect of cobalt observed in victims of beer-drinkers' cardiomyopathy was hypothyroidism (Taylor and Marks, 1978). Thyroid function tests, including uptake of [<sup>131</sup>I]iodide, were also depressed in patients receiving 0.17 to 3.9 mg/kg cobalt per day for treatment of anemia (Paley *et al.*, 1958). It has been

proposed that cobalt interferes with binding of inorganic iodide to tyrosine in the thyroid gland.

Hypersensitivity reactions have been observed in patients who received prosthetic implants made of a cobalt alloy and in industrial workers exposed to cobalt dusts (Smith and Carson, 1981). Asthma related to cobalt exposure has also been described (Cirla, 1994).

Most inhalation of cobalt is by workers in the refining and alloy production industries (NIOSH, 1981). The dusts may be in the form of the metal, its alloys, or its salts, but most often the oxide form is present. Consequently, no toxicity studies exist on exposure to pure cobalt metal or to cobalt sulfate. Exposure appears to cause pulmonary fibrosis, splenic enlargement, dermatitis, and losses of appetite and sense of smell (Dorsit *et al.*, 1970). Cobalt is used in the cemented tungsten carbide industry and is thought to be primarily responsible for pulmonary "hard metal disease," consisting of upper respiratory tract irritation, pneumonitis, and pulmonary fibrosis (NIOSH, 1981). However, the actual role of inhaled cobalt versus an interaction of cobalt and other inhaled particles remains a subject of debate (Swennen *et al.*, 1993).

## **REPRODUCTIVE AND DEVELOPMENTAL EFFECTS**

### ***Experimental Animals***

Sprague-Dawley rats maintained on diets containing 265 ppm cobalt for 98 days showed degenerative changes in the testis; these changes were considered secondary to hypoxia (Mollenhaur *et al.*, 1985). Decreases in sperm motility and/or increased abnormal sperm were noted in mice, but not in rats, exposed to 3 mg/m<sup>3</sup> or higher in 13-week inhalation studies with cobalt sulfate (NTP, 1991). Following 13 weeks of chronic exposure to 100 to 400 ppm cobalt chloride in drinking water, male CD-1 mice showed marked dose-related decreases in fertility, testicular weight, and sperm concentration and motility, and increases in circulating levels of testosterone (Pedigo *et al.*, 1988).

Cobalt has been shown to cross the placenta; cobalt chloride and nitrite salt solutions induced fetal cleft

palates when injected alone into mouse dams, but inhibited cleft formation caused by cortisone or phenytoin (Kasirsky *et al.*, 1969; Mitala *et al.*, 1978). Oral exposure of rats to cobalt chloride at daily doses of 5.4 or 21.8 mg cobalt/kg body weight from gestation day 14 through lactation day 21 resulted in stunted growth and/or decreased pup survival, although adverse effects were also evident in the dams at both doses (Domingo *et al.*, 1985). In contrast, Paternain *et al.* (1988) reported that doses of up to 100 mg/kg cobalt chloride administered by gavage to pregnant Sprague-Dawley rats once per day on days 6 to 15 of gestation did not result in significant fetotoxicity or teratogenicity. Similarly, Seidenberg *et al.* (1986) reported no effect on mouse fetal growth or mortality in dams given daily doses of 81.7 mg cobalt/kg on days 8 to 12 of pregnancy.

### **Humans**

Cobalt has not been shown to cause significant teratogenic or reproductive effects in humans (Smith and Carson, 1981). No clinical effects were noted in the babies of women who had taken cobalt chloride to counter anemia while pregnant (Jacobziner and Raybin, 1961).

## **CARCINOGENICITY**

### **Experimental Animals**

There have been no reports of adequate chronic inhalation toxicity or carcinogenicity studies with soluble or insoluble cobalt salts or metal powders (IARC, 1991). Wehner *et al.* (1977) found no increase in tumors in Syrian golden hamsters exposed to 10 mg/m<sup>3</sup> cobalt oxide dust for 7 hours per day, 5 days per week, for life; however, the study was faulted for poor survival (IARC, 1991). Cobalt oxide has been studied by intratracheal administration to groups of 50 male and 50 female Sprague-Dawley rats (Steinhoff and Mohr, 1991). Doses of 2 or 10 mg/kg were given in 19 treatments at 2-week intervals and in 10 treatments at 4-week intervals over 2 years. Two groups of 50 male and 50 female controls received saline or no treatment. Approximately 80% of the material was within the particle size range of 5 to 40 µm. At the end of the study an unspecified bronchioalveolar proliferation was noted in 51 of 100 low-dose rats (male and females combined), in 70 of 100 high-dose rats, and in no controls. One male and one female from the low-dose groups developed a

benign lung tumor, and one high-dose female had a bronchioalveolar carcinoma. Three adenocarcinomas and two bronchioalveolar adenomas were observed in high-dose males. No lung tumors occurred in the controls. In a similar but smaller study by the same group, cobalt oxide was found to enhance the lung tumor yield of benzo[a]pyrene treatment (Steinhoff and Mohr, 1991).

Sarcomas in rats have been observed at the site of injection of cobalt salts or cobalt metal powder (IARC, 1991). Heath (1956, 1960) gave an unspecified strain of rats a single injection of 0.28 mg cobalt metal powder in fowl serum into the thigh muscle. Within 2 weeks, atypical myoblasts were observed (Heath, 1960), and between 5 and 12 months, malignant neoplasms developed at the injection site in 17 of 30 rats; 11 were rhabdomyosarcomas (Heath, 1956). Gilman (1962) reported a similar neoplastic response to injections of cobalt sulfide and cobalt oxide in an unspecified strain of rats but saw no neoplasms in an unspecified strain of mice. These materials are relatively insoluble, and Abbracchio *et al.* (1982) suggested that intracellular solubilization of relatively insoluble cobalt salts would favor cellular transformation. Heath and Webb (1967) determined that cobalt is bound intracellularly in primary rhabdomyosarcomas induced by intramuscular injection of metallic cobalt, with 70% to 90% of the bound cobalt found in the nucleus. Further fractionation studies demonstrated that 50% of the nuclear cobalt is bound in the nucleolus (Webb *et al.*, 1972). Similar injection studies have given little evidence of cobalt-induced cancer in mice, hamsters, or guinea pigs (Christensen and Poulsen, 1994).

There is only one report of the formation of neoplasms after injection of a soluble cobalt salt. Shabaan *et al.* (1977) observed fibrosarcomas in 14 of 40 male Wistar rats 8 months to 1 year after administration of 40 mg/kg cobalt chloride by subcutaneous injection once per day for 10 days. Four of these neoplasms were not at the site of injection.

### **Humans**

Cobalt has been used in hundreds of patients as part of an alloy with chromium and molybdenum in prosthetic implants. During the first 14 years of its use for this purpose, no fibrosarcomas were identified in the recipients (McKee, 1971); however, a number of cases of malignant neoplasia have been reported

since that time at the sites of metal-containing fracture plates or joint prostheses, some of which contained cobalt (IARC, 1991).

The IARC (1991) considered the available data inadequate to establish an association between cancer and cobalt exposure to humans. At that time there were two epidemiological studies that were considered adequate for evaluation (Mur *et al.*, 1987; Hogstedt and Alexandersson, 1990). The Mur *et al.* (1987) cohort study was composed of 1,143 workers who were employed for at least a year between 1950 and 1980 in a French electrochemical plant producing cobalt and sodium. For workers employed only in cobalt production, the standard mortality ratio for lung cancer was 466 (95% confidence interval from 146 to 1,064) based on four cases. Hogstedt and Alexandersson (1990) studied a cohort of 3,163 male Swedish workers with at least 1 year of exposure to cobalt-containing, hard-metal dust ore between 1940 and 1982. There were 17 cases of lung cancer versus 12.7 expected (SMR, 134; 95% CI 77 to 213). Interpretation of both studies was made difficult by concurrent exposures to other substances including arsenic and nickel in the French plant and tungsten carbide in the Swedish facility.

Since the IARC evaluation, a follow-up study of the French electrochemical plant workers was completed which extended the period of observation from 1981 to 1988. No additional lung cancers were observed. Based on this and other factors, the authors concluded that the data no longer supported an association of cobalt exposure with lung cancer (Moulin *et al.*, 1993). In contrast, Lasfargues *et al.* (1994) reported on a cohort mortality study carried out on workers at a French hard-metal plant. The study specifically addressed lung cancer risks in relation to cobalt exposure and included 709 male workers who had at least 1 year of employment at the plant and who died between the years 1956 and 1989. While overall mortality was not increased, death due to lung cancer was significantly elevated (SMR=213), with 10 cases observed. This excess was associated with high cobalt exposure, but no effect of employment duration was noted. Smoking did not account for the observed incidence of lung cancer.

## GENETIC TOXICITY

Genetic toxicity data for cobalt sulfate heptahydrate are limited to a single publication. Zeiger *et al.* (1992) reported the results of a mutagenicity study with cobalt sulfate heptahydrate which showed a weakly positive response in *Salmonella typhimurium* strain TA100 in the absence of exogenous metabolic activation as well as with hamster or rat liver S9; the authors reported no induction of mutations in strain TA98 or TA1535, with or without S9.

Few studies with other cobalt compounds have been reported. The literature on genetic and related effects of cobalt compounds was reviewed by Beyersmann and Hartwig (1992). Most of the bacterial mutagenicity test results included in this review were negative. However, some positive results were reported for mammalian cell DNA damage studies, including the observation of DNA strand breaks in human cells (McLean *et al.*, 1982; Hamilton-Koch *et al.*, 1986; Hartwig *et al.*, 1990) and sister chromatid exchange induction in human (Anderson, 1983) and hamster cells (Hartwig *et al.*, 1991) treated *in vitro* with cobalt chloride in the absence of exogenous metabolic systems. The authors discussed the possible role of hydroxyl and superoxide radical formation in the generation of DNA breaks (Beyersmann and Hartwig, 1992). Morita *et al.* (1991) reported a weak response in an *in vitro* test designed to detect increased frequencies of 6-thioguanine-resistant mutant FM3A cell colonies. At a concentration of  $2 \times 10^{-4}$  M cobalt chloride (which induced a 50% decrease in cell survival), an increased number of mutant colonies (approximately four to five times the control number) was observed. At concentrations higher and lower than  $2 \times 10^{-4}$  M, the mutagenic response was weaker. The authors suggested, based upon results from the testing of other known mutagens in this assay, that metal ions such as cobalt require relatively high concentrations and long exposure periods to induce an effect and that the induced mutagenic response obtained is weak and seen over a narrow dose range. In the *Drosophila* wing spot test, cobalt chloride was demonstrated to induce a significant, dose-dependent increase in somatic recombination in third instar larvae exposed to cobalt chloride concentrations of 2 to 10 mM during development to the adult stage (Ogawa *et al.*, 1994).

## STUDY RATIONALE

Cobalt sulfate was nominated by the National Cancer Institute for study based on a lack of information on the toxicity of soluble cobalt salts. The more common cobalt(II) form and the inhalation route were selected for study to mimic worker exposure. Prechronic studies were previously reported (Bucher *et al.*, 1990;

NTP, 1991) with a spectrum of lesions noted in the respiratory tract of rats and mice. Polycythemia was also observed in rats. A NOAEL was not reached in these studies using doses as low as 0.3 mg/m<sup>3</sup>. This report documents the findings of 2-year inhalation exposure studies with cobalt sulfate heptahydrate in F344/N rats and B6C3F<sub>1</sub> mice.



## MATERIALS AND METHODS

### PROCUREMENT AND CHARACTERIZATION OF COBALT SULFATE HEPTAHYDRATE

Cobalt sulfate heptahydrate was obtained from Curtin Matheson Scientific (Kansas City, MO) in one lot (412092). Identity and purity analyses were conducted by the analytical chemistry laboratory, Midwest Research Institute (Kansas City, MO) (Appendix F). Reports on analyses performed in support of the cobalt sulfate heptahydrate studies are on file at the National Institute of Environmental Health Sciences.

The chemical, a red, crystalline solid, was identified as cobalt sulfate heptahydrate by infrared, ultraviolet, and/or visible spectroscopy. The purity of lot 412092 was determined by elemental analysis, Karl Fischer water analysis, and spark source mass spectroscopy. Elemental analyses for sulfur and hydrogen were in agreement with the theoretical values for cobalt sulfate heptahydrate, but results for cobalt were slightly low. Karl Fischer water analysis indicated  $44.6\% \pm 0.5\%$  water. Spark source mass spectroscopy indicated 140 ppm nickel present as an impurity; all other impurities had a combined total of less than 175 ppm. The overall purity was determined to be approximately 99%.

Literature references indicate that cobalt sulfate heptahydrate is stable as a bulk chemical when stored protected from light at normal temperatures. The heptahydrate dehydrates to the hexahydrate at  $41.5^\circ\text{C}$  and to the monohydrate when heated to  $71^\circ\text{C}$ , with no further changes expected below the decomposition temperature ( $708^\circ\text{C}$ ). Therefore, an accelerated stability study was not conducted. To ensure stability, the bulk chemical was stored in its original shipping containers, metal cans, at room temperature. Stability was monitored during the studies using elemental analysis by inductively coupled plasma/atomic emission spectroscopy (ICP/AES) normalized against a cobalt standard (National Institute of Standards and

Technology, Gaithersburg, MD); no degradation of the bulk chemical was detected.

### AEROSOL GENERATION AND EXPOSURE SYSTEM

Cobalt sulfate heptahydrate was generated and delivered from an aqueous solution by a system composed of three main components: a compressed-air-driven nebulizer (Model PN7002; RETEC Development Laboratory, Portland, OR), an aerosol charge neutralizer, and an aerosol distribution system. Cobalt sulfate heptahydrate in deionized water was siphoned from the bulk reservoir to the nebulizer reservoir and then aspirated into the nebulizer chamber and expelled as a stream. Shear forces broke the stream into droplets that were evaporated to leave dry particles of cobalt sulfate heptahydrate. The aerosol generation and delivery system included primary and secondary compressed-air-driven nebulizers. The aerosol generated by the compressed-air-driven nebulizer was passed through the aerosol charge neutralizer to remove static charge that formed on the aerosol particles during generation. Detailed descriptions of the inhalation chambers and the vapor generation system are provided in Appendix F.

A distribution line carried aerosol to the Hazleton 2000 inhalation exposure chambers (Harford Systems Division of Lab Products, Inc., Aberdeen, MD) on both sides of the exposure room. At each chamber, aerosol moving through the chamber inlet was further diluted with HEPA-filtered air to the appropriate concentration for the chamber.

### AEROSOL CONCENTRATION MONITORING

The chamber concentrations of cobalt sulfate heptahydrate were monitored by computer-controlled real-time aerosol monitors (Model RAM-1; MIE, Inc.,

Bedford, MA). Chamber aerosol concentrations were sampled at least once per hour during each exposure day. Throughout the studies, the background concentrations of total suspended particles in the control chambers were less than the limit of detection. The RAM-1 voltage output was calibrated against cobalt sulfate heptahydrate concentrations of chamber filter samples. Solutions of filter samples in 2% nitric acid were analyzed quantitatively for cobalt sulfate heptahydrate by ICP/AES. The ICP/AES was calibrated with a solution of standard cobalt diluted with nitric acid. Stability studies performed with X-ray diffraction analyses of samples from the 0.3 and 3.0 mg/m<sup>3</sup> chambers indicated that cobalt sulfate hexahydrate was the primary species delivered to the chambers. Chamber concentration uniformity was maintained throughout the 2-year studies. A summary of chamber concentrations is presented in Table F1.

## CHAMBER ATMOSPHERE CHARACTERIZATION

The time required for the chamber concentration to reach 90% of the target value following the beginning of exposure ( $T_{90}$ ) and the time required for the chamber concentration to reach 10% of the target value following termination of the exposure ( $T_{10}$ ) were determined for each exposure chamber. Without animals present,  $T_{90}$  values ranged from 9 to 11 minutes for rats and from 7 to 12 minutes for mice;  $T_{10}$  ranged from 8 to 9 minutes for rats and mice. With animals present,  $T_{90}$  values ranged from 11 to 16 minutes for rats and from 8 to 12 minutes for mice;  $T_{10}$  ranged from 12 to 13 minutes for rats and from 11 to 12 minutes for mice. A  $T_{90}$  of 12 minutes was selected for the 2-year studies.

Aerosol size distribution was determined monthly for each exposure chamber with a Mercer-style seven-stage impactor (In-Tox Products, Albuquerque, NM). Samples were analyzed for cobalt sulfate heptahydrate with ICP/AES. The relative mass on each impactor stage was analyzed by probit analysis; the mass median aerodynamic diameter for the aerosol was within the specified range of 1 to 3  $\mu\text{m}$  (Tables F2 and F3).

Studies of cobalt sulfate heptahydrate degradation and monitoring for impurities were conducted throughout the 2-year studies with ICP/AES. No degradation of cobalt sulfate heptahydrate was observed during the

studies. Cageboards were used after the first 8 weeks of the studies to control ammonia in the exposure chambers.

## 2-YEAR STUDIES

### Study Design

Groups of 50 male and 50 female rats and mice were exposed to aqueous aerosols containing 0, 0.3, 1.0, or 3.0 mg/m<sup>3</sup> cobalt sulfate heptahydrate for 6 hours plus  $T_{90}$  (12 minutes) per day, 5 days per week, for 105 weeks.

The exposure concentrations for the 2-year cobalt sulfate heptahydrate studies were based on the findings of 16-day and 13-week studies reported previously (NTP, 1991). The most sensitive tissue was the larynx, with squamous metaplasia observed in rats and mice at the lowest exposure concentration of 0.3 mg/m<sup>3</sup>. A NOAEL was not reached for this tissue. Inflammatory polyps, some nearly obstructing the esophagus, were observed at 10 and 30 mg/m<sup>3</sup> in rats, while these lesions at the 0.3 and 1.0 mg/m<sup>3</sup> exposure concentrations were composed of mild or minimal squamous metaplasia and/or chronic inflammation in both rats and mice. The severity of the laryngeal changes and other lesions in the respiratory tract at 3.0 mg/m<sup>3</sup> was not considered life threatening, and, therefore, exposure concentrations of 0.3, 1.0, and 3.0 mg/m<sup>3</sup> were chosen for the 2-year study with rats and mice.

### Source and Specification of Animals

Male and female F344/N rats and B6C3F<sub>1</sub> mice were obtained from Simonsen Laboratories (Gilroy, CA) for use in the 2-year studies. Rats and mice were quarantined for 14 days before the beginning of the studies. Five male and five female rats and mice were selected for parasite evaluation and gross observation of disease. Serology samples were collected for viral screening. Rats and mice were approximately 6 weeks old at the beginning of the studies. The health of the animals was monitored during the studies according to the protocols of the NTP Sentinel Animal Program (Appendix H).

### Animal Maintenance

Rats and mice were housed individually. Feed was available *ad libitum* except during exposure periods, and water was available *ad libitum*. Cages and racks

were rotated weekly. Further details of animal maintenance are given in Table 1. Information on feed composition and contaminants is provided in Appendix G.

### Clinical Examinations and Pathology

All animals were observed twice daily. Clinical findings and body weights were recorded initially, at weeks 5, 9, and 13 (clinical findings) or weekly for 13 weeks (body weights), monthly through week 92, every 2 weeks thereafter, and at the end of the studies.

A complete necropsy and microscopic examination were performed on all rats and mice. At necropsy, all organs and tissues were examined for grossly visible lesions, and all major tissues were fixed and preserved in 10% neutral buffered formalin, processed and trimmed, embedded in paraffin, sectioned to a thickness of 5 to 6  $\mu\text{m}$ , and stained with hematoxylin and eosin for microscopic examination. For all paired organs (i.e., adrenal gland, kidney, ovary), samples from each organ were examined. Tissues examined microscopically are listed in Table 1.

Microscopic evaluations were completed by the study laboratory pathologist, and the pathology data were entered into the Toxicology Data Management System. The slides, paraffin blocks, and residual wet tissues were sent to the NTP Archives for inventory, slide/block match, and wet tissue audit. The slides, individual animal data records, and pathology tables were evaluated by an independent quality assessment laboratory. The individual animal records and tables were compared for accuracy, the slide and tissue counts were verified, and the histotechnique was evaluated. For the 2-year studies, a quality assessment pathologist evaluated slides from all tumors and all potential target organs which included the adrenal medulla, lung, larynx, nose, and all neoplasms in all

groups except testicular neoplasms for male and female rats. For male and female mice, the quality assessment pathologist evaluated slides from all tumors and all potential target organs which included the larynx, liver, lung, nose, and trachea, and all neoplasms in all organs. Additionally, all thyroid glands were reviewed for incidences of proliferative lesions of the follicular cells. Renal and iliac lymph nodes of male mice were reviewed when the diagnosis of lymphoid hyperplasia occurred. Ovaries of female mice were reviewed when the diagnoses of cyst, bilateral cyst, or corpus luteum cyst occurred.

The quality assessment report and the reviewed slides were submitted to the NTP Pathology Working Group (PWG) chairperson, who reviewed the selected tissues and addressed any inconsistencies in the diagnoses made by the laboratory and quality assessment pathologists. Representative histopathology slides containing examples of lesions related to chemical administration, examples of disagreements in diagnoses between the laboratory and quality assessment pathologist, or lesions of general interest were presented by the chairperson to the PWG for review. The PWG consisted of the quality assessment pathologist and other pathologists experienced in rodent toxicologic pathology. This group examined the tissues usually without any knowledge of dose groups or previously rendered diagnoses. When the PWG consensus differed from the opinion of the laboratory pathologist, the diagnosis was changed. Final diagnoses for reviewed lesions represent a consensus between the laboratory pathologist, reviewing pathologist(s), and the PWG. Details of these review procedures have been described, in part, by Maronpot and Boorman (1982) and Boorman *et al.* (1985). For subsequent analyses of the pathology data, the decision of whether to evaluate the diagnosed lesions for each tissue type separately or combined was generally based on the guidelines of McConnell *et al.* (1986).

**TABLE 1**  
**Experimental Design and Materials and Methods in the 2-Year Inhalation Studies**  
**of Cobalt Sulfate Heptahydrate**

---

**Study Laboratory**

Battelle Pacific Northwest Laboratories  
(Richland, WA)

**Strain and Species**

Rats: F344/N  
Mice: B6C3F<sub>1</sub>

**Animal Source**

Simonsen Laboratories  
(Gilroy, CA)

**Time Held Before Studies**

14 days

**Average Age When Studies Began**

6 weeks

**Date of First Exposure**

Rats: 30 August 1990  
Mice: 23 August 1990

**Duration of Exposure**

6 hours plus T<sub>90</sub> (12 minutes) per day, 5 days per week, for 105 weeks

**Date of Last Exposure**

Rats: 28 August 1992  
Mice: 21 August 1992

**Necropsy Dates**

Rats: 1-4 September 1992  
Mice: 24-27 August 1992

**Average Age at Necropsy**

111 weeks

**Size of Study Groups**

50 males and 50 females

**Method of Distribution**

Animals were distributed randomly into groups of approximately equal initial mean body weights; cages were distributed randomly into groups from another computer-generated list of random numbers.

**Animals per Cage**

1

---

**TABLE 1**  
**Experimental Design and Materials and Methods in the 2-Year Inhalation Studies**  
**of Cobalt Sulfate Heptahydrate**

---

**Method of Animal Identification**

Tail tattoo

**Diet**

NIH-07 open formula pellet diet (Zeigler Brothers, Inc., Gardners, PA), available *ad libitum* except during exposure periods, changed weekly

**Water Distribution**

Tap water (Richland municipal supply) via automatic watering system (Edstrom Industries, Waterford, WI), available *ad libitum*

**Cages**

Stainless-steel wire-bottom (Hazleton System, Inc., Aberdeen, MD), changed weekly

**Bedding**

Cageboard (Bunzl Cincinnati Paper Co., Cincinnati, OH), changed daily (15 October 1990 to study termination)

**Chamber Air Supply Filters**

Single HEPA (Flanders Filters, Inc., San Rafael, CA)

**Chambers**

Stainless-steel with excreta pan suspended below each cage unit (Harford System Division of Lab Products, Inc., Aberdeen, MD), changed weekly

**Chamber Environment**

Temperature: 21.3°–26.6° C (rats); 19.5°–27.1° C (mice)

Relative humidity: 31%–89% (rats); 28%–93% (mice)

Room fluorescent light: 12 hours/day

Chamber air changes: 9-23/hour

**Exposure Concentrations**

0, 0.3, 1.0, or 3.0 mg/m<sup>3</sup>

**Type and Frequency of Observation**

Observed twice daily; animals were weighed and clinical findings were recorded initially, at weeks 5, 9, and 13 (clinical findings) or weekly for 13 weeks (body weights), monthly through week 92, every 2 weeks thereafter, and at the end of the studies.

**Method of Sacrifice**

CO<sub>2</sub> anesthetization

**Necropsy**

Necropsy performed on all animals

**Histopathology**

Complete histopathology was performed on all rats and mice. In addition to gross lesions and tissue masses, the tissues examined included: adrenal gland, bone with marrow, brain, clitoral gland, esophagus, gallbladder (mice), harderian gland (rats), heart, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, larynx, liver, lungs/bronchi, lymph nodes (mandibular, mesenteric, bronchial, mediastinal), mammary gland (except male mice), nose, oral cavity (rats), ovary, pancreas, pancreatic islets, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, sciatic nerve, seminal vesicle, skin, spinal cord, spleen, stomach (forestomach and glandular), testes/epididymides, thymus, thyroid gland, trachea, urinary bladder, and uterus.

---

## STATISTICAL METHODS

### Survival Analyses

The probability of survival was estimated by the product-limit procedure of Kaplan and Meier (1958) and is presented in the form of graphs. Animals found dead of other than natural causes or pregnant were censored from the survival analyses; animals dying from natural causes were not censored. Statistical analyses for possible dose-related effects on survival used Cox's (1972) method for testing two groups for equality and Tarone's (1975) life table test to identify dose-related trends. All reported P values for the survival analyses are two sided.

### Calculation of Incidence

The incidences of neoplasms or nonneoplastic lesions as presented in Tables A1, A5, B1, B5, C1, C5, D1, and D5 are given as the number of animals bearing such lesions at a specific anatomic site and the number of animals with that site examined microscopically. For calculation of statistical significance, the incidences of most neoplasms (Tables A3, B3, C3, and D3) and all nonneoplastic lesions are given as the numbers of animals affected at each site examined microscopically. However, when macroscopic examination was required to detect neoplasms in certain tissues (e.g., harderian gland, intestine, mammary gland, and skin) before microscopic evaluation, or when neoplasms had multiple potential sites of occurrence (e.g., leukemia or lymphoma), the denominators consist of the number of animals on which a necropsy was performed. Tables A3, B3, C3, and D3 also give the survival-adjusted neoplasm rate for each group and each site-specific neoplasm, i.e., the Kaplan-Meier estimate of the neoplasm incidence that would have been observed at the end of the study in the absence of mortality from all other competing risks (Kaplan and Meier, 1958).

### Analysis of Neoplasm Incidences

The majority of neoplasms in these studies were considered to be incidental to the cause of death or not rapidly lethal. Thus, the primary statistical method used was logistic regression analysis, which assumed that the diagnosed neoplasms were discovered as the result of death from an unrelated cause and thus did not affect the risk of death. In this approach, neoplasm prevalence was modeled as a logistic function

of chemical exposure and time. Both linear and quadratic terms in time were incorporated initially, and the quadratic term was eliminated if the fit of the model was not significantly enhanced. The neoplasm incidences of exposed and control groups were compared on the basis of the likelihood score test for the regression coefficient of dose. This method of adjusting for intercurrent mortality is the prevalence analysis of Dinse and Lagakos (1983), further described and illustrated by Dinse and Haseman (1986). When neoplasms are incidental, this comparison of the time-specific neoplasm prevalences also provides a comparison of the time-specific neoplasm incidences (McKnight and Crowley, 1984).

In addition to logistic regression, other methods of statistical analysis were used, and the results of these tests are summarized in the appendixes. These methods include the life table test (Cox, 1972; Tarone, 1975), appropriate for rapidly lethal neoplasms, and the Fisher exact test and the Cochran-Armitage trend test (Armitage, 1971; Gart *et al.*, 1979), procedures based on the overall proportion of neoplasm-bearing animals.

Tests of significance included pairwise comparisons of each exposed group with controls and a test for an overall dose-related trend. Continuity-corrected tests were used in the analysis of neoplasm incidence, and reported P values are one sided. The procedures described in the preceding paragraphs were also used to evaluate selected nonneoplastic lesions. For further discussion of these statistical methods, refer to Haseman (1984).

### Analysis of Nonneoplastic Lesion Incidences

Because all nonneoplastic lesions in this study were considered to be incidental to the cause of death or not rapidly lethal, the primary statistical analysis used was a logistic regression analysis in which nonneoplastic lesion prevalence was modeled as a logistic function of chemical exposure and time.

### Analysis of Continuous Variables

Average severity values were analyzed for significance using the Mann-Whitney U test (Hollander and Wolfe, 1973).

### Historical Control Data

Although the concurrent control group is always the first and most appropriate control group used for evaluation, historical control data can be helpful in the overall assessment of neoplasm incidence in certain instances. Consequently, neoplasm incidences from the NTP historical control database, which is updated yearly, are included in the NTP reports for neoplasms appearing to show compound-related effects.

### QUALITY ASSURANCE METHODS

The studies were conducted in compliance with Food and Drug Administration Good Laboratory Practice Regulations (21 CFR, Part 58). In addition, as records from the studies were submitted to the NTP Archives, these studies were audited retrospectively by an independent quality assurance contractor. Separate audits covering completeness and accuracy of the pathology data, pathology specimens, final pathology tables, and a draft of this NTP Technical Report were conducted. Audit procedures and findings are presented in the reports and are on file at NIEHS. The audit findings were reviewed and assessed by NTP staff, so all comments had been resolved or were otherwise addressed during the preparation of this Technical Report.

### GENETIC TOXICOLOGY

The genetic toxicity of cobalt sulfate heptahydrate was assessed by testing the ability of the chemical to induce mutations in various strains of *Salmonella typhimurium*. The protocols for these studies and the results are given in Appendix E.

The genetic toxicity studies of cobalt sulfate heptahydrate are part of a larger effort by the NTP to develop a database that would permit the evaluation of carcinogenicity in experimental animals from the molecular structure and the effects of the chemical in short-term *in vitro* and *in vivo* genetic toxicity tests. These genetic toxicity tests were originally developed to study mechanisms of chemical-induced DNA damage and to predict carcinogenicity in animals, based on the electrophilicity theory of chemical mutagenesis and the somatic mutation theory of cancer (Miller and Miller, 1977; Straus, 1981; Crawford, 1985).

There is a strong correlation between a chemical's potential electrophilicity (structural alert to DNA reactivity), mutagenicity in *Salmonella*, and carcinogenicity in rodents. The combination of electrophilicity and *Salmonella* mutagenicity is highly correlated with the induction of carcinogenicity in rats and mice and/or at multiple tissue sites (Ashby and Tennant, 1991). Other *in vitro* genetic toxicity tests correlate less well with rodent carcinogenicity (Tennant *et al.*, 1987; Zeiger *et al.*, 1990), although these other tests can provide information on the types of DNA and chromosome effects that can be induced by the chemical being investigated. Data from NTP studies show that a positive response in *Salmonella* is the most predictive *in vitro* test for rodent carcinogenicity (89% of the *Salmonella* mutagens are rodent carcinogens), and that there is no complementarity among the *in vitro* genetic toxicity tests. That is, no battery of tests that included the *Salmonella* test improved the predictivity of the *Salmonella* test alone.



## RESULTS

### RATS

#### Survival

Estimates of 2-year survival probabilities for male and female rats are shown in Table 2 and in the Kaplan-Meier survival curves (Figure 1). Survival of exposed males and females was similar to that of the chamber controls.

#### Body Weights and Clinical Findings

Mean body weights of exposed male and female rats were similar to those of the chamber controls throughout the study (Figure 2 and Tables 3 and 4). Irregular breathing was observed more frequently in female rats exposed to 3.0 mg/m<sup>3</sup> than in the chamber controls or other exposed groups.

**TABLE 2**  
**Survival of Rats in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate**

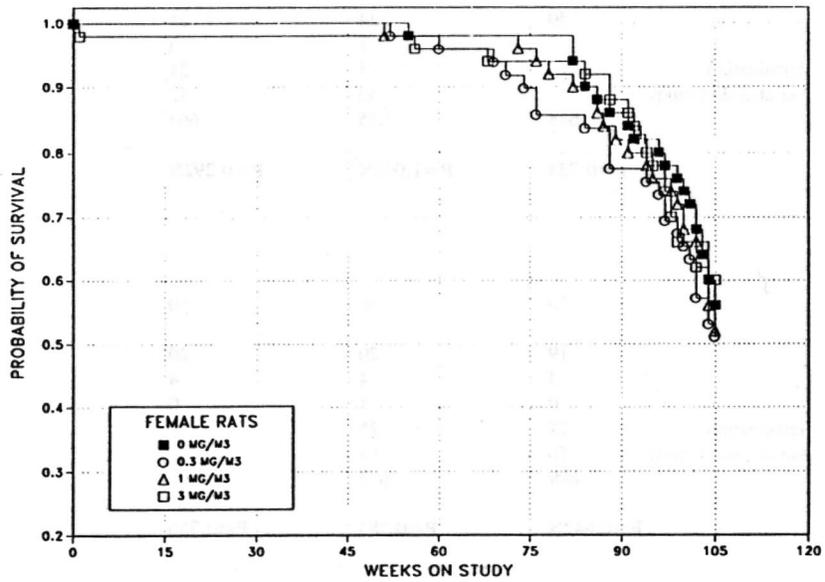
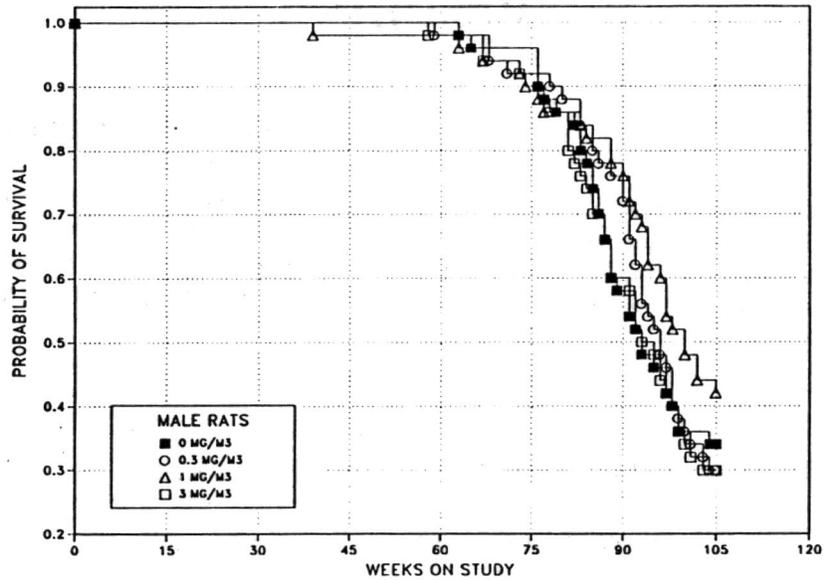
	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Male</b>				
Animals initially in study	50	50	50	50
Moribund	30	34	26	34
Natural deaths	3	1	3	1
Animals surviving to study termination	17	15	21	15
Percent probability of survival at end of study <sup>a</sup>	34	30	42	30
Mean survival (days) <sup>b</sup>	648	655	663	643
Survival analysis <sup>c</sup>	P=0.723	P=1.000N	P=0.292N	P=0.876
<b>Female</b>				
Animals initially in study	50	50	50	50
Moribund	19	20	20	17
Natural deaths	3	4	4	3
Pregnant <sup>d</sup>	0	1	0	0
Animals surviving to study termination	28	25	26	30
Percent probability of survival at end of study	56	51	52	60
Mean survival (days)	699	677	691	684
Survival analysis	P=0.642N	P=0.583	P=0.756	P=0.959N

<sup>a</sup> Kaplan-Meier determinations

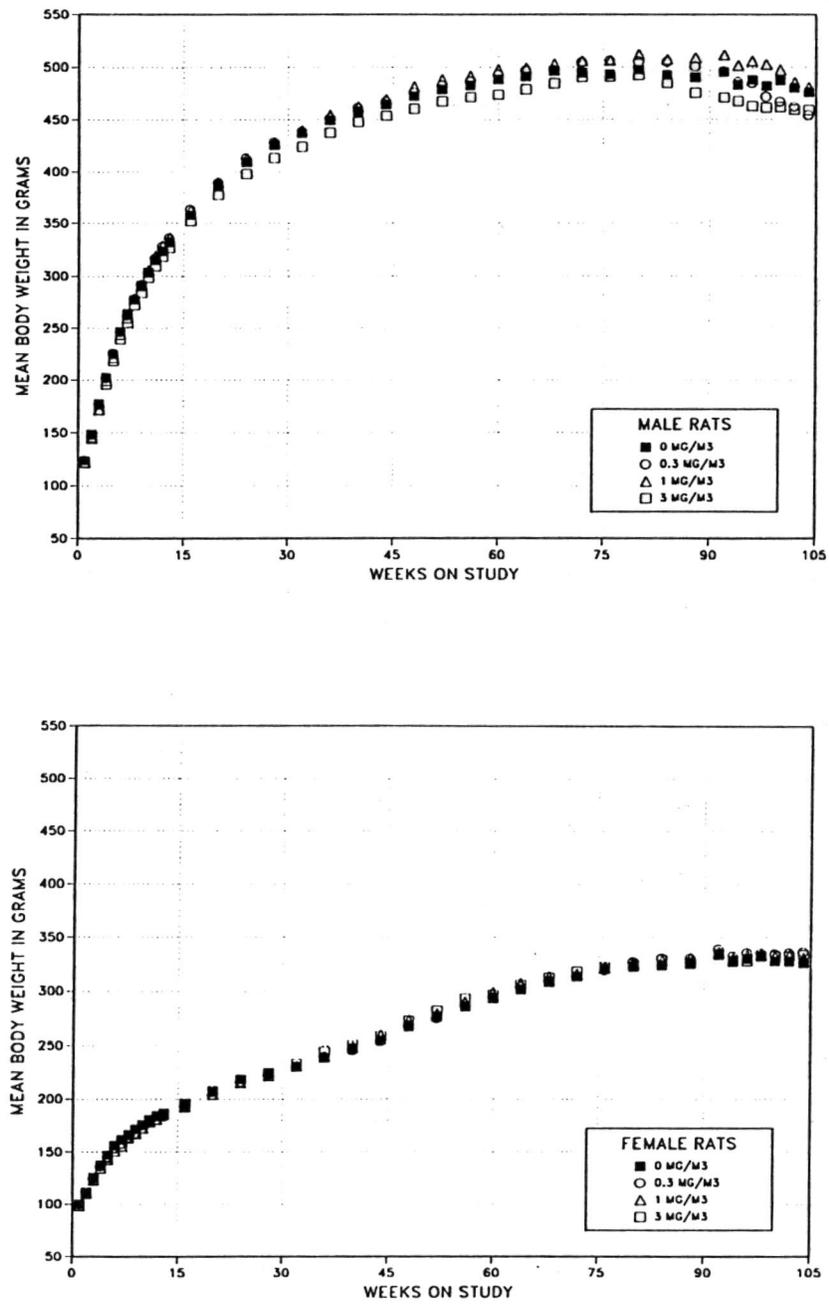
<sup>b</sup> Mean of all deaths (uncensored, censored, and terminal sacrifice)

<sup>c</sup> The result of the life table trend test (Tarone, 1975) is in the chamber control column, and the results of the life table pairwise comparisons (Cox, 1972) with the chamber controls are in the exposed group columns. A negative trend or lower mortality in an exposure group is indicated by **N**.

<sup>d</sup> Censored from survival analyses



**FIGURE 1**  
**Kaplan-Meier Survival Curves for Male and Female Rats**  
**Exposed to Cobalt Sulfate Heptahydrate by Inhalation for 2 Years**



**FIGURE 2**  
**Growth Curves for Male and Female Rats**  
**Exposed to Cobalt Sulfate Heptahydrate by Inhalation for 2 Years**

**TABLE 3**  
**Mean Body Weights and Survival of Male Rats in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

Weeks on Study	Chamber Control		0.3 mg/m <sup>3</sup>			1.0 mg/m <sup>3</sup>			3.0 mg/m <sup>3</sup>		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	124	50	124	100	50	122	99	50	122	99	50
2	149	50	148	100	50	145	97	50	146	98	50
3	177	50	177	100	50	172	97	50	172	97	50
4	202	50	202	100	50	200	99	50	196	97	50
5	225	50	226	101	50	221	99	50	219	97	50
6	247	50	247	100	50	244	99	50	240	97	50
7	264	50	263	99	50	260	98	50	255	97	50
8	277	50	278	100	50	279	101	50	273	98	50
9	291	50	292	100	50	291	100	50	284	98	50
10	303	50	304	100	50	306	101	50	299	98	50
11	315	50	317	101	50	320	102	50	310	98	50
12	324	50	328	101	50	329	102	50	319	99	50
13	332	50	336	101	50	336	101	50	327	98	50
16	358	50	364	102	50	363	101	50	352	98	50
20	387	50	390	101	50	390	101	50	377	98	50
24	409	50	413	101	50	412	101	50	398	97	50
28	425	50	428	101	50	429	101	50	413	97	50
32	437	50	439	100	50	440	101	50	424	97	50
36	450	50	452	101	50	455	101	50	438	97	50
40	458	50	462	101	50	463	101	49	448	98	50
44	464	50	468	101	50	470	101	49	454	98	50
48	473	50	476	101	50	482	102	49	461	98	50
52	479	50	483	101	50	488	102	49	468	98	50
56	483	50	487	101	50	492	102	49	472	98	50
60	489	50	493	101	49	498	102	49	474	97	49
64	491	49	497	101	49	499	102	48	479	98	49
68	497	48	498	100	49	503	101	47	485	98	47
72	495	48	505	102	46	506	102	47	491	99	47
76	493	48	506	103	46	506	103	45	491	100	46
80	498	43	505	102	45	512	103	43	493	99	43
84	493	40	505	103	42	507	103	42	485	98	38
88	490	32	501	102	39	509	104	41	476	97	33
92	495	27	497	100	33	512	103	36	471	95	29
94	483	24	486	101	28	502	104	34	468	97	25
96	488	23	485	99	26	506	104	31	463	95	24
98	482	21	472	98	23	503	104	27	462	96	21
100	487	18	467	96	19	498	102	26	462	95	18
102	481	18	462	96	17	486	101	24	460	96	16
104	476	18	454	95	16	481	101	22	459	96	15
<b>Mean for weeks</b>											
1-13	248		249	100		248	100		243	98	
14-52	434		438	101		439	101		423	97	
53-104	489		489	100		501	102		474	97	

**TABLE 4**  
**Mean Body Weights and Survival of Female Rats in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

Weeks on Study	Chamber Control		0.3 mg/m <sup>3</sup>			1.0 mg/m <sup>3</sup>			3.0 mg/m <sup>3</sup>		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	100	50	100	100	50	99	98	50	99	98	50
2	112	50	112	100	50	110	99	50	111	99	49
3	126	50	125	99	50	123	98	50	123	98	49
4	138	50	136	99	50	134	98	50	134	98	49
5	147	50	145	99	50	143	97	50	144	98	49
6	156	50	154	99	50	151	97	50	154	99	49
7	162	50	158	98	50	156	97	50	158	98	49
8	166	50	164	99	50	163	98	50	166	100	49
9	171	50	168	98	50	168	98	50	170	99	49
10	176	50	173	98	50	173	98	50	176	100	49
11	179	50	178	99	50	179	100	50	181	101	49
12	184	50	181	99	49	181	99	50	184	100	49
13	186	50	184	99	49	186	100	50	187	100	49
16	195	50	193	99	49	193	99	50	196	101	49
20	207	50	204	99	49	205	99	50	208	101	49
24	219	50	216	99	49	215	99	50	219	100	49
28	224	50	223	99	49	222	99	50	225	100	49
32	230	50	230	100	49	231	100	50	233	101	49
36	238	50	240	101	49	241	101	50	244	103	49
40	247	50	246	100	49	249	101	50	251	102	49
44	255	50	254	100	49	260	102	50	259	101	49
48	267	50	269	101	49	273	102	50	273	102	49
52	276	50	275	100	49	279	101	49	282	102	49
56	286	49	288	101	48	290	102	49	293	103	49
60	293	49	294	100	48	299	102	49	297	101	48
64	302	49	304	101	47	307	102	49	306	101	48
68	308	49	310	100	47	313	102	49	312	101	48
72	314	49	314	100	45	316	101	49	318	102	47
76	321	49	320	100	44	322	100	48	323	101	47
80	323	49	328	102	42	326	101	46	325	101	47
84	324	47	331	102	42	329	101	45	329	102	47
88	326	44	331	102	41	331	102	42	327	101	46
92	334	42	339	102	38	337	101	40	335	100	43
94	327	41	333	102	38	331	101	40	328	100	41
96	330	41	336	102	37	334	101	38	328	99	39
98	333	39	336	101	34	334	100	38	333	100	37
100	328	38	335	102	33	333	102	36	333	102	33
102	328	36	336	103	31	334	102	34	333	102	33
104	326	32	337	103	27	331	102	32	334	102	31
<b>Mean for weeks</b>											
1-13	154		152	99		151	98		153	99	
14-52	236		235	100		237	100		239	101	
53-104	319		323	101		323	101		322	101	

### ***Pathology and Statistical Analyses***

This section describes the statistically significant or biologically noteworthy changes in the incidences of neoplasms and/or nonneoplastic lesions of the lung, adrenal medulla, nose, and larynx. Summaries of the incidences of neoplasms and nonneoplastic lesions, individual animal tumor diagnoses, statistical analyses of primary neoplasms that occurred with an incidence of at least 5% in at least one animal group, and historical incidences for the neoplasms mentioned in this section are presented in Appendix A for male rats and Appendix B for female rats.

**Lung:** In all exposed groups of male and female rats, the incidences of proteinosis, alveolar epithelial metaplasia, granulomatous alveolar inflammation, and interstitial fibrosis were significantly greater than those in the chamber controls (Tables 5, A5, and B5). In general, these lung lesions increased in incidence and severity with increased exposure to cobalt sulfate heptahydrate. The incidence of squamous metaplasia in 1.0 mg/m<sup>3</sup> females was significantly greater than in the chamber control group. Multifocally, throughout the lungs, pulmonary architecture was distorted by a combination of inflammatory cells, fibrosis, and epithelial metaplasia. Lesions tended to be subpleural, peripheral, and/or along larger blood vessels and airways. Granulomatous inflammation was characterized by accumulations of alveolar macrophages with foamy cytoplasm, occasional multinucleated giant cells and cholesterol clefts, cell debris and few neutrophils. In these areas, the alveolar interstitium and occasionally the overlying pleura were variably thickened by dense fibrous connective tissue which often effaced alveoli (Plates 1 and 2). Although a diffuse change, aggregates of homogeneous to granular eosinophilic material within alveolar lumens (alveolar proteinosis) were often pronounced within the areas of chronic inflammation. Metaplasia of the alveolar epithelium in alveoli within and at the periphery of foci of inflammation was characterized by replacement of normal Type I epithelial cells with plump cuboidal or ciliated columnar epithelial cells. The incidences of alveolar epithelial hyperplasia in all groups of exposed males and in females exposed to 3.0 mg/m<sup>3</sup> and atypical alveolar epithelial hyperplasia in 3.0 mg/m<sup>3</sup> females were significantly greater than those in the chamber control groups.

The combined incidence of alveolar/bronchiolar neoplasms (adenoma and/or carcinoma) was significantly greater in 3.0 mg/m<sup>3</sup> males than that in the chamber controls and exceeded the historical control range (Tables 5 and A3). In females exposed to 1.0 or 3.0 mg/m<sup>3</sup>, the incidences of alveolar/bronchiolar neoplasms were significantly greater than those in the chamber control group and exceeded the historical control ranges (Tables 5, B3, and B4a). Although the incidences of alveolar/bronchiolar adenoma in 3.0 mg/m<sup>3</sup> males and alveolar/bronchiolar carcinoma in 1.0 mg/m<sup>3</sup> males were not significantly increased, they exceeded the historical control ranges for inhalation studies (Tables 5, A3, and A4a).

The spectrum of alveolar/bronchiolar neoplasms and nonneoplastic proliferative lesions observed within the lungs of exposed rats was broad. While many of these lesions were highly cellular and morphologically similar to those observed spontaneously, others were predominantly fibrotic, squamous, or mixtures of alveolar/bronchiolar epithelium and squamous or fibrous components. Hyperplasia generally represented an increase in numbers of epithelial cells along alveolar walls with maintenance of normal alveolar architecture (Plates 3 and 4). Multiple hyperplastic lesions were often observed in animals receiving higher concentrations of cobalt sulfate heptahydrate. The benign neoplasms typical of those observed spontaneously were generally distinct masses that often compressed surrounding tissue (Plates 5 and 6). Component epithelial cells were often arranged in acinar and/or irregular papillary structures and occasionally in a solid cellular pattern. These epithelial cells were typically uniform and similar to hyperplastic counterparts. Malignant alveolar/bronchiolar neoplasms had similar cellular patterns but were generally larger and had one or more of the following histologic features: heterogeneous growth pattern, cellular pleomorphism and/or atypia, and local invasion or metastasis (Plate 7).

In addition to these more typical proliferative lesions, there were "fibroproliferative" lesions ranging from less than 1 mm to greater than 1 cm in diameter. Generally, these lesions had a rounded outline and a central fibrous core containing dispersed glandular (alveolar) structures lined by uniformly cuboidal epithelial cells. Aggregates of mostly necrotic

**TABLE 5**  
**Incidences of Neoplasms and Nonneoplastic Lesions of the Lung in Rats in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Male</b>				
Number Examined Microscopically	50	50	48	50
Alveolar Epithelium, Hyperplasia <sup>a</sup>	9 (1.8) <sup>b</sup>	20* (2.0)	20* (2.1)	23** (2.0)
Alveolar Epithelium, Hyperplasia, Atypical	0	1 (2.0)	2 (3.0)	2 (4.0)
Metaplasia, Squamous	0	1 (1.0)	4 (2.0)	2 (3.0)
Alveolar Epithelium, Metaplasia	0	50** (1.9)	48** (3.1)	49** (3.7)
Inflammation, Granulomatous	2 (1.0)	50** (1.9)	48** (3.1)	50** (3.7)
Interstitialium, Fibrosis	1 (1.0)	50** (1.9)	48** (3.1)	49** (3.7)
Proteinosis	0	16** (1.4)	40** (2.3)	47** (3.4)
Cyst	0	0	0	1 (4.0)
Alveolar/bronchiolar Adenoma <sup>c</sup>				
Overall rate <sup>d</sup>	1/50 (2%)	4/50 (8%)	1/48 (2%)	6/50 (12%)
Adjusted rate <sup>e</sup>	2.3%	17.7%	2.4%	28.4%
Terminal rate <sup>f</sup>	0/17 (0%)	2/15 (13%)	0/21 (0%)	2/15 (13%)
First incidence (days)	568	589	611	638
Logistic regression test <sup>g</sup>	P=0.051	P=0.179	P=0.753	P=0.055
Alveolar/bronchiolar Carcinoma <sup>h</sup>				
Overall rate	0/50 (0%)	0/50 (0%)	3/48 (6%)	1/50 (2%)
Adjusted rate	0.0%	0.0%	11.3%	6.7%
Terminal rate	0/17 (0%)	0/15 (0%)	1/21 (5%)	1/15 (7%)
First incidence (days)	— <sup>i</sup>	—	652	734 (T)
Logistic regression test	P=0.360	—	P=0.136	P=0.475
Alveolar/bronchiolar Adenoma or Carcinoma <sup>j</sup>				
Overall rate	1/50 (2%)	4/50 (8%)	4/48 (8%)	7/50 (14%)
Adjusted rate	2.3%	17.7%	13.4%	33.9%
Terminal rate	0/17 (0%)	2/15 (13%)	1/21 (5%)	3/15 (20%)
First incidence (days)	568	589	611	638
Logistic regression test	P=0.032	P=0.179	P=0.163	P=0.029
<b>Female</b>				
Number Examined Microscopically	50	49	50	50
Alveolar Epithelium, Hyperplasia	15 (1.4)	7 (1.6)	20 (1.8)	33** (2.0)
Alveolar Epithelium, Hyperplasia, Atypical	0	0	3 (3.7)	5* (3.2)
Metaplasia, Squamous	0	1 (2.0)	8** (2.3)	3 (1.7)
Alveolar Epithelium, Metaplasia	2 (1.0)	47** (2.0)	50** (3.6)	49** (3.9)
Inflammation, Granulomatous	9 (1.0)	47** (2.0)	50** (3.6)	49** (3.9)
Interstitialium, Fibrosis	7 (1.0)	47** (2.0)	50** (3.6)	49** (3.9)
Proteinosis	0	36** (1.2)	49** (2.8)	49** (3.9)
Cyst	0	0	1 (4.0)	0
Alveolar/bronchiolar Adenoma <sup>k</sup>				
Overall rate	0/50 (0%)	1/49 (2%)	10/50 (20%)	9/50 (18%)
Adjusted rate	0.0%	3.4%	36.4%	30.0%
Terminal rate	0/28 (0%)	0/25 (0%)	9/26 (35%)	9/30 (30%)
First incidence (days)	—	714	692	735 (T)
Logistic regression test	P=0.001	P=0.480	P< 0.001	P=0.003

**TABLE 5**  
**Incidences of Neoplasms and Nonneoplastic Lesions of the Lung in Rats in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Female (continued)</b>				
Alveolar/bronchiolar Carcinoma <sup>l</sup>				
Overall rate	0/50 (0%)	2/49 (4%)	6/50 (12%)	6/50 (12%)
Adjusted rate	0.0%	8.0%	20.2%	17.5%
Terminal rate	0/28 (0%)	2/25 (8%)	4/26 (15%)	4/30 (13%)
First incidence (days)	—	735 (T)	694	610
Logistic regression test	P=0.023	P=0.213	P=0.015	P=0.017
Alveolar/bronchiolar Adenoma or Carcinoma <sup>m</sup>				
Overall rate	0/50 (0%)	3/49 (6%)	15/50 (30%)	15/50 (30%)
Adjusted rate	0.0%	11.2%	50.6%	46.1%
Terminal rate	0/28 (0%)	2/25 (8%)	12/26 (46%)	13/30 (43%)
First incidence (days)	—	714	692	610
Logistic regression test	P < 0.001	P=0.096	P < 0.001	P < 0.001
Squamous Cell Carcinoma				
Overall rate	0/50 (0%)	0/49 (0%)	1/50 (2%)	1/50 (2%)
Alveolar/bronchiolar Adenoma, Alveolar/bronchiolar Carcinoma, or Squamous Cell Carcinoma				
Overall rate	0/50 (0%)	3/49 (6%)	16/50 (32%)	16/50 (32%)
Adjusted rate	0.0%	11.2%	54.1%	49.2%
Terminal rate	0/28 (0%)	2/25 (8%)	13/26 (50%)	14/30 (47%)
First incidence (days)	—	714	692	610
Logistic regression test	P < 0.001	P=0.096	P < 0.001	P < 0.001

\* Significantly different ( $P \leq 0.05$ ) from the chamber control by the logistic regression test

\*\*  $P \leq 0.01$

(T) Terminal sacrifice

<sup>a</sup> Number of animals with lesion

<sup>b</sup> Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked

<sup>c</sup> Historical incidence for 2-year inhalation studies with chamber controls (mean  $\pm$  standard deviation): 17/654 (2.6%  $\pm$  3.6%); range 0%-10%

<sup>d</sup> Number of animals with neoplasm per number of animals with lung examined microscopically

<sup>e</sup> Kaplan-Meier estimated neoplasm incidence at the end of the study after adjustment for intercurrent mortality

<sup>f</sup> Observed incidence in animals surviving until the end of the study

<sup>g</sup> In the chamber control column are the P values associated with the trend test. In the exposed group columns are the P values corresponding to the pairwise comparisons between the chamber controls and that exposed group. The logistic regression test regards lesions in animals dying prior to terminal kill as nonfatal.

<sup>h</sup> Historical incidence: 6/654 (0.9%  $\pm$  1.0%); range 0%-2%

<sup>i</sup> Not applicable; no neoplasms in animal group

<sup>j</sup> Historical incidence: 23/654 (3.5%  $\pm$  3.7%); range 0%-10%

<sup>k</sup> Historical incidence: 7/650 (1.1%  $\pm$  1.6%); range 0%-4%

<sup>l</sup> Historical incidence: 0/650

<sup>m</sup> Historical incidence: 7/650 (1.1%  $\pm$  1.6%); range 0%-4%

inflammatory cells were also present in adjacent alveoli and often within the glandular structures. Peripherally, the fibroproliferative lesions had one to several layers of epithelium which coursed along and often extended into adjacent alveoli, frequently forming papillary projections (Plates 8, 9, and 10). These epithelial cells were often slightly pleomorphic with occasional mitotic figures. The smallest of these lesions were usually observed adjacent to areas of chronic inflammation. Small lesions with modest amounts of peripheral epithelial proliferation were diagnosed as atypical hyperplasia, while larger lesions with florid epithelial proliferation, marked cellular pleomorphism, and/or local invasion were diagnosed as alveolar/bronchiolar carcinomas (Plate 11).

While squamous epithelium is not normally observed within the lung, squamous metaplasia of alveolar/bronchiolar epithelium is a relatively common response to pulmonary injury and occurred in a number of rats in this study (Table 5). Squamous metaplasia was a minor change consisting of a small cluster of alveoli in which the normal epithelium was replaced by multiple layers of flattened squamous epithelial cells (Plate 12) that occasionally formed keratin. One 3.0 mg/m<sup>3</sup> male and one 1.0 mg/m<sup>3</sup> female had a large cystic squamous lesion rimmed by a variably thick (a few to many cell layers) band of viable squamous epithelium with a large central core of keratin (Plate 13). These were diagnosed as cysts. In one 1.0 mg/m<sup>3</sup> and one 3.0 mg/m<sup>3</sup> female, proliferative squamous lesions had cystic areas but also more solid areas of pleomorphic cells and invasion into the adjacent lung; these lesions were considered to be squamous cell carcinomas (Plate 14). In general, diagnoses of squamous lesions were made only when the lesion composition was almost entirely squamous epithelium. However, squamous metaplasia/differentiation was a variable component of other alveolar/bronchiolar proliferative lesions (Plate 15), including the fibroproliferative lesions, and was clearly a part of the spectrum of lesions resulting from exposure to cobalt sulfate heptahydrate.

*Adrenal Medulla:* The incidence of benign pheochromocytoma in 3.0 mg/m<sup>3</sup> females was significantly greater than that in the chamber controls and exceeded the historical range for inhalation studies (Tables 6, B3, and B4b). The incidences of benign, complex, or malignant pheochromocytoma (combined) in 1.0 mg/m<sup>3</sup> males and in 3.0 mg/m<sup>3</sup> females were significantly greater than those in the chamber controls and exceeded the historical control ranges (Tables 6, A3, A4b, B3, and B4b).

The incidences of bilateral pheochromocytoma in exposed males slightly exceeded that in the chamber control group. The incidence of hyperplasia was not significantly increased in exposed males or females. Focal hyperplasia and pheochromocytoma are considered to constitute a morphological continuum in the adrenal medulla. Focal hyperplasia consisted of irregular, small foci of small- to normal-sized medullary cells arranged in packets or solid clusters slightly larger than normal; compression of surrounding parenchyma was minimal or absent. Benign pheochromocytomas were well-delineated masses often with altered architecture and variable compression of surrounding parenchyma. Neoplastic cells were arranged in variably sized aggregates, clusters, and/or variably thick trabecular cords. Larger neoplasms usually exhibited greater cellular pleomorphism and atypia than smaller neoplasms. Malignant pheochromocytomas were identified when there was invasion of or beyond the adrenal capsule or when distant metastases were observed. Although a very common spontaneous neoplasm in male F344/N rats, pheochromocytomas have a lower spontaneous occurrence in females. In this study, the incidence of pheochromocytoma in 3.0 mg/m<sup>3</sup> females was considered related to the administration of cobalt sulfate heptahydrate. The marginally increased incidence of pheochromocytoma in males was considered an uncertain finding because it occurred only in the 1.0 mg/m<sup>3</sup> group and was not supported by increased incidence or severity of hyperplasia.

**TABLE 6**  
**Incidences of Neoplasms and Nonneoplastic Lesions of the Adrenal Medulla in Rats**  
**in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Male</b>				
Number Examined Microscopically	50	50	49	50
Hyperplasia <sup>a</sup>	34 (2.0) <sup>b</sup>	23* (2.5)	29 (2.1)	30 (2.1)
Benign Bilateral Pheochromocytoma				
Overall rate	1/50 (2%)	4/50 (8%)	6/49 (12%)	5/50 (10%)
Benign Pheochromocytoma (includes benign bilateral pheochromocytoma) <sup>c</sup>				
Overall rate <sup>d</sup>	14/50 (28%)	19/50 (38%)	23/49 (47%)	20/50 (40%)
Adjusted rate <sup>e</sup>	51.0%	70.0%	71.9%	71.4%
Terminal rate <sup>f</sup>	6/17 (35%)	8/15 (53%)	13/21 (62%)	8/15 (53%)
First incidence (days)	534	541	526	526
Logistic regression test <sup>g</sup>	P=0.172	P=0.226	P=0.069	P=0.126
Benign, Complex, or Malignant Pheochromocytoma (includes benign bilateral pheochromocytoma) <sup>h</sup>				
Overall rate	15/50 (30%)	19/50 (38%)	25/49 (51%)	20/50 (40%)
Adjusted rate	52.1%	70.0%	74.1%	71.4%
Terminal rate	6/17 (35%)	8/15 (53%)	13/21 (62%)	8/15 (53%)
First incidence (days)	534	541	526	526
Logistic regression test	P=0.218	P=0.295	P=0.045	P=0.180
<b>Female</b>				
Number Examined Microscopically	48	49	50	48
Hyperplasia	8 (1.6)	7 (2.3)	11 (2.1)	13 (2.0)
Benign Pheochromocytoma <sup>i</sup>				
Overall rate	2/48 (4%)	1/49 (2%)	3/50 (6%)	8/48 (17%)
Adjusted rate	5.1%	3.1%	9.3%	26.4%
Terminal rate	0/27 (0%)	0/25 (0%)	1/26 (4%)	7/29 (24%)
First incidence (days)	666	702	694	709
Logistic regression test	P=0.004	P=0.498N	P=0.512	P=0.043
Benign, Complex, or Malignant Pheochromocytoma <sup>j</sup>				
Overall rate	2/48 (4%)	1/49 (2%)	4/50 (8%)	10/48 (21%)
Adjusted rate	5.1%	3.1%	11.7%	31.5%
Terminal rate	0/27 (0%)	0/25 (0%)	1/26 (4%)	8/29 (28%)
First incidence (days)	666	702	685	663
Logistic regression test	P< 0.001	P=0.498N	P=0.323	P=0.014

\* Significantly different ( $P \leq 0.05$ ) from the chamber control by the logistic regression test

<sup>a</sup> Number of animals with lesion

<sup>b</sup> Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked

<sup>c</sup> Historical incidence for 2-year inhalation studies with chamber controls (mean  $\pm$  standard deviation): 163/623 (26.2%  $\pm$  13.2%); range 0%-50%

<sup>d</sup> Number of animals with neoplasm per number of animals with adrenal medulla examined microscopically

<sup>e</sup> Kaplan-Meier estimated neoplasm incidence at the end of the study after adjustment for intercurrent mortality

<sup>f</sup> Observed incidence in animals surviving until the end of the study

<sup>g</sup> In the chamber control column are the P values associated with the trend test. In the exposed group columns are the P values corresponding to the pairwise comparisons between the chamber controls and that exposed group. The logistic regression test regards lesions in animals dying prior to terminal kill as nonfatal. A lower incidence in an exposure group is indicated by N.

<sup>h</sup> Historical incidence: 176/623 (28.3%  $\pm$  12.0%); range 8%-50%

<sup>i</sup> Historical incidence: 35/608 (5.8%  $\pm$  4.9%); range 0%-14%

<sup>j</sup> Historical incidence: 39/608 (6.4%  $\pm$  4.4%); range 2%-14%

**Nose:** The incidences of hyperplasia of the lateral wall of the nose and atrophy of the olfactory epithelium in all exposed groups of males and females were significantly greater than those in the chamber controls, and the severities of these lesions increased with increasing exposure concentration (Tables 7, A5, and B5). The incidences of squamous metaplasia of the lateral wall of the nose and metaplasia of the olfactory epithelium in 3.0 mg/m<sup>3</sup> males and females were significantly greater than those in the chamber controls.

Although the incidence and severity of nasal lesions increased with increased exposure to cobalt sulfate heptahydrate, they involved limited portions of nasal epithelium and none were severe. Hyperplasia and

squamous metaplasia were minimal to mild, unilateral or bilateral, and involved the transitional epithelium along the walls and turbinates of the anterior nasal passage. Hyperplasia was characterized by an increase in thickness of the epithelium from the normal one to two layers to two or more layers, while squamous metaplasia represented areas where the normal transitional epithelium was replaced by multiple layers of flattened epithelial cells. More posterior in the nose, along the dorsal meatus, atrophy of the olfactory epithelium was characterized by loss of cell layers and disorganization of remaining epithelium, and in some instances, increased prominence of sensory cell nuclei. Metaplasia was characterized by replacement of olfactory epithelium with respiratory-type ciliated columnar epithelium.

**TABLE 7**  
**Incidences of Nonneoplastic Lesions of the Nose and Larynx in Rats in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Male</b>				
Nose <sup>a</sup>	50	50	49	50
Lateral Wall, Hyperplasia <sup>b</sup>	2 (1.5) <sup>c</sup>	14**(1.4)	21**(1.5)	20**(1.6)
Lateral Wall, Metaplasia, Squamous	1 (1.0)	3 (1.3)	5 (1.4)	8* (2.0)
Olfactory Epithelium, Atrophy	8 (1.1)	24**(1.4)	42**(1.5)	48**(2.5)
Olfactory Epithelium, Metaplasia	5 (1.2)	1 (3.0)	5 (1.8)	30**(1.9)
Larynx	50	49	48	50
Epiglottis, Metaplasia, Squamous	0	10**(1.3)	37**(1.8)	50**(2.8)
<b>Female</b>				
Nose	50	49	50	50
Lateral Wall, Hyperplasia	1 (1.0)	8* (1.3)	26**(1.4)	38**(1.7)
Lateral Wall, Metaplasia, Squamous	1 (1.0)	1 (3.0)	4 (1.3)	10**(1.4)
Olfactory Epithelium, Atrophy	5 (1.4)	29**(1.2)	46**(1.6)	47**(2.9)
Olfactory Epithelium, Metaplasia	2 (2.0)	2 (1.5)	3 (1.7)	40**(2.3)
Larynx	50	49	50	50
Epiglottis, Metaplasia, Squamous	1 (1.0)	22**(1.1)	39**(1.4)	48**(2.6)

\* Significantly different ( $P \leq 0.05$ ) from the chamber control by the logistic regression test

\*\*  $P \leq 0.01$

<sup>a</sup> Number of animals with tissue examined microscopically

<sup>b</sup> Number of animals with lesion

<sup>c</sup> Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked

*Larynx:* The incidences of squamous metaplasia of the epiglottis in all exposed groups of males and females were significantly greater than those in the chamber controls, and the severity of this lesion increased with increasing exposure concentration (Tables 7, A5, and B5). Squamous metaplasia was limited to the base

of the epiglottis and was not a severe lesion in exposed rats. It was characterized by replacement of the ciliated respiratory epithelium by one or more layers of flattened epithelial cells overlying a basal layer of cuboidal cells. Keratinization was sometimes observed.

## MICE

### Survival

Estimates of 2-year survival probabilities for male and female mice are shown in Table 8 and in the Kaplan-Meier survival curves (Figure 3). Survival of exposed males and females was similar to that of the chamber controls.

### Body Weights and Clinical Findings

Mean body weights are given in Figure 4 and Tables 9 and 10. Mean body weights of 3.0 mg/m<sup>3</sup> male mice

were less than those of the chamber controls from week 96 until the end of the study. The mean body weights of all exposed female mice were generally greater than those of the chamber controls from week 20 until the end of the study. Irregular breathing was observed slightly more frequently in female mice exposed to 1.0 mg/m<sup>3</sup> than in the chamber controls or other exposed groups.

**TABLE 8**  
**Survival of Mice in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate**

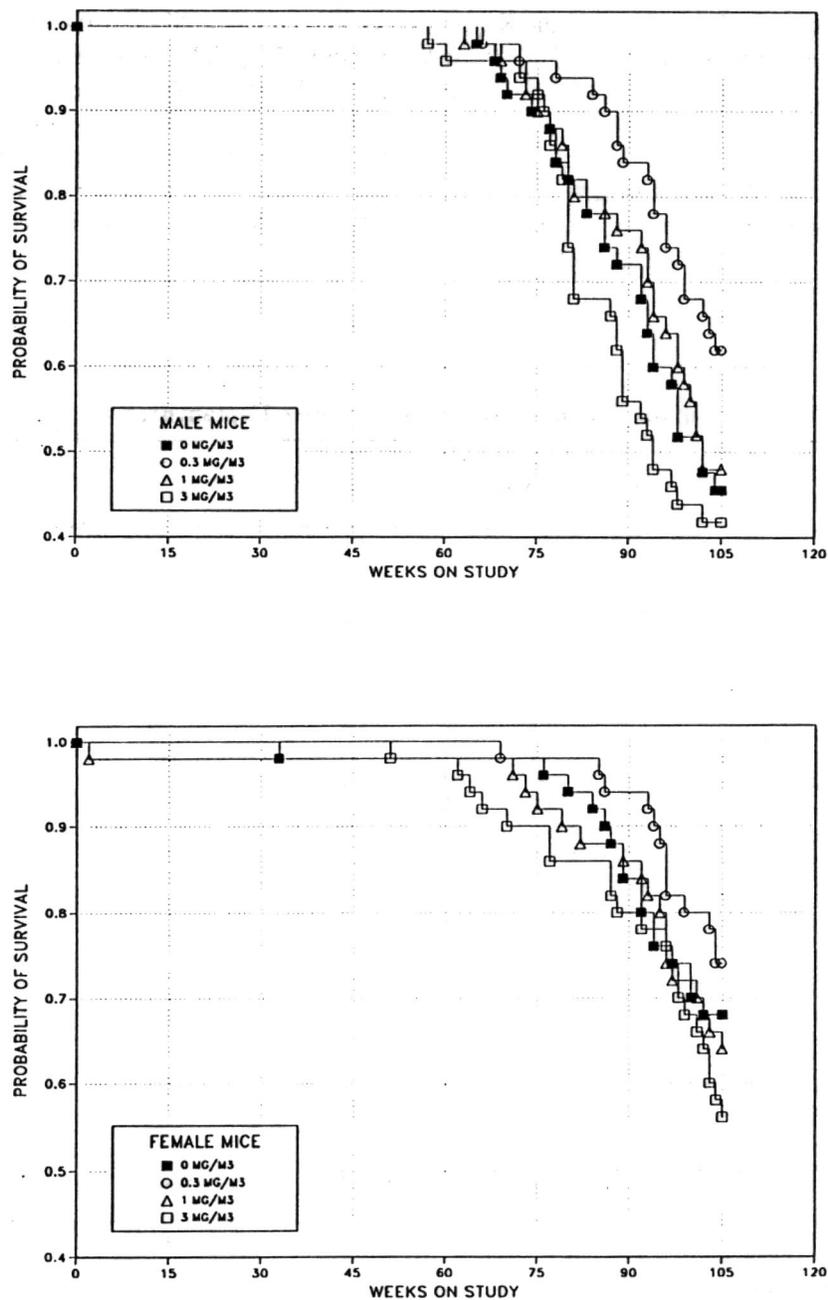
	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Male</b>				
Animals initially in study	50	50	50	50
Accidental deaths <sup>a</sup>	1	0	0	1
Moribund	19	16	17	23
Natural deaths	8	3	9	6
Animals surviving to study termination	22	31	24	20
Percent probability of survival at end of study <sup>b</sup>	46	62	48	42
Mean survival (days) <sup>c</sup>	662	695	670	643
Survival analysis <sup>d</sup>	P=0.104	P=0.088N	P=0.861N	P=0.577
<b>Female</b>				
Animals initially in study	50	50	50	50
Moribund	11	10	13	16
Natural deaths	5	3	5	6
Animals surviving to study termination	34	37	32	28
Percent probability of survival at end of study	68	74	64	56
Mean survival (days)	694	713	685	680
Survival analysis	P=0.102	P=0.529N	P=0.855	P=0.327

<sup>a</sup> Censored from survival analyses

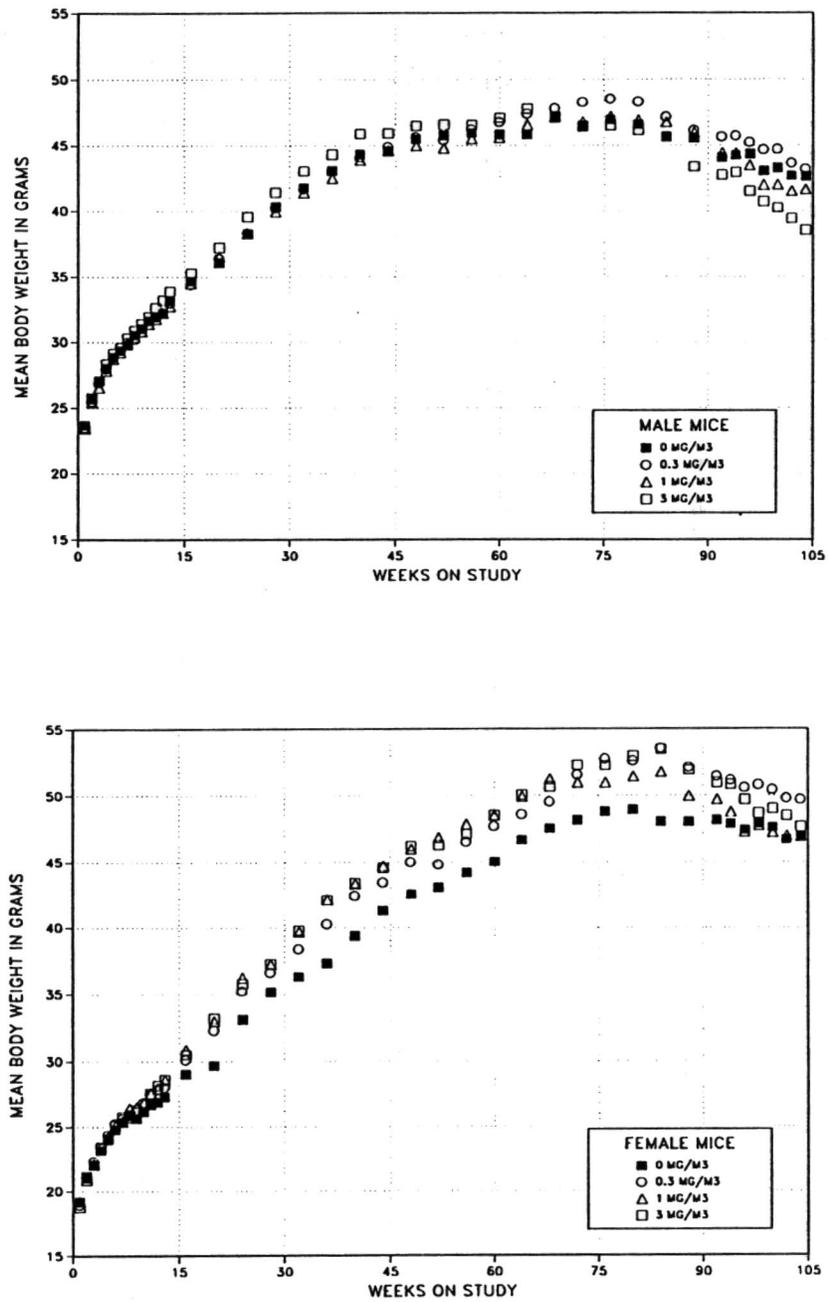
<sup>b</sup> Kaplan-Meier determinations

<sup>c</sup> Mean of all deaths (uncensored, censored, and terminal sacrifice)

<sup>d</sup> The result of the life table trend test (Tarone, 1975) is in the chamber control column, and the results of the life table pairwise comparisons (Cox, 1972) with the chamber controls are in the exposed group columns. A lower mortality in an exposure group is indicated by **N**.



**FIGURE 3**  
**Kaplan-Meier Survival Curves for Male and Female Mice**  
**Exposed to Cobalt Sulfate Heptahydrate by Inhalation for 2 Years**



**FIGURE 4**  
Growth Curves for Male and Female Mice Exposed to Cobalt Sulfate Heptahydrate by Inhalation for 2 Years

**TABLE 9**  
**Mean Body Weights and Survival of Male Mice in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

Weeks on Study	Chamber Control		0.3 mg/m <sup>3</sup>			1.0 mg/m <sup>3</sup>			3.0 mg/m <sup>3</sup>		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	23.7	50	23.5	99	50	23.4	99	50	23.4	99	50
2	25.8	50	25.5	99	50	25.4	98	50	25.7	100	50
3	27.0	50	26.9	100	50	26.6	99	50	27.1	100	50
4	28.0	50	27.9	100	50	27.8	99	50	28.3	101	50
5	28.8	50	28.8	100	50	28.7	100	50	29.1	101	50
6	29.3	50	29.6	101	50	29.2	100	50	29.6	101	50
7	29.8	50	30.0	101	50	30.0	101	50	30.3	102	50
8	30.5	50	30.2	99	50	30.4	100	50	30.9	101	50
9	31.0	50	30.8	99	50	30.8	99	50	31.4	101	50
10	31.6	50	31.3	99	50	31.4	99	50	31.9	101	50
11	32.0	50	31.9	100	50	31.8	99	50	32.7	102	50
12	32.2	50	32.2	100	50	32.2	100	50	33.2	103	50
13	33.1	50	33.0	100	50	32.7	99	50	33.9	102	50
16	34.7	50	34.4	99	50	34.5	99	50	35.3	102	50
20	36.1	50	36.4	101	50	36.6	101	50	37.2	103	50
24	38.3	50	38.4	100	50	38.3	100	50	39.6	103	50
28	40.3	50	40.3	100	50	40.0	99	50	41.4	103	50
32	41.8	50	41.7	100	50	41.4	99	50	43.1	103	50
36	43.1	50	43.1	100	50	42.5	99	50	44.3	103	50
40	44.3	50	44.1	100	50	43.9	99	50	45.9	104	50
44	44.6	50	44.9	101	50	44.7	100	50	45.9	103	50
48	45.5	50	45.6	100	50	45.0	99	50	46.5	102	50
52	45.8	50	45.4	99	50	44.8	98	50	46.6	102	50
56	45.9	50	46.2	101	50	45.5	99	50	46.5	101	50
60	45.8	50	46.8	102	50	45.6	100	50	47.1	103	48
64	45.8	50	47.4	104	50	46.6	102	49	47.8	104	48
68	47.1	48	47.8	102	49	47.2	100	49	47.2	100	48
72	46.4	46	48.3	104	48	46.8	101	48	46.5	100	48
76	46.9	45	48.5	103	48	47.2	101	45	46.5	99	46
80	46.6	42	48.3	104	47	46.9	101	43	46.1	99	40
84	45.6	39	47.2	104	47	46.7	102	40	45.7	100	34
88	45.5	37	46.1	101	45	46.0	101	38	43.4	95	32
92	44.1	36	45.6	103	42	44.4	101	38	42.8	97	28
94	44.2	32	45.7	103	41	44.4	101	35	42.9	97	26
96	44.3	30	45.2	102	39	43.5	98	33	41.5	94	24
98	43.0	29	44.7	104	37	42.0	98	32	40.7	95	22
100	43.3	25	44.7	103	34	42.0	97	29	40.3	93	21
102	42.7	25	43.7	102	34	41.5	97	26	39.5	93	21
104	42.6	23	43.2	101	32	41.6	98	24	38.5	90	20
<b>Mean for weeks</b>											
1-13	29.4		29.4	100		29.3	100		29.8	101	
14-52	41.5		41.4	100		41.2	99		42.6	103	
53-104	45.0		46.2	103		44.9	100		43.9	98	

**TABLE 10**  
**Mean Body Weights and Survival of Female Mice in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

Weeks on Study	Chamber Control		0.3 mg/m <sup>3</sup>			1.0 mg/m <sup>3</sup>			3.0 mg/m <sup>3</sup>		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	19.2	50	18.9	98	50	19.2	100	50	18.7	97	50
2	21.2	50	21.1	100	50	21.1	100	50	20.8	98	50
3	22.0	50	22.3	101	50	22.2	101	49	22.1	101	50
4	23.2	50	23.5	101	50	23.4	101	49	23.5	101	50
5	24.0	50	24.4	102	50	24.3	101	49	24.1	100	50
6	24.8	50	25.3	102	50	25.1	101	49	25.1	101	50
7	25.4	50	25.6	101	50	25.5	100	49	25.8	102	50
8	26.0	50	25.9	100	50	26.5	102	49	25.7	99	50
9	25.6	50	26.2	102	50	26.6	104	49	26.4	103	50
10	26.2	50	26.9	103	50	26.9	103	49	26.8	102	50
11	26.7	50	27.5	103	50	27.6	103	49	27.6	103	50
12	26.9	50	27.9	104	50	28.0	104	49	28.2	105	50
13	27.3	50	28.0	103	50	28.6	105	49	28.6	105	50
16	29.1	50	30.1	103	50	30.9	106	49	30.5	105	50
20	29.7	50	32.3	109	50	33.0	111	49	33.2	112	50
24	33.1	50	35.3	107	50	36.3	110	49	35.8	108	50
28	35.2	50	36.6	104	50	37.3	106	49	37.3	106	50
32	36.3	50	38.4	106	50	39.7	109	49	39.8	110	50
36	37.3	49	40.3	108	50	42.1	113	49	42.1	113	50
40	39.4	49	42.4	108	50	43.3	110	49	43.4	110	50
44	41.3	49	43.4	105	50	44.7	108	49	44.5	108	50
48	42.5	49	45.0	106	50	46.0	108	49	46.2	109	50
52	43.0	49	44.8	104	50	46.9	109	49	46.3	108	49
56	44.2	49	46.5	105	50	47.9	108	49	47.1	107	49
60	45.0	49	47.7	106	50	48.5	108	49	48.5	108	49
64	46.7	49	48.6	104	50	49.9	107	49	50.1	107	47
68	47.5	49	49.5	104	50	51.3	108	49	50.7	107	46
72	48.2	49	51.6	107	49	51.0	106	48	52.3	109	45
76	48.8	49	52.8	108	49	51.0	105	46	52.3	107	45
80	48.9	48	52.6	108	49	51.4	105	45	53.0	108	43
84	48.1	46	53.5	111	49	51.8	108	44	53.5	111	43
88	48.1	44	52.1	108	47	50.0	104	44	51.9	108	41
92	48.2	42	51.5	107	47	49.7	103	43	51.0	106	40
94	47.9	40	51.2	107	46	48.8	102	41	50.8	106	39
96	47.4	38	50.6	107	44	47.3	100	40	49.7	105	39
98	47.9	37	50.8	106	41	47.8	100	36	48.7	102	37
100	47.6	37	50.5	106	40	47.2	99	36	49.0	103	34
102	46.7	35	49.8	107	40	47.0	101	35	48.5	104	33
104	46.9	34	49.7	106	39	46.9	100	33	47.7	102	30
<b>Mean for weeks</b>											
1-13	24.5		24.9	102		25.0	102		24.9	102	
14-52	36.7		38.9	106		40.0	109		39.9	109	
53-104	47.4		50.6	107		49.2	104		50.3	106	

### ***Pathology and Statistical Analyses***

This section describes the statistically significant or biologically noteworthy changes in the incidences of neoplasms and/or nonneoplastic lesions of the lung, nose, larynx, thyroid gland, and liver. Summaries of the incidences of neoplasms and nonneoplastic lesions, individual animal tumor diagnoses, statistical analyses of primary neoplasms that occurred with an incidence of at least 5% in at least one animal group, and historical incidences for the neoplasms mentioned in this section are presented in Appendix C for male mice and Appendix D for female mice.

*Lung:* In all exposed groups of males and females, the incidences of cytoplasmic vacuolization of the bronchi were significantly greater than those in the chamber control groups (Tables 11, C5, and D5). The incidences of diffuse histiocytic cell infiltration in 3.0 mg/m<sup>3</sup> males and of focal histiocytic cell infiltration in 3.0 mg/m<sup>3</sup> females were significantly greater than those in the chamber controls.

Cytoplasmic vacuolization of the bronchial epithelium was a minimal change of unknown biological significance confined to the epithelial cells lining the apex of the bronchial bifurcation. The affected cells were somewhat larger than normal with a diffusely clear to finely vacuolated cytoplasm. Histiocyte infiltration was characterized by one or more histiocytes with foamy cytoplasm within variable numbers of alveolar lumens. Focal infiltrate was a localized accumulation of histiocytes, while diffuse infiltrate was more widely scattered. The histiocyte infiltrate was very commonly seen in lungs with alveolar/bronchiolar neoplasms, and the increased incidences of infiltrate in the lungs of exposed animals were considered to reflect the higher incidences of lung neoplasms in these animals rather than a primary effect of cobalt sulfate heptahydrate exposure.

The incidences of alveolar/bronchiolar neoplasms (adenoma and/or carcinoma) in 3.0 mg/m<sup>3</sup> males and females and the combined incidence of alveolar/bronchiolar neoplasms in 1.0 mg/m<sup>3</sup> females were significantly greater than those in the chamber control groups and generally exceeded the historical control ranges for inhalation studies (Tables 11, C3, C4a, D3, and D4a). In exposed males and females, the incidences of all lung neoplasms occurred with positive trends.

Unlike in the rat, all the alveolar/bronchiolar proliferative lesions observed within the lungs of exposed mice were typical of those observed spontaneously. Hyperplasia generally represented an increase in numbers of epithelial cells along alveolar walls which retained normal alveolar structure. Adenomas generally were distinct masses that often compressed surrounding tissue (Plate 16). Component cells were arranged in acinar and/or irregular papillary structures and occasionally in a solid cellular pattern. These cells were typically uniform and similar to hyperplastic counterparts. Malignant alveolar/bronchiolar neoplasms had similar cellular patterns but were generally larger (Plate 17) and had one or more of the following: heterogeneous growth pattern, cellular pleomorphism, and/or atypia and local invasion or metastasis.

Although similar in appearance to “spontaneous” lung neoplasms in chamber controls, alveolar/bronchiolar neoplasms in mice exposed to cobalt sulfate heptahydrate had different molecular lesions in the *Kras* gene (Appendix I). Of the *K-ras* mutations detected at the second base of codon 12, a higher frequency (5/9, 55%) of G to T transversions was detected compared to concurrent (0/1) and historical control lung neoplasms (1/24, 4%). *K-ras* codon 61 CTA or CGA mutations were not present in cobalt sulfate heptahydrate-induced lung neoplasms.

**TABLE 11**  
**Incidences of Neoplasms and Nonneoplastic Lesions of the Lung in Mice in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Male</b>				
Number Examined Microscopically	50	50	50	50
Infiltration Cellular, Diffuse, Histiocyte <sup>a</sup>	1 (3.0) <sup>b</sup>	2 (3.0)	4 (2.3)	10**(1.5)
Infiltration Cellular, Focal, Histiocyte	10 (2.7)	5 (2.6)	8 (3.0)	17 (2.7)
Bronchus, Cytoplasmic Vacuolization	0	18**(1.0)	34**(1.0)	38**(1.0)
Alveolar Epithelium Hyperplasia	0	4 (2.3)	4 (1.8)	4 (2.3)
Alveolar/bronchiolar Adenoma <sup>c</sup>				
Overall rate <sup>d</sup>	9/50 (18%)	12/50 (24%)	13/50 (26%)	18/50 (36%)
Adjusted rate <sup>e</sup>	30.4%	30.9%	41.1%	54.6%
Terminal rate <sup>f</sup>	4/22 (18%)	6/31 (19%)	7/24 (29%)	7/20 (35%)
First incidence (days)	600	460	548	524
Logistic regression test <sup>g</sup>	P=0.018	P=0.353	P=0.256	P=0.027
Alveolar/bronchiolar Carcinoma <sup>h</sup>				
Overall rate	4/50 (8%)	5/50 (10%)	7/50 (14%)	11/50 (22%)
Adjusted rate	13.2%	16.1%	25.3%	43.7%
Terminal rate	2/22 (9%)	5/31 (16%)	4/24 (17%)	7/20 (35%)
First incidence (days)	449	733 (T)	687	552
Logistic regression test	P=0.006	P=0.528	P=0.273	P=0.033
Alveolar/bronchiolar Adenoma or Carcinoma <sup>i</sup>				
Overall rate	11/50 (22%)	14/50 (28%)	19/50 (38%)	28/50 (56%)
Adjusted rate	35.5%	36.5%	56.5%	78.8%
Terminal rate	5/22 (23%)	8/31 (26%)	10/24 (42%)	13/20 (65%)
First incidence (days)	449	460	548	524
Logistic regression test	P< 0.001	P=0.345	P=0.071	P< 0.001
<b>Female</b>				
Number Examined Microscopically	50	50	50	50
Infiltration Cellular, Diffuse, Histiocyte	0	0	0	4 (3.3)
Infiltration Cellular, Focal, Histiocyte	2 (2.0)	5 (1.8)	7 (2.9)	10* (2.4)
Bronchus, Cytoplasmic Vacuolization	0	6* (1.0)	31**(1.0)	43**(1.0)
Alveolar Epithelium Hyperplasia	2 (1.5)	3 (1.3)	0	5 (2.0)
Alveolar/bronchiolar Adenoma <sup>j</sup>				
Overall rate	3/50 (6%)	6/50 (12%)	9/50 (18%)	10/50 (20%)
Adjusted rate	8.8%	15.0%	25.2%	32.8%
Terminal rate	3/34 (9%)	4/37 (11%)	6/32 (19%)	8/28 (29%)
First incidence (days)	734 (T)	664	649	706
Logistic regression test	P=0.024	P=0.287	P=0.057	P=0.024
Alveolar/bronchiolar Carcinoma <sup>k</sup>				
Overall rate	1/50 (2%)	1/50 (2%)	4/50 (8%)	9/50 (18%)
Adjusted rate	2.9%	2.7%	9.2%	25.3%
Terminal rate	1/34 (3%)	1/37 (3%)	1/32 (3%)	4/28 (14%)
First incidence (days)	734 (T)	734 (T)	495	536
Logistic regression test	P< 0.001	P=0.743N	P=0.201	P=0.009

**TABLE 11**  
**Incidences of Neoplasms and Nonneoplastic Lesions of the Lung in Mice in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Female</b> (continued)				
Alveolar/bronchiolar Adenoma or Carcinoma <sup>1</sup>				
Overall rate	4/50 (8%)	7/50 (14%)	13/50 (26%)	18/50 (36%)
Adjusted rate	11.8%	17.5%	32.6%	50.2%
Terminal rate	4/34 (12%)	5/37 (14%)	7/32 (22%)	11/28 (39%)
First incidence (days)	734 (T)	664	495	536
Logistic regression test	P < 0.001	P = 0.318	P = 0.016	P < 0.001

\* Significantly different ( $P \leq 0.05$ ) from the chamber control by the logistic regression test

\*\*  $P \leq 0.01$

(T) Terminal sacrifice

<sup>a</sup> Number of animals with lesion

<sup>b</sup> Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked

<sup>c</sup> Historical incidence for 2-year NTP inhalation studies with chamber control groups (mean  $\pm$  standard deviation): 141/947 (14.9%  $\pm$  7.0%); range 6%-36%

<sup>d</sup> Number of animals with neoplasm per number of animals with lung examined microscopically

<sup>e</sup> Kaplan-Meier estimated neoplasm incidence at the end of the study after adjustment for intercurrent mortality

<sup>f</sup> Observed incidence in animals surviving until the end of the study

<sup>g</sup> In the chamber control column are the P values associated with the trend test. In the exposed group columns are the P values corresponding to the pairwise comparisons between the chamber controls and that exposed group. The logistic regression test regards lesions in animals dying prior to terminal kill as nonfatal. A lower incidence in an exposure group is indicated by N.

<sup>h</sup> Historical incidence: 75/947 (7.9%  $\pm$  5.7%); range 0%-16%

<sup>i</sup> Historical incidence: 205/947 (21.7%  $\pm$  8.0%); range 10%-42%

<sup>j</sup> Historical incidence: 61/939 (6.5%  $\pm$  3.2%); range 0%-14%

<sup>k</sup> Historical incidence: 38/939 (4.1%  $\pm$  3.2%); range 0%-12%

<sup>l</sup> Historical incidence: 97/939 (10.3%  $\pm$  3.7%); range 0%-16%

**Nose:** The incidences of atrophy of the olfactory epithelium in 1.0 and 3.0 mg/m<sup>3</sup> males and females and hyperplasia of the olfactory epithelium in 3.0 mg/m<sup>3</sup> males and females were significantly greater than those in the chamber controls. The incidences of suppurative inflammation in 3.0 mg/m<sup>3</sup> males and in 1.0 mg/m<sup>3</sup> females were significantly greater than those in the chamber controls (Tables 12, C5, and D5). The nasal lesions in mice were less severe than in the rats and involved limited segments of the olfactory epithelium located further back in the nasal passage. Atrophy of the olfactory epithelium was characterized by loss of cell layers (sensory cells) and a decrease in the number of axons in the lamina propria. Hyperplasia of the olfactory epithelium was observed only in animals exposed to 3.0 mg/m<sup>3</sup> and was characterized by increased numbers of sensory cells that were usually arranged in nests or rosettes.

The suppurative inflammation involved only a few animals and was a very mild change. It primarily involved animals that died prior to the end of the study and consisted of a focal aggregate of inflammatory cells.

**Larynx:** The incidences of squamous metaplasia in all exposed groups of males and females were significantly greater than those in the chamber controls (Tables 12, C5, and D5). Squamous metaplasia was limited to the base of the epiglottis and was not a severe lesion in exposed mice. It was characterized by replacement of the ciliated respiratory epithelium by one or more layers of flattened epithelial cells overlying a basal layer of cuboidal cells. Keratinization was sometimes observed.

**TABLE 12**  
**Incidences of Nonneoplastic Lesions of the Nose and Larynx in Mice in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Male</b>				
Nose <sup>a</sup>	50	50	48	49
Olfactory Epithelium, Atrophy <sup>b</sup>	0	0	29**(1.2) <sup>c</sup>	48**(1.8)
Olfactory Epithelium, Hyperplasia	0	0	0	10**(1.0)
Inflammation, Suppurative	0	1 (3.0)	0	6* (2.2)
Larynx	48	49	48	49
Metaplasia, Squamous	0	37**(1.0)	48**(1.0)	44**(1.0)
<b>Female</b>				
Nose	50	50	49	48
Olfactory Epithelium, Atrophy	0	2 (1.5)	12**(1.0)	46**(1.5)
Olfactory Epithelium, Hyperplasia	0	0	0	30**(1.3)
Inflammation, Suppurative	0	1 (1.0)	5* (1.6)	4 (1.5)
Larynx	50	49	47	50
Metaplasia, Squamous	0	45**(1.0)	40**(1.0)	50**(1.1)

\* Significantly different ( $P \leq 0.05$ ) from the chamber control by the logistic regression test

\*\*  $P \leq 0.01$

<sup>a</sup> Number of animals with tissue examined microscopically

<sup>b</sup> Number of animals with lesion

<sup>c</sup> Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked

**Thyroid Gland:** The incidences of follicular cell hyperplasia in all exposed groups of males were significantly greater than the incidence in the chamber controls (chamber control, 3/49; 0.3 mg/m<sup>3</sup>, 17/50; 1.0 mg/m<sup>3</sup>, 11/50; 3.0 mg/m<sup>3</sup>, 10/50; Table C5). Minimal hyperplasias are commonly observed in untreated male and female mice, suggesting that the rate in the concurrent chamber control group is low. The severity of most hyperplasias in these mice was minimal to mild and did not differ between chamber control and exposed groups. The incidence of hyperplasia did not increase with exposure to cobalt sulfate heptahydrate, nor was the incidence of neoplasms of the follicular cells increased.

**Liver:** High incidences of chronic inflammation, karyomegaly, oval cell hyperplasia, and regeneration occurred in all groups of male mice and were usually observed together in the same liver (Tables 13 and C5). These changes were generally mild to moderate

in severity and observed throughout the liver (usually not within proliferative lesions), but they appeared most pronounced in the portal regions. Similar lesions were observed in only a few females, and the severity was also much less than that observed in most males (Tables 13 and D5). This spectrum of lesions is consistent with those observed with *Helicobacter hepaticus* infection (Appendix J). Liver sections from four of five male mice with liver lesions were positive for bacterial organisms consistent with *H. hepaticus* when examined using Steiner's modification of the Warthin Starry silver stain.

The incidences of hemangiosarcoma in all exposed groups of male mice and in 1.0 mg/m<sup>3</sup> in female mice exceeded the range observed in historical controls for inhalation studies (Tables 13, C3, and C4b). In addition, the incidence of hemangiosarcoma in 1.0 mg/m<sup>3</sup> males was significantly greater than that in

**TABLE 13**  
**Incidences of Neoplasms and Nonneoplastic Lesions of the Liver of Mice in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Male</b>				
Number Examined Microscopically	50	50	50	50
Inflammation, Chronic <sup>a</sup>	33 (1.3) <sup>b</sup>	36 (1.6)	40 (1.7)	39 (1.3)
Karyomegaly	39 (2.3)	35 (2.8)	39 (2.7)	43 (2.7)
Regeneration	32 (2.3)	30 (2.7)	35 (2.4)	38 (2.8)
Bile Duct, Hyperplasia	0	3 (1.3)	6* (1.7)	4 (2.5)
Oval Cell, Hyperplasia	38 (2.6)	36 (2.8)	40 (2.7)	44 (2.7)
Hemangiosarcoma <sup>c</sup>				
Overall rate <sup>d</sup>	2/50 (4%)	4/50 (8%)	8/50 (16%)	7/50 (14%)
Adjusted rate <sup>e</sup>	9.1%	11.5%	23.5%	25.0%
Terminal rate <sup>f</sup>	2/22 (9%)	2/31 (6%)	2/24 (8%)	3/20 (15%)
First incidence (days)	733 (T)	685	523	502
Logistic regression test <sup>g</sup>	P=0.078	P=0.441	P=0.050	P=0.069
<b>Female</b>				
Number Examined Microscopically	50	50	50	49
Inflammation, Chronic	6 (1.7)	1 (1.0)	1 (1.0)	2 (2.0)
Karyomegaly	4 (2.8)	2 (1.5)	0	1 (2.0)
Oval Cell, Hyperplasia	2 (2.0)	1 (2.0)	0	0
Hemangiosarcoma <sup>h</sup>				
Overall rate	1/50 (2%)	0/50 (0%)	3/50 (6%)	0/49 (0%)
Adjusted rate	2.9%	0.0%	7.3%	0.0%
Terminal rate	1/34 (3%)	0/37 (0%)	1/32 (3%)	0/28 (0%)
First incidence (days)	734 (T)	— <sup>i</sup>	524	—
Logistic regression test	P=0.431N	P=0.483N	P=0.318	P=0.539N

\* Significantly different (P≤0.05) from the chamber control by the logistic regression test

(T) Terminal sacrifice

<sup>a</sup> Number of animals with lesion

<sup>b</sup> Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked

<sup>c</sup> Historical incidence for 2-year NTP inhalation studies with chamber control groups (mean ± standard deviation): 12/947 (1.3% ± 1.7%); range 0%-6%

<sup>d</sup> Number of animals with neoplasm per number of animals with liver examined microscopically

<sup>e</sup> Kaplan-Meier estimated neoplasm incidence at the end of the study after adjustment for intercurrent mortality

<sup>f</sup> Observed incidence in animals surviving until the end of the study

<sup>g</sup> In the chamber control column are the P values associated with the trend test. In the exposed group columns are the P values corresponding to the pairwise comparisons between the chamber controls and that exposed group. The logistic regression test regards lesions in animals dying prior to terminal kill as nonfatal. A negative trend or lower incidence in an exposure group is indicated by N.

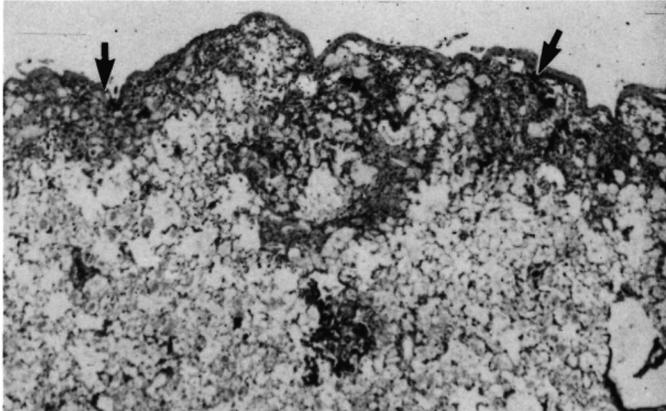
<sup>h</sup> Historical incidence: 5/937 (0.5% ± 1.0%); range 0%-3%

<sup>i</sup> Not applicable; no neoplasms in animal group

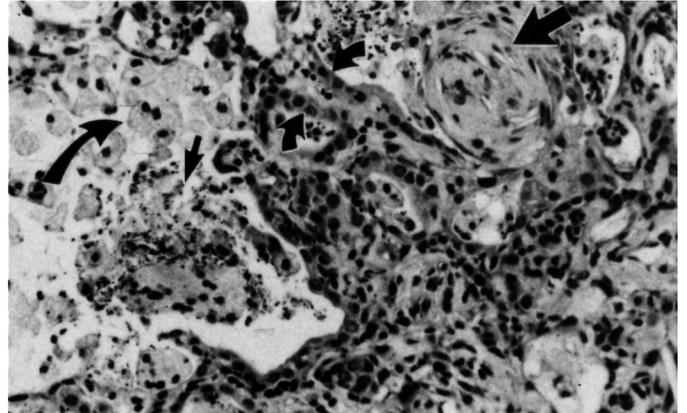
the chamber controls. Hemangiosarcomas were morphologically similar to those observed spontaneously and consisted of multiple variably sized blood-filled spaces that were separated by cords of hepatocytes and lined by plump endothelial cells.

## GENETIC TOXICOLOGY

Cobalt sulfate heptahydrate (3 to 10,000 µg/mL) was mutagenic in *Salmonella typhimurium* strain TA100 in the absence of S9 metabolic activation, and with 5% hamster or rat liver S9; no mutagenicity was detected in strain TA98 or TA1535, with or without S9 (Zeiger *et al.*, 1992; Table E1).



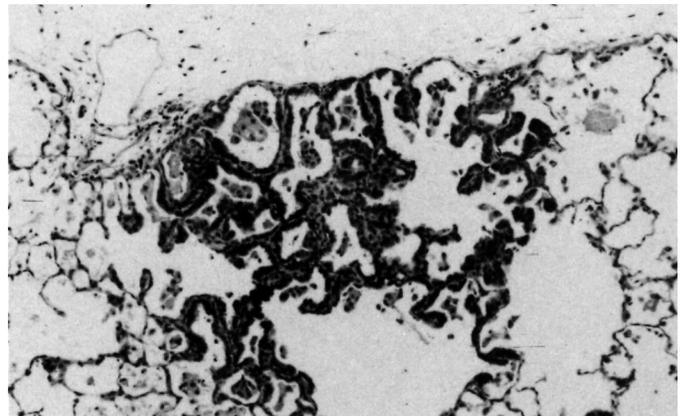
**PLATE 1**  
 Low magnification of a typical area of chronic inflammation (arrows) in the lung of a female F344/N rat exposed to 3.0mg/m<sup>3</sup> cobalt sulfate heptahydrate by inhalation for 2 years. H&E; 20×



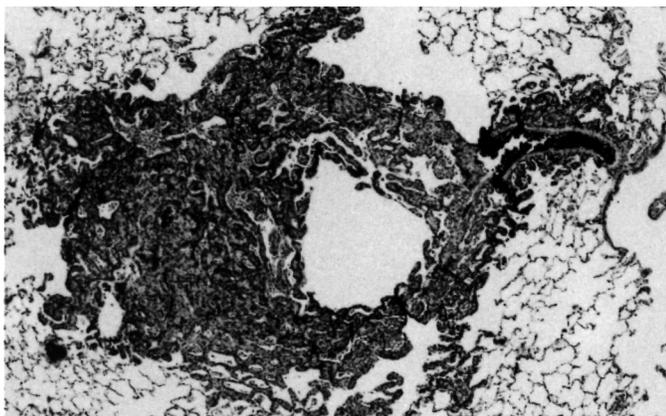
**PLATE 2**  
 Higher magnification of an area of chronic inflammation. Note the areas of fibrosis (large arrow), foamy alveolar macrophages (large curved arrow), necrotic cellular debris (small arrow), and epithelial hyperplasia (curved arrows) in the lung of a female F344/N rat exposed to 3.0 mg/m<sup>3</sup> cobalt sulfate heptahydrate by inhalation for 2 years. H&E; 160×



**PLATE 3**  
 Hyperplasia (arrow) in the lung of a female F344/N rat exposed to 3.0 mg/m<sup>3</sup> cobalt sulfate heptahydrate by inhalation for 2 years. H&E; 20×

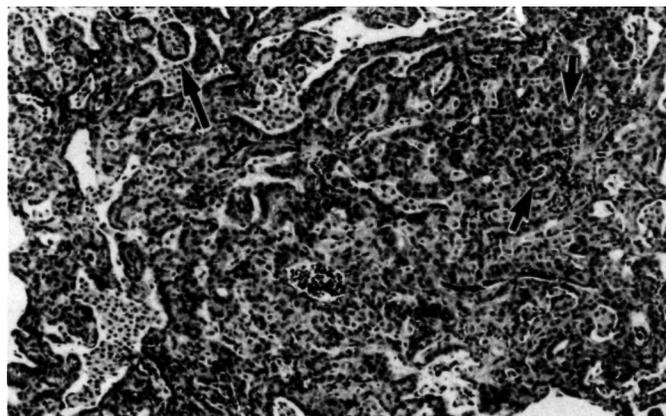


**PLATE 4**  
 Higher magnification of Plate 3. Note the proliferation of cells along the alveolar walls, but normal alveolar structure is maintained. H&E; 100×



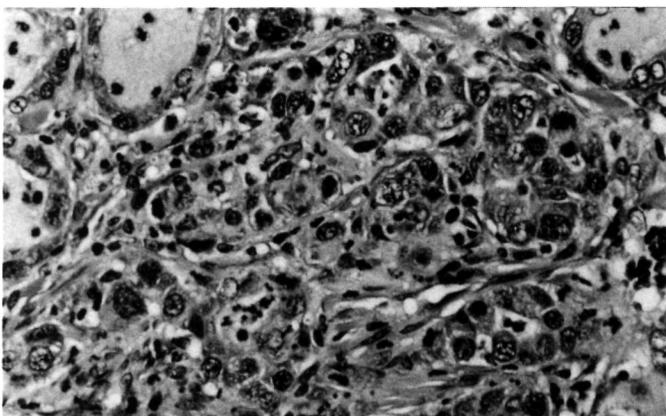
**PLATE 5**

Alveolar/bronchiolar adenoma in the lung of a male F344/N rat exposed to 3.0 mg/m<sup>3</sup> cobalt sulfate heptahydrate by inhalation for 2 years. H&E; 26×



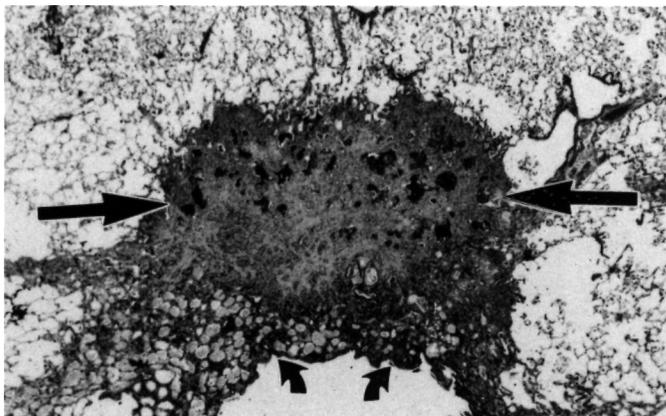
**PLATE 6**

Higher magnification of Plate 5. Component cells are arranged in acini (small arrows) and papillary projections (large arrow). H&E; 66×



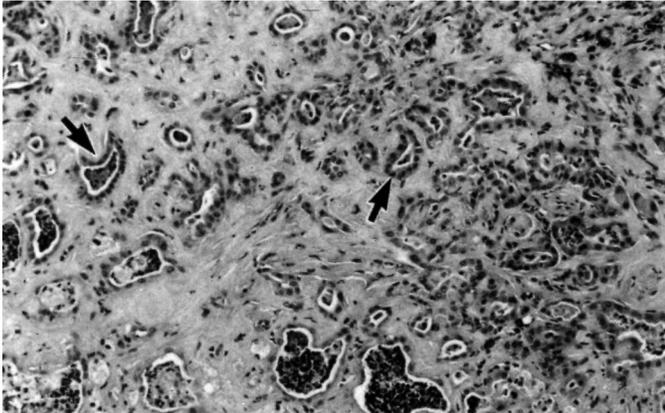
**PLATE 7**

Alveolar/bronchiolar carcinoma in the lung of a female F344/N rat exposed to 3.0 mg/m<sup>3</sup> cobalt sulfate heptahydrate by inhalation for 2 years. Note the variation in the size of the cells comprising acini at this high magnification. H&E; 200×



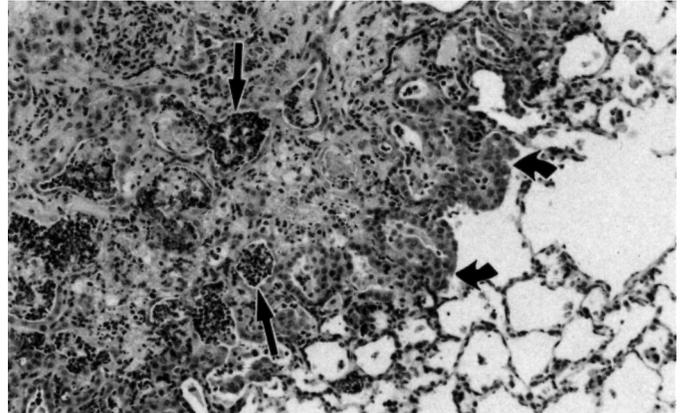
**PLATE 8**

Atypical hyperplasia (arrows) in the lung of a female F344/N rat exposed to 1.0 mg/m<sup>3</sup> cobalt sulfate heptahydrate by inhalation for 2 years. The lesion is located within an area of chronic inflammation (curved arrows). H&E; 16×



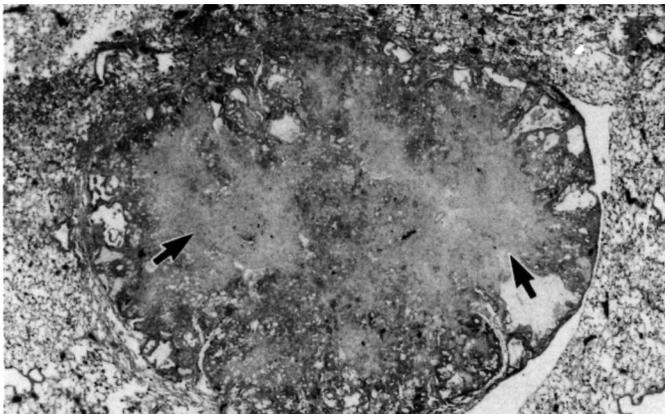
**PLATE 9**

Higher magnification of Plate 8. Note the glandular structures (arrows) lined by cuboidal epithelium within the fibrotic core. H&E; 80×



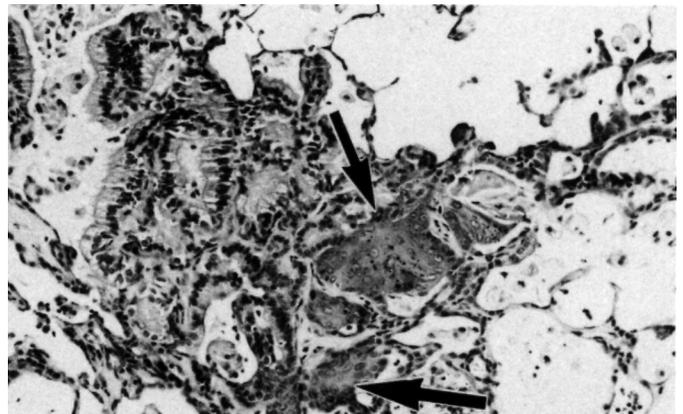
**PLATE 10**

High magnification of the border of an atypical hyperplasia in the lung of a male F344/N rat exposed to 3.0 mg/m<sup>3</sup> cobalt sulfate heptahydrate by inhalation for 2 years. Note the necrotic debris within the glandular structure (arrows) and the proliferative epithelium at the periphery (curved arrows). H&E; 80×



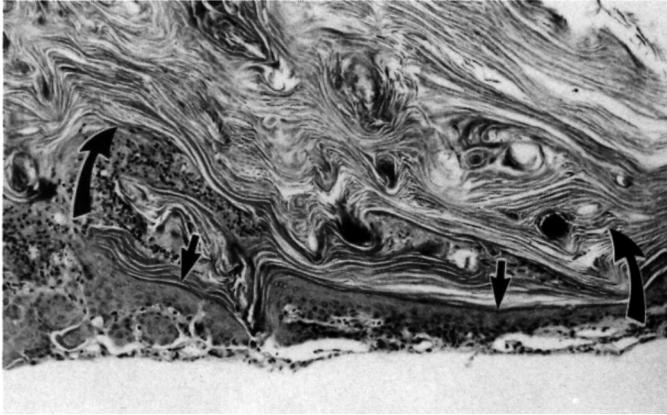
**PLATE 11**

Alveolar/bronchiolar carcinoma with abundant fibrous connective tissue (arrows) in the lung of a male F344/N rat exposed to 1.0 mg/m<sup>3</sup> cobalt sulfate heptahydrate by inhalation for 2 years. H&E; 10×



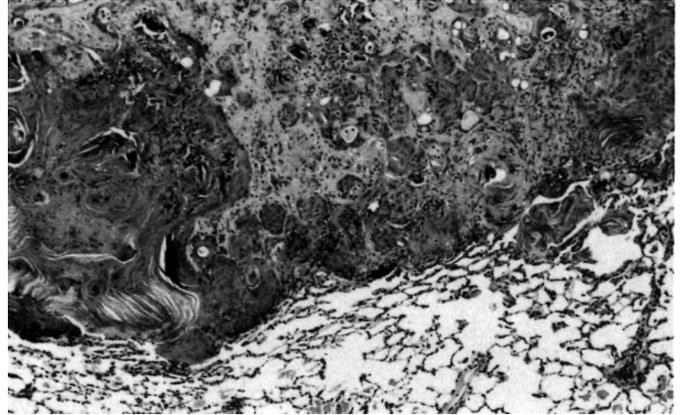
**PLATE 12**

Squamous metaplasia along the alveolar wall consisting of several layers of squamous epithelium (arrows) in the lung of a female F344/N rat exposed to 3.0 mg/m<sup>3</sup> cobalt sulfate heptahydrate by inhalation for 2 years. H&E; 100×



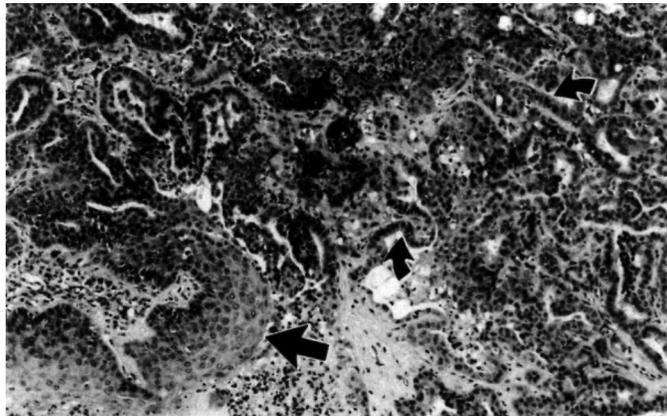
**PLATE 13**

Squamous cyst rimmed by a variably thick wall of squamous epithelium (large arrows) and filled with keratinous material (curved arrows) in the lung of a male F344/N rat exposed to 3.0 mg/m<sup>3</sup> cobalt sulfate heptahydrate by inhalation for 2 years. H&E; 66×



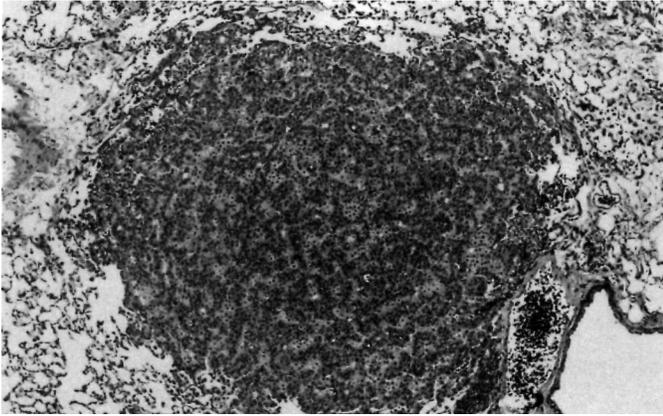
**PLATE 14**

High magnification of a squamous cell carcinoma in the lung of a female F344/N rat exposed to 3.0 mg/m<sup>3</sup> cobalt sulfate heptahydrate by inhalation for 2 years. H&E; 40×



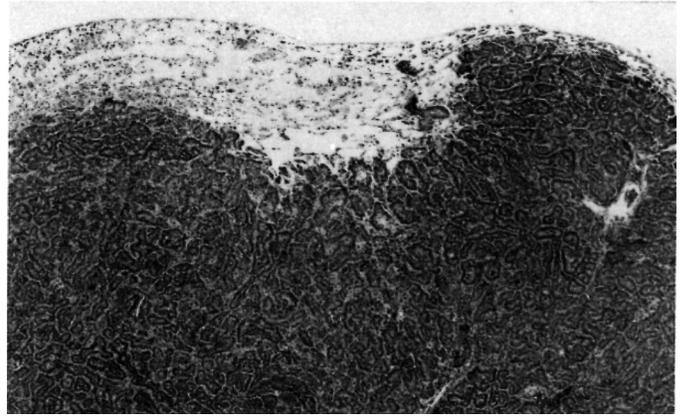
**PLATE 15**

Alveolar/bronchiolar carcinoma with an area of alveolar/bronchiolar epithelium to the right (curved arrows) and squamous differentiation to the left (arrow) in the lung of a male F344/N rat exposed to 1.0 mg/m<sup>3</sup> cobalt sulfate heptahydrate by inhalation for 2 years. H&E; 66×



**PLATE 16**

Alveolar/bronchiolar adenoma in the lung of a female B6C3F<sub>1</sub> mouse exposed to 3.0 mg/m<sup>3</sup> cobalt sulfate heptahydrate by inhalation for 2 years. H&E; 40×



**PLATE 17**

Section of an alveolar/bronchiolar carcinoma with irregular and variably sized acinar structures in a female B6C3F<sub>1</sub> mouse exposed to 3.0 mg/m<sup>3</sup> cobalt sulfate heptahydrate by inhalation for 2 years. H&E; 26×

## DISCUSSION AND CONCLUSIONS

This report presents the findings and conclusions of 2-year inhalation studies with cobalt sulfate heptahydrate. A companion report (NTP, 1991) discusses the findings of 16-day and 13-week inhalation studies conducted prior to the 2-year studies at the same laboratory. In all studies, the respiratory tract was the primary site of nonneoplastic lesions and neoplasms. In the 13-week studies, laryngeal lesions ranged from mild squamous metaplasia with or without chronic inflammation at concentrations ultimately selected for the 2-year studies, to large inflammatory polyps present in rats exposed to higher concentrations. Although other respiratory tract lesions were present, the larynx appeared to be the most sensitive to cobalt sulfate heptahydrate exposure, and lesions in this tissue were the determining factor in exposure concentration selection for the 2-year studies.

The highest concentration ( $3.0 \text{ mg/m}^3$ ) chosen for the 2-year studies did not affect survival or body weight gains of rats or survival of mice in either the 13-week or 2-year studies. The polycythemia noted in rats in the 13-week study was very mild at  $3.0 \text{ mg/m}^3$ , and there was no indication that this effect worsened to the point of causing clinical effects with longer exposure, although no hematologic measures were performed during the 2-year study. Similarly, there was no indication that the lesions observed in rats and mice in the 13-week studies in the larynx progressed in extent or changed in character with the prolonged exposures. There was no evidence of laryngeal polyp formation in rats, and the metaplastic and inflammatory changes in rats remained greater than in mice.

In contrast to the findings in the larynx, prolonged exposure to cobalt sulfate heptahydrate aerosol appeared to cause a progressive injury to the nose of rats and mice and to the lung of rats. Olfactory epithelial degeneration occurred primarily in rats and mice exposed to 10 and  $30 \text{ mg/m}^3$  in the 13-week studies, but olfactory epithelial atrophy was increased at even the lowest concentration ( $0.3 \text{ mg/m}^3$ ) in rats and at  $1.0 \text{ mg/m}^3$  in mice in the 2-year studies. Lesions in the lungs of rats changed markedly in character with the prolonged exposure in the 2-year study. Inflam-

mation in the alveoli of rats was much more severe and occurred at lower concentrations than in the prechronic studies, and proteinosis was moderate to marked in the 2-year study rats and not noted in the prechronic study. Interstitial fibrosis is known to be a rather slowly developing lesion, but the extent of this lesion and its occurrence in essentially all rats at all exposure concentrations was not predicted based on the findings of the 13-week study. The alveolar epithelium of rats also displayed a spectrum of proliferative changes ranging from metaplasia through hyperplasia and atypical hyperplasia, and extending to neoplasia.

The spectrum of proliferative lung lesions observed in rats in the 2-year study ranged from highly cellular proliferations (typical of spontaneous lesions) to fibroproliferative and squamous lesions not typical of spontaneous lesions, and morphologic variants in between. The biological behavior of "typical" lung lesions, and to a lesser extent, squamous lesions, is fairly well documented. However, little is known about the biology of fibroproliferative lesions. In this study, many of the smaller lesions were identified within and/or adjacent to areas of chronic inflammation and fibrosis; however, it was clear that these lesions represented proliferative lesions distinct from the inflammation. Based upon the morphologic spectrum observed, it appears that their growth is progressive. There was, however, no clear morphologic correlate signaling autonomy of growth (i.e., consistent with a benign neoplasm) for these fibroproliferative lesions. Therefore, unless growth alterations consistent with a malignant neoplasm were present, all fibroproliferative lesions were diagnosed as atypical hyperplasia. There were several animals that had malignant neoplasms with a very prominent fibrous component; presumably, some of these progressed from atypical hyperplasias. In many respects, the range of proliferative lesions within the lungs of exposed rats resembled those observed in NTP studies of particulates (talc and the nickel compounds; NTP, 1993, 1996a,b,c), and it is clear that all the morphologic variants of proliferative lesions represent a response to cobalt sulfate heptahydrate.

Nonneoplastic lesions in the lungs of mice exposed to cobalt sulfate heptahydrate did not appear to differ appreciably from those expected in mice based on the results of the prechronic study. The lesions were confined primarily to histiocytic infiltration, and there was an absence of fibrosis and only minimal evidence of the nonneoplastic proliferative lesions noted in exposed rats. Most of the diagnoses of histiocytic infiltration were noted in animals that also had an alveolar/bronchiolar neoplasm; this is a frequent observation in mice with lung neoplasms and is not necessarily related to exposure to cobalt sulfate heptahydrate. Thus, it is not possible to clearly attribute the presence of histiocytic infiltration to cobalt sulfate heptahydrate exposure. Nonetheless, the lung changes were clearly much less severe than those seen in rats and differed markedly in character.

While rats and mice exhibited quite different nonneoplastic pulmonary responses to cobalt sulfate heptahydrate, exposed male and female rats and mice developed alveolar/bronchiolar adenomas and carcinomas. The distinction between these neoplasms is largely based on size, and both categories of this neoplasm were increased in exposed male and female rats and mice. In all groups, the neoplasms appeared with a significant positive trend, and the incidences in the 3.0 mg/m<sup>3</sup> groups exceeded the historical control ranges in the respective groups. The magnitude of the neoplastic response was somewhat less in male rats than in the other groups.

The incidences of follicular cell hyperplasia of the thyroid gland were moderately increased in all exposed groups of male mice, although no dose response was observed. Hypothyroidism has been noted in humans who also exhibited cardiomyopathy associated with consumption of cobalt-contaminated beer (Taylor and Marks, 1978).

Incidences of pheochromocytoma of the adrenal medulla were increased in female rats exposed to cobalt sulfate heptahydrate. Pheochromocytomas are relatively common in male F344/N rats, occurring with an historical rate of about 30% in inhalation studies. The historical inhalation chamber control rate in females is much lower (6%), and the incidence in the concurrent chamber control was 4%. While the incidences of this neoplasm were increased in exposed males and females, the strength of the response was

much greater in females, and the increase in males was judged equivocal. In the NTP database of chemical carcinogenesis studies of nearly 450 chemicals, pheochromocytomas were part of a carcinogenic response in only 13 rat studies, five of which were inhalation studies. Although the historical control rates of pheochromocytomas do not appreciably differ between inhalation and dosed feed studies, a positive response is more likely to occur in inhalation studies than in studies using other routes of exposure. The reasons for this are not clear. Of the five other positive inhalation studies, two were with nickel compounds (oxide and subsulfide) and one with the particulate, talc.

Although the mechanisms responsible for induction of pheochromocytomas in rats are not understood, it is worth considering whether the adrenal gland and the pulmonary responses to cobalt sulfate heptahydrate in the rat might represent nonspecific responses to the physical inhalation and pulmonary accumulation of a particle, rather than a chemical-specific response. Measures of the possible accumulation of cobalt in the lung were not taken during these studies, although urinary cobalt concentrations have demonstrated dose-related absorption in the prechronic studies. Nickel sulfate hexahydrate is a highly water-soluble salt, as is cobalt sulfate heptahydrate. In similar studies, nickel sulfate hexahydrate did not show evidence of exposure-concentration-related accumulation in the lung of rats or mice exposed to concentrations as high as 30 mg/m<sup>3</sup> (NTP, 1996c). In contrast, the less soluble nickel subsulfide (NTP, 1996b) and the highly insoluble nickel oxide (NTP, 1996a) did accumulate in the lung. Thus, given the similar solubility and use of exposure concentrations ten-fold lower than those used with nickel sulfate hexahydrate, it is unlikely that cobalt would accumulate in the lung unless there was specific toxicity to pulmonary clearance mechanisms. The absence of nonneoplastic changes associated with cobalt sulfate heptahydrate inhalation by mice would argue against impaired clearance. The rather extensive and progressive pulmonary toxicity in the rat could have resulted in impaired clearance of cobalt, but it is unlikely that the toxicity represented a simple inflammatory and fibrotic response to an "overload" situation as has been postulated with chemically inert particles (Morrow *et al.*, 1991). The fact that the entire respiratory tract demonstrated a toxic response to cobalt sulfate heptahydrate argues convincingly that

the chemical has inherent toxicity and is not acting through secondary mechanisms related to its inhalation as a particle.

A number of factors need to be considered to properly address the relationship of these findings to typical human exposures to cobalt. The segments of the human population with the highest potential exposure to significant airborne cobalt concentrations are workers in the hard metal industry, coal mining, and those involved in ore processing (USDHHS, 1992). In these situations cobalt may exist in various forms, primarily as cobalt powder or cobalt oxide. These agents are less soluble than cobalt sulfate heptahydrate, and the toxic response of the respiratory system would likely depend on the combination of inherent toxicity, solubility in biological fluids, and residence time in the tissue. The carcinogenic potential of various cobalt compounds has been perhaps best demonstrated in injection studies in experimental animals (reviewed in IARC, 1991), and both insoluble and soluble forms have been shown to produce injection-site neoplasms.

The present demonstration of alveolar/bronchiolar neoplasms in rats and mice exposed to cobalt sulfate heptahydrate by inhalation confirms the findings of the injection studies and suggests that cobalt is inherently carcinogenic. These findings also lend credence to the epidemiological investigations of Mur *et al.* (1987), Hogstedt and Alexandersson (1990), and Lasfargues *et al.* (1994) that reported increased risks for lung cancer among workers producing cobalt and exposed to cobalt in the hard metal industry. Cobalt concentrations in the urine of workers in the Italian hard metal industry were found to be as high as 0.21 µg/mL at the end of the work shift (Sabbioni *et al.*, 1994). Ichikawa *et al.* (1985) reported even higher concentrations (0.39 µg/mL) in Japanese workers. In prechronic inhalation studies reported previously (NTP, 1991), average urinary cobalt concentrations in rats exposed to 0.3, 1.0, and 3.0 mg/m<sup>3</sup>, respectively, were 0.14, 0.32, and 1.77 µg/mL. If urine cobalt concentrations roughly approximate relative inhalation exposures to cobalt, then the results from the current 2-year rat and mouse studies appear similar to occupational exposure levels and suggest that humans and rodents may be similarly sensitive to cobalt carcinogenesis.

The mechanisms of cobalt-induced carcinogenesis are not well understood. The genotoxicity of cobalt compounds has been established in a variety of eukaryotic test systems (reviewed in IARC, 1991), and cobalt has been shown under certain conditions to catalyze the production of oxygen-based free radicals that may underlie some of the observed adverse genetic events (Shi *et al.*, 1993). The observation of a larger than usual number of G to T transversions at the second base of codon 12 of those mouse lung neoplasms carrying a mutated K-ras gene (Appendix I) is also consistent with oxidative injury. Similar increases in G to T transversions were seen in lung neoplasms from mice exposed to ozone (NTP, 1994)

The potential contribution of the sulfate moiety to the carcinogenic response is worthy of consideration in that exposures of humans to concentrated inorganic acid mists are recognized as causing respiratory tract neoplasms, primarily in the larynx (IARC, 1992). There are no experimental animal carcinogenicity studies with sulfuric acid mists *per se* (IARC, 1992), but nickel sulfate hexahydrate was studied by inhalation as mentioned earlier (NTP, 1996c). In this instance, there was no evidence of carcinogenicity of nickel sulfate hexahydrate to the respiratory tract or other tissues despite the fact that other nickel salts are carcinogenic. Additionally, nickel sulfate hexahydrate was studied at an equivalent exposure concentration to that which caused significant increases in lung neoplasms in mice exposed to cobalt sulfate heptahydrate (1.0 mg/m<sup>3</sup>). Thus, there seems to be little evidence to suggest that the sulfate moiety contributed significantly to the carcinogenic response.

Based on retrospective analyses, *Helicobacter hepaticus* was determined to have infected mice in 12 recent NTP 2-year studies (Appendix J). Of the 12 studies, mice (primarily males) from nine studies (including this study of cobalt sulfate heptahydrate) had a *H. hepaticus*-associated hepatitis. Qualitatively, the hepatitis and silver-staining organisms within the liver were similar among the nine studies. In a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) based assay, *H. hepaticus* was identified in studies from which adequately preserved (frozen) liver tissue was available. In general, efforts to identify *H. hepaticus* from tissue fixed in formalin for over a week were not successful

(Malarkey *et al.*, 1997), which was the case for this study of cobalt sulfate heptahydrate. However, because of the presence of the typical liver lesions and silver-staining helical organisms, mice from the study were presumed to be infected with *H. hepaticus*.

Increases in the incidences of hepatocellular neoplasms in male mice have been shown to be associated with *H. hepaticus* infection when hepatitis is also present (Ward *et al.*, 1994; Fox *et al.*, 1996; Appendix J). Additionally, in NTP studies with *H. hepaticus*-associated hepatitis, increased incidences of hemangiosarcoma were seen in the livers of male mice (Appendix J). Because of the latter association, interpretation of the increased incidences of hemangiosarcoma in the liver of male mice was confounded. Incidences of lesions at other sites in this study of cobalt sulfate heptahydrate were not considered to have been significantly impacted by the infection with *H. hepaticus* or its associated hepatitis (Appendix J).

## CONCLUSIONS

Under the conditions of these 2-year inhalation studies, there was *some evidence of carcinogenic activity*\* of cobalt sulfate heptahydrate in male F344/N rats based on increased incidences of alveolar/bronchiolar neoplasms. Marginal increases in incidences of pheochromocytomas of the adrenal medulla may have been related to exposure to cobalt sulfate heptahydrate. There was *clear evidence of carcinogenic activity* in female F344/N rats based on increased incidences of alveolar/bronchiolar neoplasms and pheochromocytomas of the adrenal medulla in groups exposed to cobalt sulfate heptahydrate. There was *clear evidence of carcinogenic activity* of cobalt sulfate heptahydrate in male and female B6C3F<sub>1</sub> mice based on increased incidences of alveolar/bronchiolar neoplasms.

Exposure to cobalt sulfate heptahydrate caused a spectrum of inflammatory, fibrotic, and proliferative lesions in the respiratory tract of male and female rats and mice.

---

\* Explanation of Levels of Evidence of Carcinogenic Activity is on page 10. A summary of the Technical Reports Review Subcommittee comments and the public discussion on this Technical Report appears on page 12.

## REFERENCES

- Abbracchio, M.P., Heck, J.D., and Costa, M. (1982). The phagocytosis and transforming activity of crystalline metal sulfide particles are related to their negative surface charge. *Carcinogenesis* **3**, 175-180.
- American Conference of Governmental Industrial Hygienists (ACGIH) (1996). *1996 Threshold Limit Values for Chemical Substances and Physical Agents and Biological Exposure Indices*. Cincinnati, OH.
- Anderson, O. (1983). Effects of coal combustion products and metal compounds on sister chromatid exchanges in a macrophagelike cell line. *Environ. Health Perspect.* **47**, 239-253.
- Armitage, P. (1971). *Statistical Methods in Medical Research*, pp. 362-365. John Wiley and Sons, New York.
- Ashby, J., and Tennant, R.W. (1991). Definitive relationships among chemical structure, carcinogenicity and mutagenicity for 301 chemicals tested by the U.S. NTP. *Mutat. Res.* **257**, 229-306.
- Baker, P.F., Meves, H., and Ridgway, E.B. (1973). Effects of manganese and other agents on the calcium uptake that follows depolarization of squid axons. *J. Physiol.* **231**, 511-526.
- Barrett, K.W. (1983). Report from the Mitre Corporation to the U.S. Environmental Protection Agency.
- Beyersmann, D., and Hartwig, A. (1992). The genetic toxicology of cobalt. *Toxicol. Appl. Pharmacol.* **115**, 137-145.
- Boorman, G.A., Montgomery, C.A., Jr., Eustis, S.L., Wolfe, M.J., McConnell, E.E., and Hardisty, J.F. (1985). Quality assurance in pathology for rodent carcinogenicity studies. In *Handbook of Carcinogen Testing* (H.A. Milman and E.K. Weisburger, Eds.), pp. 345-357. Noyes Publications, Park Ridge, NJ.
- Bucher, J.R., Elwell, M.R., Thompson, M.B., Chou, B.J., Renne, R., and Ragan, H.A. (1990). Inhalation toxicity studies of cobalt sulfate in F344/N rats and B6C3F1 mice. *Fundam. Appl. Toxicol.* **15**, 357-372.
- Buttlaire, D.H., Czuba, B.A., Stevens, T.H., Lee, Y.C., and Himes, R.H. (1980). Manganous ion binding to tubulin. *J. Biol. Chem.* **255**, 2164-2168.
- Christensen, J.M., and Poulsen, O.M. (1994). A 1982-1992 surveillance programme on Danish pottery painters. Biological levels and health effects following exposure to soluble or insoluble cobalt compounds in cobalt blue dyes. *Sci. Total Environ.* **150**, 95-104.
- Cirila, A.M. (1994). Cobalt-related asthma: Clinical and immunological aspects. *Sci. Total Environ.* **150**, 85-94.
- Code of Federal Regulations (CFR). **21**, Part 58.
- Cox, D.R. (1972). Regression models and life-tables. *J. R. Stat. Soc.* **B34**, 187-220.
- Crawford, B.D. (1985). Perspectives on the somatic mutation model of carcinogenesis. In *Advances in Modern Environmental Toxicology. Mechanisms and Toxicity of Chemical Carcinogens and Mutagens* (M.A. Mehlman, W.G. Flamm, and R.J. Lorentzen, Eds.), pp. 13-59. Princeton Scientific Publishing Co., Princeton, NJ.
- De Bie, E., and Doyen, P. (1962). Cobalt oxides and salts. *Cobalt* **15**, 3-13.
- De Matteis, F., and Gibbs, A.H. (1977). Inhibition of haem synthesis caused by cobalt in rat liver. Evidence for two different sites of action. *Biochem. J.* **162**, 213-216.

- Dingle, J.T., Heath, J.C., Webb, M., and Daniel, M. (1962). The biological action of cobalt and other metals. II. The mechanism of the respiratory inhibition produced by cobalt in mammalian tissues. *Biochim. Biophys. Acta* **65**, 34-46.
- Dinse, G.E., and Haseman, J.K. (1986). Logistic regression analysis of incidental-tumor data from animal carcinogenicity experiments. *Fundam. Appl. Toxicol.* **6**, 44-52.
- Dinse, G.E., and Lagakos, S.W. (1983). Regression analysis of tumour prevalence data. *Appl. Stat.* **32**, 236-248.
- Domingo, J.L. (1989). Cobalt in the environment and its toxicological implications. *Rev. Environ. Contam. Toxicol.* **108**, 105-123.
- Domingo, J.L., Llobet, J.M., and Bernat, R. (1984a). Nutritional and toxicological study of cobalt administered to rats in their drinking water. *Rev. Toxicol.* **1**, 43-54.
- Domingo, J.L., Llobet, J.M., and Bernat, R. (1984b). A study of the effects of cobalt administered orally to rats. *Arch. Farmacol. Toxicol.* **10**, 13-20.
- Domingo, J.L., Paternain, J.L., Llobet, J.M., and Corbella, J. (1985). Effects of cobalt on postnatal development and late gestation in rats upon oral administration. *Rev. Esp. Fisiol.* **41**, 293-298.
- Dorsit, G., Girard, R., Rousset, H., Brune, J., Wiesendanger, T., Tolot, F., Bourret, J., and Galy, P. (1970). Fibrose pulmonaire chez 3 sujets d'une meme usine exposes aux poussières de cobalt et de carbure de tungstène. Les troubles pulmonaires de l'industrie des métaux durs. *Sem. Hop.* **46**, 3363-3376.
- Eaton, R.P. (1972). Cobalt chloride-induced hyperlipemia in the rat: Effects on intermediary metabolism. *Am. J. Physiol.* **222**, 1550-1557.
- Edel, J., Sabbioni, E., Pietra, R., Rossi, A., Torre, M., Rizzato, G., and Fraioli, P. (1990). Trace metal lung disease: In vitro interaction of hard metals with human lung and plasma components. *Sci. Total Environ.* **95**, 107-117.
- Finch, C.A. (1980). Drugs effective in iron deficiency and other hypochromic anemias. In *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, 6th ed. (A.G. Gilman, L.S. Goodman, A. Gilman, S.E. Mayer, and K.L. Melmon, Eds.), pp. 1315-1330. Macmillan Publishing Co., New York.
- Fox, J.G., Li, X., Yan, L., Cahill, R.J., Hurley, R., Lewis, R., and Murphy, J.C. (1996). Chronic proliferative hepatitis in A/JCr mice associated with persistent *Helicobacter hepaticus* infection: A model of *Helicobacter*-induced carcinogenesis. *Infect. Immun.* **64**, 1548-1558.
- Gart, J.J., Chu, K.C., and Tarone, R.E. (1979). Statistical issues in interpretation of chronic bioassay tests for carcinogenicity. *JNCI* **62**, 957-974.
- Gilman, J.P.W. (1962). Metal carcinogenesis. II. A study on the carcinogenic activity of cobalt, copper, iron, and nickel compounds. *Cancer Res.* **22**, 158-162.
- Gregus, Z., and Klaassen, C.D. (1986). Disposition of metals in rats: A comparative study of fecal, urinary, and biliary excretion and tissue distribution of eighteen metals. *Toxicol. Appl. Pharmacol.* **85**, 24-38.
- Grice, H.C., Goodman, T., Munro, I.C., Wiberg, G.S., and Morrison, A.B. (1969). Myocardial toxicity of cobalt in the rat. *Ann. N. Y. Acad. Sci.* **156**, 189-194.
- Hamilton, E.I. (1994). The geobiochemistry of cobalt. *Sci. Total Environ.* **150**, 7-39.
- Hamilton-Koch, W., Snyder, R.D., and Lavelle, J.M. (1986). Metal-induced DNA damage and repair in human diploid fibroblasts and Chinese hamster ovary cells. *Chem. Biol. Interact.* **59**, 17-28.
- Hammond, P.B., and Beliles, R.P. (1980). Metals. In *Casarett and Doull's Toxicology*, 2nd ed. (J. Doull, C.D. Klaassen, and M.O. Amdur, Eds.), pp. 442-443. Macmillan Publishing Co., New York.
- Harding, H.E. (1950). Notes on the toxicology of cobalt metal. *Br. J. Ind. Med.* **7**, 76-78.

- Hartwig, A., Kasten, U., Boakye-Dankwa, K., Schlepegrell, R., and Beyersmann, D. (1990). Uptake and genotoxicity of micromolar concentrations of cobalt chloride in mammalian cells. *Toxicol. Environ. Chem.* **28**, 205-215.
- Hartwig, A., Snyder, R.D., Schlepegrell, R., and Beyersmann, D. (1991). Modulation by Co(II) of UV-induced DNA repair, mutagenesis and sister-chromatid exchanges in mammalian cells. *Mutat. Res.* **248**, 177-185.
- Haseman, J.K. (1984). Statistical issues in the design, analysis and interpretation of animal carcinogenicity studies. *Environ. Health Perspect.* **58**, 385-392.
- Heath, J.C. (1956). The production of malignant tumours by cobalt in the rat. *Br. J. Cancer* **10**, 668-673.
- Heath, J.C. (1960). The histogenesis of malignant tumours induced by cobalt in the rat. *Br. J. Cancer* **14**, 478-482.
- Heath, J.C., and Webb, M. (1967). Content and intracellular distribution of the inducing metal in the primary rhabdomyosarcomata induced in the rat by cobalt, nickel and cadmium. *Br. J. Cancer* **21**, 768-779.
- Hogstedt, C., and Alexandersson, R. (1990). Mortality among hard metal workers [in German, English summary]. *Arbete och Hälsa* **21**, 1-26.
- Hollander, M., and Wolfe, D.A. (1973). *Nonparametric Statistical Methods*, pp. 120-123. John Wiley and Sons, New York.
- Ichikawa, Y., Kusaka, Y., and Goto, S. (1985). Biological monitoring of cobalt exposure, based on cobalt concentrations in blood and urine. *Int. Arch. Occup. Environ. Health* **55**, 269-276.
- International Agency for Research on Cancer (IARC) (1991). *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Chlorinated Drinking-water; Chlorination By-products; Some Other Halogenated Compounds; Cobalt and Cobalt Compounds*, Vol. 52. IARC, Lyon, France.
- International Agency for Research on Cancer (IARC) (1992). *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Occupational Exposures to Mists and Vapours from Strong Inorganic Acids; and Other Industrial Chemicals*, Vol. 54. IARC, Lyon, France.
- Jacobziner, H., and Raybin, H.W. (1961). Poison control. Accidental cobalt poisoning. *Arch. Pediatr.* **78**, 200-205.
- Jasmin, G. (1974). Anaphylactoid edema induced in rats by nickel and cobalt salts. *Proc. Soc. Exp. Biol. Med.* **147**, 289-292.
- Jennette, K.W. (1981). The role of metals in carcinogenesis: Biochemistry and metabolism. *Environ. Health Perspect.* **40**, 233-252.
- Jensen, A.A., and Tüchsen, F. (1990). Cobalt exposure and cancer risk. *Crit. Rev. Toxicol.* **20**, 427-437.
- Johansson, A., Curstedt, T., Robertson, B., and Camner, P. (1984). Lung morphology and phospholipids after experimental inhalation of soluble cadmium, copper, and cobalt. *Environ. Res.* **34**, 295-309.
- Johansson, A., Lundborg, M., Wiernik, A., Jarstrand, C., and Camner, P. (1986). Rabbit alveolar macrophages after long-term inhalation of soluble cobalt. *Environ. Res.* **41**, 488-496.
- Kaplan, E.L., and Meier, P. (1958). Nonparametric estimation from incomplete observations. *J. Am. Stat. Assoc.* **53**, 457-481.
- Kasirsky, G., Sherman, W.T., Gautieri, R.F., and Mann, D.E., Jr. (1969). Cobalt-cortisone interrelationships in the induction and inhibition of cleft palate in mice. *J. Pharm. Sci.* **58**, 766-767.
- Kazantzis, G. (1981). Role of cobalt, iron, lead, manganese, mercury, platinum, selenium, and titanium in carcinogenesis. *Environ. Health Perspect.* **40**, 143-161.

- Kerfoot, E.J., Fredrick, W.G., and Domeier, E. (1975). Cobalt metal inhalation studies on miniature swine. *Am. Ind. Hyg. Assoc. J.* **36**, 17-25.
- Kichina, M.M. (1974). Cobalt and titanium levels in animals under the influence of cobalt sulfate. *Sb. Rab. Leningr. Vet. Inst.* **38**, 83-87.
- Korman, E.F., Ward, J.F., and Myers, L.S. (1978). Development of toxicology of energy-related pollutants. *DOE Symposium Series* **47**, 383-395.
- Krasovskii, G.N., and Fridlyand, S.A. (1971). Experimental data for the validation of the maximum permissible concentration of cobalt in water bodies. *Hyg. Sanit.* **36**, 277-279.
- Lasfargues, G., Wild, P., Moulin, J.J., Hammon, B., Rosmorduc, B., du Noyer, C.R., Lavandier, M., and Moline, J. (1994). Lung cancer mortality in a French cohort of hard-metal workers. *Am. J. Ind. Med.* **26**, 585-595.
- Llobet, J.M., Domingo, J.L., and Corbella, J. (1986). Comparison of the effectiveness of several chelators after single administration on the toxicity, excretion and distribution of cobalt. *Arch. Toxicol.* **58**, 278-281.
- McConnell, E.E., Solleveld, H.A., Swenberg, J.A., and Boorman, G.A. (1986). Guidelines for combining neoplasms for evaluation of rodent carcinogenesis studies. *JNCI* **76**, 283-289.
- McKee, G.K. (1971). Carcinogenic properties of wear particles from prostheses made in cobalt-chromium alloy. *Lancet* **1**, 750.
- McKnight, B., and Crowley, J. (1984). Tests for differences in tumor incidence based on animal carcinogenesis experiments. *J. Am. Stat. Assoc.* **79**, 639-648.
- McLean, J.R., McWilliams, R.S., Kaplan, J.G., and Birnboim, H.C. (1982). Rapid detection of DNA strand breaks in human peripheral blood cells and animal organs following treatment with physical and chemical agents. In *Progress in Mutation Research: Chemical Mutagenesis, Human Population Monitoring and Genetic Risk Assessment* (K.C. Bora, G.R. Douglas, and E.R. Nestmann, Eds.), Vol. 3, pp. 137-141. Elsevier Biomedical Press, Amsterdam.
- Maines, M.D., and Kappas, A. (1976). Studies on the mechanism of induction of haem oxygenase by cobalt and other metal ions. *Biochem. J.* **154**, 125-131.
- Malarkey, D.E., Ton, T.-V., Hailey, J.R., and Devereux, T.R. (1997). A PCR-RFLP method for the detection of *Helicobacter hepaticus* in frozen or fixed liver from B6C3F<sub>1</sub> mice. *Toxicol. Pathol.* **25**, 606-612.
- Maronpot, R.R., and Boorman, G.A. (1982). Interpretation of rodent hepatocellular proliferative alterations and hepatocellular tumors in chemical safety assessment. *Toxicol. Pathol.* **10**, 71-80.
- The Merck Index* (1983). 10th ed. (M. Windholz, Ed.), p. 347. Merck and Company, Rahway, NJ.
- The Merck Index* (1989). 11th ed. (S. Budavari, Ed.), p. 2439. Merck and Company, Rahway, NJ.
- Miller, J.A., and Miller, E.C. (1977). Ultimate chemical carcinogens as reactive mutagenic electrophiles. In *Origins of Human Cancer* (H.H. Hiatt, J.D. Watson, and J.A. Winsten, Eds.), pp. 605-627. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Miller, M.E., Howard, D., Stohlman, F., Jr., and Flanagan, P. (1974). Mechanism of erythropoietin production by cobaltous chloride. *Blood* **44**, 339-346.

- Mitala, J.J., Mann, D.E., Jr., and Gautieri, R.F. (1978). Influence of cobalt (dietary), cobalamins, and inorganic cobalt salts on phenytoin- and cortisone-induced teratogenesis in mice. *J. Pharm. Sci.* **67**, 377-380.
- Mollenhauer, H.H., Corrier, D.E., Clark, D.E., Hare, M.F., and Elisalde, M.H. (1985). Effects of dietary cobalt on testicular structure. *Virchows Arch. Cell Pathol.* **49**, 241-248.
- Morgan, G.B., Ozolins, G., and Tabor, E.C. (1970). Air pollution surveillance systems. *Science* **170**, 289-296.
- Morin, Y., and Daniel, P. (1967). Quebec beer-drinkers' cardiomyopathy: Etiological considerations. *Can. Med. Assoc. J.* **97**, 926-928.
- Morita, H., Umeda, M., and Ogawa, H.I. (1991). Mutagenicity of various chemicals including nickel and cobalt compounds in cultured mouse FM3A cells. *Mutat. Res.* **261**, 131-137.
- Morrow, P.E., Muhle, H., and Mermelstein, R. (1991). Chronic inhalation study findings as a basis for proposing a new occupational dust exposure limit. *J. Am. Coll. Toxicol.* **10**, 279-290.
- Moulin, J.J., Wild, P., Mur, J.M., Fournier Betz, M., and Mercier-Gallay, M. (1993). A mortality study of cobalt production workers: An extension of the follow-up. *Am. J. Ind. Med.* **23**, 281-288.
- Mur, J.M., Moulin, J.J., Charruyer-Seinerra, M.P., and Lafitte, J. (1987). A cohort mortality study among cobalt and sodium workers in an electrochemical plant. *Am. J. Ind. Med.* **11**, 75-81.
- Murdock, H.R., Jr. (1959). Studies on the pharmacology of cobalt chloride. *J. Am. Pharm. Assoc.* **48**, 140-142.
- National Cancer Institute (NCI) (1976). Guidelines for Carcinogen Bioassay in Small Rodents. Technical Report Series No. 1. NIH Publication No. 76-801. U.S. Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, Bethesda, MD.
- National Institute for Occupational Safety and Health (NIOSH) (1981). Criteria for Controlling Occupational Exposure to Cobalt. Occupational Hazard Assessment. DHHS (NIOSH) Publication No. 82-107. U.S. Department of Health and Human Services, National Institute for Occupational Safety and Health, Washington, DC.
- National Institutes of Health (NIH) (1978). Open Formula Rat and Mouse Ration (NIH-07). Specification NIH-11-1335. U.S. Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, Bethesda, MD.
- National Research Council (NRC) (1977). Drinking Water and Health. National Academy of Sciences, Washington, DC.
- National Toxicology Program (NTP) (1991). Toxicity Studies of Cobalt Sulfate Heptahydrate (CAS No. 10026-24-1) in F344/N Rats and B6C3F<sub>1</sub> Mice (Inhalation Studies). Toxicity Study Report Series No. 5. NIH Publication No. 91-3124. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.
- National Toxicology Program (NTP) (1993). Toxicology and Carcinogenesis Studies of Talc (CAS No. 14807-96-6) in F344/N Rats and B6C3F<sub>1</sub> Mice (Inhalation Studies). Technical Report Series No. 421. NIH Publication No. 93-3152. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

- National Toxicology Program (NTP) (1994). Toxicology and Carcinogenesis Studies of Ozone (CAS No. 10028-15-6/64091-91-4) in F344/N Rats and B6C3F<sub>1</sub> Mice (Inhalation Studies). Technical Report Series No. 440. NIH Publication No. 95-3371. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.
- National Toxicology Program (NTP) (1996a). Toxicology and Carcinogenesis Studies of Nickel Oxide (CAS No. 1313-99-1) in F344/N Rats and B6C3F<sub>1</sub> Mice (Inhalation Studies). Technical Report Series No. 451. NIH Publication No. 96-3367. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.
- National Toxicology Program (NTP) (1996b). Toxicology and Carcinogenesis Studies of Nickel Subulfide (CAS No. 12035-72-2) in F344/N Rats and B6C3F<sub>1</sub> Mice (Inhalation Studies). Technical Report Series No. 453. NIH Publication No. 96-3369. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.
- National Toxicology Program (NTP) (1996c). Toxicology and Carcinogenesis Studies of Nickel Sulfate Hexahydrate (CAS No. 10101-97-0) in F344/N Rats and B6C3F<sub>1</sub> Mice (Inhalation Studies). Technical Report Series No. 454. NIH Publication No. 96-3370. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.
- Ogawa, H.I., Shibahara, T., Iwata, H., Okada, T., Tsuruta, S., Kakimoto, K., Sakata, K., Kato, Y., Ryo, H., Itoh, T., and Fujikawa, K. (1994). Genotoxic activities in vivo of cobaltous chloride and other metal chlorides as assayed in the *Drosophila* wing spot test. *Mutat. Res.* **320**, 133-140.
- Paley, K.R., Sobel, E.S., and Yalow, R.S. (1958). Effect of oral and intravenous cobaltous chloride on thyroid function. *J. Clin. Endocrinol. Metab.* **18**, 850-859.
- Paternain, J.L., Domingo, J.L., and Corbella, J. (1988). Developmental toxicity of cobalt in the rat. *J. Toxicol. Environ. Health* **24**, 193-200.
- Pedigo, N.G., George, W.J., and Anderson, M.B. (1988). Effects of acute and chronic exposure to cobalt on male reproduction in mice. *Reprod. Toxicol.* **2**, 45-53.
- Phillips, C.E. (1980). Intracellularly injected cobaltous ions accumulate at synaptic densities. *Science* **207**, 1477-1479.
- Popov, L.N. (1977). Izuchenie vlianiia malykh koncentratsii aerosolia metallichesкого koba'ta na organizm zhivotnykh v gigencheskom eksperimente. *Gig. Sanit.* **4**, 97-98.
- Sabbioni, E., Minoia, C., Pietra, R., Mosconi, G., Forni, A., and Scansetti, G. (1994). Metal determinations in biological specimens of diseased and non-diseased hard metal workers. *Sci. Total Environ.* **150**, 41-54.
- Scansetti, G., Botta, G.C., Spinelli, P., Reviglione, L., and Ponzetti, C. (1994). Absorption and excretion of cobalt in the hard metal industry. *Sci. Total Environ.* **150**, 141-144.
- Sederholm, T., Kouvalainen, K., and Lamberg, B.A. (1968). Cobalt-induced hypothyroidism and polycythemia in lipid nephrosis. *Acta Med. Scand.* **184**, 301-306.
- Seidenberg, J.M., Anderson, D.G., and Becker, R.A. (1986). Validation of an in vivo developmental toxicity screen in the mouse. *Teratog. Carcinog. Mutagen.* **6**, 361-374.
- Shabaan, A.A., Marks, V., Lancaster, M.C., and Dufeu, G.N. (1977). Fibrosarcomas induced by cobalt chloride (CoCl<sub>2</sub>) in rats. *Lab. Anim.* **11**, 43-46.
- Shi, X., Dalal, N.S., and Kasprzak, K.S. (1993). Generation of free radicals from model lipid hydroperoxides and H<sub>2</sub>O<sub>2</sub> by Co(II) in the presence of cysteinyl and histidyl chelators. *Chem. Res. Toxicol.* **6**, 277-283.

- Sinclair, P.R., Sinclair, J.F., Bonkowsky, H.L., Gibbs, A.H., and De Matteis, F. (1982). Formation of cobalt protoporphyrin by chicken hepatocytes in culture. Relationship to decrease of 5-aminolaevulinatase synthase caused by cobalt. *Biochem. Pharmacol.* **31**, 993-999.
- Smith, I.C., and Carson, B.L., Eds. (1981). *Cobalt. Trace Metals in the Environment*, Vol. 6. Ann Arbor Science Publishers, Ann Arbor, MI.
- Smith, R.P. (1980). Toxic responses of the blood. In *Casarett and Doull's Toxicology*, 2nd ed. (J. Doull, C.D. Klaassen, and M.O. Amdur, Eds.), pp. 311-331. Macmillan Publishing Co., New York.
- Smith, T., Edmonds, C.J., and Barnaby, C.F. (1972). Absorption and retention of cobalt in man by whole-body counting. *Health Phys.* **22**, 359-367.
- Speijers, G.J., Krajnc, E.I., Berkvens, J.M., and van Logten, M.J. (1982). Acute oral toxicity of inorganic cobalt compounds in rats. *Food Chem. Toxicol.* **20**, 311-314.
- Stelzer, K.J., and Klaassen, C.D. (1985). Effect of cobalt on biliary excretion of bilirubin and glutathione. *J. Toxicol. Environ. Health* **15**, 813-822.
- Steinhoff, D., and Mohr, U. (1991). On the question of a carcinogenic action of cobalt-containing compounds. *Exp. Pathol.* **41**, 169-174.
- Straus, D.S. (1981). Somatic mutation, cellular differentiation, and cancer causation. *JNCI* **67**, 233-241.
- Swennen, B., Buchet, J.-P., Stănescu, D., Lison, D., and Lauwerys, R. (1993). Epidemiological survey of workers exposed to cobalt oxides, cobalt salts, and cobalt metal. *Br. J. Ind. Med.* **50**, 835-842.
- Tarone, R.E. (1975). Tests for trend in life table analysis. *Biometrika* **62**, 679-682.
- Taylor, A., and Marks, V. (1978). Cobalt: A review. *J. Hum. Nutr.* **32**, 165-177.
- Telib, M. (1972). Effects of cobaltous chloride in laboratory animals: I. The histological and electron microscopical changes in the islets of rabbits. *Endokrinologie* **60**, 81-102.
- Tennant, R.W., Margolin, B.H., Shelby, M.D., Zeiger, E., Haseman, J.K., Spalding, J., Caspary, W., Resnick, M., Stasiewicz, S., Anderson, B., and Minor, R. (1987). Prediction of chemical carcinogenicity in rodents from *in vitro* genetic toxicity assays. *Science* **236**, 933-941.
- Tephly, T.R., and Hibbeln, P. (1971). The effect of cobalt chloride administration on the synthesis of hepatic microsomal cytochrome P-450. *Biochem. Biophys. Res. Commun.* **42**, 589-595.
- U.S. Department of Health and Human Services (USDHHS) (1992). Toxicological Profile for Cobalt. U.S. Department of Health and Human Services, Public Health Service, Agency for Toxic Substances and Disease Registry.
- Ward, J.M., Fox, J.G., Anver, M.R., Haines, D.C., George, C.V., Collins, M.J., Jr., Gorelick, P.L., Nagashima, K., Gonda, M.A., Gilden, R.V., Tully, J.G., Russell, R.J., Benveniste, R.E., Paster, B.J., Dewhirst, F.E., Donovan, J.C., Anderson, L.M., and Rice, J.M. (1994). Chronic active hepatitis and associated liver tumors in mice caused by a persistent bacterial infection with a novel *Helicobacter* species. *J. Natl. Cancer Inst.* **86**, 1222-1227.
- Webb, M., Heath, J.C., and Hopkins, T. (1972). Intranuclear distribution of the inducing metal in primary rhabdomyosarcomata induced in the rat by nickel, cobalt, and cadmium. *Br. J. Cancer* **26**, 274-278.
- Wehner, A.P., Busch, R.H., Olson, R.J., and Craig, D.K. (1977). Chronic inhalation of cobalt oxide and cigarette smoke by hamsters. *Am. Ind. Hyg. Assoc. J.* **38**, 338-346.

Zeiger, E., Haseman, J.K., Shelby, M.D., Margolin, B.H., and Tennant, R.W. (1990). Evaluation of four in vitro genetic toxicity tests for predicting rodent carcinogenicity: Confirmation of earlier results with 41 additional chemicals. *Environ. Mol. Mutagen.* **16** (Suppl. 18), 1-14.

Zeiger, E., Anderson, B., Haworth, S., Lawlor, T., and Mortelmans, K. (1992). Salmonella mutagenicity tests: V. Results from the testing of 311 chemicals. *Environ. Mol. Mutagen.* **19** (Suppl. 21), 2-141.

## **APPENDIX A**

### **SUMMARY OF LESIONS IN MALE RATS IN THE 2-YEAR INHALATION STUDY OF COBALT SULFATE HEPTAHYDRATE**

<b>TABLE A1</b>	<b>Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate . . . . .</b>	<b>64</b>
<b>TABLE A2</b>	<b>Individual Animal Tumor Pathology of Male Rats in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate . . . . .</b>	<b>68</b>
<b>TABLE A3</b>	<b>Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate . . . . .</b>	<b>92</b>
<b>TABLE A4a</b>	<b>Historical Incidence of Alveolar/bronchiolar Neoplasms in Chamber Control Male F344/N Rats . . . . .</b>	<b>99</b>
<b>TABLE A4b</b>	<b>Historical Incidence of Neoplasms of the Adrenal Medulla in Chamber Control Male F344/N Rats . . . . .</b>	<b>99</b>
<b>TABLE A5</b>	<b>Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate . . . . .</b>	<b>100</b>

**TABLE A1**  
**Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate<sup>a</sup>**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Disposition Summary</b>				
Animals initially in study	50	50	50	50
Early deaths				
Moribund	30	34	26	34
Natural deaths	3	1	3	1
Survivors				
Terminal sacrifice	17	15	21	15
Animals examined microscopically	50	50	50	50
<b>Alimentary System</b>				
Intestine large, colon	(49)	(50)	(49)	(49)
Intestine large, cecum	(48)	(49)	(48)	(49)
Intestine small, duodenum	(50)	(50)	(48)	(49)
Intestine small, jejunum	(49)	(49)	(47)	(49)
Intestine small, ileum	(50)	(49)	(47)	(48)
Liver	(50)	(50)	(48)	(50)
Carcinoma, metastatic, seminal vesicle		1 (2%)		
Hepatocellular carcinoma		1 (2%)		
Hepatocellular adenoma	1 (2%)		1 (2%)	1 (2%)
Sarcoma, metastatic, uncertain primary site		1 (2%)		
Mesentery	(21)	(14)	(11)	(14)
Carcinoma, metastatic, seminal vesicle		1 (7%)		
Sarcoma, metastatic, uncertain primary site		1 (7%)		
Fat, lipoma			1 (9%)	
Pancreas	(50)	(50)	(48)	(50)
Adenoma			1 (2%)	1 (2%)
Carcinoma			1 (2%)	
Carcinoma, metastatic, seminal vesicle		1 (2%)		
Mixed tumor malignant				1 (2%)
Sarcoma, metastatic, uncertain primary site		1 (2%)		
Salivary glands	(50)	(50)	(50)	(50)
Stomach, forestomach	(50)	(50)	(49)	(50)
Stomach, glandular	(50)	(50)	(48)	(50)
Schwannoma malignant				1 (2%)
<b>Cardiovascular System</b>				
Heart	(50)	(50)	(50)	(50)
Carcinoma, metastatic, seminal vesicle		1 (2%)		
<b>Endocrine System</b>				
Adrenal cortex	(50)	(50)	(48)	(50)
Adenoma			1 (2%)	1 (2%)
Carcinoma		1 (2%)	1 (2%)	
Adrenal medulla	(50)	(50)	(49)	(50)
Pheochromocytoma malignant		2 (4%)	2 (4%)	2 (4%)
Pheochromocytoma complex	1 (2%)		1 (2%)	
Pheochromocytoma benign	13 (26%)	15 (30%)	17 (35%)	15 (30%)
Bilateral, pheochromocytoma benign	1 (2%)	4 (8%)	6 (12%)	5 (10%)

**TABLE A1**  
**Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Endocrine System (continued)</b>				
Islets, pancreatic	(50)	(50)	(48)	(50)
Adenoma	1 (2%)	4 (8%)	5 (10%)	5 (10%)
Adenoma, multiple	1 (2%)	1 (2%)		
Carcinoma	3 (6%)	4 (8%)	4 (8%)	7 (14%)
Pituitary gland	(49)	(49)	(50)	(49)
Pars distalis, adenoma	43 (88%)	40 (82%)	42 (84%)	41 (84%)
Thyroid gland	(49)	(50)	(48)	(50)
Bilateral, C-cell, adenoma				1 (2%)
C-cell, adenoma	4 (8%)	2 (4%)	3 (6%)	3 (6%)
C-cell, carcinoma	3 (6%)	3 (6%)	4 (8%)	3 (6%)
Follicular cell, adenoma		1 (2%)		1 (2%)
Follicular cell, carcinoma	1 (2%)	2 (4%)		
<b>General Body System</b>				
Peritoneum		(2)	(1)	(1)
Sarcoma, metastatic, uncertain primary site		1 (50%)		
<b>Genital System</b>				
Epididymis	(50)	(50)	(50)	(50)
Preputial gland	(50)	(50)	(49)	(50)
Adenoma			1 (2%)	1 (2%)
Carcinoma	2 (4%)	2 (4%)		3 (6%)
Bilateral, adenoma			1 (2%)	
Bilateral, carcinoma	1 (2%)			
Prostate	(50)	(50)	(49)	(50)
Adenoma	1 (2%)	2 (4%)	4 (8%)	1 (2%)
Seminal vesicle	(50)	(50)	(49)	(49)
Carcinoma		1 (2%)		
Sarcoma, metastatic, uncertain primary site		1 (2%)		
Testes	(50)	(50)	(50)	(50)
Bilateral, interstitial cell, adenoma	26 (52%)	18 (36%)	26 (52%)	19 (38%)
Interstitial cell, adenoma	9 (18%)	13 (26%)	7 (14%)	10 (20%)
<b>Hematopoietic System</b>				
Bone marrow	(50)	(50)	(48)	(50)
Lymph node	(8)	(9)	(9)	(9)
Lymph node, bronchial	(45)	(30)	(41)	(49)
Lymph node, mandibular	(46)	(47)	(47)	(49)
Lymph node, mesenteric	(50)	(50)	(48)	(50)
Lymph node, mediastinal	(47)	(46)	(44)	(49)
Carcinoma, metastatic, seminal vesicle		1 (2%)		
Spleen	(50)	(50)	(49)	(50)
Carcinoma, metastatic, seminal vesicle		1 (2%)		
Fibroma	1 (2%)			
Sarcoma, metastatic, uncertain primary site		1 (2%)		
Thymus	(45)	(42)	(47)	(48)
Thymoma benign			1 (2%)	

**TABLE A1**  
**Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Integumentary System</b>				
Mammary gland	(30)	(34)	(36)	(38)
Fibroadenoma	3 (10%)	1 (3%)	2 (6%)	3 (8%)
Fibroadenoma, multiple				1 (3%)
Skin	(50)	(48)	(50)	(50)
Basal cell adenoma	2 (4%)			
Basal cell carcinoma				1 (2%)
Keratoacanthoma	2 (4%)	5 (10%)		2 (4%)
Keratoacanthoma, multiple				1 (2%)
Sarcoma, metastatic, uncertain primary site		1 (2%)		
Squamous cell papilloma				1 (2%)
Sebaceous gland, adenoma	1 (2%)	1 (2%)		
Subcutaneous tissue, fibroma		1 (2%)	2 (4%)	2 (4%)
Subcutaneous tissue, fibrosarcoma		2 (4%)	1 (2%)	2 (4%)
Subcutaneous tissue, lipoma		1 (2%)	1 (2%)	1 (2%)
Subcutaneous tissue, sarcoma				1 (2%)
Subcutaneous tissue, schwannoma malignant	1 (2%)			
<b>Musculoskeletal System</b>				
Bone	(50)	(50)	(50)	(50)
Chordoma	1 (2%)	1 (2%)		
Osteosarcoma		3 (6%)		
Skeletal muscle		(2)	(1)	(1)
Carcinoma, metastatic, seminal vesicle		1 (50%)		
Sarcoma				1 (100%)
Sarcoma, metastatic, uncertain primary site		1 (50%)		
<b>Nervous System</b>				
Brain	(50)	(50)	(50)	(50)
Astrocytoma malignant	1 (2%)		1 (2%)	
Spinal cord			(1)	
<b>Respiratory System</b>				
Larynx	(50)	(49)	(48)	(50)
Lung	(50)	(50)	(48)	(50)
Alveolar/bronchiolar adenoma	1 (2%)	4 (8%)	1 (2%)	5 (10%)
Alveolar/bronchiolar adenoma, multiple				1 (2%)
Alveolar/bronchiolar carcinoma			3 (6%)	1 (2%)
Carcinoma, metastatic, seminal vesicle		1 (2%)		
Chordoma, metastatic, bone	1 (2%)			
Osteosarcoma, metastatic, bone		1 (2%)		
Sarcoma, metastatic, skeletal muscle				1 (2%)
Nose	(50)	(50)	(49)	(50)
Nasopharyngeal duct, squamous cell carcinoma				1 (2%)
Pleura	(1)			
Trachea	(50)	(50)	(48)	(50)
<b>Special Senses System</b>				
Zymbal's gland	(1)			(2)
Carcinoma	1 (100%)			2 (100%)

**TABLE A1**  
**Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Urinary System</b>				
Kidney	(50)	(50)	(48)	(50)
Sarcoma, metastatic, uncertain primary site		1 (2%)		
Renal tubule, adenoma	1 (2%)		1 (2%)	
Urinary bladder	(50)	(50)	(48)	(50)
<b>Systemic Lesions</b>				
Multiple organs <sup>b</sup>	(50)	(50)	(50)	(50)
Leukemia mononuclear	30 (60%)	32 (64%)	29 (58%)	28 (56%)
Mesothelioma malignant	3 (6%)	2 (4%)	2 (4%)	2 (4%)
<b>Neoplasm Summary</b>				
Total animals with primary neoplasms <sup>c</sup>	50	50	50	50
Total primary neoplasms	159	169	173	178
Total animals with benign neoplasms	48	46	47	48
Total benign neoplasms	111	113	124	122
Total animals with malignant neoplasms	38	41	35	38
Total malignant neoplasms	48	56	49	56
Total animals with metastatic neoplasms	1	3		1
Total metastatic neoplasms	1	18		1
Total animals with malignant neoplasms of uncertain primary site		1		

<sup>a</sup> Number of animals examined microscopically at the site and the number of animals with neoplasm

<sup>b</sup> Number of animals with any tissue examined microscopically

<sup>c</sup> Primary neoplasms: all neoplasms except metastatic neoplasms













**TABLE A2**  
**Individual Animal Tumor Pathology of Male Rats in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate:**  
**0.3 mg/m<sup>3</sup>**

<b>Number of Days on Study</b>	4 4 4 4 5 5 5 5 5 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6
	0 7 7 9 4 5 7 7 8 8 0 1 2 2 3 3 3 3 3 4 4 4 5 6 6
	8 1 5 5 1 4 6 9 9 9 0 0 9 9 1 1 2 8 8 5 6 9 2 3 9
<b>Carcass ID Number</b>	2 2
	3 2 0 4 3 0 2 0 2 4 3 3 2 2 3 4 1 1 4 5 0 3 4 1 4
	3 0 7 6 7 6 9 5 7 9 6 0 2 6 9 0 9 1 7 0 4 2 5 5 8
<b>Alimentary System</b>	
Esophagus	+ +
Intestine large, colon	+ +
Intestine large, rectum	+ +
Intestine large, cecum	+ A
Intestine small, duodenum	+ +
Intestine small, jejunum	+ A
Intestine small, ileum	+ A
Liver	+ +
Carcinoma, metastatic, seminal vesicle	X
Hepatocellular carcinoma	
Sarcoma, metastatic, uncertain primary site	
Mesentery	+ +
Carcinoma, metastatic, seminal vesicle	X
Sarcoma, metastatic, uncertain primary site	
Pancreas	+ +
Carcinoma, metastatic, seminal vesicle	X
Sarcoma, metastatic, uncertain primary site	
Salivary glands	+ +
Stomach, forestomach	+ +
Stomach, glandular	+ +
<b>Cardiovascular System</b>	
Heart	+ +
Carcinoma, metastatic, seminal vesicle	X
<b>Endocrine System</b>	
Adrenal cortex	+ +
Carcinoma	
Adrenal medulla	+ +
Pheochromocytoma malignant	
Pheochromocytoma benign	
Bilateral, pheochromocytoma benign	
Islets, pancreatic	+ +
Adenoma	X
Adenoma, multiple	
Carcinoma	
Parathyroid gland	+ +
Pituitary gland	+ +
Pars distalis, adenoma	X X
Thyroid gland	+ +
C-cell, adenoma	
C-cell, carcinoma	
Follicular cell, adenoma	
Follicular cell, carcinoma	
<b>General Body System</b>	
Peritoneum	+ +
Sarcoma, metastatic, uncertain primary site	X



































**TABLE A3**  
**Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Adrenal Medulla: Benign Pheochromocytoma</b>				
Overall rate <sup>a</sup>	14/50 (28%)	19/50 (38%)	23/49 (47%)	20/50 (40%)
Adjusted rate <sup>b</sup>	51.0%	70.0%	71.9%	71.4%
Terminal rate <sup>c</sup>	6/17 (35%)	8/15 (53%)	13/21 (62%)	8/15 (53%)
First incidence (days)	534	541	526	526
Life table test <sup>d</sup>	P=0.166	P=0.220	P=0.214	P=0.134
Logistic regression test <sup>d</sup>	P=0.172	P=0.226	P=0.069	P=0.126
Cochran-Armitage test <sup>d</sup>	P=0.229			
Fisher exact test <sup>d</sup>		P=0.198	P=0.041	P=0.146
<b>Adrenal Medulla: Benign, Complex, or Malignant Pheochromocytoma</b>				
Overall rate	15/50 (30%)	19/50 (38%)	25/49 (51%)	20/50 (40%)
Adjusted rate	52.1%	70.0%	74.1%	71.4%
Terminal rate	6/17 (35%)	8/15 (53%)	13/21 (62%)	8/15 (53%)
First incidence (days)	534	541	526	526
Life table test	P=0.206	P=0.285	P=0.188	P=0.182
Logistic regression test	P=0.218	P=0.295	P=0.045	P=0.180
Cochran-Armitage test	P=0.279			
Fisher exact test		P=0.263	P=0.027	P=0.201
<b>Bone: Osteosarcoma</b>				
Overall rate	0/50 (0%)	3/50 (6%)	0/50 (0%)	0/50 (0%)
Adjusted rate	0.0%	11.4%	0.0%	0.0%
Terminal rate	0/17 (0%)	0/15 (0%)	0/21 (0%)	0/15 (0%)
First incidence (days)	— <sup>e</sup>	631	—	—
Life table test	P=0.258N	P=0.146	—	—
Logistic regression test	P=0.257N	P=0.123	—	—
Cochran-Armitage test	P=0.255N			
Fisher exact test		P=0.121	—	—
<b>Lung: Alveolar/bronchiolar Adenoma</b>				
Overall rate	1/50 (2%)	4/50 (8%)	1/48 (2%)	6/50 (12%)
Adjusted rate	2.3%	17.7%	2.4%	28.4%
Terminal rate	0/17 (0%)	2/15 (13%)	0/21 (0%)	2/15 (13%)
First incidence (days)	568	589	611	638
Life table test	P=0.042	P=0.187	P=0.726N	P=0.056
Logistic regression test	P=0.051	P=0.179	P=0.753	P=0.055
Cochran-Armitage test	P=0.055			
Fisher exact test		P=0.181	P=0.742	P=0.056
<b>Lung: Alveolar/bronchiolar Carcinoma</b>				
Overall rate	0/50 (0%)	0/50 (0%)	3/48 (6%)	1/50 (2%)
Adjusted rate	0.0%	0.0%	11.3%	6.7%
Terminal rate	0/17 (0%)	0/15 (0%)	1/21 (5%)	1/15 (7%)
First incidence (days)	—	—	652	734 (T)
Life table test	P=0.355	—	P=0.181	P=0.475
Logistic regression test	P=0.360	—	P=0.136	P=0.475
Cochran-Armitage test	P=0.382			
Fisher exact test		—	P=0.114	P=0.500

**TABLE A3**  
**Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Lung: Alveolar/bronchiolar Adenoma or Carcinoma</b>				
Overall rate	1/50 (2%)	4/50 (8%)	4/48 (8%)	7/50 (14%)
Adjusted rate	2.3%	17.7%	13.4%	33.9%
Terminal rate	0/17 (0%)	2/15 (13%)	1/21 (5%)	3/15 (20%)
First incidence (days)	568	589	611	638
Life table test	P=0.027	P=0.187	P=0.263	P=0.030
Logistic regression test	P=0.032	P=0.179	P=0.163	P=0.029
Cochran-Armitage test	P=0.038			
Fisher exact test		P=0.181	P=0.168	P=0.030
<b>Mammary Gland: Fibroadenoma</b>				
Overall rate	3/50 (6%)	1/50 (2%)	2/50 (4%)	4/50 (8%)
Adjusted rate	17.6%	6.7%	7.6%	21.2%
Terminal rate	3/17 (18%)	1/15 (7%)	1/21 (5%)	2/15 (13%)
First incidence (days)	734 (T)	734 (T)	652	611
Life table test	P=0.199	P=0.346N	P=0.391N	P=0.449
Logistic regression test	P=0.203	P=0.346N	P=0.398N	P=0.475
Cochran-Armitage test	P=0.240			
Fisher exact test		P=0.309N	P=0.500N	P=0.500
<b>Pancreatic Islets: Adenoma</b>				
Overall rate	2/50 (4%)	5/50 (10%)	5/48 (10%)	5/50 (10%)
Adjusted rate	10.2%	21.3%	18.3%	20.5%
Terminal rate	1/17 (6%)	2/15 (13%)	3/21 (14%)	1/15 (7%)
First incidence (days)	679	471	509	611
Life table test	P=0.278	P=0.217	P=0.301	P=0.222
Logistic regression test	P=0.304	P=0.224	P=0.226	P=0.208
Cochran-Armitage test	P=0.316			
Fisher exact test		P=0.218	P=0.201	P=0.218
<b>Pancreatic Islets: Carcinoma</b>				
Overall rate	3/50 (6%)	4/50 (8%)	4/48 (8%)	7/50 (14%)
Adjusted rate	11.4%	19.8%	14.3%	33.2%
Terminal rate	1/17 (6%)	1/15 (7%)	1/21 (5%)	2/15 (13%)
First incidence (days)	526	680	657	638
Life table test	P=0.089	P=0.501	P=0.611	P=0.149
Logistic regression test	P=0.091	P=0.515	P=0.489	P=0.149
Cochran-Armitage test	P=0.110			
Fisher exact test		P=0.500	P=0.477	P=0.159
<b>Pancreatic Islets: Adenoma or Carcinoma</b>				
Overall rate	5/50 (10%)	9/50 (18%)	9/48 (19%)	11/50 (22%)
Adjusted rate	20.7%	37.6%	30.6%	44.7%
Terminal rate	2/17 (12%)	3/15 (20%)	4/21 (19%)	3/15 (20%)
First incidence (days)	526	471	509	611
Life table test	P=0.101	P=0.202	P=0.328	P=0.088
Logistic regression test	P=0.107	P=0.205	P=0.192	P=0.077
Cochran-Armitage test	P=0.127			
Fisher exact test		P=0.194	P=0.172	P=0.086

**TABLE A3**  
**Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Pituitary Gland (Pars Distalis): Adenoma</b>				
Overall rate	43/49 (88%)	40/49 (82%)	42/50 (84%)	41/49 (84%)
Adjusted rate	97.4%	100.0%	89.3%	95.1%
Terminal rate	15/16 (94%)	15/15 (100%)	16/21 (76%)	13/15 (87%)
First incidence (days)	435	471	467	401
Life table test	P=0.455	P=0.340N	P=0.121N	P=0.509N
Logistic regression test	P=0.485N	P=0.269N	P=0.376N	P=0.386N
Cochran-Armitage test	P=0.474N			
Fisher exact test		P=0.288N	P=0.403N	P=0.387N
<b>Preputial Gland: Carcinoma</b>				
Overall rate	3/50 (6%)	2/50 (4%)	0/49 (0%)	3/50 (6%)
Adjusted rate	14.7%	11.1%	0.0%	12.8%
Terminal rate	1/17 (6%)	0/15 (0%)	0/21 (0%)	1/15 (7%)
First incidence (days)	679	701	—	511
Life table test	P=0.472	P=0.512N	P=0.087N	P=0.632
Logistic regression test	P=0.486	P=0.229N	P=0.093N	P=0.657
Cochran-Armitage test	P=0.500			
Fisher exact test		P=0.500N	P=0.125N	P=0.661N
<b>Preputial Gland: Adenoma or Carcinoma</b>				
Overall rate	3/50 (6%)	2/50 (4%)	2/49 (4%)	4/50 (8%)
Adjusted rate	14.7%	11.1%	6.8%	15.8%
Terminal rate	1/17 (6%)	0/15 (0%)	0/21 (0%)	1/15 (7%)
First incidence (days)	679	701	654	511
Life table test	P=0.292	P=0.512N	P=0.380N	P=0.478
Logistic regression test	P=0.305	P=0.229N	P=0.329N	P=0.494
Cochran-Armitage test	P=0.320			
Fisher exact test		P=0.500N	P=0.510N	P=0.500
<b>Prostate Gland: Adenoma</b>				
Overall rate	1/50 (2%)	2/50 (4%)	4/49 (8%)	1/50 (2%)
Adjusted rate	5.9%	10.7%	17.1%	6.7%
Terminal rate	1/17 (6%)	1/15 (7%)	3/21 (14%)	1/15 (7%)
First incidence (days)	734 (T)	680	673	734 (T)
Life table test	P=0.537N	P=0.481	P=0.250	P=0.736
Logistic regression test	P=0.533N	P=0.508	P=0.251	P=0.736
Cochran-Armitage test	P=0.500N			
Fisher exact test		P=0.500	P=0.175	P=0.753N
<b>Skin: Keratoacanthoma</b>				
Overall rate	2/50 (4%)	5/50 (10%)	0/50 (0%)	3/50 (6%)
Adjusted rate	11.1%	17.8%	0.0%	20.0%
Terminal rate	1/17 (6%)	1/15 (7%)	0/21 (0%)	3/15 (20%)
First incidence (days)	727	589	—	734 (T)
Life table test	P=0.602	P=0.231	P=0.196N	P=0.437
Logistic regression test	P=0.608N	P=0.227	P=0.183N	P=0.445
Cochran-Armitage test	P=0.582N			
Fisher exact test		P=0.218	P=0.247N	P=0.500

**TABLE A3**  
**Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Skin: Squamous Cell Papilloma or Keratoacanthoma</b>				
Overall rate	2/50 (4%)	5/50 (10%)	0/50 (0%)	4/50 (8%)
Adjusted rate	11.1%	17.8%	0.0%	26.7%
Terminal rate	1/17 (6%)	1/15 (7%)	0/21 (0%)	4/15 (27%)
First incidence (days)	727	589	—	734 (T)
Life table test	P=0.385	P=0.231	P=0.196N	P=0.272
Logistic regression test	P=0.400	P=0.227	P=0.183N	P=0.271
Cochran-Armitage test	P=0.429			
Fisher exact test		P=0.218	P=0.247N	P=0.339
<b>Skin: Squamous Cell Papilloma, Keratoacanthoma, Basal Cell Adenoma, or Basal Cell Carcinoma</b>				
Overall rate	4/50 (8%)	5/50 (10%)	0/50 (0%)	5/50 (10%)
Adjusted rate	22.2%	17.8%	0.0%	33.3%
Terminal rate	3/17 (18%)	1/15 (7%)	0/21 (0%)	5/15 (33%)
First incidence (days)	727	589	—	734 (T)
Life table test	P=0.406	P=0.493	P=0.040N	P=0.413
Logistic regression test	P=0.420	P=0.526	P=0.033N	P=0.414
Cochran-Armitage test	P=0.458			
Fisher exact test		P=0.500	P=0.059N	P=0.500
<b>Skin (Subcutaneous Tissue): Fibrosarcoma or Sarcoma</b>				
Overall rate	0/50 (0%)	2/50 (4%)	1/50 (2%)	3/50 (6%)
Adjusted rate	0.0%	9.8%	3.3%	16.9%
Terminal rate	0/17 (0%)	1/15 (7%)	0/21 (0%)	1/15 (7%)
First incidence (days)	—	646	673	692
Life table test	P=0.113	P=0.245	P=0.553	P=0.113
Logistic regression test	P=0.113	P=0.246	P=0.509	P=0.109
Cochran-Armitage test	P=0.132			
Fisher exact test		P=0.247	P=0.500	P=0.121
<b>Skin (Subcutaneous Tissue): Fibroma, Fibrosarcoma, or Sarcoma</b>				
Overall rate	0/50 (0%)	3/50 (6%)	2/50 (4%)	5/50 (10%)
Adjusted rate	0.0%	15.4%	7.9%	25.9%
Terminal rate	0/17 (0%)	1/15 (7%)	1/21 (5%)	2/15 (13%)
First incidence (days)	—	646	673	663
Life table test	P=0.033	P=0.120	P=0.293	P=0.031
Logistic regression test	P=0.031	P=0.126	P=0.272	P=0.028
Cochran-Armitage test	P=0.044			
Fisher exact test		P=0.121	P=0.247	P=0.028
<b>Testes: Adenoma</b>				
Overall rate	35/50 (70%)	31/50 (62%)	33/50 (66%)	29/50 (58%)
Adjusted rate	94.3%	90.5%	88.7%	92.7%
Terminal rate	15/17 (88%)	12/15 (80%)	17/21 (81%)	13/15 (87%)
First incidence (days)	568	475	534	526
Life table test	P=0.369N	P=0.321N	P=0.093N	P=0.319N
Logistic regression test	P=0.236N	P=0.142N	P=0.185N	P=0.145N
Cochran-Armitage test	P=0.179N			
Fisher exact test		P=0.263N	P=0.415N	P=0.149N

**TABLE A3**  
**Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Thyroid Gland (C-cell): Adenoma</b>				
Overall rate	4/49 (8%)	2/50 (4%)	3/48 (6%)	4/50 (8%)
Adjusted rate	18.8%	13.3%	12.1%	20.3%
Terminal rate	2/17 (12%)	2/15 (13%)	2/21 (10%)	2/15 (13%)
First incidence (days)	645	734 (T)	649	586
Life table test	P=0.391	P=0.358N	P=0.368N	P=0.602
Logistic regression test	P=0.406	P=0.316N	P=0.426N	P=0.634
Cochran-Armitage test	P=0.447			
Fisher exact test		P=0.329N	P=0.512N	P=0.631N
<b>Thyroid Gland (C-cell): Carcinoma</b>				
Overall rate	3/49 (6%)	3/50 (6%)	4/48 (8%)	3/50 (6%)
Adjusted rate	12.4%	13.6%	13.6%	15.3%
Terminal rate	1/17 (6%)	1/15 (7%)	1/21 (5%)	2/15 (13%)
First incidence (days)	435	576	582	562
Life table test	P=0.569	P=0.647	P=0.597	P=0.631
Logistic regression test	P=0.587N	P=0.656N	P=0.479	P=0.655N
Cochran-Armitage test	P=0.582N			
Fisher exact test		P=0.651N	P=0.488	P=0.651N
<b>Thyroid Gland (C-cell): Adenoma or Carcinoma</b>				
Overall rate	7/49 (14%)	5/50 (10%)	6/48 (13%)	7/50 (14%)
Adjusted rate	29.5%	25.9%	22.3%	34.1%
Terminal rate	3/17 (18%)	3/15 (20%)	3/21 (14%)	4/15 (27%)
First incidence (days)	435	576	582	562
Life table test	P=0.394	P=0.408N	P=0.349N	P=0.558
Logistic regression test	P=0.430	P=0.353N	P=0.479N	P=0.609
Cochran-Armitage test	P=0.466			
Fisher exact test		P=0.365N	P=0.516N	P=0.597N
<b>Thyroid Gland (Follicular Cell): Adenoma or Carcinoma</b>				
Overall rate	1/49 (2%)	3/50 (6%)	0/48 (0%)	1/50 (2%)
Adjusted rate	5.9%	16.0%	0.0%	6.7%
Terminal rate	1/17 (6%)	1/15 (7%)	0/21 (0%)	1/15 (7%)
First incidence (days)	734 (T)	680	—	734 (T)
Life table test	P=0.453N	P=0.288	P=0.458N	P=0.736
Logistic regression test	P=0.448N	P=0.309	P=0.458N	P=0.736
Cochran-Armitage test	P=0.423N			
Fisher exact test		P=0.316	P=0.505N	P=0.747N
<b>All Organs: Mononuclear Cell Leukemia</b>				
Overall rate	30/50 (60%)	32/50 (64%)	29/50 (58%)	28/50 (56%)
Adjusted rate	77.3%	80.6%	74.9%	75.6%
Terminal rate	9/17 (53%)	8/15 (53%)	12/21 (57%)	8/15 (53%)
First incidence (days)	453	475	435	463
Life table test	P=0.468N	P=0.501	P=0.199N	P=0.507N
Logistic regression test	P=0.313N	P=0.433	P=0.467N	P=0.420N
Cochran-Armitage test	P=0.297N			
Fisher exact test		P=0.418	P=0.500N	P=0.420N

**TABLE A3**  
**Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>All Organs: Malignant Mesothelioma</b>				
Overall rate	3/50 (6%)	2/50 (4%)	2/50 (4%)	2/50 (4%)
Adjusted rate	10.2%	11.1%	6.5%	9.3%
Terminal rate	0/17 (0%)	1/15 (7%)	0/21 (0%)	1/15 (7%)
First incidence (days)	589	681	649	603
Life table test	P=0.523N	P=0.477N	P=0.397N	P=0.517N
Logistic regression test	P=0.498N	P=0.494N	P=0.507N	P=0.501N
Cochran-Armitage test	P=0.491N			
Fisher exact test		P=0.500N	P=0.500N	P=0.500N
<b>All Organs: Osteosarcoma</b>				
Overall rate	0/50 (0%)	3/50 (6%)	0/50 (0%)	0/50 (0%)
Adjusted rate	0.0%	11.4%	0.0%	0.0%
Terminal rate	0/17 (0%)	0/15 (0%)	0/21 (0%)	0/15 (0%)
First incidence (days)	—	631	—	—
Life table test	P=0.258N	P=0.146	—	—
Logistic regression test	P=0.257N	P=0.123	—	—
Cochran-Armitage test	P=0.255N			
Fisher exact test		P=0.121	—	—
<b>All Organs: Benign Neoplasms</b>				
Overall rate	48/50 (96%)	46/50 (92%)	47/50 (94%)	48/50 (96%)
Adjusted rate	97.9%	100.0%	97.9%	100.0%
Terminal rate	16/17 (94%)	15/15 (100%)	20/21 (95%)	15/15 (100%)
First incidence (days)	435	471	467	401
Life table test	P=0.317	P=0.419N	P=0.125N	P=0.437
Logistic regression test	P=0.385	P=0.319N	P=0.517N	P=0.687
Cochran-Armitage test	P=0.434			
Fisher exact test		P=0.339N	P=0.500N	P=0.691N
<b>All Organs: Malignant Neoplasms</b>				
Overall rate	38/50 (76%)	42/50 (84%)	35/50 (70%)	38/50 (76%)
Adjusted rate	89.9%	93.1%	78.9%	87.5%
Terminal rate	13/17 (76%)	12/15 (80%)	12/21 (57%)	10/15 (67%)
First incidence (days)	435	408	272	401
Life table test	P=0.490	P=0.386	P=0.115N	P=0.469
Logistic regression test	P=0.396N	P=0.241	P=0.329N	P=0.592N
Cochran-Armitage test	P=0.395N			
Fisher exact test		P=0.227	P=0.326N	P=0.592N

**TABLE A3**  
**Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>All Organs: Benign or Malignant Neoplasms</b>				
Overall rate	50/50 (100%)	50/50 (100%)	50/50 (100%)	50/50 (100%)
Adjusted rate	100.0%	100.0%	100.0%	100.0%
Terminal rate	17/17 (100%)	15/15 (100%)	21/21 (100%)	15/15 (100%)
First incidence (days)	435	408	272	401
Life table test	P=0.361	P=0.510N	P=0.146N	P=0.438
Logistic regression test	— <sup>f</sup>	—	—	—
Cochran-Armitage test	—	—	—	—
Fisher exact test	—	P=1.000N	P=1.000N	P=1.000N

(T)Terminal sacrifice

<sup>a</sup> Number of neoplasm-bearing animals/number of animals examined. Denominator is number of animals examined microscopically for adrenal gland, bone, lung, pancreatic islets, pituitary gland, preputial gland, prostate gland, testes, and thyroid gland; for other tissues, denominator is number of animals necropsied.

<sup>b</sup> Kaplan-Meier estimated neoplasm incidence at the end of the study after adjustment for intercurrent mortality

<sup>c</sup> Observed incidence at terminal kill

<sup>d</sup> Beneath the chamber control incidence are the P values associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the chamber controls and that exposed group. The life table test regards neoplasms in animals dying prior to terminal kill as being (directly or indirectly) the cause of death. The logistic regression test regards these lesions as nonfatal. The Cochran-Armitage and Fisher exact tests compare directly the overall incidence rates. For all tests, a negative trend or a lower incidence in an exposure group is indicated by N.

<sup>e</sup> Not applicable; no neoplasms in animal group

<sup>f</sup> Value of statistic cannot be computed.

**TABLE A4a**  
**Historical Incidence of Alveolar/bronchiolar Neoplasms in Chamber Control Male F344/N Rats<sup>a</sup>**

Study	Incidence in Controls		
	Adenoma	Carcinoma	Adenoma or Carcinoma
<b>Historical Incidence at Battelle Pacific Northwest Laboratories</b>			
<i>o</i> -Chlorobenzalmalononitrile (CS2)	4/50	0/50	4/50
Acetonitrile	1/48	1/48	2/48
2-Chloroacetophenone	1/49	1/49	2/49
<i>l</i> -Epinephrine Hydrochloride	4/50	1/50	5/50
Chloroethane	0/50	0/50	0/50
Hexachlorocyclopentadiene	5/50	0/50	5/50
Ozone	1/50	1/50	2/50
<b>Overall Historical Incidence</b>			
Total	17/654 (2.6%)	6/654 (0.9%)	23/654 (3.5%)
Standard deviation	3.6%	1.0%	3.7%
Range	0%-10%	0%-2%	0%-10%

<sup>a</sup> Data as of 12 May 1995

**TABLE A4b**  
**Historical Incidence of Neoplasms of the Adrenal Medulla in Chamber Control Male F344/N Rats<sup>a</sup>**

Study	Incidence in Controls	
	Benign Pheochromocytoma	Benign, Complex, or Malignant Pheochromocytoma <sup>b</sup>
<b>Historical Incidence at Battelle Pacific Northwest Laboratories</b>		
<i>o</i> -Chlorobenzalmalononitrile (CS2)	18/42	20/42
Acetonitrile	4/48	4/48
2-Chloroacetophenone	14/46	15/46
<i>l</i> -Epinephrine Hydrochloride	11/50	11/50
Chloroethane	8/36	8/36
Hexachlorocyclopentadiene	15/50	16/50
Ozone	17/50	17/50
<b>Overall Historical Incidence</b>		
Total	163/623 (26.2%)	176/623 (28.3%)
Standard deviation	13.2%	12.0%
Range	0%-50%	8%-50%

<sup>a</sup> Data as of 12 May 1995

<sup>b</sup> Seven unspecified pheochromocytomas are included in the overall incidence.

**TABLE A5**  
**Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate<sup>a</sup>**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Disposition Summary</b>				
Animals initially in study	50	50	50	50
Early deaths				
Moribund	30	34	26	34
Natural deaths	3	1	3	1
Survivors				
Terminal sacrifice	17	15	21	15
Animals examined microscopically	50	50	50	50
<b>Alimentary System</b>				
Esophagus	(50)	(50)	(50)	(50)
Inflammation, suppurative			1 (2%)	
Intestine small, jejunum	(49)	(49)	(47)	(49)
Inflammation, chronic active		1 (2%)		
Liver	(50)	(50)	(48)	(50)
Angiectasis	5 (10%)	1 (2%)		1 (2%)
Basophilic focus	13 (26%)	16 (32%)	23 (48%)	21 (42%)
Clear cell focus	8 (16%)	5 (10%)	8 (17%)	6 (12%)
Degeneration, cystic	10 (20%)	11 (22%)	16 (33%)	10 (20%)
Degeneration, fatty	6 (12%)	5 (10%)	5 (10%)	6 (12%)
Eosinophilic focus	2 (4%)		3 (6%)	5 (10%)
Hepatodiaphragmatic nodule	1 (2%)	4 (8%)	4 (8%)	6 (12%)
Mixed cell focus	3 (6%)	3 (6%)	4 (8%)	5 (10%)
Necrosis		2 (4%)	1 (2%)	1 (2%)
Regeneration	3 (6%)	1 (2%)	1 (2%)	1 (2%)
Thrombosis	1 (2%)	1 (2%)		
Bile duct, hyperplasia	41 (82%)	42 (84%)	34 (71%)	35 (70%)
Centrilobular, necrosis	17 (34%)	19 (38%)	5 (10%)	11 (22%)
Mesentery	(21)	(14)	(11)	(14)
Fat, hemorrhage	1 (5%)		1 (9%)	
Fat, necrosis	20 (95%)	11 (79%)	9 (82%)	13 (93%)
Pancreas	(50)	(50)	(48)	(50)
Angiectasis		1 (2%)		
Atrophy	25 (50%)	25 (50%)	20 (42%)	28 (56%)
Basophilic focus	3 (6%)	2 (4%)	6 (13%)	3 (6%)
Hyperplasia	2 (4%)	1 (2%)	4 (8%)	1 (2%)
Metaplasia, hepatocyte		1 (2%)		
Salivary glands	(50)	(50)	(50)	(50)
Atrophy	2 (4%)	2 (4%)		1 (2%)
Stomach, forestomach	(50)	(50)	(49)	(50)
Hyperplasia, basal cell		1 (2%)		
Hyperplasia, squamous		1 (2%)		
Necrosis	6 (12%)	5 (10%)	10 (20%)	3 (6%)
Stomach, glandular	(50)	(50)	(48)	(50)
Inflammation, acute		1 (2%)		
Mineralization	1 (2%)	2 (4%)	1 (2%)	1 (2%)
Necrosis	3 (6%)	2 (4%)	5 (10%)	2 (4%)
Tongue	(1)			(2)
Hyperplasia, squamous	1 (100%)			1 (50%)
Epithelium, cyst				1 (50%)
Tooth			(1)	(1)
Developmental malformation			1 (100%)	1 (100%)

<sup>a</sup> Number of animals examined microscopically at the site and the number of animals with lesion

**TABLE A5**  
**Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Cardiovascular System</b>				
Blood vessel				(1)
Aorta, mineralization				1 (100%)
Heart	(50)	(50)	(50)	(50)
Cardiomyopathy	38 (76%)	46 (92%)	45 (90%)	38 (76%)
Atrium, thrombosis	2 (4%)	4 (8%)	1 (2%)	4 (8%)
<b>Endocrine System</b>				
Adrenal cortex	(50)	(50)	(48)	(50)
Hyperplasia	26 (52%)	24 (48%)	26 (54%)	24 (48%)
Hypertrophy	2 (4%)	5 (10%)	5 (10%)	5 (10%)
Necrosis	2 (4%)	1 (2%)		3 (6%)
Thrombosis			1 (2%)	
Vacuolization cytoplasmic	6 (12%)	4 (8%)	3 (6%)	3 (6%)
Adrenal medulla	(50)	(50)	(49)	(50)
Cyst				1 (2%)
Hyperplasia	34 (68%)	23 (46%)	29 (59%)	30 (60%)
Islets, pancreatic	(50)	(50)	(48)	(50)
Hyperplasia		1 (2%)		
Parathyroid gland	(48)	(48)	(49)	(49)
Hyperplasia	1 (2%)	1 (2%)	3 (6%)	1 (2%)
Pituitary gland	(49)	(49)	(50)	(49)
Pars distalis, hyperplasia	4 (8%)	5 (10%)	4 (8%)	5 (10%)
Thyroid gland	(49)	(50)	(48)	(50)
C-cell, hyperplasia	31 (63%)	32 (64%)	32 (67%)	34 (68%)
Follicular cell, hyperplasia	2 (4%)		1 (2%)	1 (2%)
<b>General Body System</b>				
None				
<b>Genital System</b>				
Epididymis	(50)	(50)	(50)	(50)
Granuloma sperm	1 (2%)	3 (6%)		
Preputial gland	(50)	(50)	(49)	(50)
Hyperplasia	1 (2%)			
Inflammation, chronic active	11 (22%)	10 (20%)	3 (6%)	12 (24%)
Prostate	(50)	(50)	(49)	(50)
Hyperplasia	12 (24%)	8 (16%)	5 (10%)	9 (18%)
Inflammation, chronic active	3 (6%)	4 (8%)	1 (2%)	1 (2%)
Necrosis				1 (2%)
Seminal vesicle	(50)	(50)	(49)	(49)
Inflammation, chronic active			1 (2%)	
Necrosis				1 (2%)
Testes	(50)	(50)	(50)	(50)
Atrophy	2 (4%)	4 (8%)	5 (10%)	
Artery, inflammation, chronic active	1 (2%)	1 (2%)	2 (4%)	3 (6%)
Interstitial cell, hyperplasia	13 (26%)	10 (20%)	6 (12%)	10 (20%)

**TABLE A5**  
**Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Hematopoietic System</b>				
Bone marrow	(50)	(50)	(48)	(50)
Necrosis		1 (2%)		
Lymph node	(8)	(9)	(9)	(9)
Iliac, ectasia				1 (11%)
Iliac, hemorrhage				1 (11%)
Pancreatic, ectasia			1 (11%)	
Renal, hemorrhage			1 (11%)	
Lymph node, bronchial	(45)	(30)	(41)	(49)
Inflammation, suppurative			1 (2%)	
Lymph node, mandibular	(46)	(47)	(47)	(49)
Infiltration cellular, plasma cell				1 (2%)
Lymph node, mediastinal	(47)	(46)	(44)	(49)
Hemorrhage		1 (2%)		1 (2%)
Spleen	(50)	(50)	(49)	(50)
Accessory spleen	1 (2%)	1 (2%)	1 (2%)	1 (2%)
Depletion cellular			1 (2%)	
Fibrosis	16 (32%)	16 (32%)	12 (24%)	13 (26%)
Hematopoietic cell proliferation	1 (2%)	1 (2%)	1 (2%)	
Hyperplasia, focal				1 (2%)
Necrosis	2 (4%)		1 (2%)	2 (4%)
<b>Integumentary System</b>				
Mammary gland	(30)	(34)	(36)	(38)
Galactocele	2 (7%)	1 (3%)		1 (3%)
Hyperplasia, atypical			1 (3%)	
Skin	(50)	(48)	(50)	(50)
Cyst epithelial inclusion	1 (2%)			
Inflammation, chronic	3 (6%)	1 (2%)	1 (2%)	1 (2%)
Inflammation, chronic active	1 (2%)	2 (4%)		3 (6%)
Subcutaneous tissue, edema				1 (2%)
<b>Musculoskeletal System</b>				
Bone	(50)	(50)	(50)	(50)
Fibrous osteodystrophy			2 (4%)	
Hyperostosis	2 (4%)	2 (4%)		
<b>Nervous System</b>				
Brain	(50)	(50)	(50)	(50)
Developmental malformation		1 (2%)		
Gliosis	1 (2%)	1 (2%)	1 (2%)	
Mineralization		1 (2%)		
Necrosis	2 (4%)	1 (2%)		
<b>Respiratory System</b>				
Larynx	(50)	(49)	(48)	(50)
Epiglottis, metaplasia, squamous		10 (20%)	37 (77%)	50 (100%)

**TABLE A5**  
**Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Respiratory System</b> (continued)				
Lung	(50)	(50)	(48)	(50)
Cyst				1 (2%)
Foreign body				1 (2%)
Hemorrhage	1 (2%)			
Hyperplasia, atypical			1 (2%)	1 (2%)
Infiltration cellular, histiocyte		1 (2%)	1 (2%)	1 (2%)
Inflammation, suppurative				1 (2%)
Metaplasia, squamous		1 (2%)	4 (8%)	2 (4%)
Mineralization				1 (2%)
Proteinosis		16 (32%)	40 (83%)	47 (94%)
Thrombosis		1 (2%)		1 (2%)
Alveolar epithelium, hyperplasia	9 (18%)	20 (40%)	20 (42%)	23 (46%)
Alveolar epithelium, hyperplasia, atypical		1 (2%)	2 (4%)	2 (4%)
Alveolar epithelium, metaplasia		50 (100%)	48 (100%)	49 (98%)
Alveolus, inflammation, granulomatous	2 (4%)	50 (100%)	48 (100%)	50 (100%)
Artery, mediastinum, mineralization				1 (2%)
Bronchiole, inflammation, chronic				1 (2%)
Interstitialium, fibrosis	1 (2%)	50 (100%)	48 (100%)	49 (98%)
Mediastinum, inflammation, suppurative			1 (2%)	
Nose	(50)	(50)	(49)	(50)
Inflammation, chronic active	1 (2%)			
Inflammation, suppurative	4 (8%)	15 (30%)	5 (10%)	6 (12%)
Metaplasia, squamous	1 (2%)			
Thrombosis	14 (28%)	18 (36%)	3 (6%)	9 (18%)
Glands, cyst	1 (2%)			
Lateral wall, hyperplasia	2 (4%)	14 (28%)	21 (43%)	20 (40%)
Lateral wall, inflammation				1 (2%)
Lateral wall, metaplasia, squamous	1 (2%)	3 (6%)	5 (10%)	8 (16%)
Olfactory epithelium, atrophy	8 (16%)	24 (48%)	42 (86%)	48 (96%)
Olfactory epithelium, metaplasia	5 (10%)	1 (2%)	5 (10%)	30 (60%)
Respiratory epithelium, hyperplasia, focal	6 (12%)	1 (2%)	3 (6%)	2 (4%)
Respiratory epithelium, inflammation	4 (8%)			1 (2%)
Respiratory epithelium, metaplasia, squamous	1 (2%)			1 (2%)
Pleura	(1)			
Trachea	(50)	(50)	(48)	(50)
Mineralization				1 (2%)
<b>Special Senses System</b>				
Eye	(5)		(1)	(1)
Cataract	5 (100%)		1 (100%)	1 (100%)
Retina, atrophy	5 (100%)		1 (100%)	1 (100%)
<b>Urinary System</b>				
Kidney	(50)	(50)	(48)	(50)
Cyst	1 (2%)			1 (2%)
Hydronephrosis		1 (2%)		1 (2%)
Infarct	4 (8%)	1 (2%)	1 (2%)	3 (6%)
Nephropathy	49 (98%)	49 (98%)	48 (100%)	50 (100%)
Thrombosis			1 (2%)	
Papilla, necrosis				1 (2%)
Renal tubule, hyperplasia	2 (4%)	4 (8%)	2 (4%)	2 (4%)
Urinary bladder	(50)	(50)	(48)	(50)
Necrosis				1 (2%)
Transitional epithelium, hyperplasia				1 (2%)



**APPENDIX B**  
**SUMMARY OF LESIONS IN FEMALE RATS**  
**IN THE 2-YEAR INHALATION STUDY**  
**OF COBALT SULFATE HEPTAHYDRATE**

<b>TABLE B1</b>	<b>Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate . . . . .</b>	<b>106</b>
<b>TABLE B2</b>	<b>Individual Animal Tumor Pathology of Female Rats in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate . . . . .</b>	<b>110</b>
<b>TABLE B3</b>	<b>Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate . . . . .</b>	<b>128</b>
<b>TABLE B4a</b>	<b>Historical Incidence of Alveolar/bronchiolar Neoplasms in Chamber Control Female F344/N Rats . . . . .</b>	<b>134</b>
<b>TABLE B4b</b>	<b>Historical Incidence of Neoplasms of the Adrenal Medulla in Chamber Control Female F344/N Rats . . . . .</b>	<b>134</b>
<b>TABLE B5</b>	<b>Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate . . . . .</b>	<b>135</b>

**TABLE B1**  
**Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate<sup>a</sup>**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Disposition Summary</b>				
Animals initially in study	50	50	50	50
Early deaths				
Moribund	19	20	20	17
Natural deaths	3	4	4	3
Survivors				
Terminal sacrifice	28	25	26	30
Pregnant		1		
Animals examined microscopically	50	49	50	50
<b>Alimentary System</b>				
Intestine large, colon	(48)	(45)	(48)	(48)
Intestine large, cecum	(48)	(45)	(47)	(47)
Intestine small, duodenum	(49)	(46)	(48)	(48)
Intestine small, ileum	(49)	(45)	(47)	(47)
Liver	(50)	(49)	(50)	(49)
Hemangiosarcoma			1 (2%)	
Histiocytic sarcoma			1 (2%)	
Pheochromocytoma malignant, metastatic, adrenal medulla			1 (2%)	
Mesentery	(5)	(8)	(6)	(13)
Sarcoma stromal, metastatic, uterus		1 (13%)		
Oral mucosa	(1)	(2)		
Pharyngeal, squamous cell papilloma	1 (100%)	2 (100%)		
Pancreas	(49)	(49)	(50)	(48)
Salivary glands	(50)	(49)	(50)	(50)
Adenoma				1 (2%)
Sarcoma, metastatic, eye				1 (2%)
Stomach, forestomach	(50)	(49)	(50)	(50)
Leiomyoma				1 (2%)
Stomach, glandular	(49)	(49)	(49)	(48)
Carcinoid tumor benign		1 (2%)		
<b>Cardiovascular System</b>				
Heart	(50)	(49)	(50)	(50)
Carcinoma, metastatic, lung				1 (2%)
Carcinoma, metastatic, thyroid gland	1 (2%)			
Schwannoma benign	1 (2%)	2 (4%)	2 (4%)	1 (2%)
<b>Endocrine System</b>				
Adrenal cortex	(49)	(49)	(50)	(49)
Adenoma		1 (2%)		1 (2%)
Carcinoma, metastatic, kidney	1 (2%)			
Adrenal medulla	(48)	(49)	(50)	(48)
Pheochromocytoma malignant			1 (2%)	1 (2%)
Pheochromocytoma complex				1 (2%)
Pheochromocytoma benign	2 (4%)	1 (2%)	3 (6%)	7 (15%)
Bilateral, pheochromocytoma benign				1 (2%)
Islets, pancreatic	(49)	(49)	(50)	(48)
Adenoma	4 (8%)	1 (2%)		
Carcinoma	2 (4%)		1 (2%)	

**TABLE B1**  
**Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Endocrine System (continued)</b>				
Pituitary gland	(50)	(49)	(50)	(49)
Pars distalis, adenoma	40 (80%)	39 (80%)	38 (76%)	40 (82%)
Thyroid gland	(49)	(49)	(50)	(48)
C-cell, adenoma	6 (12%)	6 (12%)	7 (14%)	3 (6%)
C-cell, carcinoma	3 (6%)		2 (4%)	3 (6%)
Follicular cell, adenoma		1 (2%)		1 (2%)
<b>General Body System</b>				
None				
<b>Genital System</b>				
Clitoral gland	(50)	(48)	(48)	(49)
Adenoma	5 (10%)	7 (15%)	3 (6%)	3 (6%)
Carcinoma	2 (4%)	3 (6%)	4 (8%)	3 (6%)
Bilateral, adenoma			2 (4%)	
Ovary	(50)	(49)	(50)	(49)
Granulosa cell tumor benign	1 (2%)			
Bilateral, granulosa-theca tumor benign		1 (2%)		
Uterus	(50)	(49)	(50)	(49)
Histiocytic sarcoma				1 (2%)
Leiomyoma				1 (2%)
Polyp stromal	8 (16%)	7 (14%)	10 (20%)	4 (8%)
Polyp stromal, multiple			1 (2%)	
Sarcoma stromal	1 (2%)	2 (4%)		
<b>Hematopoietic System</b>				
Bone marrow	(49)	(49)	(50)	(50)
Lymph node	(2)	(3)	(3)	(2)
Lymph node, bronchial	(30)	(30)	(37)	(37)
Lymph node, mandibular	(44)	(40)	(47)	(45)
Sarcoma, metastatic, eye				1 (2%)
Sarcoma, metastatic, skin				1 (2%)
Lymph node, mesenteric	(49)	(49)	(50)	(48)
Lymph node, mediastinal	(43)	(38)	(43)	(45)
Spleen	(49)	(48)	(50)	(49)
Histiocytic sarcoma				1 (2%)
Thymus	(42)	(45)	(45)	(44)
<b>Integumentary System</b>				
Mammary gland	(50)	(49)	(50)	(49)
Adenoma	2 (4%)			
Carcinoma	2 (4%)	2 (4%)	5 (10%)	4 (8%)
Carcinoma, multiple	1 (2%)			1 (2%)
Fibroadenoma	18 (36%)	18 (37%)	13 (26%)	20 (41%)
Fibroadenoma, multiple	4 (8%)	4 (8%)	7 (14%)	7 (14%)

**TABLE B1**  
**Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Integumentary System</b> (continued)				
Skin	(50)	(49)	(50)	(50)
Basal cell adenoma				1 (2%)
Squamous cell papilloma	1 (2%)			
Sebaceous gland, adenoma		1 (2%)		1 (2%)
Subcutaneous tissue, fibroma				1 (2%)
Subcutaneous tissue, fibrosarcoma			1 (2%)	
Subcutaneous tissue, lipoma	2 (4%)			
Subcutaneous tissue, sarcoma				1 (2%)
<b>Musculoskeletal System</b>				
Bone	(50)	(49)	(50)	(50)
Sarcoma, metastatic, eye				1 (2%)
Skeletal muscle			(1)	(1)
Carcinoma, metastatic, lung				1 (100%)
Rhabdomyosarcoma			1 (100%)	
<b>Nervous System</b>				
Brain	(50)	(49)	(50)	(50)
Astrocytoma benign				1 (2%)
Astrocytoma malignant		1 (2%)		
Oligodendroglioma benign			1 (2%)	
<b>Respiratory System</b>				
Larynx	(50)	(49)	(50)	(50)
Lung	(50)	(49)	(50)	(50)
Alveolar/bronchiolar adenoma		1 (2%)	9 (18%)	7 (14%)
Alveolar/bronchiolar adenoma, multiple			1 (2%)	2 (4%)
Alveolar/bronchiolar carcinoma		2 (4%)	6 (12%)	6 (12%)
Carcinoma, metastatic, mammary gland	1 (2%)			
Histiocytic sarcoma				1 (2%)
Pheochromocytoma malignant, metastatic, adrenal medulla			1 (2%)	
Sarcoma, metastatic, eye				1 (2%)
Sarcoma, metastatic, skin				1 (2%)
Sarcoma, metastatic, uncertain primary site		1 (2%)		
Squamous cell carcinoma			1 (2%)	1 (2%)
Mediastinum, alveolar/bronchiolar carcinoma, metastatic, lung				1 (2%)
Nose	(50)	(49)	(50)	(50)
Pleura		(1)		
<b>Special Senses System</b>				
Eye	(1)	(5)	(3)	(3)
Sarcoma				1 (33%)
Zymbal's gland		(1)	(1)	
Carcinoma		1 (100%)	1 (100%)	

**TABLE B1**  
**Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Urinary System</b>				
Kidney	(49)	(49)	(50)	(48)
Histiocytic sarcoma				1 (2%)
Lipoma	1 (2%)			1 (2%)
Mesenchymal tumor benign		1 (2%)		
Bilateral, renal tubule, carcinoma, multiple	1 (2%)			
Urinary bladder	(49)	(49)	(50)	(47)
<b>Systemic Lesions</b>				
Multiple organs <sup>b</sup>	(50)	(49)	(50)	(50)
Histiocytic sarcoma			1 (2%)	1 (2%)
Leukemia mononuclear	15 (30%)	16 (33%)	19 (38%)	10 (20%)
Lymphoma malignant				1 (2%)
Mesothelioma malignant		1 (2%)		
<b>Neoplasm Summary</b>				
Total animals with primary neoplasms <sup>c</sup>	48	47	50	46
Total primary neoplasms	123	122	141	139
Total animals with benign neoplasms	45	44	47	44
Total benign neoplasms	96	94	97	105
Total animals with malignant neoplasms	25	23	32	27
Total malignant neoplasms	27	28	44	34
Total animals with metastatic neoplasms	3	3	1	3
Total metastatic neoplasms	3	3	2	9
Total animals with malignant neoplasms of uncertain primary site		1		

<sup>a</sup> Number of animals examined microscopically at the site and the number of animals with neoplasm

<sup>b</sup> Number of animals with any tissue examined microscopically

<sup>c</sup> Primary neoplasms: all neoplasms except metastatic neoplasms



































**TABLE B2**  
**Individual Animal Tumor Pathology of Female Rats in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate:**  
**3.0 mg/m<sup>3</sup>**

<b>Number of Days on Study</b>	7 7	
	3 3	
	5 5 6 6 6 6 6 6 6 6 6 6 6 6 6 6 7 7 7 7 7 7 7 7	
<b>Carcass ID Number</b>	7 7	Total
	3 3 0 0 0 0 1 2 2 2 2 3 3 4 4 5 1 1 2 2 3 3 4 4 4	Tissues/
	5 8 2 3 5 6 5 0 3 4 5 1 2 5 6 0 3 4 2 7 4 7 0 1 8	Tumors
<b>Urinary System</b>		
Kidney	+ +	48
Histiocytic sarcoma		1
Lipoma		1
Urinary bladder	+ +	47
<b>Systemic Lesions</b>		
Multiple organs	+ +	50
Histiocytic sarcoma		1
Leukemia mononuclear		10
Lymphoma malignant		1

**TABLE B3**  
**Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Adrenal Medulla: Benign Pheochromocytoma</b>				
Overall rate <sup>a</sup>	2/48 (4%)	1/49 (2%)	3/50 (6%)	8/48 (17%)
Adjusted rate <sup>b</sup>	5.1%	3.1%	9.3%	26.4%
Terminal rate <sup>c</sup>	0/27 (0%)	0/25 (0%)	1/26 (4%)	7/29 (24%)
First incidence (days)	666	702	694	709
Life table test <sup>d</sup>	P=0.006	P=0.546N	P=0.483	P=0.054
Logistic regression test <sup>d</sup>	P=0.004	P=0.498N	P=0.512	P=0.043
Cochran-Armitage test <sup>d</sup>	P=0.003			
Fisher exact test <sup>d</sup>		P=0.492N	P=0.520	P=0.045
<b>Adrenal Medulla: Benign, Complex, or Malignant Pheochromocytoma</b>				
Overall rate	2/48 (4%)	1/49 (2%)	4/50 (8%)	10/48 (21%)
Adjusted rate	5.1%	3.1%	11.7%	31.5%
Terminal rate	0/27 (0%)	0/25 (0%)	1/26 (4%)	8/29 (28%)
First incidence (days)	666	702	685	663
Life table test	P=0.001	P=0.546N	P=0.325	P=0.019
Logistic regression test	P< 0.001	P=0.498N	P=0.323	P=0.014
Cochran-Armitage test	P< 0.001			
Fisher exact test		P=0.492N	P=0.359	P=0.014
<b>Clitoral Gland: Adenoma</b>				
Overall rate	5/50 (10%)	7/48 (15%)	5/48 (10%)	3/49 (6%)
Adjusted rate	16.0%	23.4%	17.8%	8.8%
Terminal rate	2/28 (7%)	4/24 (17%)	3/26 (12%)	1/30 (3%)
First incidence (days)	727	652	727	680
Life table test	P=0.160N	P=0.289	P=0.567	P=0.360N
Logistic regression test	P=0.182N	P=0.286	P=0.581	P=0.384N
Cochran-Armitage test	P=0.189N			
Fisher exact test		P=0.351	P=0.603	P=0.369N
<b>Clitoral Gland: Carcinoma</b>				
Overall rate	2/50 (4%)	3/48 (6%)	4/48 (8%)	3/49 (6%)
Adjusted rate	7.1%	9.3%	13.4%	10.0%
Terminal rate	2/28 (7%)	1/24 (4%)	2/26 (8%)	3/30 (10%)
First incidence (days)	735 (T)	610	694	735 (T)
Life table test	P=0.548	P=0.439	P=0.305	P=0.532
Logistic regression test	P=0.504	P=0.459	P=0.301	P=0.532
Cochran-Armitage test	P=0.499			
Fisher exact test		P=0.480	P=0.319	P=0.490
<b>Clitoral Gland: Adenoma or Carcinoma</b>				
Overall rate	7/50 (14%)	10/48 (21%)	8/48 (17%)	6/49 (12%)
Adjusted rate	22.5%	31.1%	27.0%	18.2%
Terminal rate	4/28 (14%)	5/24 (21%)	5/26 (19%)	4/30 (13%)
First incidence (days)	727	610	694	680
Life table test	P=0.234N	P=0.207	P=0.429	P=0.480N
Logistic regression test	P=0.280N	P=0.205	P=0.432	P=0.545N
Cochran-Armitage test	P=0.291N			
Fisher exact test		P=0.266	P=0.465	P=0.516N

**TABLE B3**  
**Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Lung: Alveolar/bronchiolar Adenoma</b>				
Overall rate	0/50 (0%)	1/49 (2%)	10/50 (20%)	9/50 (18%)
Adjusted rate	0.0%	3.4%	36.4%	30.0%
Terminal rate	0/28 (0%)	0/25 (0%)	9/26 (35%)	9/30 (30%)
First incidence (days)	— <sup>e</sup>	714	692	735 (T)
Life table test	P=0.003	P=0.468	P< 0.001	P=0.003
Logistic regression test	P=0.001	P=0.480	P< 0.001	P=0.003
Cochran-Armitage test	P=0.002			
Fisher exact test		P=0.495	P< 0.001	P=0.001
<b>Lung: Alveolar/bronchiolar Carcinoma</b>				
Overall rate	0/50 (0%)	2/49 (4%)	6/50 (12%)	6/50 (12%)
Adjusted rate	0.0%	8.0%	20.2%	17.5%
Terminal rate	0/28 (0%)	2/25 (8%)	4/26 (15%)	4/30 (13%)
First incidence (days)	—	735 (T)	694	610
Life table test	P=0.033	P=0.213	P=0.015	P=0.022
Logistic regression test	P=0.023	P=0.213	P=0.015	P=0.017
Cochran-Armitage test	P=0.022			
Fisher exact test		P=0.242	P=0.013	P=0.013
<b>Lung: Alveolar/bronchiolar Adenoma or Carcinoma</b>				
Overall rate	0/50 (0%)	3/49 (6%)	15/50 (30%)	15/50 (30%)
Adjusted rate	0.0%	11.2%	50.6%	46.1%
Terminal rate	0/28 (0%)	2/25 (8%)	12/26 (46%)	13/30 (43%)
First incidence (days)	—	714	692	610
Life table test	P< 0.001	P=0.101	P< 0.001	P< 0.001
Logistic regression test	P< 0.001	P=0.096	P< 0.001	P< 0.001
Cochran-Armitage test	P< 0.001			
Fisher exact test		P=0.117	P< 0.001	P< 0.001
<b>Lung: Alveolar/bronchiolar Adenoma, Alveolar/bronchiolar Carcinoma, or Squamous Cell Carcinoma</b>				
Overall rate	0/50 (0%)	3/49 (6%)	16/50 (32%)	16/50 (32%)
Adjusted rate	0.0%	11.2%	54.1%	49.2%
Terminal rate	0/28 (0%)	2/25 (8%)	13/26 (50%)	14/30 (47%)
First incidence (days)	—	714	692	610
Life table test	P< 0.001	P=0.101	P< 0.001	P< 0.001
Logistic regression test	P< 0.001	P=0.096	P< 0.001	P< 0.001
Cochran-Armitage test	P< 0.001			
Fisher exact test		P=0.117	P< 0.001	P< 0.001
<b>Mammary Gland: Fibroadenoma</b>				
Overall rate	22/50 (44%)	22/49 (45%)	20/50 (40%)	27/50 (54%)
Adjusted rate	54.6%	67.3%	56.5%	70.5%
Terminal rate	11/28 (39%)	15/25 (60%)	12/26 (46%)	19/30 (63%)
First incidence (days)	569	610	510	386
Life table test	P=0.291	P=0.386	P=0.524N	P=0.280
Logistic regression test	P=0.147	P=0.439	P=0.435N	P=0.182
Cochran-Armitage test	P=0.153			
Fisher exact test		P=0.545	P=0.420N	P=0.212

**TABLE B3**  
**Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Mammary Gland: Fibroadenoma or Adenoma</b>				
Overall rate	23/50 (46%)	22/49 (45%)	20/50 (40%)	27/50 (54%)
Adjusted rate	55.6%	67.3%	56.5%	70.5%
Terminal rate	11/28 (39%)	15/25 (60%)	12/26 (46%)	19/30 (63%)
First incidence (days)	569	610	510	386
Life table test	P=0.334	P=0.452	P=0.457N	P=0.343
Logistic regression test	P=0.179	P=0.535	P=0.349N	P=0.244
Cochran-Armitage test	P=0.186			
Fisher exact test		P=0.537N	P=0.343N	P=0.274
<b>Mammary Gland: Carcinoma</b>				
Overall rate	3/50 (6%)	2/49 (4%)	5/50 (10%)	5/50 (10%)
Adjusted rate	8.5%	5.4%	15.9%	15.9%
Terminal rate	1/28 (4%)	0/25 (0%)	2/26 (8%)	4/30 (13%)
First incidence (days)	638	526	695	693
Life table test	P=0.247	P=0.555N	P=0.333	P=0.370
Logistic regression test	P=0.216	P=0.471N	P=0.341	P=0.342
Cochran-Armitage test	P=0.217			
Fisher exact test		P=0.510N	P=0.357	P=0.357
<b>Mammary Gland: Adenoma or Carcinoma</b>				
Overall rate	5/50 (10%)	2/49 (4%)	5/50 (10%)	5/50 (10%)
Adjusted rate	13.2%	5.4%	15.9%	15.9%
Terminal rate	1/28 (4%)	0/25 (0%)	2/26 (8%)	4/30 (13%)
First incidence (days)	572	526	695	693
Life table test	P=0.423	P=0.280N	P=0.589	P=0.621N
Logistic regression test	P=0.390	P=0.182N	P=0.626	P=0.622
Cochran-Armitage test	P=0.393			
Fisher exact test		P=0.226N	P=0.630N	P=0.630N
<b>Mammary Gland: Fibroadenoma, Adenoma, or Carcinoma</b>				
Overall rate	25/50 (50%)	23/49 (47%)	24/50 (48%)	29/50 (58%)
Adjusted rate	59.2%	68.1%	65.0%	74.0%
Terminal rate	12/28 (43%)	15/25 (60%)	14/26 (54%)	20/30 (67%)
First incidence (days)	569	526	510	386
Life table test	P=0.324	P=0.511	P=0.528	P=0.348
Logistic regression test	P=0.160	P=0.527N	P=0.513N	P=0.242
Cochran-Armitage test	P=0.168			
Fisher exact test		P=0.459N	P=0.500N	P=0.274
<b>Pancreatic Islets: Adenoma</b>				
Overall rate	4/49 (8%)	1/49 (2%)	0/50 (0%)	0/48 (0%)
Adjusted rate	14.3%	4.0%	0.0%	0.0%
Terminal rate	4/28 (14%)	1/25 (4%)	0/26 (0%)	0/30 (0%)
First incidence (days)	735 (T)	735 (T)	—	—
Life table test	P=0.049N	P=0.212N	P=0.071N	P=0.053N
Logistic regression test	P=0.049N	P=0.212N	P=0.071N	P=0.053N
Cochran-Armitage test	P=0.058N			
Fisher exact test		P=0.181N	P=0.056N	P=0.061N

**TABLE B3**  
**Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Pancreatic Islets: Adenoma or Carcinoma</b>				
Overall rate	6/49 (12%)	1/49 (2%)	1/50 (2%)	0/48 (0%)
Adjusted rate	19.6%	4.0%	3.4%	0.0%
Terminal rate	5/28 (18%)	1/25 (4%)	0/26 (0%)	0/30 (0%)
First incidence (days)	572	735 (T)	727	—
Life table test	P=0.023N	P=0.078N	P=0.073N	P=0.015N
Logistic regression test	P=0.025N	P=0.069N	P=0.059N	P=0.019N
Cochran-Armitage test	P=0.027N			
Fisher exact test		P=0.056N	P=0.053N	P=0.014N
<b>Pituitary Gland (Pars Distalis): Adenoma</b>				
Overall rate	40/50 (80%)	39/49 (80%)	38/50 (76%)	40/49 (82%)
Adjusted rate	86.8%	95.0%	90.3%	90.8%
Terminal rate	22/28 (79%)	23/25 (92%)	22/26 (85%)	26/30 (87%)
First incidence (days)	569	420	510	386
Life table test	P=0.350N	P=0.336	P=0.530	P=0.491N
Logistic regression test	P=0.450	P=0.451	P=0.474N	P=0.449
Cochran-Armitage test	P=0.450			
Fisher exact test		P=0.579N	P=0.405N	P=0.520
<b>Thyroid Gland (C-cell): Adenoma</b>				
Overall rate	6/49 (12%)	6/49 (12%)	7/50 (14%)	3/48 (6%)
Adjusted rate	21.4%	19.1%	24.5%	8.3%
Terminal rate	6/28 (21%)	3/25 (12%)	5/26 (19%)	1/30 (3%)
First incidence (days)	735 (T)	517	722	611
Life table test	P=0.139N	P=0.540	P=0.449	P=0.220N
Logistic regression test	P=0.177N	P=0.597	P=0.469	P=0.254N
Cochran-Armitage test	P=0.184N			
Fisher exact test		P=0.620N	P=0.516	P=0.254N
<b>Thyroid Gland (C-cell): Carcinoma</b>				
Overall rate	3/49 (6%)	0/49 (0%)	2/50 (4%)	3/48 (6%)
Adjusted rate	10.0%	0.0%	6.5%	9.3%
Terminal rate	2/28 (7%)	0/25 (0%)	0/26 (0%)	2/30 (7%)
First incidence (days)	721	—	709	686
Life table test	P=0.365	P=0.147N	P=0.528N	P=0.651N
Logistic regression test	P=0.340	P=0.140N	P=0.511N	P=0.653
Cochran-Armitage test	P=0.323			
Fisher exact test		P=0.121N	P=0.490N	P=0.651
<b>Thyroid Gland (C-cell): Adenoma or Carcinoma</b>				
Overall rate	9/49 (18%)	6/49 (12%)	9/50 (18%)	6/48 (13%)
Adjusted rate	30.7%	19.1%	29.4%	17.0%
Terminal rate	8/28 (29%)	3/25 (12%)	5/26 (19%)	3/30 (10%)
First incidence (days)	721	517	709	611
Life table test	P=0.277N	P=0.376N	P=0.543	P=0.263N
Logistic regression test	P=0.339N	P=0.326N	P=0.578	P=0.303N
Cochran-Armitage test	P=0.357N			
Fisher exact test		P=0.288N	P=0.584N	P=0.303N

**TABLE B3**  
**Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Uterus: Stromal Polyp</b>				
Overall rate	8/50 (16%)	7/49 (14%)	11/50 (22%)	4/50 (8%)
Adjusted rate	23.9%	20.0%	35.6%	13.3%
Terminal rate	5/28 (18%)	3/25 (12%)	8/26 (31%)	4/30 (13%)
First incidence (days)	569	483	600	735 (T)
Life table test	P=0.113N	P=0.585N	P=0.254	P=0.159N
Logistic regression test	P=0.146N	P=0.467N	P=0.294	P=0.189N
Cochran-Armitage test	P=0.144N			
Fisher exact test		P=0.517N	P=0.306	P=0.178N
<b>Uterus: Stromal Polyp or Stromal Sarcoma</b>				
Overall rate	9/50 (18%)	9/49 (18%)	11/50 (22%)	4/50 (8%)
Adjusted rate	26.3%	23.6%	35.6%	13.3%
Terminal rate	5/28 (18%)	3/25 (12%)	8/26 (31%)	4/30 (13%)
First incidence (days)	569	362	600	735 (T)
Life table test	P=0.059N	P=0.507	P=0.341	P=0.108N
Logistic regression test	P=0.071N	P=0.513N	P=0.387	P=0.126N
Cochran-Armitage test	P=0.074N			
Fisher exact test		P=0.584	P=0.402	P=0.117N
<b>All Organs: Mononuclear Cell Leukemia</b>				
Overall rate	15/50 (30%)	16/49 (33%)	19/50 (38%)	10/50 (20%)
Adjusted rate	37.4%	41.1%	47.7%	25.6%
Terminal rate	5/28 (18%)	5/25 (20%)	7/26 (27%)	3/30 (10%)
First incidence (days)	385	483	526	470
Life table test	P=0.107N	P=0.367	P=0.242	P=0.217N
Logistic regression test	P=0.091N	P=0.529	P=0.273	P=0.166N
Cochran-Armitage test	P=0.095N			
Fisher exact test		P=0.473	P=0.263	P=0.178N
<b>All Organs: Benign Neoplasms</b>				
Overall rate	45/50 (90%)	44/49 (90%)	47/50 (94%)	44/50 (88%)
Adjusted rate	93.7%	95.6%	100.0%	97.8%
Terminal rate	25/28 (89%)	23/25 (92%)	26/26 (100%)	29/30 (97%)
First incidence (days)	569	420	510	386
Life table test	P=0.259N	P=0.318	P=0.275	P=0.420N
Logistic regression test	P=0.483N	P=0.566	P=0.248	P=0.636N
Cochran-Armitage test	P=0.418N			
Fisher exact test		P=0.617N	P=0.357	P=0.500N
<b>All Organs: Malignant Neoplasms</b>				
Overall rate	25/50 (50%)	23/49 (47%)	32/50 (64%)	27/50 (54%)
Adjusted rate	58.3%	54.9%	72.0%	65.0%
Terminal rate	11/28 (39%)	8/25 (32%)	14/26 (54%)	16/30 (53%)
First incidence (days)	385	362	355	470
Life table test	P=0.472	P=0.513	P=0.129	P=0.464
Logistic regression test	P=0.341	P=0.376N	P=0.119	P=0.402
Cochran-Armitage test	P=0.337			
Fisher exact test		P=0.459N	P=0.113	P=0.421

**TABLE B3**  
**Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>All Organs: Benign or Malignant Neoplasms</b>				
Overall rate	48/50 (96%)	47/49 (96%)	50/50 (100%)	46/50 (92%)
Adjusted rate	96.0%	95.9%	100.0%	97.9%
Terminal rate	26/28 (93%)	23/25 (92%)	26/26 (100%)	29/30 (97%)
First incidence (days)	385	362	355	386
Life table test	P=0.216N	P=0.310	P=0.280	P=0.365N
Logistic regression test	P=0.228N	P=0.655N	P=0.254	P=0.450N
Cochran-Armitage test	P=0.180N			
Fisher exact test		P=0.684N	P=0.247	P=0.339N

(T)Terminal sacrifice

- <sup>a</sup> Number of neoplasm-bearing animals/number of animals examined. Denominator is number of animals examined microscopically for adrenal gland, clitoral gland, lung, pancreatic islets, pituitary gland, thyroid gland, and uterus; for other tissues, denominator is number of animals necropsied.
- <sup>b</sup> Kaplan-Meier estimated neoplasm incidence at the end of the study after adjustment for intercurrent mortality
- <sup>c</sup> Observed incidence at terminal kill
- <sup>d</sup> Beneath the chamber control incidence are the P values associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the chamber controls and that exposed group. The life table test regards neoplasms in animals dying prior to terminal kill as being (directly or indirectly) the cause of death. The logistic regression test regards these lesions as nonfatal. The Cochran-Armitage and Fisher exact tests compare directly the overall incidence rates. For all tests, a negative trend or a lower incidence in an exposure group is indicated by N.
- <sup>e</sup> Not applicable; no neoplasms in animal group

**TABLE B4a**  
**Historical Incidence of Alveolar/bronchiolar Neoplasms in Chamber Control Female F344/N Rats<sup>a</sup>**

Study	Incidence in Controls		
	Adenoma	Carcinoma	Adenoma or Carcinoma
<b>Historical Incidence at Battelle Pacific Northwest Laboratories</b>			
<i>o</i> -Chlorobenzalmononitrile (CS <sub>2</sub> )	2/49	0/49	2/49
Acetonitrile	0/48	0/48	0/48
2-Chloroacetophenone	1/49	0/49	1/49
<i>l</i> -Epinephrine Hydrochloride	0/50	0/50	0/50
Chloroethane	0/50	0/50	0/50
Hexachlorocyclopentadiene	1/50	0/50	1/50
Ozone	0/50	0/50	0/50
<b>Overall Historical Incidence</b>			
Total	7/650 (1.1%)	0/650 (0.0%)	7/650 (1.1%)
Standard deviation	1.6%		1.6%
Range	0%-4%		0%-4%

<sup>a</sup> Data as of 12 May 1995

**TABLE B4b**  
**Historical Incidence of Neoplasms of the Adrenal Medulla in Chamber Control Female F344/N Rats<sup>a</sup>**

Study	Incidence in Controls	
	Benign Pheochromocytoma	Benign, Complex, or Malignant Pheochromocytoma <sup>b</sup>
<b>Historical Incidence at Battelle Pacific Northwest Laboratories</b>		
<i>o</i> -Chlorobenzalmononitrile (CS <sub>2</sub> )	5/37	5/37
Acetonitrile	1/48	1/48
2-Chloroacetophenone	5/49	5/49
<i>l</i> -Epinephrine Hydrochloride	1/50	1/50
Chloroethane	1/35	1/35
Hexachlorocyclopentadiene	6/47	6/47
Ozone	6/50	6/50
<b>Overall Historical Incidence</b>		
Total	35/608 (5.8%)	39/608 (6.4%)
Standard deviation	4.9%	4.4%
Range	0%-14%	2%-14%

<sup>a</sup> Data as of 12 May 1995

<sup>b</sup> One unspecified pheochromocytoma is included in the overall incidence.

**TABLE B5**  
**Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate<sup>a</sup>**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Disposition Summary</b>				
Animals initially in study	50	50	50	50
Early deaths				
Moribund	19	20	20	17
Natural deaths	3	4	4	3
Survivors				
Terminal sacrifice	28	25	26	30
Pregnant		1		
Animals examined microscopically	50	49	50	50
<b>Alimentary System</b>				
Esophagus	(50)	(49)	(50)	(50)
Inflammation, suppurative			1 (2%)	
Intestine large, cecum	(48)	(45)	(47)	(47)
Necrosis			1 (2%)	
Liver	(50)	(49)	(50)	(49)
Angiectasis	6 (12%)	4 (8%)	1 (2%)	2 (4%)
Basophilic focus	39 (78%)	37 (76%)	45 (90%)	41 (84%)
Clear cell focus	7 (14%)	6 (12%)	5 (10%)	11 (22%)
Cyst			1 (2%)	
Degeneration, fatty	8 (16%)	9 (18%)	8 (16%)	2 (4%)
Eosinophilic focus	4 (8%)	1 (2%)	1 (2%)	2 (4%)
Hepatodiaphragmatic nodule	6 (12%)	6 (12%)	8 (16%)	4 (8%)
Inflammation, chronic active		1 (2%)		
Mixed cell focus	11 (22%)	11 (22%)	18 (36%)	15 (31%)
Necrosis		1 (2%)		
Regeneration		1 (2%)	2 (4%)	
Thrombosis	1 (2%)			
Bile duct, hyperplasia	5 (10%)	6 (12%)	9 (18%)	7 (14%)
Centrilobular, necrosis	5 (10%)	6 (12%)	7 (14%)	5 (10%)
Mesentery	(5)	(8)	(6)	(13)
Inflammation, chronic active				1 (8%)
Artery, inflammation, chronic active		1 (13%)		
Fat, necrosis	5 (100%)	6 (75%)	6 (100%)	12 (92%)
Pancreas	(49)	(49)	(50)	(48)
Atrophy	22 (45%)	11 (22%)	16 (32%)	16 (33%)
Basophilic focus				3 (6%)
Hyperplasia				1 (2%)
Artery, inflammation			1 (2%)	
Salivary glands	(50)	(49)	(50)	(50)
Atrophy	3 (6%)	4 (8%)	1 (2%)	
Stomach, forestomach	(50)	(49)	(50)	(50)
Hyperplasia, basal cell		1 (2%)		
Hyperplasia, squamous			1 (2%)	
Necrosis	3 (6%)	2 (4%)	1 (2%)	1 (2%)
Stomach, glandular	(49)	(49)	(49)	(48)
Mineralization	1 (2%)	1 (2%)	1 (2%)	1 (2%)
Necrosis	4 (8%)		1 (2%)	
Tooth		(2)		(1)
Developmental malformation		1 (50%)		
Inflammation, chronic active		1 (50%)		1 (100%)

<sup>a</sup> Number of animals examined microscopically at the site and the number of animals with lesion

**TABLE B5**  
**Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Cardiovascular System</b>				
Blood vessel			(1)	
Aorta, inflammation, chronic active			1 (100%)	
Heart	(50)	(49)	(50)	(50)
Cardiomyopathy	29 (58%)	24 (49%)	29 (58%)	23 (46%)
Atrium, thrombosis		1 (2%)		
<b>Endocrine System</b>				
Adrenal cortex	(49)	(49)	(50)	(49)
Hyperplasia	21 (43%)	21 (43%)	25 (50%)	23 (47%)
Hypertrophy	5 (10%)	3 (6%)	8 (16%)	9 (18%)
Necrosis	1 (2%)			
Vacuolization cytoplasmic	11 (22%)	5 (10%)	10 (20%)	12 (24%)
Adrenal medulla	(48)	(49)	(50)	(48)
Hyperplasia	8 (17%)	7 (14%)	11 (22%)	13 (27%)
Pituitary gland	(50)	(49)	(50)	(49)
Pars distalis, angiectasis			2 (4%)	
Pars distalis, cyst				1 (2%)
Pars distalis, hyperplasia	5 (10%)	5 (10%)	8 (16%)	5 (10%)
Thyroid gland	(49)	(49)	(50)	(48)
C-cell, hyperplasia	37 (76%)	42 (86%)	37 (74%)	38 (79%)
Follicular cell, hyperplasia	2 (4%)			1 (2%)
<b>General Body System</b>				
None				
<b>Genital System</b>				
Clitoral gland	(50)	(48)	(48)	(49)
Hyperplasia	2 (4%)	1 (2%)	1 (2%)	2 (4%)
Inflammation, chronic active	2 (4%)	6 (13%)	2 (4%)	6 (12%)
Ovary	(50)	(49)	(50)	(49)
Cyst	5 (10%)	1 (2%)	3 (6%)	
Uterus	(50)	(49)	(50)	(49)
Hydrometra				2 (4%)
Cervix, hypertrophy		1 (2%)		
<b>Hematopoietic System</b>				
Bone marrow	(49)	(49)	(50)	(50)
Atrophy	2 (4%)			1 (2%)
Hyperplasia, histiocytic			1 (2%)	1 (2%)
Lymph node	(2)	(3)	(3)	(2)
Renal, hemorrhage		1 (33%)		
Lymph node, mandibular	(44)	(40)	(47)	(45)
Infiltration cellular, plasma cell				1 (2%)
Lymph node, mesenteric	(49)	(49)	(50)	(48)
Hemorrhage		1 (2%)		1 (2%)
Infiltration cellular, eosinophil				1 (2%)
Lymph node, mediastinal	(43)	(38)	(43)	(45)
Hemorrhage	1 (2%)			1 (2%)
Infiltration cellular, plasma cell			1 (2%)	1 (2%)

**TABLE B5**  
**Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Hematopoietic System</b> (continued)				
Spleen	(49)	(48)	(50)	(49)
Accessory spleen	1 (2%)	2 (4%)		
Fibrosis		4 (8%)	2 (4%)	
Hematopoietic cell proliferation	3 (6%)	2 (4%)	4 (8%)	3 (6%)
Hemorrhage	2 (4%)		3 (6%)	1 (2%)
Hyperplasia, focal				1 (2%)
Necrosis				1 (2%)
Thrombosis				1 (2%)
<b>Integumentary System</b>				
Mammary gland	(50)	(49)	(50)	(49)
Galactocele		2 (4%)	1 (2%)	1 (2%)
Inflammation, chronic active				1 (2%)
Skin	(50)	(49)	(50)	(50)
Hyperkeratosis		1 (2%)		
Inflammation, chronic				1 (2%)
Inflammation, chronic active		4 (8%)	3 (6%)	3 (6%)
<b>Musculoskeletal System</b>				
Bone	(50)	(49)	(50)	(50)
Hyperostosis	4 (8%)	5 (10%)	5 (10%)	3 (6%)
<b>Nervous System</b>				
Brain	(50)	(49)	(50)	(50)
Gliosis				1 (2%)
Hemorrhage	2 (4%)			
<b>Respiratory System</b>				
Larynx	(50)	(49)	(50)	(50)
Epiglottis, metaplasia, squamous	1 (2%)	22 (45%)	39 (78%)	48 (96%)
Lung	(50)	(49)	(50)	(50)
Congestion, chronic		1 (2%)		
Cyst			1 (2%)	
Metaplasia, osseous				1 (2%)
Metaplasia, squamous		1 (2%)	8 (16%)	3 (6%)
Pigmentation, hemosiderin		1 (2%)		
Proteinosis		36 (73%)	49 (98%)	49 (98%)
Alveolar epithelium, hyperplasia	15 (30%)	7 (14%)	20 (40%)	33 (66%)
Alveolar epithelium, hyperplasia, atypical			3 (6%)	5 (10%)
Alveolar epithelium, metaplasia	2 (4%)	47 (96%)	50 (100%)	49 (98%)
Alveolus, inflammation, granulomatous	9 (18%)	47 (96%)	50 (100%)	49 (98%)
Interstitialium, fibrosis	7 (14%)	47 (96%)	50 (100%)	49 (98%)
Perivascular, inflammation, chronic active				1 (2%)

**TABLE B5**  
**Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Respiratory System</b> (continued)				
Nose	(50)	(49)	(50)	(50)
Inflammation, suppurative	6 (12%)	10 (20%)	2 (4%)	4 (8%)
Thrombosis	4 (8%)	4 (8%)	3 (6%)	2 (4%)
Lateral wall, hyperplasia	1 (2%)	8 (16%)	26 (52%)	38 (76%)
Lateral wall, metaplasia, squamous	1 (2%)	1 (2%)	4 (8%)	10 (20%)
Nasolacrimal duct, metaplasia, squamous		1 (2%)		
Olfactory epithelium, atrophy	5 (10%)	29 (59%)	46 (92%)	47 (94%)
Olfactory epithelium, metaplasia	2 (4%)	2 (4%)	3 (6%)	40 (80%)
Respiratory epithelium, hyperplasia, focal	1 (2%)			
Respiratory epithelium, metaplasia, squamous	2 (4%)			
Pleura		(1)		
<b>Special Senses System</b>				
Eye	(1)	(5)	(3)	(3)
Cataract	1 (100%)	4 (80%)	3 (100%)	2 (67%)
Cornea, edema		1 (20%)		
Retina, atrophy	1 (100%)	4 (80%)	3 (100%)	1 (33%)
<b>Urinary System</b>				
Kidney	(49)	(49)	(50)	(48)
Hyperplasia, stromal				1 (2%)
Infarct	1 (2%)		2 (4%)	2 (4%)
Nephropathy	47 (96%)	48 (98%)	45 (90%)	48 (100%)
Renal tubule, hyperplasia			1 (2%)	

## **APPENDIX C**

### **SUMMARY OF LESIONS IN MALE MICE IN THE 2-YEAR INHALATION STUDY OF COBALT SULFATE HEPTAHYDRATE**

<b>TABLE C1</b>	<b>Summary of the Incidence of Neoplasms in Male Mice in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate . . . . .</b>	<b>140</b>
<b>TABLE C2</b>	<b>Individual Animal Tumor Pathology of Male Mice in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate . . . . .</b>	<b>144</b>
<b>TABLE C3</b>	<b>Statistical Analysis of Primary Neoplasms in Male Mice in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate . . . . .</b>	<b>164</b>
<b>TABLE C4a</b>	<b>Historical Incidence of Alveolar/bronchiolar Neoplasms in Chamber Control Male B6C3F<sub>1</sub> Mice . . . . .</b>	<b>168</b>
<b>TABLE C4b</b>	<b>Historical Incidence of Hemangiosarcoma of the Liver in Chamber Control Male B6C3F<sub>1</sub> Mice . . . . .</b>	<b>168</b>
<b>TABLE C5</b>	<b>Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate . . . . .</b>	<b>169</b>

**TABLE C1**  
**Summary of the Incidence of Neoplasms in Male Mice in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate<sup>a</sup>**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Disposition Summary</b>				
Animals initially in study	50	50	50	50
Early deaths				
Accidental deaths	1			1
Moribund	19	16	17	23
Natural deaths	8	3	9	6
Survivors				
Terminal sacrifice	22	31	24	20
Animals examined microscopically	50	50	50	50
<b>Alimentary System</b>				
Intestine large, cecum	(45)	(49)	(45)	(46)
Intestine small, duodenum	(45)	(49)	(42)	(45)
Polyp adenomatous		1 (2%)		
Intestine small, jejunum	(45)	(48)	(42)	(45)
Carcinoma		1 (2%)		
Intestine small, ileum	(45)	(49)	(43)	(44)
Liver	(50)	(50)	(50)	(50)
Hemangiosarcoma	2 (4%)	4 (8%)	8 (16%)	7 (14%)
Hepatoblastoma	4 (8%)		2 (4%)	2 (4%)
Hepatocellular carcinoma	16 (32%)	32 (64%)	29 (58%)	26 (52%)
Hepatocellular carcinoma, multiple	7 (14%)	1 (2%)	1 (2%)	4 (8%)
Hepatocellular adenoma	14 (28%)	12 (24%)	16 (32%)	6 (12%)
Hepatocellular adenoma, multiple	8 (16%)	9 (18%)	9 (18%)	7 (14%)
Hepatocholangiocarcinoma	1 (2%)			
Histiocytic sarcoma	1 (2%)			1 (2%)
Sarcoma				1 (2%)
Mesentery	(3)	(4)	(5)	(4)
Hemangiosarcoma		1 (25%)		1 (25%)
Hepatocellular carcinoma, metastatic, liver			1 (20%)	
Hepatocholangiocarcinoma, metastatic, liver	1 (33%)			
Sarcoma, metastatic, liver				1 (25%)
Pancreas	(48)	(50)	(49)	(50)
Histiocytic sarcoma	1 (2%)			
Sarcoma, metastatic, liver				1 (2%)
Stomach, forestomach	(49)	(50)	(50)	(50)
Sarcoma, metastatic, liver				1 (2%)
Squamous cell carcinoma		2 (4%)		
Squamous cell papilloma			1 (2%)	
Stomach, glandular	(48)	(50)	(50)	(50)
Histiocytic sarcoma	1 (2%)			
Serosa, hepatocellular carcinoma, metastatic, liver		1 (2%)		
<b>Cardiovascular System</b>				
Heart	(50)	(50)	(50)	(50)
Alveolar/bronchiolar carcinoma, metastatic, lung				1 (2%)
Hemangiosarcoma		1 (2%)		
Sarcoma, metastatic, liver				1 (2%)

**TABLE C1**  
**Summary of the Incidence of Neoplasms in Male Mice in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Endocrine System</b>				
Adrenal cortex	(49)	(50)	(49)	(50)
Hepatocolangiocarcinoma, metastatic, liver	1 (2%)			
Adrenal medulla	(48)	(50)	(49)	(50)
Hepatocolangiocarcinoma, metastatic, liver	1 (2%)			
Pheochromocytoma benign	1 (2%)			1 (2%)
Islets, pancreatic	(48)	(50)	(49)	(50)
Adenoma		1 (2%)		
Thyroid gland	(49)	(50)	(50)	(50)
Follicular cell, adenoma				1 (2%)
Follicular cell, carcinoma			1 (2%)	
<b>General Body System</b>				
Peritoneum	(1)			
Hepatocolangiocarcinoma, metastatic, liver	1 (100%)			
Tissue NOS	(1)		(1)	
Hemangioma	1 (100%)			
<b>Genital System</b>				
Epididymis	(49)	(50)	(50)	(50)
Leiomyoma			1 (2%)	
Testes	(49)	(50)	(50)	(50)
Interstitial cell, adenoma		1 (2%)		
<b>Hematopoietic System</b>				
Bone marrow	(49)	(50)	(50)	(50)
Hemangiosarcoma		2 (4%)		1 (2%)
Lymph node	(1)	(1)	(2)	(2)
Iliac, sarcoma, metastatic, liver				1 (50%)
Renal, histiocytic sarcoma	1 (100%)			
Lymph node, bronchial	(21)	(24)	(28)	(25)
Alveolar/bronchiolar carcinoma, metastatic, lung				1 (4%)
Histiocytic sarcoma	1 (5%)			
Sarcoma, metastatic, liver				1 (4%)
Lymph node, mesenteric	(49)	(48)	(43)	(47)
Hemangiosarcoma		1 (2%)		
Hepatoblastoma, metastatic, liver	1 (2%)			
Histiocytic sarcoma	1 (2%)			
Sarcoma, metastatic, liver				1 (2%)
Lymph node, mediastinal	(43)	(28)	(31)	(35)
Alveolar/bronchiolar carcinoma, metastatic, lung				1 (3%)
Hepatocellular carcinoma, metastatic, liver	1 (2%)			
Hepatocolangiocarcinoma, metastatic, liver	1 (2%)			
Histiocytic sarcoma	1 (2%)			
Sarcoma, metastatic, liver				1 (3%)
Spleen	(47)	(50)	(49)	(50)
Hemangiosarcoma	1 (2%)	3 (6%)	1 (2%)	2 (4%)
Histiocytic sarcoma	1 (2%)			1 (2%)
Thymus	(28)	(31)	(33)	(28)

**TABLE C1**  
**Summary of the Incidence of Neoplasms in Male Mice in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Integumentary System</b>				
Skin	(49)	(50)	(49)	(49)
Hemangioma	1 (2%)			
Hemangiosarcoma	1 (2%)	1 (2%)		2 (4%)
Subcutaneous tissue, carcinoma				1 (2%)
Subcutaneous tissue, sarcoma	1 (2%)			
<b>Musculoskeletal System</b>				
Bone	(50)	(50)	(50)	(50)
Hemangiosarcoma				1 (2%)
Skeletal muscle	(1)			(2)
Alveolar/bronchiolar carcinoma, metastatic, lung				1 (50%)
Hepatocolangiocarcinoma, metastatic, liver	1 (100%)			
<b>Nervous System</b>				
None				
<b>Respiratory System</b>				
Larynx	(48)	(49)	(48)	(49)
Lung	(50)	(50)	(50)	(50)
Alveolar/bronchiolar adenoma	9 (18%)	9 (18%)	11 (22%)	13 (26%)
Alveolar/bronchiolar adenoma, multiple		3 (6%)	2 (4%)	5 (10%)
Alveolar/bronchiolar carcinoma	3 (6%)	5 (10%)	5 (10%)	9 (18%)
Alveolar/bronchiolar carcinoma, multiple	1 (2%)		2 (4%)	2 (4%)
Hemangiosarcoma, metastatic, liver				1 (2%)
Hepatoblastoma, metastatic, liver	1 (2%)			
Hepatocellular carcinoma, metastatic, liver	5 (10%)	5 (10%)	6 (12%)	7 (14%)
Histiocytic sarcoma	1 (2%)			
Nose	(50)	(50)	(48)	(49)
Trachea	(49)	(50)	(50)	(50)
<b>Special Senses System</b>				
Harderian gland	(4)	(4)	(4)	(6)
Adenoma	4 (100%)	2 (50%)	2 (50%)	1 (17%)
<b>Urinary System</b>				
Kidney	(49)	(50)	(50)	(50)
Alveolar/bronchiolar carcinoma, metastatic, lung				1 (2%)
Histiocytic sarcoma				1 (2%)
Renal tubule, adenoma		1 (2%)		1 (2%)
<b>Systemic Lesions</b>				
Multiple organs <sup>b</sup>	(50)	(50)	(50)	(50)
Histiocytic sarcoma	1 (2%)			1 (2%)
Lymphoma malignant	1 (2%)	1 (2%)	1 (2%)	

**TABLE C1**  
**Summary of the Incidence of Neoplasms in Male Mice in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Neoplasm Summary</b>				
Total animals with primary neoplasms <sup>c</sup>	49	48	47	46
Total primary neoplasms	77	94	92	95
Total animals with benign neoplasms	30	30	30	27
Total benign neoplasms	38	39	42	35
Total animals with malignant neoplasms	33	39	41	38
Total malignant neoplasms	39	55	50	60
Total animals with metastatic neoplasms	7	6	7	9
Total metastatic neoplasms	14	6	7	21

<sup>a</sup> Number of animals examined microscopically at the site and the number of animals with neoplasm

<sup>b</sup> Number of animals with any tissue examined microscopically

<sup>c</sup> Primary neoplasms: all neoplasms except metastatic neoplasms











**TABLE C2**  
**Individual Animal Tumor Pathology of Male Mice in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate:**  
**Chamber Control**

<b>Number of Days on Study</b>	7 7	
	0 1 2 3	
	8 1 5 3 3 3 3 3 3 3 3 3 3 3 3 4 4 4 4 4 4 4 4 4	
<b>Carcass ID Number</b>	0 0	Total
	2 3 4 0 1 1 1 2 2 2 2 3 3 4 4 0 0 0 1 3 3 3 3 4 4	Tissues/
	2 6 0 8 1 4 8 0 1 3 4 0 5 1 5 2 3 6 7 1 4 7 9 6 9	Tumors
<b>Special Senses System</b>		
Eye		1
Harderian gland		+
Adenoma		X
<b>Urinary System</b>		
Kidney	+ +	49
Urinary bladder	+ +	46
<b>Systemic Lesions</b>		
Multiple organs	+ +	50
Histiocytic sarcoma	X	1
Lymphoma malignant		1





























**TABLE C3**  
**Statistical Analysis of Primary Neoplasms in Male Mice in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Harderian Gland: Adenoma</b>				
Overall rate <sup>a</sup>	4/50 (8%)	2/50 (4%)	2/50 (4%)	1/50 (2%)
Adjusted rate <sup>b</sup>	13.2%	6.5%	8.3%	4.5%
Terminal rate <sup>c</sup>	1/22 (5%)	2/31 (6%)	2/24 (8%)	0/20 (0%)
First incidence (days)	600	733 (T)	733 (T)	686
Life table test <sup>d</sup>	P=0.270N	P=0.221N	P=0.306N	P=0.239N
Logistic regression test <sup>d</sup>	P=0.229N	P=0.307N	P=0.324N	P=0.192N
Cochran-Armitage test <sup>d</sup>	P=0.185N			
Fisher exact test <sup>d</sup>		P=0.339N	P=0.339N	P=0.181N
<b>Liver: Hemangiosarcoma</b>				
Overall rate	2/50 (4%)	4/50 (8%)	8/50 (16%)	7/50 (14%)
Adjusted rate	9.1%	11.5%	23.5%	25.0%
Terminal rate	2/22 (9%)	2/31 (6%)	2/24 (8%)	3/20 (15%)
First incidence (days)	733 (T)	685	523	502
Life table test	P=0.036	P=0.500	P=0.071	P=0.064
Logistic regression test	P=0.078	P=0.441	P=0.050	P=0.069
Cochran-Armitage test	P=0.096			
Fisher exact test		P=0.339	P=0.046	P=0.080
<b>Liver: Hepatocellular Adenoma</b>				
Overall rate	22/50 (44%)	21/50 (42%)	25/50 (50%)	13/50 (26%)
Adjusted rate	71.4%	57.5%	74.0%	42.3%
Terminal rate	14/22 (64%)	16/31 (52%)	16/24 (67%)	5/20 (25%)
First incidence (days)	470	614	440	533
Life table test	P=0.192N	P=0.102N	P=0.473	P=0.123N
Logistic regression test	P=0.061N	P=0.290N	P=0.389	P=0.067N
Cochran-Armitage test	P=0.026N			
Fisher exact test		P=0.500N	P=0.344	P=0.046N
<b>Liver: Hepatocellular Carcinoma</b>				
Overall rate	23/50 (46%)	33/50 (66%)	30/50 (60%)	30/50 (60%)
Adjusted rate	60.8%	69.5%	66.5%	71.2%
Terminal rate	9/22 (41%)	17/31 (55%)	10/24 (42%)	9/20 (45%)
First incidence (days)	482	460	440	502
Life table test	P=0.074	P=0.398	P=0.262	P=0.094
Logistic regression test	P=0.471	P=0.017	P=0.097	P=0.143
Cochran-Armitage test	P=0.303			
Fisher exact test		P=0.035	P=0.115	P=0.115
<b>Liver: Hepatocellular Adenoma or Carcinoma</b>				
Overall rate	38/50 (76%)	41/50 (82%)	43/50 (86%)	38/50 (76%)
Adjusted rate	89.8%	85.2%	93.2%	83.9%
Terminal rate	18/22 (82%)	24/31 (77%)	21/24 (88%)	13/20 (65%)
First incidence (days)	470	460	440	502
Life table test	P=0.134	P=0.161N	P=0.425	P=0.338
Logistic regression test	P=0.375N	P=0.284	P=0.155	P=0.591
Cochran-Armitage test	P=0.399N			
Fisher exact test		P=0.312	P=0.154	P=0.592N

**TABLE C3**  
**Statistical Analysis of Primary Neoplasms in Male Mice in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Liver: Hepatoblastoma</b>				
Overall rate	4/50 (8%)	0/50 (0%)	2/50 (4%)	2/50 (4%)
Adjusted rate	11.8%	0.0%	7.6%	10.0%
Terminal rate	1/22 (5%)	0/31 (0%)	1/24 (4%)	2/20 (10%)
First incidence (days)	533	— <sup>e</sup>	705	733 (T)
Life table test	P=0.596	P=0.043N	P=0.313N	P=0.390N
Logistic regression test	P=0.567N	P=0.095N	P=0.342N	P=0.345N
Cochran-Armitage test	P=0.549N			
Fisher exact test		P=0.059N	P=0.339N	P=0.339N
<b>Liver: Hepatocellular Carcinoma or Hepatoblastoma</b>				
Overall rate	27/50 (54%)	33/50 (66%)	31/50 (62%)	31/50 (62%)
Adjusted rate	66.6%	69.5%	67.7%	73.8%
Terminal rate	10/22 (45%)	17/31 (55%)	10/24 (42%)	10/20 (50%)
First incidence (days)	482	460	440	502
Life table test	P=0.106	P=0.458N	P=0.436	P=0.190
Logistic regression test	P=0.500N	P=0.062	P=0.221	P=0.328
Cochran-Armitage test	P=0.414			
Fisher exact test		P=0.154	P=0.272	P=0.272
<b>Liver: Hepatocellular Adenoma, Hepatocellular Carcinoma, or Hepatoblastoma</b>				
Overall rate	40/50 (80%)	41/50 (82%)	44/50 (88%)	39/50 (78%)
Adjusted rate	90.3%	85.2%	93.5%	86.2%
Terminal rate	18/22 (82%)	24/31 (77%)	21/24 (88%)	14/20 (70%)
First incidence (days)	470	460	440	502
Life table test	P=0.134	P=0.100N	P=0.488	P=0.383
Logistic regression test	P=0.340N	P=0.423	P=0.204	P=0.494N
Cochran-Armitage test	P=0.376N			
Fisher exact test		P=0.500	P=0.207	P=0.500N
<b>Lung: Alveolar/bronchiolar Adenoma</b>				
Overall rate	9/50 (18%)	12/50 (24%)	13/50 (26%)	18/50 (36%)
Adjusted rate	30.4%	30.9%	41.1%	54.6%
Terminal rate	4/22 (18%)	6/31 (19%)	7/24 (29%)	7/20 (35%)
First incidence (days)	600	460	548	524
Life table test	P=0.005	P=0.589	P=0.308	P=0.024
Logistic regression test	P=0.018	P=0.353	P=0.256	P=0.027
Cochran-Armitage test	P=0.029			
Fisher exact test		P=0.312	P=0.235	P=0.035
<b>Lung: Alveolar/bronchiolar Carcinoma</b>				
Overall rate	4/50 (8%)	5/50 (10%)	7/50 (14%)	11/50 (22%)
Adjusted rate	13.2%	16.1%	25.3%	43.7%
Terminal rate	2/22 (9%)	5/31 (16%)	4/24 (17%)	7/20 (35%)
First incidence (days)	449	733 (T)	687	552
Life table test	P=0.004	P=0.603N	P=0.313	P=0.031
Logistic regression test	P=0.006	P=0.528	P=0.273	P=0.033
Cochran-Armitage test	P=0.021			
Fisher exact test		P=0.500	P=0.262	P=0.045

**TABLE C3**  
**Statistical Analysis of Primary Neoplasms in Male Mice in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Lung: Alveolar/bronchiolar Adenoma or Carcinoma</b>				
Overall rate	11/50 (22%)	14/50 (28%)	19/50 (38%)	28/50 (56%)
Adjusted rate	35.5%	36.5%	56.5%	78.8%
Terminal rate	5/22 (23%)	8/31 (26%)	10/24 (42%)	13/20 (65%)
First incidence (days)	449	460	548	524
Life table test	P < 0.001	P=0.544N	P=0.122	P < 0.001
Logistic regression test	P < 0.001	P=0.345	P=0.071	P < 0.001
Cochran-Armitage test	P < 0.001			
Fisher exact test		P=0.322	P=0.063	P < 0.001
<b>Spleen: Hemangiosarcoma</b>				
Overall rate	1/47 (2%)	3/50 (6%)	1/49 (2%)	2/50 (4%)
Adjusted rate	3.0%	8.5%	3.1%	10.0%
Terminal rate	0/22 (0%)	1/31 (3%)	0/24 (0%)	2/20 (10%)
First incidence (days)	651	685	681	733 (T)
Life table test	P=0.454	P=0.419	P=0.739N	P=0.450
Logistic regression test	P=0.524	P=0.337	P=0.754N	P=0.465
Cochran-Armitage test	P=0.596			
Fisher exact test		P=0.332	P=0.742N	P=0.523
<b>All Organs: Hemangiosarcoma</b>				
Overall rate	3/50 (6%)	6/50 (12%)	8/50 (16%)	9/50 (18%)
Adjusted rate	11.8%	17.6%	23.5%	31.1%
Terminal rate	2/22 (9%)	4/31 (13%)	2/24 (8%)	4/20 (20%)
First incidence (days)	651	685	523	502
Life table test	P=0.025	P=0.423	P=0.139	P=0.047
Logistic regression test	P=0.061	P=0.344	P=0.103	P=0.054
Cochran-Armitage test	P=0.079			
Fisher exact test		P=0.243	P=0.100	P=0.061
<b>All Organs: Hemangioma or Hemangiosarcoma</b>				
Overall rate	5/50 (10%)	6/50 (12%)	8/50 (16%)	9/50 (18%)
Adjusted rate	20.7%	17.6%	23.5%	31.1%
Terminal rate	4/22 (18%)	4/31 (13%)	2/24 (8%)	4/20 (20%)
First incidence (days)	651	685	523	502
Life table test	P=0.060	P=0.528N	P=0.338	P=0.149
Logistic regression test	P=0.120	P=0.605N	P=0.289	P=0.162
Cochran-Armitage test	P=0.160			
Fisher exact test		P=0.500	P=0.277	P=0.194
<b>All Organs: Benign Neoplasms</b>				
Overall rate	30/50 (60%)	30/50 (60%)	30/50 (60%)	27/50 (54%)
Adjusted rate	84.8%	74.3%	77.7%	73.0%
Terminal rate	17/22 (77%)	21/31 (68%)	16/24 (67%)	11/20 (55%)
First incidence (days)	470	460	440	524
Life table test	P=0.276	P=0.086N	P=0.430N	P=0.538
Logistic regression test	P=0.437N	P=0.353N	P=0.542N	P=0.444N
Cochran-Armitage test	P=0.279N			
Fisher exact test		P=0.581N	P=0.581N	P=0.343N

**TABLE C3**  
**Statistical Analysis of Primary Neoplasms in Male Mice in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>All Organs: Malignant Neoplasms</b>				
Overall rate	33/50 (66%)	39/50 (78%)	41/50 (82%)	38/50 (76%)
Adjusted rate	73.8%	79.4%	85.1%	87.8%
Terminal rate	11/22 (50%)	21/31 (68%)	17/24 (71%)	15/20 (75%)
First incidence (days)	449	460	440	502
Life table test	P=0.053	P=0.374N	P=0.270	P=0.141
Logistic regression test	P=0.412	P=0.042	P=0.037	P=0.205
Cochran-Armitage test	P=0.318			
Fisher exact test		P=0.133	P=0.055	P=0.189
<b>All Organs: Benign or Malignant Neoplasms</b>				
Overall rate	49/50 (98%)	48/50 (96%)	47/50 (94%)	46/50 (92%)
Adjusted rate	100.0%	96.0%	95.9%	95.8%
Terminal rate	22/22 (100%)	29/31 (94%)	22/24 (92%)	18/20 (90%)
First incidence (days)	449	460	440	502
Life table test	P=0.114	P=0.028N	P=0.283N	P=0.435
Logistic regression test	P=0.238N	P=0.527N	P=0.297N	P=0.279N
Cochran-Armitage test	P=0.143N			
Fisher exact test		P=0.500N	P=0.309N	P=0.181N

(T)Terminal sacrifice

<sup>a</sup> Number of neoplasm-bearing animals/number of animals examined. Denominator is number of animals examined microscopically for liver, lung, and spleen; for other tissues, denominator is number of animals necropsied.

<sup>b</sup> Kaplan-Meier estimated neoplasm incidence at the end of the study after adjustment for intercurrent mortality

<sup>c</sup> Observed incidence at terminal kill

<sup>d</sup> Beneath the chamber control incidence are the P values associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the chamber controls and that exposed group. The life table test regards neoplasms in animals dying prior to terminal kill as being (directly or indirectly) the cause of death. The logistic regression test regards these lesions as nonfatal. The Cochran-Armitage and Fisher exact tests compare directly the overall incidence rates. For all tests, a negative trend or a lower incidence in an exposure group is indicated by N.

<sup>e</sup> Not applicable; no neoplasms in animal group

**TABLE C4a**  
**Historical Incidence of Alveolar/bronchiolar Neoplasms in Chamber Control Male B6C3F<sub>1</sub> Mice<sup>a</sup>**

Study	Incidence in Controls		
	Adenoma	Carcinoma	Adenoma or Carcinoma
<b>Historical Incidence at Battelle Pacific Northwest Laboratories</b>			
1,3-Butadiene	18/50	5/50	21/50
Acetonitrile	6/50	4/50	10/50
Allyl Glycidyl Ether	7/50	0/50	7/50
2-Chloroacetophenone	7/50	6/50	11/50
<i>l</i> -Epinephrine Hydrochloride	11/50	5/50	15/50
Chloroethane	3/50	2/50	5/50
Hexachlorocyclopentadiene	11/49	0/49	11/49
<i>o</i> -Chlorobenzalmalononitrile (CS2)	7/49	7/49	14/49
Ozone	6/50	8/50	14/50
<b>Overall Historical Incidence</b>			
Total	141/947 (14.9%)	75/947 (7.9%)	205/947 (21.7%)
Standard deviation	7.0%	5.7%	8.0%
Range	6%-36%	0%-16%	10%-42%

<sup>a</sup> Data as of 12 May 1995

**TABLE C4b**  
**Historical Incidence of Hemangiosarcoma of the Liver in Chamber Control Male B6C3F<sub>1</sub> Mice<sup>a</sup>**

Study	Incidence in Controls	
	Adenoma	Carcinoma
<b>Historical Incidence at Battelle Pacific Northwest Laboratories</b>		
1,3-Butadiene		0/50
Acetonitrile		1/50
Allyl Glycidyl Ether		0/49
2-Chloroacetophenone		0/50
<i>l</i> -Epinephrine Hydrochloride		1/50
Chloroethane		0/50
Hexachlorocyclopentadiene		0/50
<i>o</i> -Chlorobenzalmalononitrile (CS2)		0/49
Ozone		0/50
<b>Overall Historical Incidence</b>		
Total		12/947 (1.3%)
Standard deviation		1.7%
Range		0%-6%

<sup>a</sup> Data as of 12 May 1995

**TABLE C5**  
**Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate<sup>a</sup>**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Disposition Summary</b>				
Animals initially in study	50	50	50	50
Early deaths				
Accidental deaths	1			1
Moribund	19	16	17	23
Natural deaths	8	3	9	6
Survivors				
Terminal sacrifice	22	31	24	20
Animals examined microscopically	50	50	50	50
<b>Alimentary System</b>				
Gallbladder	(37)	(41)	(38)	(38)
Degeneration, hyaline	1 (3%)	1 (2%)	2 (5%)	
Infiltration cellular, lymphocyte			1 (3%)	
Intestine small, duodenum	(45)	(49)	(42)	(45)
Hyperplasia, lymphoid			1 (2%)	
Inflammation, chronic			1 (2%)	
Necrosis, focal			1 (2%)	
Liver	(50)	(50)	(50)	(50)
Basophilic focus	1 (2%)			
Clear cell focus		3 (6%)	2 (4%)	
Clear cell focus, multiple			1 (2%)	
Cyst				1 (2%)
Eosinophilic focus	8 (16%)	7 (14%)	9 (18%)	2 (4%)
Eosinophilic focus, multiple	1 (2%)	1 (2%)	3 (6%)	
Infiltration cellular, lymphocyte	1 (2%)			
Inflammation, chronic	33 (66%)	36 (72%)	40 (80%)	39 (78%)
Karyomegaly	39 (78%)	35 (70%)	39 (78%)	43 (86%)
Mineralization	1 (2%)			
Mitotic alteration				1 (2%)
Necrosis, focal	3 (6%)	3 (6%)	6 (12%)	1 (2%)
Regeneration	32 (64%)	30 (60%)	35 (70%)	38 (76%)
Vacuolization cytoplasmic, diffuse		3 (6%)	1 (2%)	
Vacuolization cytoplasmic, focal			1 (2%)	
Bile duct, hyperplasia		3 (6%)	6 (12%)	4 (8%)
Oval cell, hyperplasia	38 (76%)	36 (72%)	40 (80%)	44 (88%)
Mesentery	(3)	(4)	(5)	(4)
Inflammation, chronic	1 (33%)			1 (25%)
Mineralization			1 (20%)	
Artery, fibrosis	1 (33%)			1 (25%)
Artery, inflammation, chronic		1 (25%)		
Fat, necrosis	1 (33%)	2 (50%)	3 (60%)	
Pancreas	(48)	(50)	(49)	(50)
Acinus, atrophy		1 (2%)		
Stomach, forestomach	(49)	(50)	(50)	(50)
Cyst			1 (2%)	
Hyperplasia, squamous	4 (8%)	2 (4%)		2 (4%)
Inflammation				1 (2%)

<sup>a</sup> Number of animals examined microscopically at the site and the number of animals with lesion

**TABLE C5**  
**Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Alimentary System</b> (continued)				
Stomach, glandular	(48)	(50)	(50)	(50)
Mineralization				1 (2%)
Tooth				(1)
Inflammation				1 (100%)
<b>Cardiovascular System</b>				
Heart	(50)	(50)	(50)	(50)
Cardiomyopathy	1 (2%)	4 (8%)	2 (4%)	1 (2%)
Inflammation, chronic			1 (2%)	
Artery, inflammation, chronic			1 (2%)	
Atrium, thrombosis	1 (2%)	2 (4%)	3 (6%)	2 (4%)
<b>Endocrine System</b>				
Adrenal cortex	(49)	(50)	(49)	(50)
Accessory adrenal cortical nodule			1 (2%)	
Hyperplasia	3 (6%)	4 (8%)	5 (10%)	2 (4%)
Inflammation		1 (2%)		
Necrosis		1 (2%)		
Necrosis, diffuse			1 (2%)	
Adrenal medulla	(48)	(50)	(49)	(50)
Hyperplasia	1 (2%)			5 (10%)
Necrosis, diffuse			1 (2%)	
Thyroid gland	(49)	(50)	(50)	(50)
Inflammation				1 (2%)
Follicular cell, hyperplasia	3 (6%)	17 (34%)	11 (22%)	10 (20%)
<b>General Body System</b>				
Tissue NOS	(1)		(1)	
Inflammation, chronic			1 (100%)	
<b>Genital System</b>				
Epididymis	(49)	(50)	(50)	(50)
Degeneration				1 (2%)
Granuloma sperm	1 (2%)			
Inflammation, chronic			1 (2%)	2 (4%)
Mineralization		2 (4%)	1 (2%)	
Penis	(1)			
Inflammation, chronic	1 (100%)			
Preputial gland	(49)	(50)	(50)	(49)
Cyst	1 (2%)	2 (4%)	3 (6%)	1 (2%)
Inflammation, chronic	13 (27%)	12 (24%)	9 (18%)	5 (10%)
Inflammation, suppurative		2 (4%)	2 (4%)	
Prostate	(46)	(50)	(44)	(49)
Inflammation	2 (4%)		2 (5%)	1 (2%)
Seminal vesicle	(48)	(50)	(49)	(49)
Inflammation, chronic			1 (2%)	
Testes	(49)	(50)	(50)	(50)
Atrophy		2 (4%)		2 (4%)
Degeneration, focal			1 (2%)	

**TABLE C5**  
**Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Hematopoietic System</b>				
Lymph node	(1)	(1)	(2)	(2)
Iliac, hyperplasia, lymphoid		1 (100%)		1 (50%)
Iliac, infiltration cellular, histiocyte			1 (50%)	
Renal, hyperplasia, lymphoid		1 (100%)		
Lymph node, bronchial	(21)	(24)	(28)	(25)
Infiltration cellular, plasma cell		1 (4%)		
Infiltration cellular, histiocyte	1 (5%)			1 (4%)
Lymph node, mesenteric	(49)	(48)	(43)	(47)
Hematopoietic cell proliferation			1 (2%)	
Hemorrhage	2 (4%)	2 (4%)	4 (9%)	3 (6%)
Hyperplasia, lymphoid		1 (2%)		2 (4%)
Inflammation, chronic	1 (2%)		2 (5%)	2 (4%)
Lymph node, mediastinal	(43)	(28)	(31)	(35)
Hyperplasia, lymphoid		1 (4%)	4 (13%)	
Infiltration cellular, plasma cell	1 (2%)	1 (4%)		
Infiltration cellular, histiocyte	2 (5%)		1 (3%)	
Inflammation, chronic			1 (3%)	1 (3%)
Spleen	(47)	(50)	(49)	(50)
Angiectasis	1 (2%)			1 (2%)
Hematopoietic cell proliferation	10 (21%)	6 (12%)	6 (12%)	14 (28%)
Hyperplasia, lymphoid		4 (8%)		1 (2%)
<b>Integumentary System</b>				
Skin	(49)	(50)	(49)	(49)
Inflammation, chronic		1 (2%)		2 (4%)
Inflammation, suppurative		1 (2%)		
Ulcer		1 (2%)	2 (4%)	
Epidermis, hyperplasia			1 (2%)	1 (2%)
Prepuce, inflammation, chronic		1 (2%)		
Prepuce, ulcer	4 (8%)	1 (2%)	1 (2%)	
<b>Musculoskeletal System</b>				
Bone	(50)	(50)	(50)	(50)
Fracture				1 (2%)
Skeletal muscle	(1)			(2)
Inflammation, chronic, focal				1 (50%)
<b>Nervous System</b>				
None				
<b>Respiratory System</b>				
Larynx	(48)	(49)	(48)	(49)
Foreign body	1 (2%)			
Metaplasia, squamous		37 (76%)	48 (100%)	44 (90%)

**TABLE C5**  
**Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Respiratory System</b> (continued)				
Lung	(50)	(50)	(50)	(50)
Hemorrhage	1 (2%)		1 (2%)	1 (2%)
Infiltration cellular, diffuse, histiocyte	1 (2%)	2 (4%)	4 (8%)	10 (20%)
Infiltration cellular, focal, histiocyte	10 (20%)	5 (10%)	8 (16%)	17 (34%)
Inflammation, chronic		1 (2%)	1 (2%)	3 (6%)
Alveolar epithelium, hyperplasia		4 (8%)	4 (8%)	4 (8%)
Alveolar epithelium, goblet cell, metaplasia, focal		1 (2%)		
Artery, thrombosis			1 (2%)	
Bronchus, hyperplasia			1 (2%)	
Bronchus, vacuolization cytoplasmic		18 (36%)	34 (68%)	38 (76%)
Nose	(50)	(50)	(48)	(49)
Hemorrhage				1 (2%)
Inflammation, suppurative		1 (2%)		6 (12%)
Olfactory epithelium, atrophy			29 (60%)	48 (98%)
Olfactory epithelium, degeneration, hyaline			2 (4%)	2 (4%)
Olfactory epithelium, hyperplasia				10 (20%)
Olfactory epithelium, metaplasia				2 (4%)
Respiratory epithelium, degeneration, hyaline				1 (2%)
Trachea	(49)	(50)	(50)	(50)
Inflammation, chronic	1 (2%)			
<b>Special Senses System</b>				
Eye	(1)		(1)	(1)
Degeneration	1 (100%)			1 (100%)
Hemorrhage				1 (100%)
Inflammation				1 (100%)
Cornea, inflammation			1 (100%)	
Harderian gland	(4)	(4)	(4)	(6)
Inflammation, acute		1 (25%)		
<b>Urinary System</b>				
Kidney	(49)	(50)	(50)	(50)
Cyst		1 (2%)		1 (2%)
Infiltration cellular, mixed cell	5 (10%)	1 (2%)	5 (10%)	3 (6%)
Inflammation, chronic	1 (2%)		1 (2%)	
Metaplasia, osseous			1 (2%)	
Mineralization				1 (2%)
Nephropathy	11 (22%)	13 (26%)	7 (14%)	9 (18%)
Glomerulus, amyloid deposition		1 (2%)		2 (4%)
Medulla, inflammation, chronic				1 (2%)
Renal tubule, hyperplasia				1 (2%)
Urinary bladder	(46)	(49)	(45)	(48)
Inflammation	3 (7%)		3 (7%)	3 (6%)

**APPENDIX D**  
**SUMMARY OF LESIONS IN FEMALE MICE**  
**IN THE 2-YEAR INHALATION STUDY**  
**OF COBALT SULFATE HEPTAHYDRATE**

<b>TABLE D1</b>	<b>Summary of the Incidence of Neoplasms in Female Mice in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate . . . . .</b>	<b>174</b>
<b>TABLE D2</b>	<b>Individual Animal Tumor Pathology of Female Mice in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate . . . . .</b>	<b>178</b>
<b>TABLE D3</b>	<b>Statistical Analysis of Primary Neoplasms in Female Mice in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate . . . . .</b>	<b>198</b>
<b>TABLE D4a</b>	<b>Historical Incidence of Alveolar/bronchiolar Neoplasms in Chamber Control Female B6C3F<sub>1</sub> Mice . . . . .</b>	<b>203</b>
<b>TABLE D4b</b>	<b>Historical Incidence of Hemangiosarcoma of the Liver in Chamber Control Female B6C3F<sub>1</sub> Mice . . . . .</b>	<b>203</b>
<b>TABLE D5</b>	<b>Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate . . . . .</b>	<b>204</b>

**TABLE D1**  
**Summary of the Incidence of Neoplasms in Female Mice in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate<sup>a</sup>**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Disposition Summary</b>				
Animals initially in study	50	50	50	50
Early deaths				
Moribund	11	10	13	16
Natural deaths	5	3	5	6
Survivors				
Terminal sacrifice	34	37	32	28
Animals examined microscopically	50	50	50	50
<b>Alimentary System</b>				
Gallbladder	(43)	(43)	(38)	(43)
Intestine large, cecum	(49)	(49)	(48)	(47)
Leiomyoma				1 (2%)
Intestine small, duodenum	(47)	(48)	(47)	(45)
Intestine small, jejunum	(48)	(49)	(47)	(44)
Hemangiosarcoma			1 (2%)	
Intestine small, ileum	(48)	(48)	(47)	(45)
Liver	(50)	(50)	(50)	(49)
Hemangiosarcoma	1 (2%)		3 (6%)	
Hepatocellular carcinoma	12 (24%)	9 (18%)	16 (32%)	4 (8%)
Hepatocellular adenoma	7 (14%)	7 (14%)	11 (22%)	9 (18%)
Hepatocellular adenoma, multiple	1 (2%)	3 (6%)	2 (4%)	3 (6%)
Hepatocholangiocarcinoma	2 (4%)			
Histiocytic sarcoma	3 (6%)	2 (4%)		2 (4%)
Mesentery	(10)	(12)	(8)	(7)
Hemangioma				1 (14%)
Hemangiosarcoma		1 (8%)	1 (13%)	
Hepatocholangiocarcinoma, metastatic, liver	1 (10%)			
Histiocytic sarcoma		1 (8%)		
Sarcoma	1 (10%)			
Pancreas	(50)	(50)	(49)	(49)
Histiocytic sarcoma	1 (2%)			
Salivary glands	(50)	(50)	(50)	(50)
Stomach, forestomach	(50)	(50)	(49)	(50)
Squamous cell papilloma	1 (2%)	1 (2%)		1 (2%)
Stomach, glandular	(50)	(50)	(49)	(50)
Muscularis, serosa, sarcoma	1 (2%)			
<b>Cardiovascular System</b>				
Heart	(50)	(50)	(50)	(50)
Hepatocholangiocarcinoma, metastatic, liver	1 (2%)			

**TABLE D1**  
**Summary of the Incidence of Neoplasms in Female Mice in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Endocrine System</b>				
Adrenal cortex	(50)	(50)	(49)	(50)
Histiocytic sarcoma		1 (2%)		
Capsule, adenoma		1 (2%)		
Adrenal medulla	(49)	(50)	(49)	(49)
Pheochromocytoma benign		1 (2%)		
Islets, pancreatic	(50)	(50)	(49)	(49)
Adenoma	1 (2%)			1 (2%)
Pituitary gland	(48)	(47)	(47)	(48)
Pars distalis, adenoma	11 (23%)	8 (17%)	7 (15%)	8 (17%)
Thyroid gland	(50)	(49)	(49)	(49)
Follicular cell, adenoma	3 (6%)			5 (10%)
Follicular cell, carcinoma	1 (2%)		2 (4%)	
<b>General Body System</b>				
None				
<b>Genital System</b>				
Ovary	(48)	(49)	(49)	(48)
Arrhenoblastoma benign	1 (2%)			
Cystadenocarcinoma		1 (2%)		
Cystadenoma	2 (4%)	3 (6%)	3 (6%)	3 (6%)
Granulosa cell tumor benign	1 (2%)		1 (2%)	
Histiocytic sarcoma	1 (2%)	1 (2%)		1 (2%)
Luteoma	1 (2%)		1 (2%)	
Teratoma benign	1 (2%)	1 (2%)	1 (2%)	
Yolk sac carcinoma			1 (2%)	
Uterus	(50)	(50)	(49)	(49)
Hemangioma		1 (2%)	2 (4%)	
Hemangiosarcoma		1 (2%)		
Histiocytic sarcoma	1 (2%)			1 (2%)
Leiomyoma	1 (2%)			
Leiomyosarcoma				1 (2%)
Polyp stromal		2 (4%)		
<b>Hematopoietic System</b>				
Bone marrow	(50)	(50)	(49)	(50)
Histiocytic sarcoma	1 (2%)			2 (4%)
Lymph node	(3)	(6)	(3)	(4)
Histiocytic sarcoma	1 (33%)			
Lumbar, histiocytic sarcoma	1 (33%)			
Renal, histiocytic sarcoma	2 (67%)			
Lymph node, bronchial	(30)	(34)	(27)	(35)
Hepatocholangiocarcinoma, metastatic, liver	2 (7%)			
Histiocytic sarcoma	1 (3%)	1 (3%)		1 (3%)
Lymph node, mandibular	(37)	(37)	(36)	(36)
Histiocytic sarcoma	2 (5%)	2 (5%)		1 (3%)
Lymph node, mesenteric	(46)	(45)	(46)	(44)
Histiocytic sarcoma	3 (7%)	2 (4%)		1 (2%)

**TABLE D1**  
**Summary of the Incidence of Neoplasms in Female Mice in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Hematopoietic System</b> (continued)				
Lymph node, mediastinal	(41)	(36)	(28)	(34)
Alveolar/bronchiolar carcinoma, metastatic, lung				1 (3%)
Hepatocolangiocarcinoma, metastatic, liver	2 (5%)			
Histiocytic sarcoma	3 (7%)	2 (6%)		1 (3%)
Spleen	(50)	(50)	(49)	(49)
Histiocytic sarcoma	3 (6%)	2 (4%)		1 (2%)
Thymus	(41)	(44)	(41)	(41)
Histiocytic sarcoma	1 (2%)	1 (2%)		
<b>Integumentary System</b>				
Mammary gland	(47)	(50)	(50)	(50)
Adenoma				1 (2%)
Carcinoma	1 (2%)	1 (2%)	1 (2%)	1 (2%)
Skin	(49)	(50)	(50)	(50)
Hemangiosarcoma			1 (2%)	
Histiocytic sarcoma				1 (2%)
Squamous cell carcinoma	1 (2%)			
Subcutaneous tissue, mast cell tumor benign			1 (2%)	
Subcutaneous tissue, sarcoma	6 (12%)	2 (4%)		
<b>Musculoskeletal System</b>				
Skeletal muscle		(1)	(2)	(1)
Sarcoma			1 (50%)	1 (100%)
<b>Nervous System</b>				
Brain	(50)	(50)	(50)	(50)
<b>Respiratory System</b>				
Larynx	(50)	(49)	(47)	(50)
Lung	(50)	(50)	(50)	(50)
Alveolar/bronchiolar adenoma	3 (6%)	5 (10%)	8 (16%)	8 (16%)
Alveolar/bronchiolar adenoma, multiple		1 (2%)	1 (2%)	2 (4%)
Alveolar/bronchiolar carcinoma	1 (2%)	1 (2%)	4 (8%)	9 (18%)
Hepatocellular carcinoma, metastatic, liver	3 (6%)		5 (10%)	2 (4%)
Hepatocolangiocarcinoma, metastatic, liver	2 (4%)			
Histiocytic sarcoma	3 (6%)	2 (4%)		1 (2%)
Sarcoma, metastatic, skin	1 (2%)			
Nose	(50)	(50)	(49)	(48)
<b>Special Senses System</b>				
Harderian gland	(2)	(2)		(1)
Adenoma	2 (100%)	2 (100%)		1 (100%)

**TABLE D1**  
**Summary of the Incidence of Neoplasms in Female Mice in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Urinary System</b>				
Kidney	(50)	(50)	(50)	(50)
Histiocytic sarcoma	2 (4%)	1 (2%)		1 (2%)
Urinary bladder	(48)	(47)	(45)	(46)
Histiocytic sarcoma	1 (2%)			
<b>Systemic Lesions</b>				
Multiple organs <sup>b</sup>	(50)	(50)	(50)	(50)
Histiocytic sarcoma	3 (6%)	2 (4%)		3 (6%)
Lymphoma malignant	4 (8%)	7 (14%)	7 (14%)	5 (10%)
<b>Neoplasm Summary</b>				
Total animals with primary neoplasms <sup>c</sup>	42	41	43	39
Total primary neoplasms	70	61	76	68
Total animals with benign neoplasms	25	32	27	27
Total benign neoplasms	36	36	38	44
Total animals with malignant neoplasms	27	22	31	21
Total malignant neoplasms	34	25	38	24
Total animals with metastatic neoplasms	6		5	3
Total metastatic neoplasms	12		5	3

<sup>a</sup> Number of animals examined microscopically at the site and the number of animals with neoplasm

<sup>b</sup> Number of animals with any tissue examined microscopically

<sup>c</sup> Primary neoplasms: all neoplasms except metastatic neoplasms









































**TABLE D3**  
**Statistical Analysis of Primary Neoplasms in Female Mice in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Liver: Hemangiosarcoma</b>				
Overall rate <sup>a</sup>	1/50 (2%)	0/50 (0%)	3/50 (6%)	0/49 (0%)
Adjusted rate <sup>b</sup>	2.9%	0.0%	7.3%	0.0%
Terminal rate <sup>c</sup>	1/34 (3%)	0/37 (0%)	1/32 (3%)	0/28 (0%)
First incidence (days)	734 (T)	— <sup>e</sup>	524	—
Life table test <sup>d</sup>	P=0.499N	P=0.483N	P=0.298	P=0.539N
Logistic regression test <sup>d</sup>	P=0.431N	P=0.483N	P=0.318	P=0.539N
Cochran-Armitage test <sup>d</sup>	P=0.455N			
Fisher exact test <sup>d</sup>		P=0.500N	P=0.309	P=0.505N
<b>Liver: Hepatocellular Adenoma</b>				
Overall rate	8/50 (16%)	10/50 (20%)	13/50 (26%)	12/49 (24%)
Adjusted rate	22.9%	25.0%	34.0%	35.3%
Terminal rate	7/34 (21%)	8/37 (22%)	8/32 (25%)	8/28 (29%)
First incidence (days)	713	593	622	539
Life table test	P=0.103	P=0.480	P=0.150	P=0.128
Logistic regression test	P=0.174	P=0.466	P=0.152	P=0.180
Cochran-Armitage test	P=0.221			
Fisher exact test		P=0.398	P=0.163	P=0.212
<b>Liver: Hepatocellular Carcinoma</b>				
Overall rate	12/50 (24%)	9/50 (18%)	16/50 (32%)	4/49 (8%)
Adjusted rate	31.7%	22.7%	40.5%	11.8%
Terminal rate	9/34 (26%)	7/37 (19%)	9/32 (28%)	1/28 (4%)
First incidence (days)	609	666	640	667
Life table test	P=0.094N	P=0.240N	P=0.235	P=0.063N
Logistic regression test	P=0.051N	P=0.258N	P=0.238	P=0.037N
Cochran-Armitage test	P=0.036N			
Fisher exact test		P=0.312N	P=0.252	P=0.030N
<b>Liver: Hepatocellular Adenoma or Carcinoma</b>				
Overall rate	18/50 (36%)	18/50 (36%)	24/50 (48%)	16/49 (33%)
Adjusted rate	48.1%	43.3%	56.7%	43.8%
Terminal rate	15/34 (44%)	14/37 (38%)	14/32 (44%)	9/28 (32%)
First incidence (days)	609	593	622	539
Life table test	P=0.444	P=0.451N	P=0.145	P=0.530
Logistic regression test	P=0.474N	P=0.488N	P=0.137	P=0.506N
Cochran-Armitage test	P=0.374N			
Fisher exact test		P=0.582N	P=0.156	P=0.445N
<b>Lung: Alveolar/bronchiolar Adenoma</b>				
Overall rate	3/50 (6%)	6/50 (12%)	9/50 (18%)	10/50 (20%)
Adjusted rate	8.8%	15.0%	25.2%	32.8%
Terminal rate	3/34 (9%)	4/37 (11%)	6/32 (19%)	8/28 (29%)
First incidence (days)	734 (T)	664	649	706
Life table test	P=0.014	P=0.297	P=0.056	P=0.016
Logistic regression test	P=0.024	P=0.287	P=0.057	P=0.024
Cochran-Armitage test	P=0.045			
Fisher exact test		P=0.243	P=0.061	P=0.036

**TABLE D3**  
**Statistical Analysis of Primary Neoplasms in Female Mice in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Lung: Alveolar/bronchiolar Carcinoma</b>				
Overall rate	1/50 (2%)	1/50 (2%)	4/50 (8%)	9/50 (18%)
Adjusted rate	2.9%	2.7%	9.2%	25.3%
Terminal rate	1/34 (3%)	1/37 (3%)	1/32 (3%)	4/28 (14%)
First incidence (days)	734 (T)	734 (T)	495	536
Life table test	P< 0.001	P=0.743N	P=0.173	P=0.007
Logistic regression test	P< 0.001	P=0.743N	P=0.201	P=0.009
Cochran-Armitage test	P< 0.001			
Fisher exact test		P=0.753N	P=0.181	P=0.008
<b>Lung: Alveolar/bronchiolar Adenoma or Carcinoma</b>				
Overall rate	4/50 (8%)	7/50 (14%)	13/50 (26%)	18/50 (36%)
Adjusted rate	11.8%	17.5%	32.6%	50.2%
Terminal rate	4/34 (12%)	5/37 (14%)	7/32 (22%)	11/28 (39%)
First incidence (days)	734 (T)	664	495	536
Life table test	P< 0.001	P=0.322	P=0.016	P< 0.001
Logistic regression test	P< 0.001	P=0.318	P=0.016	P< 0.001
Cochran-Armitage test	P< 0.001			
Fisher exact test		P=0.262	P=0.016	P< 0.001
<b>Ovary: Cystadenoma</b>				
Overall rate	2/48 (4%)	3/49 (6%)	3/49 (6%)	3/48 (6%)
Adjusted rate	6.3%	8.3%	9.4%	10.7%
Terminal rate	2/32 (6%)	3/36 (8%)	3/32 (9%)	3/28 (11%)
First incidence (days)	734 (T)	734 (T)	734 (T)	734 (T)
Life table test	P=0.390	P=0.554	P=0.500	P=0.439
Logistic regression test	P=0.390	P=0.554	P=0.500	P=0.439
Cochran-Armitage test	P=0.487			
Fisher exact test		P=0.510	P=0.510	P=0.500
<b>Ovary: Cystadenoma or Cystadenocarcinoma</b>				
Overall rate	2/48 (4%)	4/49 (8%)	3/49 (6%)	3/48 (6%)
Adjusted rate	6.3%	11.1%	9.4%	10.7%
Terminal rate	2/32 (6%)	4/36 (11%)	3/32 (9%)	3/28 (11%)
First incidence (days)	734 (T)	734 (T)	734 (T)	734 (T)
Life table test	P=0.390	P=0.395	P=0.500	P=0.439
Logistic regression test	P=0.390	P=0.395	P=0.500	P=0.439
Cochran-Armitage test	P=0.487			
Fisher exact test		P=0.349	P=0.510	P=0.500
<b>Pituitary Gland (Pars Distalis): Adenoma</b>				
Overall rate	11/48 (23%)	8/47 (17%)	7/47 (15%)	8/48 (17%)
Adjusted rate	30.0%	22.2%	20.7%	24.7%
Terminal rate	8/33 (24%)	8/36 (22%)	5/30 (17%)	5/28 (18%)
First incidence (days)	596	734 (T)	659	675
Life table test	P=0.538N	P=0.236N	P=0.271N	P=0.426N
Logistic regression test	P=0.430N	P=0.255N	P=0.248N	P=0.343N
Cochran-Armitage test	P=0.355N			
Fisher exact test		P=0.323N	P=0.231N	P=0.305N

**TABLE D3**  
**Statistical Analysis of Primary Neoplasms in Female Mice in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Skin (Subcutaneous Tissue): Sarcoma</b>				
Overall rate	6/50 (12%)	2/50 (4%)	0/50 (0%)	0/50 (0%)
Adjusted rate	16.0%	4.3%	0.0%	0.0%
Terminal rate	4/34 (12%)	0/37 (0%)	0/32 (0%)	0/28 (0%)
First incidence (days)	225	593	—	—
Life table test	P=0.022N	P=0.116N	P=0.022N	P=0.029N
Logistic regression test	P=0.012N	P=0.210N	P=0.014N	P=0.014N
Cochran-Armitage test	P=0.015N			
Fisher exact test		P=0.134N	P=0.013N	P=0.013N
<b>Thyroid Gland (Follicular Cell): Adenoma</b>				
Overall rate	3/50 (6%)	0/49 (0%)	0/49 (0%)	5/49 (10%)
Adjusted rate	8.0%	0.0%	0.0%	16.7%
Terminal rate	2/34 (6%)	0/36 (0%)	0/32 (0%)	4/28 (14%)
First incidence (days)	619	—	—	686
Life table test	P=0.026	P=0.113N	P=0.130N	P=0.274
Logistic regression test	P=0.038	P=0.126N	P=0.125N	P=0.323
Cochran-Armitage test	P=0.046			
Fisher exact test		P=0.125N	P=0.125N	P=0.346
<b>Thyroid Gland (Follicular Cell): Adenoma or Carcinoma</b>				
Overall rate	4/50 (8%)	0/49 (0%)	2/49 (4%)	5/49 (10%)
Adjusted rate	10.6%	0.0%	6.3%	16.7%
Terminal rate	2/34 (6%)	0/36 (0%)	2/32 (6%)	4/28 (14%)
First incidence (days)	619	—	734 (T)	686
Life table test	P=0.081	P=0.056N	P=0.360N	P=0.407
Logistic regression test	P=0.108	P=0.065N	P=0.351N	P=0.462
Cochran-Armitage test	P=0.130			
Fisher exact test		P=0.061N	P=0.349N	P=0.487
<b>All Organs: Hemangiosarcoma</b>				
Overall rate	1/50 (2%)	2/50 (4%)	4/50 (8%)	0/50 (0%)
Adjusted rate	2.9%	4.8%	10.3%	0.0%
Terminal rate	1/34 (3%)	1/37 (3%)	2/32 (6%)	0/28 (0%)
First incidence (days)	734 (T)	652	524	—
Life table test	P=0.321N	P=0.536	P=0.173	P=0.539N
Logistic regression test	P=0.250N	P=0.505	P=0.184	P=0.539N
Cochran-Armitage test	P=0.263N			
Fisher exact test		P=0.500	P=0.181	P=0.500N
<b>All Organs: Hemangioma or Hemangiosarcoma</b>				
Overall rate	1/50 (2%)	3/50 (6%)	6/50 (12%)	1/50 (2%)
Adjusted rate	2.9%	7.5%	15.8%	3.6%
Terminal rate	1/34 (3%)	2/37 (5%)	3/32 (9%)	1/28 (4%)
First incidence (days)	734 (T)	652	524	734 (T)
Life table test	P=0.499N	P=0.342	P=0.057	P=0.718
Logistic regression test	P=0.406N	P=0.319	P=0.059	P=0.718
Cochran-Armitage test	P=0.403N			
Fisher exact test		P=0.309	P=0.056	P=0.753N

**TABLE D3**  
**Statistical Analysis of Primary Neoplasms in Female Mice in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>All Organs: Histiocytic Sarcoma</b>				
Overall rate	3/50 (6%)	2/50 (4%)	0/50 (0%)	3/50 (6%)
Adjusted rate	7.7%	4.8%	0.0%	7.9%
Terminal rate	0/34 (0%)	1/37 (3%)	0/32 (0%)	0/28 (0%)
First incidence (days)	656	649	—	639
Life table test	P=0.456	P=0.452N	P=0.126N	P=0.644
Logistic regression test	P=0.496	P=0.396N	P=0.118N	P=0.644N
Cochran-Armitage test	P=0.500			
Fisher exact test		P=0.500N	P=0.121N	P=0.661N
<b>All Organs: Malignant Lymphoma</b>				
Overall rate	4/50 (8%)	7/50 (14%)	7/50 (14%)	5/50 (10%)
Adjusted rate	10.8%	16.8%	19.8%	13.4%
Terminal rate	3/34 (9%)	3/37 (8%)	5/32 (16%)	1/28 (4%)
First incidence (days)	583	666	649	356
Life table test	P=0.492	P=0.327	P=0.240	P=0.429
Logistic regression test	P=0.524N	P=0.267	P=0.255	P=0.527
Cochran-Armitage test	P=0.528N			
Fisher exact test		P=0.262	P=0.262	P=0.500
<b>All Organs: Benign Neoplasms</b>				
Overall rate	25/50 (50%)	32/50 (64%)	27/50 (54%)	27/50 (54%)
Adjusted rate	65.5%	76.0%	68.9%	72.1%
Terminal rate	21/34 (62%)	27/37 (73%)	20/32 (63%)	18/28 (64%)
First incidence (days)	596	593	622	539
Life table test	P=0.185	P=0.242	P=0.336	P=0.163
Logistic regression test	P=0.408	P=0.220	P=0.390	P=0.305
Cochran-Armitage test	P=0.465N			
Fisher exact test		P=0.113	P=0.421	P=0.421
<b>All Organs: Malignant Neoplasms</b>				
Overall rate	27/50 (54%)	22/50 (44%)	31/50 (62%)	21/50 (42%)
Adjusted rate	58.5%	47.6%	68.3%	49.5%
Terminal rate	15/34 (44%)	13/37 (35%)	18/32 (56%)	7/28 (25%)
First incidence (days)	225	593	495	356
Life table test	P=0.530N	P=0.154N	P=0.268	P=0.368N
Logistic regression test	P=0.187N	P=0.266N	P=0.276	P=0.147N
Cochran-Armitage test	P=0.199N			
Fisher exact test		P=0.212N	P=0.272	P=0.158N

**TABLE D3**  
**Statistical Analysis of Primary Neoplasms in Female Mice in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>All Organs: Benign or Malignant Neoplasms</b>				
Overall rate	42/50 (84%)	41/50 (82%)	43/50 (86%)	39/50 (78%)
Adjusted rate	89.3%	87.2%	91.4%	84.8%
Terminal rate	29/34 (85%)	31/37 (84%)	28/32 (88%)	21/28 (75%)
First incidence (days)	225	593	495	356
Life table test	P=0.213	P=0.255N	P=0.387	P=0.395
Logistic regression test	P=0.366N	P=0.422N	P=0.459	P=0.353N
Cochran-Armitage test	P=0.258N			
Fisher exact test		P=0.500N	P=0.500	P=0.306N

(T) Terminal sacrifice

<sup>a</sup> Number of neoplasm-bearing animals/number of animals examined. Denominator is number of animals examined microscopically for liver, lung, ovary, pituitary gland, and thyroid gland; for other tissues, denominator is number of animals necropsied.

<sup>b</sup> Kaplan-Meier estimated neoplasm incidence at the end of the study after adjustment for intercurrent mortality

<sup>c</sup> Observed incidence at terminal kill

<sup>d</sup> Beneath the chamber control incidence are the P values associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the chamber controls and that exposed group. The life table test regards neoplasms in animals dying prior to terminal kill as being (directly or indirectly) the cause of death. The logistic regression test regards these lesions as nonfatal. The Cochran-Armitage and Fisher exact tests compare directly the overall incidence rates. For all tests, a negative trend or a lower incidence in an exposure group is indicated by N.

<sup>e</sup> Not applicable; no neoplasms in animal group

**TABLE D4a**  
**Historical Incidence of Alveolar/bronchiolar Neoplasms in Chamber Control Female B6C3F<sub>1</sub> Mice<sup>a</sup>**

Study	Incidence in Controls		
	Adenoma	Carcinoma	Adenoma or Carcinoma
<b>Historical Incidence at Battelle Pacific Northwest Laboratories</b>			
1,3-Butadiene	4/50	0/50	4/50
Acetonitrile	7/49	1/49	8/49
Allyl Glycidyl Ether	0/50	0/50	0/50
2-Chloroacetophenone	4/50	3/50	6/50
<i>l</i> -Epinephrine Hydrochloride	3/50	2/50	5/50
Chloroethane	2/49	3/49	5/49
Hexachlorocyclopentadiene	4/48	3/48	7/48
<i>o</i> -Chlorobenzalmalononitrile (CS2)	4/50	1/50	5/50
Ozone	4/50	2/50	6/50
<b>Overall Historical Incidence</b>			
Total	61/939 (6.5%)	38/939 (4.1%)	97/939 (10.3%)
Standard deviation	3.2%	3.2%	3.7%
Range	0%-14%	0%-12%	0%-16%

<sup>a</sup> Data as of 12 May 1995

**TABLE D4b**  
**Historical Incidence of Hemangiosarcoma of the Liver in Chamber Control Female B6C3F<sub>1</sub> Mice<sup>a</sup>**

Study	Incidence in Controls	
	Adenoma	Carcinoma
<b>Historical Incidence at Battelle Pacific Northwest Laboratories</b>		
1,3-Butadiene		1/49
Acetonitrile		0/49
Allyl Glycidyl Ether		0/50
2-Chloroacetophenone		0/50
<i>l</i> -Epinephrine Hydrochloride		1/50
Chloroethane		0/49
Hexachlorocyclopentadiene		0/49
<i>o</i> -Chlorobenzalmalononitrile (CS2)		0/50
Ozone		0/50
<b>Overall Historical Incidence</b>		
Total		5/937 (0.5%)
Standard deviation		1.0%
Range		0%-3%

<sup>a</sup> Data as of 12 May 1995

**TABLE D5**  
**Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate<sup>a</sup>**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Disposition Summary</b>				
Animals initially in study	50	50	50	50
Early deaths				
Moribund	11	10	13	16
Natural deaths	5	3	5	6
Survivors				
Terminal sacrifice	34	37	32	28
Animals examined microscopically	50	50	50	50
<b>Alimentary System</b>				
Intestine large, cecum	(49)	(49)	(48)	(47)
Inflammation			1 (2%)	
Intestine small, ileum	(48)	(48)	(47)	(45)
Peyer's patch, hyperplasia, lymphoid			1 (2%)	
Liver	(50)	(50)	(50)	(49)
Basophilic focus		1 (2%)		
Clear cell focus	2 (4%)	1 (2%)	1 (2%)	1 (2%)
Clear cell focus, multiple				1 (2%)
Cyst			1 (2%)	1 (2%)
Eosinophilic focus	9 (18%)	7 (14%)	8 (16%)	9 (18%)
Eosinophilic focus, multiple				1 (2%)
Hematopoietic cell proliferation		1 (2%)		1 (2%)
Hemorrhage		1 (2%)		
Infiltration cellular, lymphocyte	3 (6%)	2 (4%)	1 (2%)	1 (2%)
Infiltration cellular, mixed cell		1 (2%)		
Inflammation, chronic	6 (12%)	1 (2%)	1 (2%)	2 (4%)
Karyomegaly	4 (8%)	2 (4%)		1 (2%)
Mineralization			1 (2%)	
Necrosis	1 (2%)			
Necrosis, focal	3 (6%)	1 (2%)	2 (4%)	2 (4%)
Regeneration	1 (2%)			
Thrombosis				1 (2%)
Vacuolization cytoplasmic		1 (2%)		
Vacuolization cytoplasmic, diffuse			1 (2%)	1 (2%)
Oval cell, hyperplasia	2 (4%)	1 (2%)		
Serosa, fibrosis				1 (2%)
Mesentery	(10)	(12)	(8)	(7)
Angiectasis	1 (10%)			
Hemorrhage				1 (14%)
Infiltration cellular, lymphocyte		1 (8%)		
Inflammation, chronic		1 (8%)		
Fat, necrosis	7 (70%)	8 (67%)	7 (88%)	5 (71%)
Pancreas	(50)	(50)	(49)	(49)
Infiltration cellular, lymphocyte			1 (2%)	
Inflammation, chronic	1 (2%)	1 (2%)		
Acinus, atrophy	1 (2%)	2 (4%)		3 (6%)
Duct, cyst	1 (2%)	2 (4%)	1 (2%)	
Stomach, forestomach	(50)	(50)	(49)	(50)
Cyst		1 (2%)		
Hyperplasia, squamous	1 (2%)	3 (6%)	1 (2%)	3 (6%)
Inflammation		2 (4%)		

<sup>a</sup> Number of animals examined microscopically at the site and the number of animals with lesion

**TABLE D5**  
**Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Cardiovascular System</b>				
Heart	(50)	(50)	(50)	(50)
Mineralization	1 (2%)			
Artery, inflammation, chronic	1 (2%)		1 (2%)	
Epicardium, fibrosis				1 (2%)
<b>Endocrine System</b>				
Adrenal cortex	(50)	(50)	(49)	(50)
Accessory adrenal cortical nodule				1 (2%)
Angiectasis	1 (2%)			
Cyst, focal			1 (2%)	
Degeneration, cystic, focal		1 (2%)		
Inflammation	1 (2%)			1 (2%)
Vacuolization cytoplasmic			1 (2%)	1 (2%)
Capsule, hyperplasia			1 (2%)	
Pituitary gland	(48)	(47)	(47)	(48)
Angiectasis	2 (4%)	5 (11%)	1 (2%)	2 (4%)
Pars distalis, angiectasis	1 (2%)			
Pars distalis, hyperplasia	12 (25%)	1 (2%)	7 (15%)	6 (13%)
Thyroid gland	(50)	(49)	(49)	(49)
Cyst			1 (2%)	
Infiltration cellular, lymphocyte			1 (2%)	
Inflammation				1 (2%)
Follicular cell, hyperplasia	20 (40%)	17 (35%)	14 (29%)	19 (39%)
<b>General Body System</b>				
None				
<b>Genital System</b>				
Clitoral gland	(41)	(40)	(43)	(40)
Cyst	1 (2%)			
Inflammation	1 (2%)	1 (3%)		
Ovary	(48)	(49)	(49)	(48)
Angiectasis	1 (2%)	1 (2%)	1 (2%)	
Atrophy			2 (4%)	
Cyst	9 (19%)	12 (24%)	16 (33%)	15 (31%)
Thrombosis		1 (2%)		
Bilateral, cyst			1 (2%)	
Uterus	(50)	(50)	(49)	(49)
Angiectasis	1 (2%)	1 (2%)	1 (2%)	
Hydrometra		3 (6%)	3 (6%)	2 (4%)
Hyperplasia, cystic	37 (74%)	41 (82%)	38 (78%)	37 (76%)
Inflammation				2 (4%)
Inflammation, suppurative		1 (2%)		
Thrombosis	1 (2%)		1 (2%)	1 (2%)
Cervix, hemorrhage		1 (2%)		

**TABLE D5**  
**Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Hematopoietic System</b>				
Bone marrow	(50)	(50)	(49)	(50)
Inflammation, chronic			1 (2%)	
Lymph node	(3)	(6)	(3)	(4)
Hyperplasia, lymphoid		1 (17%)		
Iliac, ectasia			1 (33%)	
Renal, hyperplasia, lymphoid		1 (17%)		
Lymph node, bronchial	(30)	(34)	(27)	(35)
Hyperplasia, lymphoid	1 (3%)	2 (6%)	2 (7%)	3 (9%)
Infiltration cellular, histiocyte				1 (3%)
Lymph node, mandibular	(37)	(37)	(36)	(36)
Hyperplasia, lymphoid		1 (3%)		
Lymph node, mesenteric	(46)	(45)	(46)	(44)
Hyperplasia, lymphoid	1 (2%)	5 (11%)	2 (4%)	
Infiltration cellular, histiocyte	1 (2%)			
Inflammation, chronic	1 (2%)			
Necrosis		1 (2%)		
Lymph node, mediastinal	(41)	(36)	(28)	(34)
Hemorrhage				1 (3%)
Hyperplasia, lymphoid	3 (7%)	5 (14%)	1 (4%)	3 (9%)
Spleen	(50)	(50)	(49)	(49)
Hematopoietic cell proliferation	3 (6%)	11 (22%)	9 (18%)	3 (6%)
Hyperplasia, lymphoid	1 (2%)	5 (10%)	1 (2%)	2 (4%)
Thymus	(41)	(44)	(41)	(41)
Hyperplasia, lymphoid	1 (2%)	1 (2%)		
Necrosis			1 (2%)	
<b>Integumentary System</b>				
Mammary gland	(47)	(50)	(50)	(50)
Hyperplasia	3 (6%)	4 (8%)	2 (4%)	
Skin	(49)	(50)	(50)	(50)
Inflammation, chronic		1 (2%)		
Inflammation, suppurative			1 (2%)	
Ulcer		1 (2%)	1 (2%)	1 (2%)
Subcutaneous tissue, hemorrhage			1 (2%)	
Subcutaneous tissue, inflammation, chronic		1 (2%)		
<b>Musculoskeletal System</b>				
Bone	(50)	(50)	(50)	(50)
Fibrous osteodystrophy	2 (4%)	1 (2%)	1 (2%)	5 (10%)
Fracture			1 (2%)	1 (2%)
Maxilla, inflammation, chronic				1 (2%)
Skeletal muscle		(1)	(2)	(1)
Hemorrhage			1 (50%)	
<b>Nervous System</b>				
Brain	(50)	(50)	(50)	(50)
Hemorrhage				1 (2%)
Spinal cord	(1)			(1)
Degeneration				1 (100%)

**TABLE D5**  
**Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Inhalation Study**  
**of Cobalt Sulfate Heptahydrate**

	Chamber Control	0.3 mg/m <sup>3</sup>	1.0 mg/m <sup>3</sup>	3.0 mg/m <sup>3</sup>
<b>Respiratory System</b>				
Larynx	(50)	(49)	(47)	(50)
Inflammation, chronic				1 (2%)
Metaplasia, squamous		45 (92%)	40 (85%)	50 (100%)
Lung	(50)	(50)	(50)	(50)
Hemorrhage	2 (4%)	1 (2%)		4 (8%)
Infiltration cellular, diffuse, histiocyte				4 (8%)
Infiltration cellular, focal, histiocyte	2 (4%)	5 (10%)	7 (14%)	10 (20%)
Inflammation, chronic			1 (2%)	1 (2%)
Alveolar epithelium, hyperplasia	2 (4%)	3 (6%)		5 (10%)
Bronchus, vacuolization cytoplasmic		6 (12%)	31 (62%)	43 (86%)
Capillary, thrombosis, diffuse				1 (2%)
Nose	(50)	(50)	(49)	(48)
Hemorrhage			1 (2%)	
Inflammation, chronic				1 (2%)
Inflammation, suppurative		1 (2%)	5 (10%)	4 (8%)
Olfactory epithelium, atrophy		2 (4%)	12 (24%)	46 (96%)
Olfactory epithelium, degeneration, hyaline	3 (6%)	1 (2%)	2 (4%)	2 (4%)
Olfactory epithelium, hyperplasia				30 (63%)
Olfactory epithelium, metaplasia		1 (2%)	1 (2%)	
Respiratory epithelium, degeneration, hyaline	20 (40%)	16 (32%)	14 (29%)	11 (23%)
Respiratory epithelium, metaplasia, squamous				4 (8%)
<b>Special Senses System</b>				
Eye	(1)			
Degeneration	1 (100%)			
<b>Urinary System</b>				
Kidney	(50)	(50)	(50)	(50)
Cyst	1 (2%)			2 (4%)
Infarct				1 (2%)
Infiltration cellular, mixed cell	3 (6%)	1 (2%)	1 (2%)	3 (6%)
Inflammation, chronic		1 (2%)	1 (2%)	
Metaplasia, osseous		1 (2%)	1 (2%)	
Mineralization				1 (2%)
Nephropathy	5 (10%)	6 (12%)	7 (14%)	3 (6%)
Pigmentation, hemosiderin				1 (2%)
Urinary bladder	(48)	(47)	(45)	(46)
Edema		1 (2%)		



## **APPENDIX E**

# **GENETIC TOXICOLOGY**

<b>SALMONELLA MUTAGENICITY TEST PROTOCOL</b> .....	<b>210</b>
<b>RESULTS</b>	<b>210</b>
<b>TABLE E1</b> Mutagenicity of Cobalt Sulfate Heptahydrate in <i>Salmonella typhimurium</i> .....	<b>211</b>

## GENETIC TOXICOLOGY

### **SALMONELLA MUTAGENICITY TEST PROTOCOL**

Testing was performed as reported by Zeiger *et al.* (1992). Cobalt sulfate heptahydrate was sent to the laboratory as a coded aliquot from Radian Corporation (Austin, TX). It was incubated with the *Salmonella typhimurium* tester strains (TA98, TA100, and TA1535) either in buffer or S9 mix (metabolic activation enzymes and cofactors from Aroclor 1254-induced male Sprague-Dawley rat or Syrian hamster liver) for 20 minutes at 37° C. Top agar supplemented with L-histidine and d-biotin was added, and the contents of the tubes were mixed and poured onto the surfaces of minimal glucose agar plates. Histidine-independent mutant colonies arising on these plates were counted following incubation for 2 days at 37° C.

Each trial consisted of triplicate plates of concurrent positive and negative controls and of five doses of cobalt sulfate heptahydrate. The high dose was limited by experimental design to 10,000 µg/plate. All positive assays were repeated under the conditions that elicited the positive response.

In this assay, a positive response is defined as a reproducible, dose-related increase in histidine-independent (revertant) colonies in any one strain/activation combination. An equivocal response is defined as an increase in revertants that is not dose related, not reproducible, or not of sufficient magnitude to support a determination of mutagenicity. A negative response is obtained when no increase in revertant colonies is observed following chemical treatment. There is no minimum percentage or fold increase required for a chemical to be judged positive or weakly positive.

### **RESULTS**

Cobalt sulfate heptahydrate (3 to 10,000 µg/mL) was mutagenic in *S. typhimurium* strain TA100 in the absence of S9 metabolic activation, and with 5% hamster or rat liver S9; no mutagenicity was detected in strain TA98 or TA1535, with or without S9 (Zeiger *et al.*, 1992; Table E1).

**TABLE E1**  
**Mutagenicity of Cobalt Sulfate Heptahydrate in *Salmonella typhimurium*<sup>a</sup>**

Strain	Dose (µg/plate)	Revertants/plate <sup>b</sup>				
		S9			+ hamster S9	
		Trial 1	Trial 2	Trial 3	5%	5%
<b>TA100</b>	0	102 ± 6.4	113 ± 6.8	107 ± 8.5	117 ± 9.6	98 ± 6.4
	3					119 ± 11.9
	10		143 ± 4.7	134 ± 6.8		137 ± 13.7
	33		155 ± 1.7	160 ± 10.2	118 ± 5.6	162 ± 8.4
	100	201 ± 15.3	152 ± 7.9	163 ± 2.2	175 ± 1.5	176 ± 14.8
	333	217 ± 7.0	208 ± 11.7	204 ± 9.4	188 ± 6.2	163 ± 1.5
	1,000	204 ± 14.7	206 ± 17.6	152 ± 9.0	187 ± 6.1	
	3,333	126 ± 15.3			176 ± 7.3	
	10,000	101 ± 10.3 <sup>c</sup>				
	Trial summary	Positive	Weakly Positive	Weakly Positive	Weakly Positive	Weakly Positive
Positive control <sup>d</sup>	429 ± 7.8	312 ± 2.9	290 ± 20.1	922 ± 44.3	897 ± 57.6	
		<b>+ hamster S9</b>			<b>+ rat S9</b>	
		<b>10%</b>	<b>30%</b>	<b>30%</b>	<b>5%</b>	<b>5%</b>
<b>TA100</b> (continued)	0	130 ± 15.0	139 ± 11.2	159 ± 3.9	116 ± 8.7	117 ± 10.6
	10					133 ± 1.5
	33	134 ± 9.8		168 ± 15.0	143 ± 12.2	164 ± 4.7
	100	156 ± 2.8	194 ± 9.0	166 ± 0.7	176 ± 8.0	188 ± 7.5
	333	187 ± 2.5	179 ± 6.0	193 ± 4.0	189 ± 15.9	201 ± 1.2
	1,000	159 ± 5.0	176 ± 3.0	160 ± 14.0	168 ± 8.2	143 ± 24.2
	3,333	160 ± 3.1	188 ± 18.4	161 ± 5.8	146 ± 9.8	
	10,000		123 ± 3.8			
Trial summary	Equivocal	Equivocal	Negative	Weakly Positive	Weakly Positive	
Positive control	577 ± 10.9	462 ± 31.2	457 ± 22.2	909 ± 16.2	1,011 ± 25.0	
		<b>+ rat S9</b>				
		<b>10%</b>	<b>30%</b>	<b>30%</b>		
<b>TA100</b> (continued)	0	124 ± 10.7	151 ± 12.3	131 ± 3.0		
	33	122 ± 4.4		123 ± 14.8		
	100	142 ± 5.8	179 ± 16.6	144 ± 10.7		
	333	154 ± 4.9	223 ± 6.9	138 ± 8.4		
	1,000	133 ± 5.5	191 ± 0.0	137 ± 11.2		
	3,333	124 ± 4.3	202 ± 1.9	135 ± 5.3		
	10,000		176 ± 12.5			
Trial summary	Negative	Equivocal	Negative			
Positive control	556 ± 32.4	244 ± 6.7	521 ± 41.2			

**TABLE E1**  
**Mutagenicity of Cobalt Sulfate Heptahydrate in *Salmonella typhimurium***

Strain	Dose ( $\mu\text{g}/\text{plate}$ )	Revertants/plate				
		S9	+ hamster S9		+ rat S9	
			5%	10%	30%	30%
<b>TA1535</b>	0	16 $\pm$ 1.2	12 $\pm$ 3.3	11 $\pm$ 1.5		
	3		10 $\pm$ 2.6			
	10	17 $\pm$ 2.0	9 $\pm$ 2.6	8 $\pm$ 1.3		
	33	14 $\pm$ 2.3	8 $\pm$ 0.3	10 $\pm$ 1.5		
	100	9 $\pm$ 1.2	5 $\pm$ 0.6	8 $\pm$ 0.9		
	333	8 $\pm$ 1.7	4 $\pm$ 1.2	9 $\pm$ 0.3		
	1,000	9 $\pm$ 1.3		8 $\pm$ 1.5		
	Trial summary	Negative	Negative	Negative		
Positive control	213 $\pm$ 24.0	64 $\pm$ 4.9	186 $\pm$ 35.6			
<b>TA98</b>	0	22 $\pm$ 2.3	32 $\pm$ 2.2	32 $\pm$ 0.6	32 $\pm$ 3.8	24 $\pm$ 1.7
	10		36 $\pm$ 2.7	28 $\pm$ 0.9		20 $\pm$ 2.3
	33		31 $\pm$ 4.4	38 $\pm$ 2.7		24 $\pm$ 1.9
	100	26 $\pm$ 0.5	45 $\pm$ 4.1	39 $\pm$ 4.6	44 $\pm$ 4.7	23 $\pm$ 3.5
	333	25 $\pm$ 4.0	40 $\pm$ 1.2	43 $\pm$ 0.0	53 $\pm$ 4.2	34 $\pm$ 2.6
	1,000	19 $\pm$ 0.6	42 $\pm$ 2.8	48 $\pm$ 7.6	47 $\pm$ 7.3	29 $\pm$ 4.8
	3,333	16 $\pm$ 3.4			44 $\pm$ 4.7	
	10,000	5 $\pm$ 2.7 <sup>c</sup>			31 $\pm$ 3.8	
	Trial summary	Negative	Negative	Negative	Equivocal	Negative
	Positive control	372 $\pm$ 12.5	671 $\pm$ 34.6	687 $\pm$ 24.7	328 $\pm$ 4.3	542 $\pm$ 38.5
<b>TA98</b> (continued)	0	36 $\pm$ 1.8	28 $\pm$ 1.7	32 $\pm$ 3.3	32 $\pm$ 4.2	34 $\pm$ 1.5
	10	33 $\pm$ 4.9	29 $\pm$ 2.1			27 $\pm$ 1.2
	33	42 $\pm$ 3.0	38 $\pm$ 3.8			24 $\pm$ 2.3
	100	41 $\pm$ 4.7	45 $\pm$ 7.5	26 $\pm$ 1.2	44 $\pm$ 3.5	37 $\pm$ 3.5
	333	55 $\pm$ 5.6	46 $\pm$ 4.5	26 $\pm$ 0.6	54 $\pm$ 8.5	37 $\pm$ 3.3
	1,000	55 $\pm$ 2.9	33 $\pm$ 1.8	14 $\pm$ 0.6 <sup>c</sup>	28 $\pm$ 1.5	34 $\pm$ 3.8
	3,333			16 $\pm$ 4.0 <sup>c</sup>	21 $\pm$ 2.0	
	10,000			7 $\pm$ 2.6 <sup>c</sup>	24 $\pm$ 3.7	
	Trial summary	Negative	Negative	Negative	Negative	Negative
	Positive control	742 $\pm$ 14.8	451 $\pm$ 12.5	104 $\pm$ 5.9	105 $\pm$ 6.5	208 $\pm$ 15.8

<sup>a</sup> Study performed at SRI International. The detailed protocol and these data are presented in Zeiger *et al.* (1992).

<sup>b</sup> Revertants are presented as mean  $\pm$  standard error from three plates.

<sup>c</sup> Slight toxicity

<sup>d</sup> The positive controls in the absence of metabolic activation were sodium azide (TA100 and TA1535) and 4-nitro-*o*-phenylenediamine (TA98). The positive control for metabolic activation with all strains was 2-aminoanthracene.

## **APPENDIX F**

# **CHEMICAL CHARACTERIZATION AND GENERATION OF CHAMBER CONCENTRATIONS**

<b>PROCUREMENT AND CHARACTERIZATION OF COBALT SULFATE HEPTAHYDRATE</b> .....	<b>214</b>
<b>AEROSOL GENERATION AND EXPOSURE SYSTEM</b> .....	<b>214</b>
<b>AEROSOL CONCENTRATION MONITORING</b> .....	<b>215</b>
<b>CHAMBER ATMOSPHERE CHARACTERIZATION</b> .....	<b>215</b>
<b>FIGURE F1 Infrared Absorption Spectrum of Cobalt Sulfate Heptahydrate</b> .....	<b>218</b>
<b>FIGURE F2 Schematic of the Aerosol Generation and Delivery System</b> .....	<b>219</b>
<b>FIGURE F3 Schematic of the RETEC Compressed-Air-Driven Nebulizer</b> .....	<b>220</b>
<b>FIGURE F4 Inhalation Suite</b> .....	<b>221</b>
<b>FIGURE F5 Schematic of the Concentration Monitoring System</b> .....	<b>222</b>
<b>TABLE F1 Summary of Chamber Concentrations in the 2-Year Inhalation Studies of Cobalt Sulfate Heptahydrate</b> .....	<b>223</b>
<b>TABLE F2 Summary of Aerosol Size Measurements for the Rat Exposure Chambers in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate</b> .....	<b>223</b>
<b>TABLE F3 Summary of Aerosol Size Measurements for the Mouse Exposure Chambers in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate</b> .....	<b>224</b>

# CHEMICAL CHARACTERIZATION AND GENERATION OF CHAMBER CONCENTRATIONS

## PROCUREMENT AND CHARACTERIZATION OF COBALT SULFATE HEPTAHYDRATE

Cobalt sulfate heptahydrate was obtained from Curtin Matheson Scientific (Kansas City, MO) in one lot (412092), which was used during the 2-year studies. Identity and purity analyses were conducted by the analytical chemistry laboratory, Midwest Research Institute (Kansas City, MO). Reports on analyses performed in support of the cobalt sulfate heptahydrate studies are on file at the National Institute of Environmental Health Sciences.

The chemical, a red, crystalline solid, was identified as cobalt sulfate heptahydrate by infrared, ultraviolet and/or visible spectroscopy. The spectra were consistent with the structure of cobalt sulfate heptahydrate. No literature references were found. The infrared spectrum is presented in Figure F1.

The purity of lot 412092 was determined by elemental analysis, Karl Fischer water analysis, and spark source mass spectroscopy. Elemental analyses for sulfur and hydrogen were in agreement with the theoretical values for cobalt sulfate heptahydrate, but values for cobalt were slightly low. Karl Fischer water analysis indicated  $44.6\% \pm 0.5\%$  water. Spark source mass spectroscopy indicated 140 ppm nickel present as an impurity; all other impurities had a combined total of less than 175 ppm. The overall purity was determined to be approximately 99%.

Literature references indicate that cobalt sulfate heptahydrate is stable as a bulk chemical when stored protected from light at normal temperatures (*Merck Index*, 1989). The heptahydrate dehydrates to the hexahydrate at  $41.5^\circ\text{C}$  and to the monohydrate at  $71^\circ\text{C}$ , with no further changes expected below the decomposition temperature ( $708^\circ\text{C}$ ). Therefore, an accelerated stability study was not conducted. To ensure stability, the bulk chemical was stored in its original shipping containers, metal cans, at room temperature. Stability was monitored during the 2-year studies using elemental analysis by inductively coupled plasma/atomic emission spectroscopy, normalized against a cobalt standard (National Institute of Standards and Technology, Gaithersburg, MD); no degradation of the bulk chemical was detected.

## AEROSOL GENERATION AND EXPOSURE SYSTEM

A diagram of the cobalt sulfate heptahydrate aerosol generation and delivery system is shown in Figure F2. Cobalt sulfate heptahydrate aerosol was generated and delivered from an aqueous solution by a system composed of three main components: a compressed-air-driven nebulizer, an aerosol charge neutralizer, and an aerosol distribution system.

The nebulizer (Model PN7002; RETEC Development Laboratory, Portland, OR), shown in Figure F3, consisted of two orifices of different sizes aligned on opposite sides of a small chamber. Compressed air entered the chamber through the small orifice and, on entering the larger orifice, induced a negative pressure. Cobalt sulfate heptahydrate in deionized water (approximately 400 g/L) was siphoned from the bulk reservoir to the nebulizer reservoir and then aspirated into the nebulizer chamber and expelled as a stream through the larger orifice. Shear forces broke the stream into droplets that were evaporated to leave dry particles of cobalt sulfate heptahydrate. The aerosol generation and exposure system included primary and secondary compressed-air-driven nebulizers. The mass concentration of the dry particles in the feed solution was used to determine aerosol particle size. The generator output was controlled by adjusting the compressed air pressure.

The aerosol generated by the compressed-air-driven nebulizer was passed through the aerosol charge neutralizer to remove static charge that formed on the aerosol particles during generation, reducing adhesion of the droplets to the walls of the delivery system. This neutralizer consisted of a length of plastic duct with two 10-mCi  $^{63}\text{Ni}$ -plated foils suspended in the center of the tube. The activity of the foils was matched to the diameter of the duct to allow adequate time for the aerosol to approach Boltzmann equilibrium at the system flow rate.

A distribution line carried aerosol ( $20 \text{ mg/m}^3$ ) to exposure chambers on both sides of the exposure room. Aerosol was siphoned from the branches of the distribution line by pneumatic pumps (one pump per exposure chamber). The flow rate in each branch of the distribution line was controlled by an Air-Vac pump (Air-Vac Engineering, Milford, CT) and monitored by a photohelic differential pressure gauge (Dwyer Instruments, Inc., Michigan City, IN) coupled to a Venturi tube. At each chamber, aerosol moving through the chamber inlet was further diluted with HEPA-filtered air to the appropriate concentration for the chamber. A diagram of the inhalation suite is shown in Figure F4. The Hazleton 2000 inhalation exposure chambers (Harford Systems Division of Lab Products, Inc., Aberdeen, MD) were designed so that uniform aerosol concentrations could be maintained throughout each chamber with the catch pans in place. The total active mixing volume of each chamber was  $1.7 \text{ m}^3$ .

## AEROSOL CONCENTRATION MONITORING

The chamber aerosol concentrations of cobalt sulfate heptahydrate were monitored by real-time aerosol monitors (Model RAM-1; MIE, Inc., Bedford, MA) controlled by a Hewlett-Packard HP-85B computer (Hewlett-Packard Company, Palo Alto, CA). The RAM-1s detected aerosol particles ranging from 0.1 to  $20 \mu\text{m}$  in diameter. Three RAM-1s were employed in the monitoring system (Figure F5); these monitors were exchanged with different RAM-1s when the on-line monitor performance deteriorated. Chamber aerosol concentrations were sampled at least once per hour during each exposure day. Sample lines connecting the exposure chambers to the RAM-1s were designed to minimize aerosol particle losses due to settling or impaction. Throughout the 2-year studies, the background concentrations of total suspended particles in the control chambers were less than the limit of detection. A summary of chamber concentrations is presented in Table F1.

The RAM-1 voltage output was calibrated against cobalt sulfate heptahydrate concentrations of chamber filter samples. Samples were collected on Teflon<sup>®</sup>-coated, glass-fiber filters with a calibrated flow sampler. Equations for the calibration curves contained in the HP-85B computer converted the RAM-1 voltages into exposure concentrations. Solutions of filter samples in 2% nitric acid were analyzed quantitatively for cobalt sulfate heptahydrate by inductively coupled plasma/atomic emission spectroscopy (ICP/AES). Calibration samples were collected every 2 weeks. Additional samples for monitoring the accuracy of calibration were collected daily from at least one chamber monitored by each RAM-1 and were analyzed two to three times per week. The ICP/AES was calibrated with a solution of standard cobalt diluted with nitric acid.

The stability of aerosol concentrations in the  $0.3$  and  $3.0 \text{ mg/m}^3$  chambers was monitored by analyzing samples collected on Gelman A/E glass fibers using a calibrated flow sampler. X-ray diffraction analyses were performed by a Philips 3600 diffraction unit with Cu K $\alpha$  radiation. Results indicated that cobalt sulfate hexahydrate was the primary species delivered to the chambers.

## CHAMBER ATMOSPHERE CHARACTERIZATION

The time required for the chamber concentration to reach 90% of the target value following the beginning of exposure ( $T_{90}$ ) and the time required for the chamber concentration to reach 10% of the target value following termination of the exposure ( $T_{10}$ ) were determined for each exposure chamber. Without animals present,  $T_{90}$  values ranged from 9 to 11 minutes for the rat chambers and from 7 to 12 minutes for mouse

chambers;  $T_{10}$  ranged from 8 to 9 minutes for rats and mice. With animals present,  $T_{90}$  values ranged from 11 to 16 minutes for rats and from 8 to 12 minutes for mice;  $T_{10}$  ranged from 12 to 13 minutes for rats and from 11 to 12 minutes for mice. Variations in these values were considered to be due to differences in discrete sampling times, different flow rates for each chamber, fluctuations in generator output, and differing transit times for the aerosol through the delivery system. A  $T_{90}$  of 12 minutes was selected for the 2-year studies.

Aerosol size distribution was determined monthly for each exposure chamber with a Mercer-style seven-stage impactor (In-Tox Products, Albuquerque, NM). Samples were collected on glass coverslips sprayed with silicone and were analyzed for cobalt sulfate heptahydrate with ICP/AES. The relative mass on each impactor stage was analyzed by probit analysis; the mass median aerodynamic diameter for the aerosol was within the specified range of 1 to 3  $\mu\text{m}$  (Tables F2 and F3).

The uniformity of aerosol concentration in the inhalation exposure chambers was measured approximately every 3 months. Aerosol concentration was determined with the RAM-1s, with an extension tube fitted to the sampling lines to allow sampling from ports in the front and back of each chamber. Chamber concentration uniformity was acceptable throughout the studies except for measurements taken from the 0.3  $\text{mg}/\text{m}^3$  mouse exposure chamber during 1 month of the study; however, these measurements were within the specified 5% variability when repeated.

At the beginning of the studies and approximately every 90 days thereafter, the persistence of cobalt sulfate heptahydrate aerosol in the 3.0  $\text{mg}/\text{m}^3$  rat chamber with and without animals present was determined by monitoring the concentration overnight with two RAM-1s. The average time for the concentration to decrease to 1% of the target concentration was approximately 20 minutes.

Before the exposures began, a solution of cobalt sulfate heptahydrate was analyzed for purity by ICP/AES; mean concentration of cobalt sulfate heptahydrate was found to be 99% of the theoretical value. Another sample from this original solution was analyzed after approximately 10 weeks, and the concentration of cobalt sulfate heptahydrate was 103% of the theoretical value. Thus, cobalt sulfate heptahydrate in the generator reservoir solution was considered to be stable for up to 10 weeks. New formulations were prepared at approximately 8-week intervals thereafter. Because the purity information supplied by the manufacturer and determined by the analytical chemistry laboratory indicated possible sulfuric acid contamination from the manufacturing process, the pH of the cobalt sulfate heptahydrate solution in the generator reservoir was analyzed to determine the extent of contamination. The pH was approximately 4.5, compared to 6.0 for the deionized water used to prepare the solution. From the pH of 4.5, the sulfuric acid content of the solution was calculated to be 0.0004% by weight. This value corresponded to a molar concentration of  $1.6 \times 10^{-5}$  sulfuric acid.

The aerosol stoichiometry was determined by measuring the number of moles of cobalt, sulfate, and water associated with samples from the chambers and distribution line. Filters were obtained from the 3.0  $\text{mg}/\text{m}^3$  rat and mouse chambers and from the distribution line. The total mass of collected aerosol was determined by ICP/AES. The mass of water on each filter was determined gravimetrically, and the masses of cobalt and sulfate on each filter were determined as the difference between the net aerosol mass and the combined masses of cobalt and sulfate. The results indicated that the average number of moles of cobalt, sulfate, and water associated with each mole of aerosol were  $1.00 \pm 0.00$ ,  $1.01 \pm 0.01$ , and  $5.9 \pm 0.8$ , respectively. These results show that aerosol delivered to the exposure chambers is primarily cobalt sulfate hexahydrate, which is in good agreement with results obtained by X-ray diffraction.

Samples from the occupied 0.3 and 3.0  $\text{mg}/\text{m}^3$  rat exposure chambers, distribution line, and the generator reservoir were analyzed for ammonia by an Orion Model 512 ammonia electrode (Orion Research, Beverly, MA) with an internal reference. The electrode was calibrated against gravimetrically prepared ammonium

chloride solutions ranging from 0.1 to 100 mg ammonia per liter. Samples were collected on Teflon®-coated glass fibers with calibrated flow samplers. Filters were extracted with deionized water, and cobalt in the samples was quantified by ICP/AES. The filters were then extracted in an ionic strength adjustment buffer and ammonia in the sample was quantified with the ammonia-selective electrode. The concentration of ammonia relative to the amount of cobalt sulfate heptahydrate determined stoichiometrically from the ICP/AES cobalt measurements was approximately 0.9% by weight in the 0.3 mg/m<sup>3</sup> exposure chamber, approximately 1.8% in the 3.0 mg/m<sup>3</sup> chamber, and below detection limits in the distribution line and generator reservoir. The ammonia values in the exposure chambers were slightly above allowable impurity concentrations; this was attributed to the absence of cageboards during the first 8 weeks of the study. Thereafter, cageboards were used and the ammonia concentrations were expected to be significantly lower.

Due to the possibility of contamination of aerosol with carbon eroded from the organic polymers found in the components of the generation and delivery system, samples from the occupied chambers, distribution line, and reservoir were analyzed for carbon. Samples were collected from the occupied and unoccupied 0.3 and 3.0 mg/m<sup>3</sup> mouse exposure chambers, the distribution line, and the generator reservoir on Gelman A/E glass fiber filters using calibrated flow samplers. The filters were extracted with deionized water and aliquots from the extracts were analyzed for cobalt by ICP/AES. The remainder of the extract was made acidic to remove carbonate, and the extract was analyzed for total organic carbon by a Dohrmann Carbon Analyzer System (Dohrmann Division, Xertex Corporation, Santa Clara, CA). The instrument was calibrated against gravimetric standards prepared from potassium hydrogen phthalate.

The concentrations of total organic carbon in samples from the occupied exposure chamber were 8% in the 0.3 mg/m<sup>3</sup> chamber and 2.1% in the 3.0 mg/m<sup>3</sup> chamber. Carbon concentrations in other parts of the generation and delivery system and from empty chambers before exposure began were less than 0.5%. The high levels of organic carbon detected in the occupied mouse chambers were considered to be due to the presence of animals in the chambers. To verify that possibility, the analysis was repeated with samples from the occupied 0.3 and 3.0 mg/m<sup>3</sup> rat exposure chambers, the distribution line, and the generator reservoir. The results (9% and 2.1% total organic carbon in the 0.3 and 3.0 mg/m<sup>3</sup> rat chambers, respectively) were similar to those found for the mouse exposure chamber analysis, with negligible amounts again found in the generation and delivery system components. Thus, the carbon was concluded to be derived from the animals rather than from contamination of the aerosol.

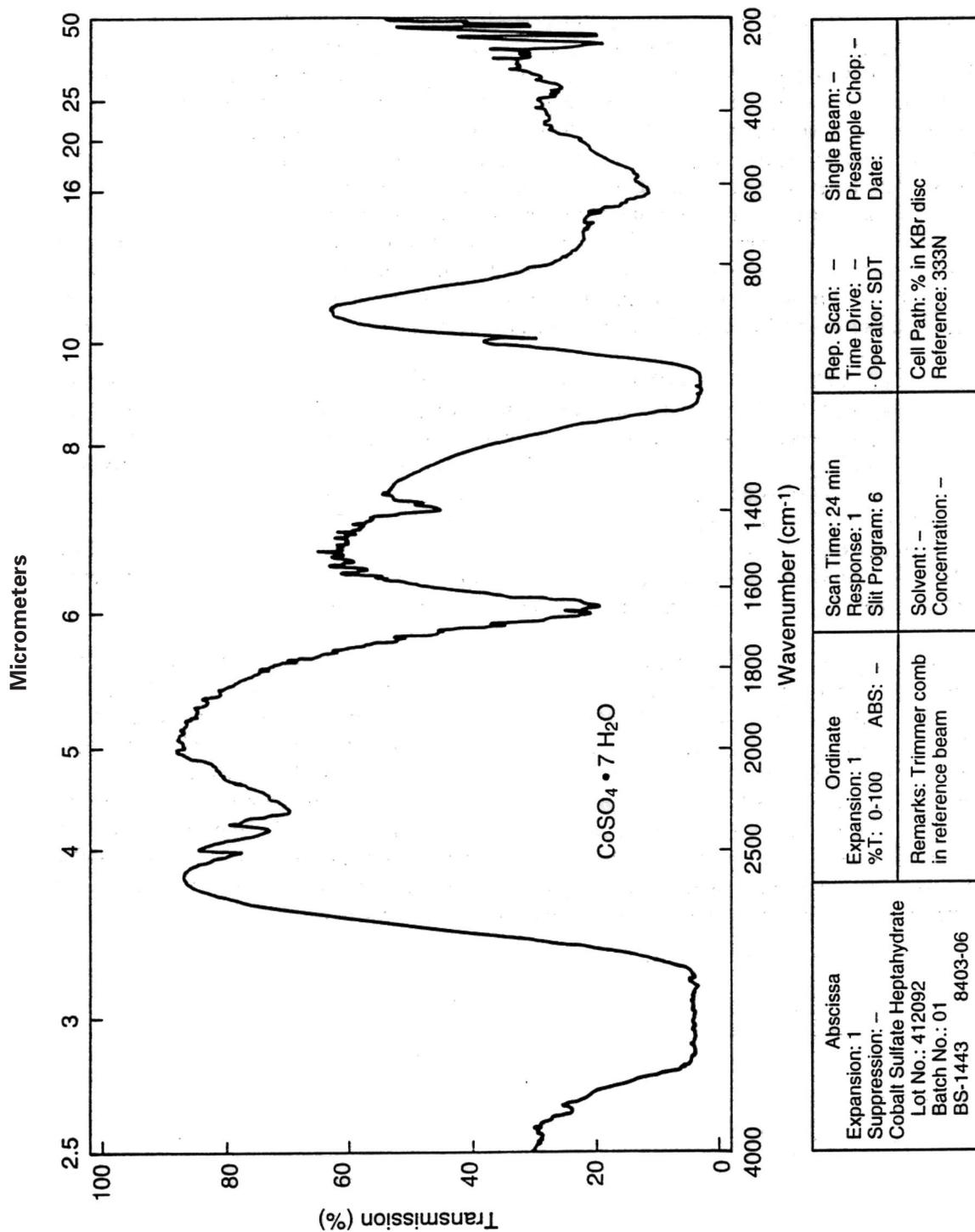


FIGURE F1  
Infrared Absorption Spectrum of Cobalt Sulfate Heptahydrate

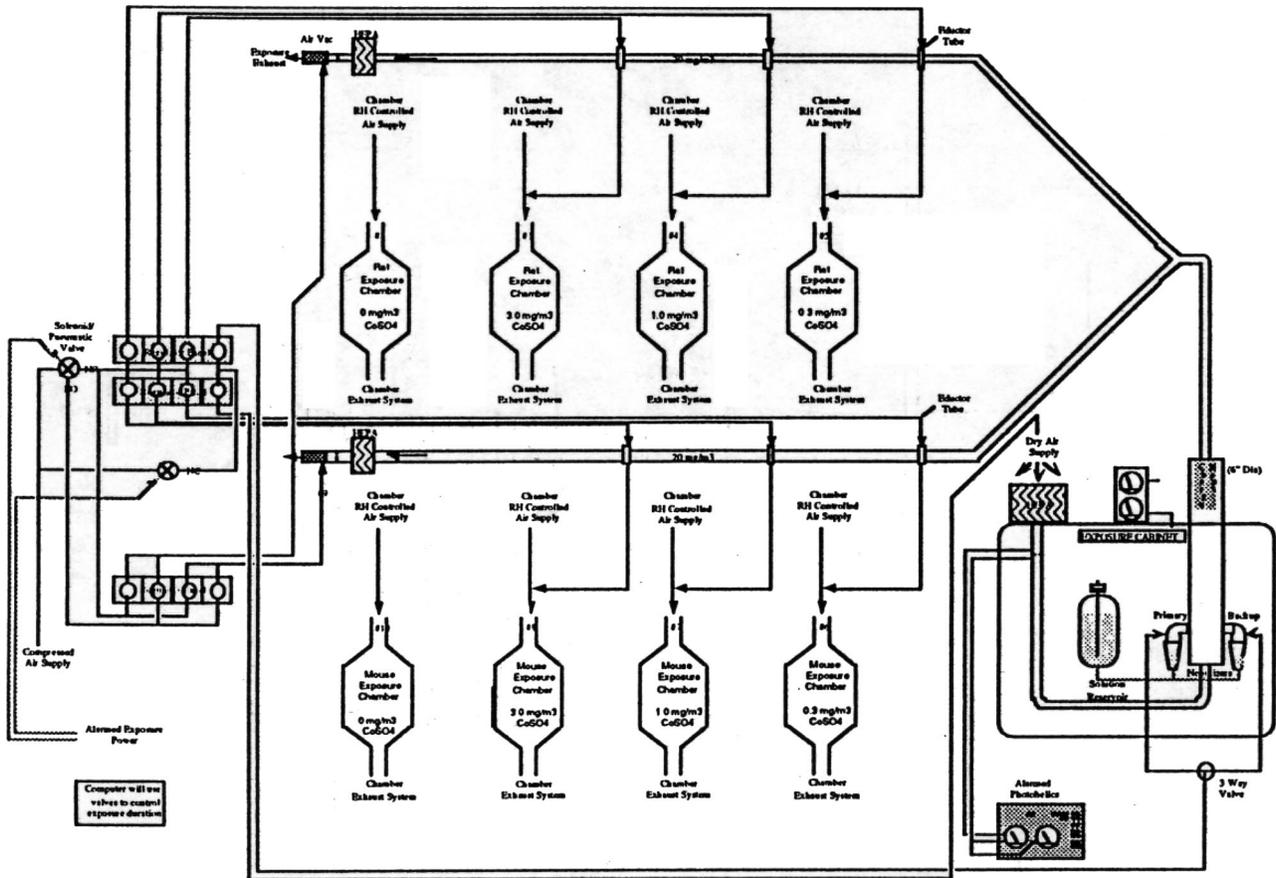
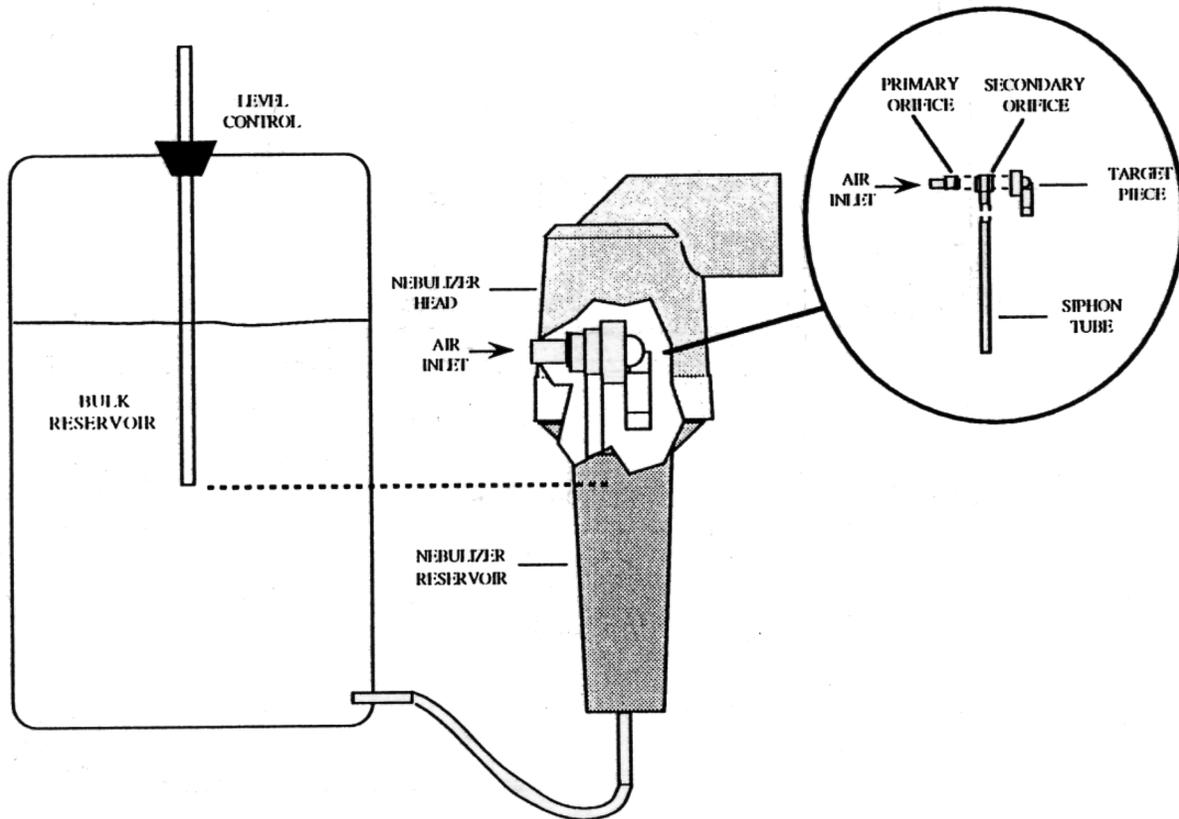


FIGURE F2  
Schematic of the Aerosol Generation and Delivery System



**FIGURE F3**  
**Schematic of the RETEC Compressed-Air-Driven Nebulizer**

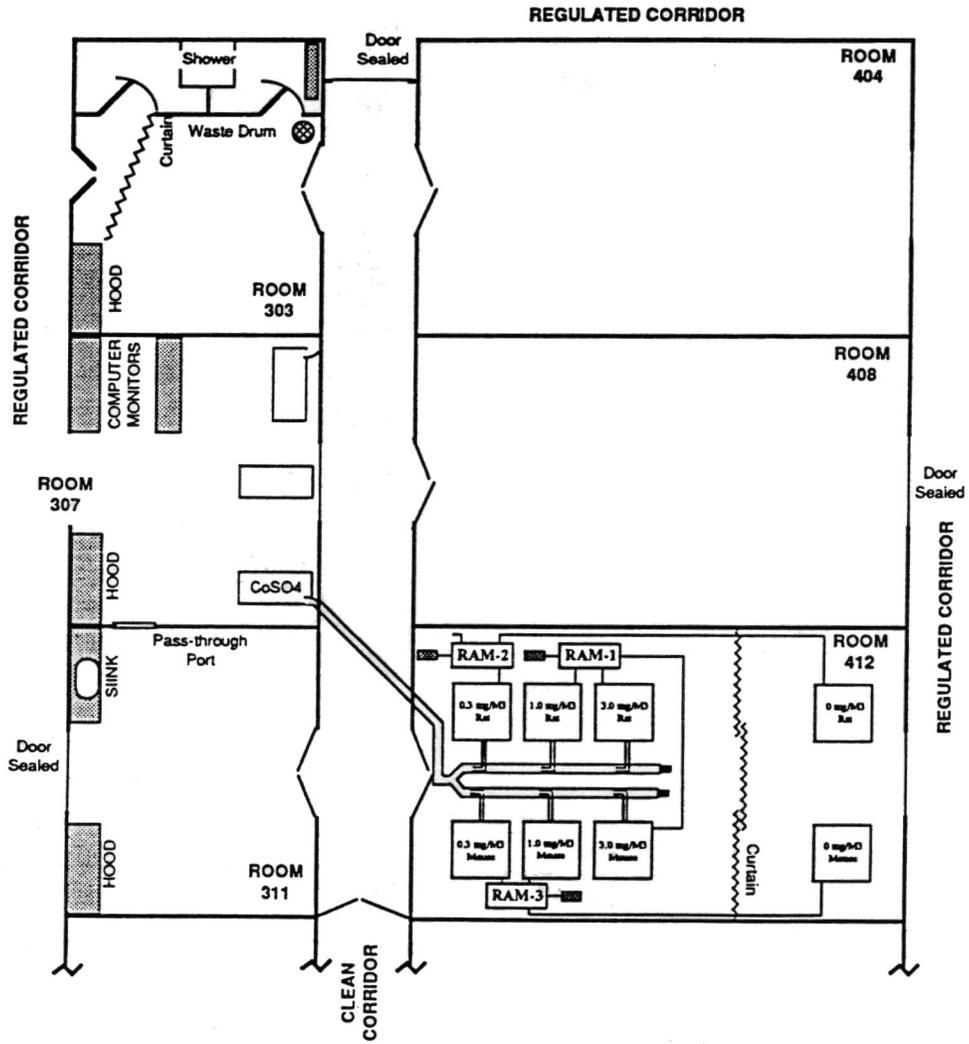


FIGURE F4  
Inhalation Suite

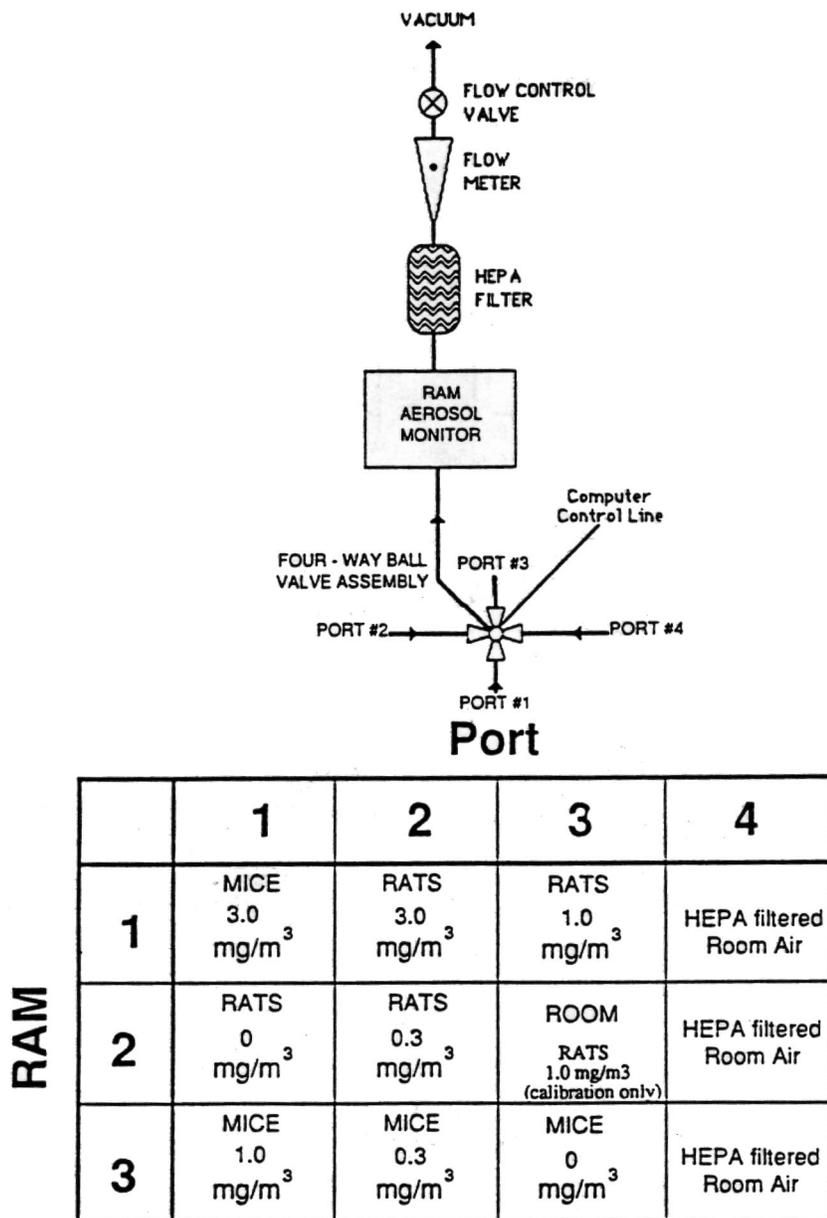


FIGURE F5  
Schematic of the Concentration Monitoring System

**TABLE F1**  
**Summary of Chamber Concentrations in the 2-Year Inhalation Studies of Cobalt Sulfate Heptahydrate**

Target Concentration (mg/m <sup>3</sup> )	Total Number of Readings	Average Concentration <sup>a</sup> (mg/m <sup>3</sup> )
<b>Rat Chambers</b>		
0.3	4,574	0.31 ± 0.03
1.0	4,574	1.03 ± 0.10
3.0	4,580	2.98 ± 0.20
<b>Mouse Chambers</b>		
0.3	4,571	0.30 ± 0.04
1.0	4,609	1.02 ± 0.08
3.0	4,605	3.01 ± 0.19

<sup>a</sup> Mean ± standard deviation**TABLE F2**  
**Summary of Aerosol Size Measurements for the Rat Exposure Chambers in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate**

Date	0.3 mg/m <sup>3</sup>		1.0 mg/m <sup>3</sup>		3.0 mg/m <sup>3</sup>	
	Mass Median Aerodynamic Diameter (µm)	Geometric Standard Deviation	Mass Median Aerodynamic Diameter (µm)	Geometric Standard Deviation	Mass Median Aerodynamic Diameter (µm)	Geometric Standard Deviation
September 1990	1.7	2.4	1.2	2.2	1.6	2.2
October 1990	1.5	2.0	1.2	2.1	1.6	2.1
November 1990	1.6	2.1	1.3	2.2	1.6	2.1
December 1990	1.3	2.1	1.1	2.0	1.1	1.9
January 1991	1.5	2.1	1.2	2.1	1.6	2.0
February 1991	1.5	2.2	1.4	2.2	1.5	2.1
March 1991	1.5	2.2	1.4	2.2	1.6	2.1
April 1991	1.6	2.1	1.4	2.2	1.6	2.1
May 1991	1.5	2.0	1.4	2.0	1.7	2.0
June 1991	1.6	2.1	1.5	2.3	1.7	2.1
July 1991	1.6	2.1	1.3	2.0	1.6	2.1
August 1991	1.5	2.0	1.4	1.9	1.7	1.9
September 1991	1.5	2.1	1.4	1.9	1.6	2.1
October 1991	1.5	2.2	1.5	2.0	1.7	2.3
November 1991	1.5	2.6	1.6	2.2	1.8	2.4
December 1991	1.3	2.2	1.5	2.0	1.7	2.3
January 1992	1.5	2.1	1.5	2.2	1.7	2.2
February 1992	1.4	2.3	1.4	2.3	1.6	2.3
March 1992	1.3	2.3	1.4	2.3	1.6	2.3
April 1992	1.6	1.9	1.5	2.3	1.6	2.3
May 1992	1.4	2.2	1.5	2.4	1.6	2.3
June 1992	1.4	2.2	1.5	2.1	1.6	2.2
July 1992	1.6	2.2	1.4	2.1	1.6	2.1
August 1992	1.5	2.2	1.3	2.2	1.6	2.2
<b>Mean ± standard deviation</b>	1.5 ± 0.10	2.2 ± 0.14	1.4 ± 0.12	2.1 ± 0.13	1.6 ± 0.12	2.2 ± 0.13

**TABLE F3**

**Summary of Aerosol Size Measurements for the Mouse Exposure Chambers in the 2-Year Inhalation Study of Cobalt Sulfate Heptahydrate**

Date	0.3 mg/m <sup>3</sup>		1.0 mg/m <sup>3</sup>		3.0 mg/m <sup>3</sup>	
	Mass Median Aerodynamic Diameter (µm)	Geometric Standard Deviation	Mass Median Aerodynamic Diameter (µm)	Geometric Standard Deviation	Mass Median Aerodynamic Diameter (µm)	Geometric Standard Deviation
September 1990	1.3	3.0	1.3	2.6	1.5	2.4
October 1990	1.6	2.6	1.7	2.2	1.9	2.1
November 1990	1.4	2.4	1.6	2.5	1.7	2.3
December 1990	1.2	2.4	— <sup>a</sup>	— <sup>a</sup>	1.1	2.2
January 1991	1.4	2.4	1.6	2.3	1.6	2.2
February 1991	1.6	2.3	1.5	2.3	1.7	2.2
March 1991	1.5	2.3	1.5	2.3	1.6	2.1
April 1991	1.6	2.4	1.5	2.4	1.8	2.3
May 1991	1.6	2.2	1.6	2.2	1.8	2.1
June 1991	1.4	2.3	1.4	2.4	1.6	2.2
July 1991	1.6	2.3	1.4	2.1	1.7	2.1
August 1991	1.6	2.1	1.5	2.1	1.6	2.2
September 1991	1.6	2.2	1.5	2.3	1.7	2.2
October 1991	1.7	2.2	1.7	2.3	1.7	2.3
November 1991	1.7	2.4	1.6	2.4	1.8	2.2
December 1991	1.7	2.2	1.6	2.1	1.7	2.3
January 1992	1.7	2.3	1.5	2.4	1.6	2.3
February 1992	1.7	2.2	1.7	2.4	1.6	2.3
March 1992	1.7	2.4	1.4	2.4	1.5	2.3
April 1992	1.7	2.5	1.6	2.5	1.5	2.6
May 1992	1.6	2.1	1.7	2.2	1.6	2.5
June 1992	1.5	2.2	1.5	2.3	1.6	2.2
July 1992	1.6	2.3	1.3	2.3	1.6	2.2
August 1992	2.0	2.3	1.5	2.3	1.5	2.2
<b>Mean ± standard deviation</b>	1.6 ± 0.16	2.3 ± 0.19	1.5 ± 0.12	2.3 ± 0.13	1.6 ± 0.15	2.3 ± 0.12

<sup>a</sup> No data available due to incomplete stage analysis

**APPENDIX G**  
**INGREDIENTS, NUTRIENT COMPOSITION,**  
**AND CONTAMINANT LEVELS**  
**IN NIH-07 RAT AND MOUSE RATION**

<b>TABLE G1</b>	<b>Ingredients of NIH-07 Rat and Mouse Ration . . . . .</b>	<b>226</b>
<b>TABLE G2</b>	<b>Vitamins and Minerals in NIH-07 Rat and Mouse Ration . . . . .</b>	<b>226</b>
<b>TABLE G3</b>	<b>Nutrient Composition of NIH-07 Rat and Mouse Ration . . . . .</b>	<b>227</b>
<b>TABLE G4</b>	<b>Contaminant Levels in NIH-07 Rat and Mouse Ration . . . . .</b>	<b>228</b>

**TABLE G1**  
**Ingredients of NIH-07 Rat and Mouse Ration<sup>a</sup>**

Ingredients <sup>b</sup>	Percent by Weight
Ground #2 yellow shelled corn	24.50
Ground hard winter wheat	23.00
Soybean meal (49% protein)	12.00
Fish meal (60% protein)	10.00
Wheat middlings	10.00
Dried skim milk	5.00
Alfalfa meal (dehydrated, 17% protein)	4.00
Corn gluten meal (60% protein)	3.00
Soy oil	2.50
Dried brewer's yeast	2.00
Dry molasses	1.50
Dicalcium phosphate	1.25
Ground limestone	0.50
Salt	0.50
Premixes (vitamin and mineral)	0.25

<sup>a</sup> NCI, 1976; NIH, 1978

<sup>b</sup> Ingredients were ground to pass through a U.S. Standard Screen No. 16 before being mixed.

**TABLE G2**  
**Vitamins and Minerals in NIH-07 Rat and Mouse Ration<sup>a</sup>**

	Amount	Source
<b>Vitamins</b>		
A	5,500,000 IU	Stabilized vitamin A palmitate or acetate
D <sub>3</sub>	4,600,000 IU	D-activated animal sterol
K <sub>3</sub>	2.8 g	Menadione
<i>d</i> - $\alpha$ -Tocopheryl acetate	20,000 IU	
Choline	560.0 g	Choline chloride
Folic acid	2.2 g	
Niacin	30.0 g	
<i>d</i> -Pantothenic acid	18.0 g	<i>d</i> -Calcium pantothenate
Riboflavin	3.4 g	
Thiamine	10.0 g	Thiamine mononitrate
B <sub>12</sub>	4,000 $\mu$ g	
Pyridoxine	1.7 g	Pyridoxine hydrochloride
Biotin	140.0 mg	<i>d</i> -Biotin
<b>Minerals</b>		
Iron	120.0 g	Iron sulfate
Manganese	60.0 g	Manganous oxide
Zinc	16.0 g	Zinc oxide
Copper	4.0 g	Copper sulfate
Iodine	1.4 g	Calcium iodate
Cobalt	0.4 g	Cobalt carbonate

<sup>a</sup> Per ton (2,000 lb) of finished product

**TABLE G3**  
**Nutrient Composition of NIH-07 Rat and Mouse Ration**

<b>Nutrient</b>	<b>Mean ± Standard Deviation</b>	<b>Range</b>	<b>Number of Samples</b>
Protein (% by weight)	23.40 ± 0.56	22.2 – 24.3	25
Crude fat (% by weight)	5.31 ± 0.19	5.00 – 5.90	25
Crude fiber (% by weight)	3.36 ± 0.33	2.60 – 4.30	25
Ash (% by weight)	6.43 ± 0.20	6.12 – 6.81	25
<b>Amino Acids (% of total diet)</b>			
Arginine	1.280 ± 0.083	1.110 – 1.390	11
Cystine	0.308 ± 0.071	0.181 – 0.400	11
Glycine	1.158 ± 0.048	1.060 – 1.220	11
Histidine	0.584 ± 0.027	0.531 – 0.630	11
Isoleucine	0.917 ± 0.033	0.867 – 0.965	11
Leucine	1.975 ± 0.051	1.850 – 2.040	11
Lysine	1.274 ± 0.049	1.200 – 1.370	11
Methionine	0.437 ± 0.109	0.306 – 0.699	11
Phenylalanine	0.999 ± 0.120	0.665 – 1.110	11
Threonine	0.904 ± 0.058	0.824 – 0.985	11
Tryptophan	0.218 ± 0.153	0.107 – 0.671	11
Tyrosine	0.685 ± 0.094	0.564 – 0.794	11
Valine	1.086 ± 0.055	0.962 – 1.170	11
<b>Essential Fatty Acids (% of total diet)</b>			
Linoleic	2.407 ± 0.227	1.830 – 2.570	10
Linolenic	0.259 ± 0.065	0.100 – 0.320	10
<b>Vitamins</b>			
Vitamin A (IU/kg)	6,738 ± 1,318	5,730 – 11,450	25
Vitamin D (IU/kg)	4,450 ± 1,382	3,000 – 6,300	4
α-Tocopherol (ppm)	35.43 ± 8.98	22.5 – 48.9	11
Thiamine (ppm)	17.48 ± 2.10	14.0 – 22.0	25
Riboflavin (ppm)	7.83 ± 0.923	6.10 – 9.00	11
Niacin (ppm)	99.22 ± 24.27	65.0 – 150.0	11
Pantothenic acid (ppm)	30.55 ± 3.52	23.0 – 34.6	11
Pyridoxine (ppm)	9.11 ± 2.53	5.60 – 14.0	11
Folic acid (ppm)	2.46 ± 0.63	1.80 – 3.70	11
Biotin (ppm)	0.268 ± 0.047	0.190 – 0.354	11
Vitamin B <sub>12</sub> (ppb)	40.5 ± 19.1	10.6 – 65.0	11
Choline (ppm)	2,991 ± 382	2,300 – 3,430	10
<b>Minerals</b>			
Calcium (%)	1.16 ± 0.10	1.00 – 1.49	25
Phosphorus (%)	0.92 ± 0.05	0.76 – 1.00	25
Potassium (%)	0.886 ± 0.063	0.772 – 0.971	9
Chloride (%)	0.529 ± 0.087	0.380 – 0.635	9
Sodium (%)	0.316 ± 0.033	0.258 – 0.371	11
Magnesium (%)	0.166 ± 0.010	0.148 – 0.181	11
Sulfur (%)	0.272 ± 0.059	0.208 – 0.420	10
Iron (ppm)	350.5 ± 87.3	255.0 – 523.0	11
Manganese (ppm)	92.48 ± 5.14	81.7 – 99.4	11
Zinc (ppm)	59.33 ± 10.2	46.1 – 81.6	11
Copper (ppm)	11.81 ± 2.50	9.09 – 15.4	11
Iodine (ppm)	3.54 ± 1.19	1.52 – 5.83	10
Chromium (ppm)	1.66 ± 0.46	0.85 – 2.09	11
Cobalt (ppm)	0.76 ± 0.23	0.49 – 1.15	7

**TABLE G4**  
**Contaminant Levels in NIH-07 Rat and Mouse Ration<sup>a</sup>**

	Mean ± Standard Deviation <sup>b</sup>	Range	Number of Samples
<b>Contaminants</b>			
Arsenic (ppm)	0.42 ± 0.20	0.10 – 0.70	25
Cadmium (ppm)	0.13 ± 0.07	0.04 – 0.20	25
Lead (ppm)	0.35 ± 0.24	0.10 – 1.00	25
Mercury (ppm) <sup>c</sup>	0.02	0.02 – 0.03	25
Selenium (ppm)	0.33 ± 0.11	0.05 – 0.40	25
Aflatoxins (ppm)	< 5.0		25
Nitrate nitrogen (ppm) <sup>d</sup>	8.99 ± 4.49	2.90 – 17.0	25
Nitrite nitrogen (ppm) <sup>d</sup>	0.15 ± 0.08	0.10 – 0.40	25
BHA (ppm) <sup>e</sup>	1.80 ± 1.94	1.00 – 10.0	25
BHT (ppm) <sup>e</sup>	1.56 ± 1.58	1.00 – 8.00	25
Aerobic plate count (CFU/g)	95,908 ± 162,569	4,100 – 710,000	25
Coliform (MPN/g)	3 ± 0.3	3 – 4	25
<i>Escherichia coli</i> (MPN/g)	< 3		25
<i>Salmonella</i> (MPN/g)	Negative		25
Total nitrosoamines (ppb) <sup>f</sup>	7.36 ± 1.75	4.70 – 11.40	25
N-Nitrosodimethylamine (ppb) <sup>f</sup>	5.40 ± 1.18	2.90 – 8.20	25
N-Nitrosopyrrolidine (ppb) <sup>f</sup>	1.96 ± 1.05	1.00 – 4.30	25
<b>Pesticides (ppm)</b>			
α-BHC	< 0.01		25
β-BHC	< 0.02		25
γ-BHC	< 0.01		25
δ-BHC	< 0.01		25
Heptachlor	< 0.01		25
Aldrin	< 0.01		25
Heptachlor epoxide	< 0.01		25
DDE	< 0.01		25
DDD	< 0.01		25
DDT	< 0.01		25
HCB	< 0.01		25
Mirex	< 0.01		25
Methoxychlor	< 0.05		25
Dieldrin	< 0.01		25
Endrin	< 0.01		25
Telodrin	< 0.01		25
Chlordane	< 0.05		25
Toxaphene	< 0.10		25
Estimated PCBs	< 0.20		25
Ronnel	< 0.01		25
Ethion	< 0.02		25
Trithion	< 0.05		25
Diazinon	< 0.10		25
Methyl parathion	< 0.02		25
Ethyl parathion	< 0.02		25
Malathion	0.23 ± 0.23	0.05 – 0.97	25
Endosulfan I	< 0.01		25
Endosulfan II	< 0.01		25
Endosulfan sulfate	< 0.03		25

<sup>a</sup> CFU = colony forming units; MPN = most probable number; BHC = hexachlorocyclohexane or benzene hexachloride

<sup>b</sup> For values less than the limit of detection, the detection limit is given as the mean.

<sup>c</sup> All values except for the September, November, and December 1991 milling dates (0.03 ppm) were less than the detection limit. The detection limit is given as the mean.

<sup>d</sup> Sources of contamination: alfalfa, grains, and fish meal

<sup>e</sup> Sources of contamination: soy oil and fish meal

<sup>f</sup> All values were corrected for percent recovery.

## **APPENDIX H**

### **SENTINEL ANIMAL PROGRAM**

<b>METHODS</b> .....	<b>230</b>
<b>TABLE H1</b> <b>Murine Virus Antibody Determinations for Rats and Mice</b> <b>in the 2-Year Studies of Cobalt Sulfate Heptahydrate</b> .....	<b>232</b>

## SENTINEL ANIMAL PROGRAM

### METHODS

Rodents used in the Carcinogenesis Program of the National Toxicology Program are produced in optimally clean facilities to eliminate potential pathogens that may affect study results. The Sentinel Animal Program is part of the periodic monitoring of animal health that occurs during the toxicologic evaluation of chemical compounds. Under this program, the disease state of the rodents is monitored via serology on sera from extra (sentinel) animals in the study rooms. These animals and the study animals are subject to identical environmental conditions. The sentinel animals come from the same production source and weanling groups as the animals used for the studies of chemical compounds.

Serum samples were collected from randomly selected rats and mice during the 2-year studies of cobalt sulfate heptahydrate. Blood from each animal was collected and allowed to clot, and the serum was separated. The samples were processed appropriately and sent to Microbiological Associates, Inc. (Bethesda, MD), for determination of antibody titers. The laboratory serology methods and viral agents for which testing was performed are tabulated below; the times at which blood was collected during the studies are also listed.

#### Method and Test

#### Time of Analysis

### RATS

#### ELISA

*Mycoplasma arthritidis*

Study termination

*Mycoplasma pulmonis*

Study termination

PVM (pneumonia virus of mice)

6, 12, and 18 months, study termination

RCV/SDA (rat coronavirus/

sialodacryoadenitis virus)

6, 12, and 18 months, study termination

Sendai

6, 12, and 18 months, study termination

#### Immunofluorescence Assay

RCV/SDA

6 months

#### Hemagglutination Inhibition

H-1 (Toolan's H-1 virus)

6, 12, and 18 months, study termination

KRV (Kilham rat virus)

6, 12, and 18 months, study termination

**MICE**

## ELISA

Ectromelia virus	6, 12, and 18 months, study termination
EDIM (epizootic diarrhea of infant mice)	6, 12, and 18 months, study termination
GDVII (mouse encephalomyelitis virus)	6, 12, and 18 months, study termination
LCM (lymphocytic choriomeningitis virus)	6, 12, and 18 months, study termination
Mouse adenoma virus-FL	6, 12, and 18 months, study termination
MHV (mouse hepatitis virus)	6, 12, and 18 months, study termination
<i>M. arthritidis</i>	Study termination
<i>M. pulmonis</i>	Study termination
PVM	6, 12, and 18 months, study termination
Reovirus 3	6, 12, and 18 months, study termination
Sendai	6, 12, and 18 months, study termination

## Immunofluorescence Assay

EDIM	18 months and study termination
LCM	6 months
MHV	Study termination
Reovirus 3	18 months and study termination

## Hemagglutination Inhibition

K (papovavirus)	6, 12, and 18 months, study termination
MVM (minute virus of mice)	6, 12, and 18 months, study termination
Polyoma virus	6, 12, and 18 months, study termination

Results of serology tests are presented in Table H1.

**TABLE H1**  
**Murine Virus Antibody Determinations for Rats and Mice in the 2-Year Studies**  
**of Cobalt Sulfate Heptahydrate**

Interval	Incidence of Antibody in Sentinel Animals	Positive Serologic Reaction for
<b>Rats</b>		
6 Months	0/16	None positive
12 Months	0/16	None positive
18 Months	0/16	None positive
Study termination	6/10	<i>M. arthritidis</i> <sup>a</sup>
<b>Mice</b>		
6 Months	0/10	None positive
12 Months	0/9	None positive
18 Months	0/10	None positive
Study termination	3/10	<i>M. arthritidis</i>

<sup>a</sup> Further evaluation of samples positive for *M. arthritidis* by immunoblot and Western blot procedures indicated that the positive titers may have been due to cross reaction with antibodies of nonpathogenic *Mycoplasma* or other agents. There were no clinical findings or histopathologic changes of *M. arthritidis* infection in animals with positive titers. Accordingly, *M. arthritidis*-positive titers were considered false positives.

**APPENDIX I**  
**K-RAS MUTATION FREQUENCY AND SPECTRA**  
**IN LUNG NEOPLASMS FROM B6C3F<sub>1</sub> MICE**  
**EXPOSED TO COBALT SULFATE HEPTAHYDRATE**  
**FOR 2 YEARS**

Robert C. Sills, Jennifer Neil, H. Lily Hong, John R. Bucher,  
 Gary A. Boorman, and Theodora R. Devereux  
 National Institute of Environmental Health Sciences  
 Research Triangle Park, North Carolina

<b>INTRODUCTION</b> .....	<b>234</b>
<b>MATERIALS AND METHODS</b> .....	<b>234</b>
<b>RESULTS</b> .....	<b>235</b>
<b>DISCUSSION</b> .....	<b>235</b>
<b>REFERENCES</b> .....	<b>237</b>
<b>TABLE I1</b> <b>K-ras Mutations in Lung Neoplasms of B6C3F<sub>1</sub> Mice</b> .....	<b>239</b>
<b>TABLE I2</b> <b>K-ras Mutation Profile in Lung Neoplasms of B6C3F<sub>1</sub> Mice</b> .....	<b>239</b>

# K-RAS MUTATION FREQUENCY AND SPECTRA IN LUNG NEOPLASMS FROM B6C3F<sub>1</sub> MICE EXPOSED TO COBALT SULFATE HEPTAHYDRATE FOR 2 YEARS

## INTRODUCTION

Lung neoplasms occur in B6C3F<sub>1</sub> mice with a typical incidence of 20% in control males and 10% in control females by 2 years of age. Molecular analysis of lung neoplasms for genetic alterations in cancer genes such as the *ras* proto-oncogene provides additional mechanistic information to help distinguish spontaneous neoplasms from chemical-induced neoplasms. For example, chemical-induced neoplasms in mice may have a higher frequency of proto-oncogene activation, particularly by point mutations in codon 12, 13, or 61 of *K-ras* genes (Sills *et al.*, 1995). The frequency of *ras* activation in these neoplasms is often greater than that detected in neoplasms occurring in control animals (Devereux *et al.*, 1991), and there is evidence for chemical specificity in the pattern of mutations. The specific types of oncogene-activating mutations induced by a chemical carcinogen often agree with what is expected based on the DNA adducts formed by the agent (Devereux *et al.*, 1993a). Even for “nongenotoxic carcinogens,” patterns of *ras* gene mutations in neoplasms can give clues about the mechanism of tumorigenesis (Devereux *et al.*, 1993b).

## MATERIALS AND METHODS

**Lung neoplasms:** Male and female B6C3F<sub>1</sub> mice were exposed to 0, 0.3, 1.0, or 3.0 mg/m<sup>3</sup> cobalt sulfate heptahydrate by inhalation for 6 hours per day, 5 days per week for 2 years. At necropsy, lung neoplasms were fixed in 10% neutral buffered formalin, routinely processed, embedded in paraffin, sectioned to a thickness of 5 µm, and stained with hematoxylin and eosin. Subsequently, six unstained serial sections (10 µm thick) were prepared from paraffin blocks containing alveolar/bronchiolar adenomas or carcinomas for isolation of DNA for polymerase chain reaction (PCR)-based assays. In order to isolate adequate amounts of DNA, lung neoplasms greater than 1 mm in diameter were identified for analysis. A total of 32 paraffin-embedded neoplasms were examined for genetic alterations in the *K-ras* gene. This included 26 neoplasms from cobalt sulfate heptahydrate-exposed mice and six neoplasms from control mice.

**DNA isolation:** The DNA isolation procedure is described in Marmur (1961) and Sills *et al.* (1995). The paraffin-embedded tissue was deparaffinized and rehydrated before digesting with proteinase K (Wright and Manos, 1990). The neoplasm tissue was digested with 10 mg/mL pronase in 1% sodium dodecyl sulfate in TNE buffer (10 mM Tris; 150 mM NaCl; and 2 mM EDTA disodium salt, pH 7.5). DNA was extracted with phenol and chloroform and precipitated with ethanol. DNA was quantified by optical density at 260 nm, and 200 ng/µL was used for amplification.

**DNA amplification:** DNA was amplified by PCR (Saiki *et al.*, 1988; Sills *et al.*, 1995); details of the use of nested primers are described in Devereux *et al.* (1991, 1993b).

**Restriction fragment length polymorphic identification:** For identification of *K-ras* mutations at codon 61, restriction fragment length polymorphism (RFLP) was used, and most of exon 2 surrounding codon 61 was amplified (Sukumuar and Barbacid, 1990). The sense primer used for amplification of exon 2 was 5'-GACATCTTAGACACAGCAGTT-3'. A restriction site for XbaI or TaqI enzyme (New England Biolaboratory, Beverly, MA) is created by the presence of an A to T or A to G mutation in the second base of codon 61. By using this technique, codon 61 CTA and CGA mutations were detected by XbaI and TaqI digestion, respectively; the normal sequence (CAA) of codon 61 is not cut by these enzymes. The reaction

was incubated at 37° C (XbaI) or 60° C (TaqI) for 2 hours. Fifteen  $\mu\text{L}$  of the mixture with bromophenol blue dye was loaded onto the 6% acrylamide tris-borate-EDTA (TBE) gel ( $8 \times 8 \text{ cm} \times 1 \text{ mm}$ ; 15 wells) (Novex, San Diego, CA). The gel was run at 100 volts for 1 hour on the Novex gel electrophoresis unit. Gels were stained with a 5  $\mu\text{g}/\text{mL}$  solution of ethidium bromide for 20 minutes and then destained in distilled water. Ethidium bromide-stained bands were visualized using a 312 nm ultraviolet viewing box and photographed.

*“Cold” single-strand conformation polymorphism analysis (SSCP):* A mixture consisting of 5  $\mu\text{L}$  of PCR product (double-stranded DNA), 0.6  $\mu\text{L}$  of 1M methylmercury hydroxide, 1  $\mu\text{L}$  of 15% W/V Ficoll (molecular weight 400,000) loading buffer containing 0.25% bromophenol blue and 0.25% xylene cyanol, and 13.4  $\mu\text{L}$  of 1X TBE buffer (Novex, San Diego, CA) was prepared to yield a total volume of 20  $\mu\text{L}$ . This nonradioactive mixture was heated to 85° C for 5 minutes and then plunged into ice prior to loading the entire 20  $\mu\text{L}$  into the gel. A 20% polyacrylamide TBE gel was used for K-ras with the matching gel electrophoresis unit (Novex, San Diego, CA). The buffer chamber was filled with 1.5X TBE buffer. The gel was run at 300 volts in a 5° C cold room until a light blue marker reached the bottom of the gel. A positive control for K-ras mutations and one undenatured DNA control (without methylmercury hydroxide and no heat) were run with unknown samples. Gels were stained with a 0.5  $\mu\text{g}/\text{mL}$  solution of ethidium bromide for 20 minutes and destained in distilled water for 5 minutes. The stained bands were visualized under a UV viewing box and photographed. For identification of K-ras mutation at codon 61, RFLP was used with XbaI enzyme digestion and “cold” SSCP analysis was performed on the same 20% gel.

*Direct sequencing:* Direct sequencing of the amplified first and second exon of the K-ras gene was performed as described by Tindall and Stankowski (1989) using previously described sequencing primers (Devereux *et al.*, 1991).

## RESULTS

In order to determine if the cobalt sulfate heptahydrate-induced neoplasms contained a K-ras mutation profile similar to that observed with “spontaneous” neoplasms, sample groups of six neoplasms consisting of adenomas and carcinomas from the chamber control, seven, eight, and 11 neoplasms from the 0.3  $\text{mg}/\text{m}^3$ , 1.0, and 3.0  $\text{mg}/\text{m}^3$  dose groups, respectively, were evaluated by PCR amplification of K-ras exon 1 or K-ras exon 2 followed by RFLP for the two codon 61 mutations CTA and CGA in the B6C3F<sub>1</sub> mouse (Table I1). SSCP was used as an alternative screening method for detection of K-ras mutations in DNA, and mutations were confirmed by direct sequencing. Mutation spectra in codons 12, 13, and 61 of the K-ras gene had some similarity to those identified in spontaneous lung neoplasms.

Of the K-ras mutations detected, a higher frequency (5/9, 55%) of G to T transversions was detected at the second base of codon 12 compared to 0/1 (0%) for chamber controls or 1/24 (4%) for NTP historical controls. K-ras codon 61 CTA or CGA mutations were not present in the cobalt sulfate heptahydrate-induced lung neoplasms. A trend toward a dose-response relationship in the frequency of K-ras mutations was observed in cobalt sulfate heptahydrate-induced lung neoplasms: 14% versus 38% versus 45% for the 0.3, 1.0, and 3.0  $\text{mg}/\text{m}^3$  doses, respectively (Table I2). There were generally no differences in the mutation frequency or spectra between benign and malignant lung neoplasms (data not shown).

## DISCUSSION

In examining the K-ras mutations detected in the cobalt sulfate heptahydrate study, a higher frequency (5/9, 55%) of G to T transversions was detected at codon 12 compared to 0/1 (0%) for chamber controls or 1/24 (4%) for historical controls. These findings are consistent with the work of Zeiger *et al.* (1992), in which cobalt sulfate heptahydrate showed a weakly positive response in *Salmonella typhimurium* strain TA100 in the absence of exogenous metabolic activation as well as with hamster or rat liver S9.

The higher number of G to T transversions at codon 12 is supportive evidence that cobalt sulfate heptahydrate may indirectly damage DNA by oxidative stress. GGT to GTT mutations appear to be infrequent in spontaneous lung neoplasms from B6C3F<sub>1</sub> mice (Table I1). However, G to T transversions are commonly detected DNA base changes associated with active oxygen species and are consistent with 8-OH-G adducts produced during oxidative damage to DNA (Tchou *et al.*, 1991; Shigenaga and Ames, 1991; Janssen *et al.*, 1993). 8-OH-G is a suspect lesion in the formation of both spontaneous cancers and those induced by various agents such as 4-nitroquinoline oxide, ionizing radiation, KBrO<sub>3</sub>, and 2-nitropropane (Floyd, 1990; Foley *et al.*, 1993). Thus, cobalt sulfate heptahydrate exposure in B6C3F<sub>1</sub> mice may have resulted in the generation of hydroxyl radicals that could have enhanced G to T transversions at the second base of codon 12. Consistent with these findings is the work of Shi *et al.* (1993), in which cobalt sulfate heptahydrate was shown to catalyze the production of oxygen-based free radicals.

The observation of similar frequencies and spectra of mutations in cobalt sulfate heptahydrate-induced alveolar/bronchiolar adenomas and carcinomas is consistent with other studies showing that *K-ras* activation occurs early and may be an initiating event in murine lung carcinogenesis (Sills *et al.*, 1995). If mutations in the *K-ras* gene occurred later, an increased frequency of *K-ras* mutations would be expected in carcinomas. In B6C3F<sub>1</sub> mice exposed to cobalt sulfate heptahydrate, specific *K-ras* mutations did not correlate with specific morphological patterns or sizes of lung neoplasms, a finding supported by Ohmori (1992).

**REFERENCES**

- Devereux, T.R., Anderson, M.W., and Belinsky, S.A. (1991). Role of *ras* protooncogene activation in the formation of spontaneous and nitrosamine-induced lung tumors in the resistant C3H mouse. *Carcinogenesis* **12**, 299-303.
- Devereux, T.R., Belinsky, S.A., Maronpot, R.R., White, C., Hegi, M., Patel, A.C., Foley, J.F., Greenwell, A., and Anderson, M.W. (1993a). Comparison of pulmonary O6-methylguanine DNA adduct levels and *K-ras* activation in lung tumors from resistant and susceptible mouse strains. *Mol. Carcinog.* **8**, 177-185.
- Devereux, T.R., Foley, J.F., Maronpot, R.R., Kari, F., and Anderson, M.W. (1993b). *Ras* proto-oncogene activation in liver and lung tumors from B6C3F<sub>1</sub> mice exposed chronically to methylene chloride. *Carcinogenesis* **14**, 795-801.
- Floyd, R.A. (1990). Role of oxygen free radicals in carcinogenesis and brain ischemia. *FASEB J.* **4**, 2587-2597.
- Foley, J., Ton, T., Maronpot, R., Butterworth, B., and Goldsworthy, T.L. (1993). Comparison of proliferating cell nuclear antigen to tritiated thymidine as a marker of proliferating hepatocytes in rats. *Environ. Health Perspect.* **101**, 199-206.
- Janssen, Y.M.W., Van Houten, B., Borm, P.J.A., and Mossman, B.T. (1993). Cell and tissue responses to oxidative damage. *Lab. Invest.* **69**, 261-274.
- Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J. Mol. Biol.* **3**, 208-218.
- Ohmori, H. (1992). Comparison of *K-ras* gene mutation among simultaneously occurring multiple urethane-induced lung tumors in individual mice. *Carcinogenesis* **13**, 851-855.
- Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich, H.A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**, 487-491.
- Shi, X., Dalal, N.S., and Kasprzak, K.S. (1993). Generation of free radicals from model lipid hydroperoxides and H<sub>2</sub>O<sub>2</sub> by Co(II) in the presence of cysteinyl and histidyl chelators. *Chem. Res. Toxicol.* **6**, 277-283.
- Shigenaga, M.K., and Ames, B.N. (1991). Assays for 8-hydroxy-2'-deoxyguanosine: A biomarker of in vivo oxidative DNA damage. *Free Radic. Biol. Med.* **10**, 211-216.
- Sills, R.C., Hong, H.L., Greenwell, A., Herbert, R.A., Boorman, G.A., and Devereux, T.R. (1995). Increased frequency of *K-ras* mutations in lung neoplasms from female B6C3F<sub>1</sub> mice exposed to ozone for 24 or 30 months. *Carcinogenesis* **16**, 1623-1628.
- Sukumuar, S., and Barbacid, M. (1990). Specific patterns of oncogene activation in transplacentally induced tumors. *Proc. Natl. Acad. Sci. USA* **87**, 718-722.

Tchou, J., Kasai, H., Shibutani, S., Chung, M.H., Laval, J., Grollamm, A.P., and Nishimura, S. (1991). 8-Oxoguanine (8-hydroxyguanine) DNA glycosylase and its substrate specificity. *Proc. Natl. Acad. Sci. USA* **88**, 4690-4694.

Tindall, K.R., and Stankowski, L.F., Jr. (1989). Molecular analysis of spontaneous mutations at the *gpt* locus in Chinese hamster ovary (AS52) cells. *Mutat. Res.* **220**, 241-253.

Wright, D.K., and Manos, M.M. (1990). Sample preparation from paraffin-embedded tissues. In *PCR Protocols: A Guide to Methods and Applications* (M.A. Innis, D.H. Gelfand, J.J. Sninsky, and T.J. White, Eds.), pp. 153-158. Academic Press, San Diego, CA.

Zeiger, E., Anderson, B., Haworth, S., Lawlor, T., and Mortelmans, K. (1992). Salmonella mutagenicity tests: V. Results from the testing of 311 chemicals. *Environ. Mol. Mutagen.* **19** (Suppl. 21), 2-141.

**TABLE I1**  
**K-ras Mutations in Lung Neoplasms of B6C3F<sub>1</sub> Mice**

Treatment	Activated K-ras (%)	Codon 12						Codon 13		Codon 61			
		GTT	GAT	TGT	CGT	CTT	ATT	CGC	GAC	CTA	CAT	CAC	CGA
Control, Historical	24/75 (33%)	1	9	4	0	0	0	3	0	0	4	1	2
Control, Chamber	1/6 (17%)	0	0	1	0	0	0	0	0	0	0	0	0
Cobalt Sulfate Heptahydrate <sup>a</sup>	9/26 (35%)	5	2	1	0	0	0	1	0	0	0	0	0
Ozone	19/27 (70%)	5	3	2	0	0	0	0	1	8	0	0	0
1,3-Butadiene	6/9 (67%)	0	0	0	0	0	0	6	0	0	0	0	0
Tetranitromethane	10/10 (100%)	0	10	0	0	0	0	0	0	0	0	0	0
Methylene Chloride	11/54 (20%)	1	1	1	0	0	0	1	0	1	1	4	1

<sup>a</sup> One animal had two neoplasms of the same type and mutation (GTT) which were counted as one neoplasm. If counted as two neoplasms, the activated K-ras would be 10/27 (37%), and there would be 6 codon 12 GTT mutations.

**TABLE I2**  
**K-ras Mutation Profile in Lung Neoplasms of B6C3F<sub>1</sub> Mice**

Treatment Concentration (mg/m <sup>3</sup> )	Activated K-ras (%)	Codon 12						Codon 13		Codon 61			
		GTT	GAT	TGT	CGT	CTT	ATT	CGC	GAC	CTA	CAT	CAC	CGA
<b>Chamber and Historical Control</b>													
	25/81 (31%)	1 (1%)	9 (11%)	5 (6%)	0	0	0	3 (4%)		0	4 (5%)	1 (1%)	2 (2%)
<b>Cobalt Sulfate Heptahydrate</b>													
0.3	1/7 (14%)	1 (14%)	0	0	0	0	0	0		0	0	0	0
1.0	3/8 (38%)	2 (25%)	0	0	0	0	0	1 (13%)		0	0	0	0
3.0	5/11 (45%)	2 (18%)	2 (18%)	1 (9%)	0	0	0	0		0	0	0	0
Total <sup>a</sup>	9/26 (35%)	5 (19%)	2 (8%)	1 (4%)	0	0	0	1 (4%)		0	0	0	0

<sup>a</sup> One animal had two neoplasms of the same type and mutation (GTT) which were counted as one neoplasm. If counted as two neoplasms, the activated K-ras would be 10/27 (37%), and there would be 6 codon 12 GTT mutations.

# APPENDIX J

## IMPACT OF *HELICOBACTER HEPATICUS* INFECTION IN B6C3F<sub>1</sub> MICE FROM 12 NTP 2-YEAR CARCINOGENESIS STUDIES

James R. Hailey<sup>1</sup>, Joseph K. Haseman<sup>1</sup>, John R. Bucher<sup>1</sup>, Ann E. Radovsky<sup>1</sup>,  
David E. Malarkey<sup>2</sup>, Richard T. Miller<sup>2</sup>, Abraham Nyska<sup>1</sup>, and Robert R. Maronpot<sup>1</sup>

<sup>1</sup>National Institute of Environmental Health Sciences  
Research Triangle Park, North Carolina

<sup>2</sup>Department of Microbiology, Pathology, and Parasitology  
College of Veterinary Medicine  
North Carolina State University  
Raleigh, North Carolina

<b>ABSTRACT</b>		<b>242</b>
<b>INTRODUCTION</b>		<b>242</b>
<b>MATERIALS AND METHODS</b>		<b>243</b>
<b>RESULTS AND DISCUSSION</b>		<b>245</b>
<b>REFERENCES</b>		<b>252</b>
<b>TABLE J1</b>	<b>Incidence of <i>Helicobacter hepaticus</i>-Associated Hepatitis in Control B6C3F<sub>1</sub> Mice from Nine NTP 2-Year Studies</b>	<b>256</b>
<b>TABLE J2</b>	<b>Identification of <i>Helicobacter hepaticus</i> with PCR-RFLP-Based Assays in Control B6C3F<sub>1</sub> Mice from 32 NTP 2-Year Studies and Three NTP 13-Week Studies</b>	<b>256</b>
<b>TABLE J3</b>	<b>Comparison of Neoplasm Incidences in Control B6C3F<sub>1</sub> Mice from <i>Helicobacter hepaticus</i>-Affected and Unaffected NTP 2-Year Studies</b>	<b>257</b>
<b>TABLE J4</b>	<b>Liver Neoplasm Incidences and Body Weights of Control B6C3F<sub>1</sub> Mice in Relation to Study Start Dates of <i>Helicobacter hepaticus</i>-Affected and Unaffected NTP 2-Year Studies</b>	<b>258</b>
<b>TABLE J5</b>	<b>Association of Liver Neoplasm Incidence and Severity of <i>Helicobacter hepaticus</i>-Associated Hepatitis in Control B6C3F<sub>1</sub> Mice from Nine Affected NTP 2-Year Studies</b>	<b>259</b>
<b>TABLE J6</b>	<b>H-<i>ras</i> Codon 61 AAA Mutations in Spontaneous Liver Neoplasms in Control B6C3F<sub>1</sub> Mice from <i>Helicobacter hepaticus</i>-Affected and Unaffected NTP 2-Year Studies</b>	<b>259</b>
<b>TABLE J7</b>	<b>Proliferating Cell Nuclear Antigen Labeling Indices in the Liver of Control B6C3F<sub>1</sub> Mice</b>	<b>260</b>
<b>TABLE J8</b>	<b>Summary of Target Sites of Carcinogenicity in B6C3F<sub>1</sub> Mice from NTP 2-Year Studies with <i>Helicobacter hepaticus</i>-Associated Hepatitis</b>	<b>261</b>

# IMPACT OF *HELICOBACTER HEPATICUS* INFECTION IN B6C3F<sub>1</sub> MICE FROM 12 NTP 2-YEAR CARCINOGENESIS STUDIES

## ABSTRACT

Male and female B6C3F<sub>1</sub> mice from 12 NTP 2-year carcinogenesis studies were found to be infected with *Helicobacter hepaticus*. Many of the male mice from nine of these studies ("affected" studies) had an associated hepatitis. The current evaluations were performed in an attempt to determine if the data from the *H. hepaticus*-affected NTP B6C3F<sub>1</sub> mouse studies were compromised and unsuitable for cancer hazard identification. The incidences of neoplasms of the liver (both hepatocellular neoplasms and hemangiosarcoma), but not of other organs in control male B6C3F<sub>1</sub> mice, were found to be increased in affected studies compared to control males from unaffected studies. The increased incidence of hepatocellular neoplasms was observed in those males exhibiting *H. hepaticus*-associated hepatitis. Other observations further differentiated control male mice from affected and unaffected studies. *H-ras* codon 61 CAA-to-AAA mutations were less common in liver neoplasms in males from affected studies compared to historical and unaffected study controls. In addition, increases in cell proliferation rates and apoptosis were observed in the livers of male mice with *H. hepaticus*-associated hepatitis. These data support the hypothesis that the increased incidence of liver neoplasms is associated with *H. hepaticus* and that hepatitis may be important in the pathogenesis. Therefore, interpretation of carcinogenic effects in the liver of B6C3F<sub>1</sub> mice may be confounded if there is *H. hepaticus*-associated hepatitis.

## INTRODUCTION

### *Helicobacter*-Induced Diseases

Since the bacterium *H. pylori* was isolated from humans in 1983, numerous *Helicobacter* species have been identified in several laboratory and domestic animal species. Their pathogenicity varies, with some species inducing significant disease while others appear merely to colonize the gastrointestinal tract. *H. pylori* is known to cause chronic gastritis and peptic ulcers in humans (Marshall and Warren, 1984; Graham, 1989; Lee *et al.*, 1993) and, more recently, has been linked to adenocarcinoma and mucosa-associated lymphoma of the stomach (Fox *et al.*, 1989; Nomura *et al.*, 1991; Parsonnet *et al.*, 1991; Wotherspoon *et al.*, 1993). Based on epidemiological and pathology findings, the International Agency for Research on Cancer (1994) has classified *H. pylori* as a group 1 carcinogen in humans. *H. hepaticus* is associated with an increase in liver neoplasm incidences in A/JCr mice (Ward *et al.*, 1994a; Fox *et al.*, 1996).

*H. hepaticus* commonly colonizes the gastrointestinal tract of many strains of mice from many sources (Fox *et al.*, 1994; Ward *et al.*, 1994b; Shames *et al.*, 1995). It has been shown to be pathogenic, with hepatitis highly prevalent in some strains of mice (A/JCr, BALB/cAnNCr, C3H/HeNCr, SJL/NCr, and SCID/NCr) (Ward *et al.*, 1994b). Intestinal colonization does not necessarily result in subsequent hepatitis, and the conditions that lead to migration of the organism from the intestine to the liver have not been determined. *H. hepaticus* appears to reside primarily within the bile canaliculi. Male mice were reported to have a greater incidence and severity of hepatitis than female mice, and this finding occurred in NTP studies as well. The recently identified *H. bilis*, like *H. hepaticus*, colonizes the biliary tract, liver, and intestine of mice. While *H. bilis* has been identified in animals with chronic hepatitis, whether it caused the hepatitis is not known (Fox *et al.*, 1995).

The pathogenesis of *H. hepaticus*-induced disease has not been fully characterized. In susceptible strains of mice, *H. hepaticus* can cause acute, focal, nonsuppurative, necrotizing hepatitis, which progresses to chronic, active hepatitis characterized by minimal necrosis, hepatocytomegaly, oval cell hyperplasia, and

cholangitis. *H. hepaticus* has been found to possess high levels of urease (Fox *et al.*, 1994). *H. hepaticus* is often isolated from the cecum and colon but is not necessarily isolated from the liver of A/JCr mice, even though these animals develop severe hepatitis. Culture supernatants from several strains of *H. hepaticus* and several other *Helicobacter* species were shown to cause cytopathic effects in a rodent hepatocyte cell line (Taylor *et al.*, 1995). Ward *et al.* (1996) suggested that autoimmunity may play a role in the progressive hepatitis and carcinogenesis in livers infected with *H. hepaticus*.

### **NTP Infectious Disease Surveillance**

In 1993, during the histological evaluation of an NTP 2-year study, pathologists identified a constellation of liver lesions (hepatitis) in control and treated male mice that was consistent with what would later be described in mice infected with *H. hepaticus* (Ward *et al.*, 1993, 1994a; Fox *et al.*, 1994). Subsequently, pathology results from all mouse studies begun since 1984 (67 two-year studies) were reviewed for diagnoses of the characteristic hepatitis; the lesions were identified in nine studies (NTP, 1998a,b,c,d,e,f). Silver stains revealed helical bacteria consistent with *Helicobacter* present in the liver of male mice in the nine studies.

Every reasonable measure is taken to prevent the occurrence of infectious diseases during NTP 2-year carcinogenicity studies. When infections occasionally occur, care is taken to identify the causal agent and its source, measures are taken to ensure that animals in later studies will not be infected, and the potential impact on biological parameters (primarily neoplastic endpoints) important in interpretation of the study is determined. To date, animals (control and treated) from a few studies have had a mild pulmonary inflammatory response presumed to be caused by an infectious agent. In other studies, there have been utero-ovarian infections with *Klebsiella* sp. (Rao *et al.*, 1987) and fungal infections of the nasal cavity. For scientifically valid reasons, interpretation of chemical-related effects was not considered significantly compromised in any of these studies. Unlike the previous infections, *H. hepaticus* involves the liver, the major metabolic organ, and has been associated with an increase in incidences of liver neoplasms in the A/JCr mouse (Ward *et al.*, 1994a). Therefore, when the contemporary epizootic of *H. hepaticus* infection in the United States affected several NTP studies, use of the data for hazard identification was questioned. The first step was to determine the extent of the infection within NTP studies and then evaluate the impact the infection had on biological parameters important in interpretation of the carcinogenic potential of test chemicals.

## **MATERIALS AND METHODS**

### **Histologic Examination**

Studies in which mice were potentially infected with *H. hepaticus* were identified by reviewing the summary pathology tables for characteristic diagnoses: oval and/or biliary epithelial hyperplasia, hepatocyte enlargement (often diagnosed as karyomegaly), chronic inflammation, and regenerative hyperplasia. All 13-week and 2-year studies begun by the NTP since 1984 and for which complete pathology data were available (67 two-year studies) were examined. Eight contemporary studies in which the characteristic lesions were not identified from pathology tables were randomly selected for histologic reevaluation. Slides containing sections of hematoxylin- and eosin-stained livers from 20 to 25 control and 20 to 25 high-dose male mice from each of seven 2-year studies and one 13-week study (10 animals from each group) were reexamined microscopically for the presence of hepatitis potentially related to *H. hepaticus* infection. Hepatitis consistent with that observed with *H. hepaticus* infection was not observed in any of these studies.

Liver sections from five or more animals from each of nine 2-year studies in which hepatitis was observed were prepared using the Warthin-Starry silver stain or Steiner's modification to identify silver-positive helical bacteria.

### PCR-RFLP Detection of *Helicobacter* DNA

Assays based on polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) were conducted at the NIEHS (Malarkey *et al.*, 1997) and the University of Missouri Research Animal Diagnostic and Investigative Laboratory (MU-RADIL) (Riley *et al.*, 1996) on liver tissue from approximately 20 animals from each of 32 NTP 2-year studies (including the nine affected studies) and three NTP 13-week studies. The majority of these studies were selected because they were begun at approximately the same time (1988-1990) as the nine affected studies. Also, two earlier studies (1984-1985; mouse life-span and *p*-nitroaniline studies) and one later study (1993; methyleugenol) were selected. The mouse life-span study was designed to evaluate the incidences of spontaneous changes associated with age; therefore, there is no NTP Technical Report. Pathology peer review is not complete for the methyleugenol study, and the NTP Technical Report (NTP, 1998g) has not been completed. Frozen tissue was available from 22 of these studies, while only formalin-fixed tissue was available for the remaining ten 2-year studies and the three 13-week studies. Most of the assays were conducted by MU-RADIL, which used *Helicobacter* genus-specific primers; MU-RADIL used restriction endonucleases on a subset of positives to determine if the species was *H. hepaticus*. DNA was isolated from frozen liver samples with a QIAamp Tissue Kit (Qiagen Inc., Chatsworth, CA) according to the manufacturer's recommendations or routine phenol/chloroform extraction (Malarkey *et al.*, 1997). DNA content and purity were determined spectrophotometrically by measuring the  $A_{260}/A_{280}$  optical density ratio. To isolate DNA from paraffin-embedded samples, five 10- $\mu$ m sections were washed twice with 1 mL xylene and twice with 500  $\mu$ L ethanol. Tissues were then dried within a vacuum centrifuge prior to DNA isolation as described above. Routine measures were taken to avoid contamination at every step from tissue collection to PCR amplification, and concurrently run controls without DNA were consistently negative.

### Statistical Analyses

Multiple regression procedures were used to compare control neoplasm rates in the nine affected studies with the 26 unaffected contemporary studies which had no histologic evidence of *H. hepaticus*-associated liver disease. While frozen liver tissue was unavailable from 13 of these 26 studies, none showed the hepatitis indicative of *H. hepaticus* and thus were assumed to be unaffected. Potential confounding factors such as body weight, date study was begun, route of administration, and animal supplier were included as covariables in the statistical analysis.

### Analysis for H-ras Codon 61 CAA-to-AAA Mutations

For analyses of formalin-fixed tissue, three to five unstained serial sections (10  $\mu$ m thick) were cut from paraffin blocks containing hepatocellular adenomas or carcinomas. Paraffin-embedded tissues were deparaffinized and rehydrated prior to being digested with proteinase k overnight at 55° C to isolate DNA. Frozen tissues were digested with 10 mg/mL pronase in 1% sodium dodecyl sulfate in TNE buffer (10 mM TRIS, 150 mM NaCl, and 2 mM EDTA; pH 7.5) overnight at 37° C; DNA was isolated by phenol chloroform extraction and precipitated with ethanol (Marmur, 1961; Sills *et al.*, 1995).

Nested primers were used for amplification of exon 2 of H-ras by PCR. The outer primers were 5'-CCA CTA AGC CTG TTG TGT TTT GCA G-3' (forward primer) and 5'-CTG TAC TGA TGG ATG TCC TCG AAG GA-3' (reverse primer). The inner primers (second round of amplification) were 5'-GAC ATC TTA GAC ACA GCA GTT-3' (forward primer) and 5'-GGT GTT GAT GGC AAA TAC-3' (reverse primer). Although the normal sequence of codon 60 is GCT, the forward PCR primer is made with a T at the penultimate 3' base to create the restriction site for MseI.

A nonradioactive RFLP method was employed to identify CAA-to-AAA mutations in the H-ras gene at codon 61 in liver neoplasms (Lee and Drinkwater, 1995). This was based on MseI enzyme restriction cutting only the sequence 5'-TTAA-3'. Thus, MseI will detect C→A conversion mutation at the first position of codon 61.

### **Analysis of PCNA and Apoptosis**

Detailed methods are included in a report by Nyska *et al.* (1997). Cell proliferation was assessed in nonneoplastic areas of the liver, kidney, and lung by determining a PCNA S-phase labeling index (the percentage of cells in S phase). The identification of apoptotic cells was based on morphologic criteria (Garewal *et al.*, 1996; Goldsworthy *et al.*, 1996) and confirmed immunohistochemically by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) procedure (Gavrieli *et al.*, 1992).

## **RESULTS AND DISCUSSION**

### **Identification of *H. hepaticus* Infection in NTP Studies**

Determining the extent of *H. hepaticus* infection involved a three-pronged approach of histologic evaluation, silver stains, and PCR-RFLP based assays; all were necessary because of the limitations identified for each. In NTP studies, and as reported in other studies (Ward *et al.*, 1994b), there were no obvious clinical signs of infection, and the only significant histologic lesion (hepatitis) was observed in the liver, primarily in males. Therefore, summary pathology tables were reviewed to identify studies that may have been affected by *H. hepaticus*-associated hepatitis. Male mice from nine studies were identified (Table J1) as having the hepatitis. Eight of the nine studies were begun during a time span of about 6 months (July 1990 to January 1991), while the other study was begun much earlier (October 1988). The hepatitis was not observed in any 13-week studies. Use of histologic evaluation for identification of infected animals has limitations, however. It is somewhat insensitive, as *H. hepaticus* has been cultured and identified by PCR-RFLP methods within livers of animals with no histological evidence of infection (Fox *et al.*, 1998). This may be explained in part by the limited sampling (two liver sections) and the sometimes focal nature of *H. hepaticus*-associated hepatitis. Also, while in the more severely affected animals the hepatitis appears somewhat characteristic, component lesions of the hepatitis are not pathognomonic, and, when the hepatitis is subtle in 2-year old animals, it is more difficult to recognize or attribute to *H. hepaticus*.

Within affected studies, the incidences of the hepatitis in male mice varied from 16% to 78% (Table J1). While generally mild to moderate, the hepatitis varied in severity from barely detectable in some animals to extensive liver involvement and regeneration in others. Only a few females were identified as having the characteristic hepatitis (Table J1). In general, the incidences and severities of *H. hepaticus*-associated hepatitis were similar between control and treated groups. This constellation of nonneoplastic liver lesions, while not pathognomonic, was certainly suggestive of an *H. hepaticus* infection, particularly when observed in control animals. Characteristic lesions included proliferation of oval and/or biliary epithelial cells, hepatocyte enlargement (diagnosed as karyomegaly), and chronic inflammation. In many instances, areas of regenerative hyperplasia were identified within diseased liver.

*Helicobacter* spp. are not usually observed on routine histologic examination of hematoxylin and eosin-stained sections of liver. The methods for confirmation of infection with *Helicobacter* include Warthin-Starry silver stain or Steiner's modification (Garvey *et al.*, 1985) of this stain for direct microscopic observation of the organisms in tissue; however, this can be a relatively insensitive technique when few organisms are present. In most instances, histologic differentiation between *Helicobacter* species is not possible. Speciation can usually be accomplished with electron microscopy, but this technique is both time consuming and labor intensive. Microbiologic culture of feces, cecal smears, and fresh or frozen liver is also possible. Currently, assays involving amplification of the DNA of the organism using PCR are the most rapid and perhaps the most sensitive methods of detection, and the use of restriction endonucleases has allowed a determination of the species present. PCR-based methods also can be used on feces, cecal contents, or liver homogenates and are most sensitive when using fresh or frozen tissue (Riley *et al.*, 1996; Malarkey *et al.*, 1997).

Using Warthin-Starry silver stains or Steiner's modification on the livers of five or more animals per study, helical bacteria (*Helicobacter*) were identified in animals from the nine affected studies. In some animals, helical bacteria were numerous, suggesting a heavy bacterial burden in these infected animals. However, even in these animals with abundant organisms, few to none were observed in proliferative hepatic lesions such as foci and neoplasms. Helical bacteria were not identified in approximately 25% of males with moderate hepatitis and were rarely identified in males without hepatitis or in females. The absence of identification of helical organisms by silver stains does not preclude infection, nor does the presence of organisms confirm *H. hepaticus*. Based upon current knowledge, however, the characteristic liver lesions in B6C3F<sub>1</sub> mice, coupled with the presence of silver-positive helical organisms, are highly suggestive of *H. hepaticus* infection.

As the NTP evaluation evolved, PCR-based assays were developed that appeared more sensitive than histologic evaluation and silver stains for identification and speciation of *Helicobacter*. Therefore, PCR-RFLP-based assays were used to confirm the presence of pathogenic *Helicobacter* (primarily *H. hepaticus*) within the nine affected studies and to determine whether there was *H. hepaticus* infection in other NTP studies. Unfortunately, none of the PCR-based assays had been specifically developed for, or proven reliable for use with, formalin-fixed tissue. Frozen tissue was available from a limited number of animals from a limited number of NTP studies, including only three of the nine affected studies. Furthermore, available frozen liver was almost always limited to tissue from a neoplasm, and, based upon results obtained with silver stains, organisms are generally not readily observed within proliferative hepatic lesions, even when organisms are abundant in adjacent liver tissue. Because the availability of frozen tissue was limited, a PCR-RFLP-based assay was developed and evaluated (Malarkey *et al.*, 1997) for use with frozen or formalin-fixed tissue.

The NIEHS and MU-RADIL laboratories conducted PCR-RFLP-based assays on 32 NTP 2-year studies and three NTP 13-week studies (data not shown); frozen tissues from 22 of the 2-year studies were available. All three bioassays in which hepatitis was identified and for which frozen tissue was available were positive for *H. hepaticus* by the PCR-RFLP-based assays (Table J2). At a third laboratory, *H. hepaticus* was also cultured from the liver tissue of animals in one of these studies (Fox *et al.*, 1998). Formalin-fixed tissues from two of the three studies were evaluated and were also positive; these tissues had been fixed in formalin for less than 48 hours. In the other six affected studies, for which only formalin-fixed tissue was available, *H. hepaticus* was identified in only 1 of 120 animals (Table J2). This decreased sensitivity was considered to be related to the prolonged formalin fixation (Malarkey *et al.*, 1997) rather than proof of an absence of *H. hepaticus*. The presence or absence of *H. hepaticus* apparently cannot be confirmed with current PCR-RFLP-based assays in liver that has been fixed in formalin for long periods (weeks or months). In the three 13-week studies with formalin-fixed tissue, only 1 of 30 animals was positive for *H. hepaticus*.

Within the three affected, PCR-RFLP-positive 2-year studies, *H. hepaticus* was often identified by PCR in frozen livers of mice that had no overt hepatitis. In fact, based upon the combined data from two studies (including PCR results from three laboratories), of 57 animals without characteristic liver lesions, 13 of 24 male mice (54%) and 17 of 33 female mice (52%) were positive for *H. hepaticus*. Furthermore, *H. hepaticus* was identified by PCR in frozen liver of several animals from three "unaffected" studies in which hepatitis typical of that associated with *H. hepaticus* was not observed (Table J2). Apparent variability occurs between various strains of mice and between individual mice from affected studies in developing hepatitis in response to *H. hepaticus* infection. One would assume that, within affected studies, most or all animals have been exposed to the organism, and even animals resistant to developing hepatitis may have organisms within the liver. This assumption is supported by the fact that animals without hepatitis are often positive with PCR-RFLP-based assays. Therefore, although alternative explanations are possible, the three PCR-RFLP-positive studies in which liver lesions are absent are assumed to be true positives. In fact, helical organisms were identified with a silver stain in one animal from one of these studies (Malarkey *et al.*, 1997). Therefore, in addition to assessing the affect of *H. hepaticus* in the nine affected 2-year

studies, the significance of a positive PCR-RFLP assay for *H. hepaticus* in the absence of liver lesions is also an important question.

### **Inconsistent Results with PCR-Based Methods**

As with any technique, the PCR-RFLP-based assays have limitations even when used to assay fresh and frozen tissue. One assessment of the variability in results of PCR and serologic analyses for *Helicobacter* among three commercial laboratories revealed significant inconsistencies (Dew *et al.*, 1997). Others (J.M. Ward and J. Thigpen, personal communications) have obtained similarly inconsistent results when sending replicate samples to different laboratories. Though the number of samples evaluated by both the NIEHS and MU-RADIL laboratories was limited, there was good, but not complete, correlation of PCR-RFLP results. Also, within the affected studies, the PCR assays were not positive in some animals with liver disease. This result may be explained, in part, by the fact that the only frozen tissues available were neoplasms; as described above, neoplasms are expected to have fewer organisms.

### **Analysis of *H. hepaticus*-Affected and Unaffected Studies for Incidence of Common Neoplasms**

To determine whether the incidences of various neoplasms were different between control groups from affected and unaffected studies, the nine affected studies were compared to 26 unaffected studies begun at relatively similar times (Table J3). There were no statistically significant differences in body weight or survival among the affected and unaffected studies. The neoplasms evaluated represent those that occurred at high enough incidences in various organs for statistically significant differences to be detected. Using multiple regression procedures, male mice in the nine affected studies were demonstrated to have a significantly ( $P < 0.05$ ) increased incidence of only two neoplasm types, both of which were in the liver (hepatocellular neoplasms and hemangiosarcoma), when compared to the unaffected studies. Because of these differences, there was also a corresponding significant difference in the overall incidence of malignant neoplasms (all sites) as well as in the overall proportion of neoplasm-bearing animals. No other tissue site showed a significant difference in the incidence of neoplasms. For female mice, the slightly increased incidence of hepatocellular neoplasms observed in the affected studies was not statistically significant.

This seemingly simple analysis is complicated by several potential confounding variables. There have been coordinate, time-related increases in body weight and in the incidence of liver neoplasms in mice in NTP studies (Haseman, 1992). Table J4 presents the liver neoplasm incidences in relation to the dates the studies began and clearly shows the increases in liver neoplasm incidences and body weights (Seilkop, 1995). In assessing differences in neoplasm incidences between *H. hepaticus*-affected and unaffected studies, the most relevant comparison would be between studies begun at approximately the same time. The starts of 20 of the 26 unaffected studies were clustered near the early part of the time frame (April 1988 to June 1990), while the starts of the affected studies were clustered toward the later end, with eight of the nine studies begun between July 1990 and January 1991; incidences of liver neoplasms in these later studies are expected to be higher based on trends in body weight alone. While the slightly increased incidences of liver neoplasms observed in female control mice in the nine affected studies is likely due to clustering in time, clearly, this alone cannot account for the increased liver neoplasm incidences observed in control male mice in the affected studies (Table J3).

Ideally, unaffected studies used in the above comparison should not only be free of histologic evidence of infection with *H. hepaticus* but should be confirmed as negative by PCR assays. Thirteen of these 26 studies could not be confirmed as negative by PCR because frozen tissue was not available; however, *H. hepaticus*-associated hepatitis was not present in any of the 26 studies. Because these and other data reported to date suggest that hepatitis is associated with neoplasm development in the liver, it seems reasonable to include those 13 studies, unconfirmed by PCR, in this analysis. The majority of the 13 studies confirmed as negative by PCR were begun much earlier than the clearly affected studies, and, therefore, comparing them alone to the nine affected studies is not reasonable. Although not presented here, a number

of comparisons were made with various groupings of studies based on the degree of confidence in their infection status. Although the outcomes of the various comparisons varied somewhat, incidences of hepatocellular neoplasms and hemangiosarcomas of the liver were consistently increased in control male mice from affected studies compared to control males from unaffected studies. Significantly increased liver neoplasm incidences generally were not observed in females. Importantly, the following data corroborate the findings and association with *H. hepaticus* identified in these analyses.

### **Analysis of Hepatitis-Positive and Hepatitis-Negative Mice for Liver Neoplasm Incidence**

Several infectious agents known to be associated with increased incidences of neoplasms cause chronic inflammation in the target tissue or organ. It is commonly hypothesized that this inflammatory process may cause or contribute to the development of neoplasms. One approach to address this was to stratify the mice from the affected studies according to the severity of hepatitis and examine liver neoplasm incidences in relation to these groupings. Thus, animals within the nine affected studies were placed into three groups: 1) animals with mild to moderate hepatitis considered related to *H. hepaticus* infection (+), 2) animals with minimal to mild hepatitis that may have been associated with *H. hepaticus* ( $\pm$ ), and 3) animals with no hepatitis that was considered to be associated with *H. hepaticus* (-). Within these groupings, the incidence of liver neoplasms was significantly increased ( $P < 0.05$ ) in males with mild to moderate *H. hepaticus*-associated hepatitis (+) when compared to animals without such hepatitis (Table J5). The neoplasm incidence in animals with minimal lesions ( $\pm$ ) was also increased. The liver neoplasm incidence in males without hepatitis (58%) was similar to the incidence (54.8%) in males from the 26 unaffected studies (Table J3). This analysis clearly suggests an association of *H. hepaticus*-associated hepatitis with increased liver neoplasm incidences. Females showed a similar trend, albeit not significant; however, these comparisons are weak because of the low numbers of females with hepatitis.

### **Analysis of H-ras Oncogene Mutations in Liver Neoplasms in Mice from Affected and Unaffected Studies**

Liver neoplasms commonly occur in control B6C3F<sub>1</sub> mice in 2-year studies. In the historical database of 333 male and female mice with liver neoplasms, 106 (32%) had H-ras codon 61 CAA-to-AAA mutations (Maronpot *et al.*, 1995). This historical control database is composed primarily of male data; however, adequate numbers of females have been assayed, and there was no significant difference in the incidences of CAA-to-AAA mutations between males and females.

In an attempt to examine further whether *H. hepaticus* infection had an effect on the development of hepatocellular neoplasms, neoplasms from control male mice from selected affected (NTP, 1998a,b,c) and unaffected (NTP, 1993, 1998h) studies were evaluated for H-ras codon 61 CAA-to-AAA mutations (Table J6). Only 6% (2/33) of the hepatocellular neoplasms from control males with hepatitis from three affected studies had this mutation. This percentage is significantly ( $P < 0.01$ ) less than the 32% (11/34) observed in males from the two unaffected studies and less than the 32% (106/333) that occurred in historical control animals. In addition, neoplasms from males without hepatitis from the affected, PCR-positive triethanolamine study (NTP, 1998a) and the unaffected, PCR-positive methyleugenol study (NTP, 1998g) were evaluated; the incidences of mutations in those groups were 3/14 (21%) and 2/17 (12%), respectively.

Neoplasms from control female mice (none had hepatitis) from affected and unaffected studies were evaluated for the CAA-to-AAA mutation (Table J6). The mutation rate was low in both the affected studies (1/25; 4%) and the unaffected study (1/11; 9%) when compared to the 32% observed in the historical control groups.

The finding of a different H-ras mutation profile in neoplasms of male mice from affected studies tends to support the association of increased neoplasm incidences with *H. hepaticus*, although there is no mechanistic understanding behind this observation. In a study of *H. hepaticus*-infected A/JCr mice, ras mutations were

not detected in the 25 hepatocellular neoplasms analyzed using a PCR/single-strand conformation polymorphism assay (Sipowicz *et al.*, 1997). Because of the low spontaneous rate of liver neoplasms in the A/JCr mouse, there are few or no conclusive data on *ras* mutations in uninfected animals, however. Point mutations at codons 12, 13, and 61 of the Ki-, Ha- and N-*ras* genes were not identified in 45 early gastric carcinomas in humans, whether or not *H. pylori* was present (Craanen *et al.*, 1995). If the increased incidence of hepatocellular neoplasms is associated with hepatitis, as many suspect, then one would expect the neoplasms from animals without hepatitis to have a similar mutational profile as that of the historical controls. The data do not provide a clear answer, because the hepatitis-free males from the affected triethanolamine study (NTP, 1998a) and the males from the methyleugenol study (NTP, 1998g), which were positive by PCR but lacked hepatitis, had mutation frequencies between those of the unaffected controls and the hepatitis-positive mice. Furthermore, mutations in neoplasms from females, none of which had hepatitis, from two affected and one unaffected study were very low compared to the historical controls. These findings were unexpected, and their significance is not understood.

### ***H. hepaticus*-Associated Alterations in Cell Kinetics**

Studies evaluating cell kinetics were completed to explore further the link between hepatitis and the increased incidence of liver neoplasms (Table J7; Nyska *et al.*, 1997). One of the major objectives was to determine whether there were differences between PCNA labeling indices in the livers of animals with hepatitis from three affected studies, cobalt sulfate heptahydrate, chloroprene, and triethanolamine (NTP, 1998a,b,c), compared to animals without hepatitis, whether from the same three affected studies or from an unaffected study, 1-trans-delta<sup>9</sup>-tetrahydrocannabinol (NTP, 1996). Male mice with hepatitis from the three affected studies had a significantly increased ( $P < 0.001$ ) labeling index, with a 24-fold increase over males from the unaffected study and a sixfold increase over males without hepatitis from the same three affected studies (Table J7). The labeling index increase in these mice was substantial and was considered biologically significant. Male mice without hepatitis from the three affected studies had a significantly greater labeling index (increased fourfold) than male mice from the unaffected study (Table J7). The significance of this finding is uncertain, as differences of a similar magnitude were observed in other comparisons. For example, the labeling index of females from the unaffected 1-trans-delta<sup>9</sup>-tetrahydrocannabinol study (Table J7; NTP, 1996) was increased fivefold over females from the PCR-positive, hepatitis-negative scopolamine hydrobromide trihydrate study (NTP, 1997). Such differences may be within the limits of normal variability for 2-year-old animals.

A second objective of the cell proliferation studies of the liver was to determine if labeling indices were increased in animals from the PCR-positive, hepatitis-negative methyleugenol (NTP, 1998g), scopolamine hydrobromide trihydrate (NTP, 1997), and mouse life-span studies compared to an unaffected PCR-negative and hepatitis-negative 1-trans-delta<sup>9</sup>-tetrahydrocannabinol study (NTP, 1996). The scopolamine hydrobromide trihydrate study was evaluated and included in the study by Nyska *et al.* (1997), while the methyleugenol and mouse life-span studies were completed later and are included in Table J7. The labeling indices of males from two of these three studies were almost identical to those of males from the unaffected study. However, the labeling index of males from the mouse life-span study is increased approximately fivefold over that of males from the unaffected study as well as fivefold over the labeling indices of males from the two like studies of scopolamine hydrobromide trihydrate and methyleugenol. This finding suggests that the increase observed in the mouse life-span study is not attributable to the presence of *H. hepaticus*, as two other studies also positive for *H. hepaticus* did not show a similar increase.

The cell proliferation data for the liver from NTP studies are consistent with data from a study by Fox *et al.* (1996) in which cell proliferation indices were evaluated at 8, 10, and 13 months in the A/JCr mouse, which is generally believed to be more susceptible to *H. hepaticus*-associated hepatitis than the B6C3F<sub>1</sub> mouse. In the study by Fox *et al.* (1996), cell proliferation rates were significantly increased at all time points in males. Some increases were observed in females in that study but did not reach statistical significance. An increased

incidence of hepatocellular neoplasms was observed only in the males. Though liver lesions were observed in females in that study, they were less severe than those in males.

In addition to the liver, cell proliferation indices (PCNA) were evaluated in the kidneys and lungs of male and female mice in affected studies versus those in unaffected studies (Nyska *et al.*, 1997). No apparent effect of *H. hepaticus* infection or the presence of hepatitis on PCNA indices was observed for the kidneys or lungs.

Apoptosis (programmed cell death) is another important parameter in evaluations of cell kinetics. The apoptotic index in the liver of male mice with hepatitis from an affected study, cobalt sulfate heptahydrate (NTP, 1998b), was significantly ( $P < 0.01$ ) greater than that observed in males from the unaffected 1-trans-delta<sup>9</sup>-tetrahydrocannabinol study and the PCR-positive, hepatitis-negative scopolamine hydrobromide trihydrate study (Nyska *et al.*, 1997). For females, there were no significant differences among the three studies.

Two 13-week studies which were begun during the same time as the nine affected studies were randomly selected for evaluation of PCNA indices. *H. hepaticus* was not identified in either of the studies by PCR-RFLP; however, as with all NTP 13-week studies, only tissue fixed in formalin for an unspecified period was available. Because of this, no true negative control group was available; therefore, the labeling index of these 19- to 20-week-old animals was compared to values cited in the literature (Eldridge and Goldsworthy, 1996) for 20-week-old B6C3F<sub>1</sub> mice. The labeling index in the NTP studies clearly was not increased (data not shown).

### **The Impact of *H. hepaticus* on the Interpretation of 2-Year Carcinogenesis Studies**

Increases in the incidences of neoplasms are associated with a number of infectious agents. The chronic inflammation caused by these agents has been hypothesized to be important in the pathogenesis of the increased neoplasm incidences (e.g., gastric cancer associated with *H. pylori*). The increased incidences of liver neoplasms in male mice from the nine affected NTP studies were observed in the animals with *H. hepaticus*-associated hepatitis. Neoplasms from males with hepatitis tended to have an H-ras mutation profile different from that of animals from unaffected studies. Further, cell replication rates at 2 years were significantly higher in males with hepatitis compared to those in males without hepatitis. The data suggest that *H. hepaticus*-associated hepatitis is associated with the increased incidences of liver neoplasms in the male B6C3F<sub>1</sub> mouse. Therefore, the most important consideration in evaluating the impact of *H. hepaticus* infection on the interpretation of study results appears to be the presence or absence of significant hepatitis.

For any carcinogenicity study, data within and specific to the individual study provide the greatest basis for an accurate interpretation. However, it is prudent to consider and evaluate all data or information which may affect the interpretation. Based upon the data presented in this and other reports, general guidelines emerge that may be useful in interpreting potential chemical-associated carcinogenic effects in *H. hepaticus*-infected B6C3F<sub>1</sub> mice. In a study with sufficient evidence of *H. hepaticus*-associated hepatitis (> 10% of the animals having the characteristic hepatitis may be a reasonable guideline), interpretation of increased incidences of liver neoplasms (hepatocellular neoplasms and hemangiosarcoma) of male mice is considered to be potentially confounded.

Altered chemical uptake and metabolism, due to the intestinal load of *H. hepaticus* and to *H. hepaticus*-associated liver disease, respectively, are possible reasons for considering that the male mouse response to chemical administration at sites other than the liver should also be considered confounded. Data do not currently exist that definitively answer this question. In this group of nine studies, however, there is no evidence to suggest that affected mice responded to chemical treatment in organs other than the liver in a manner different from mice in nonaffected studies. Within each study, there was excellent concordance in chemical-associated neoplasms between the male mice and the females, which had little or no hepatitis

(Table J8). Furthermore, analyses indicate that *H. hepaticus* is not associated with neoplastic responses outside the liver; incidences of neoplasms at sites other than the liver were not different between control groups from affected and unaffected studies (Table J3). Cell replication rates in two major organs (lung and kidney) also were not increased in control groups from affected studies compared to those from unaffected studies.

One of the more difficult issues to address is whether interpretation of a treatment-related increase in liver neoplasm incidences in the female mouse is confounded when *H. hepaticus*-associated hepatitis is present within the male mice in the study. Most evidence to date links hepatitis with the increased liver neoplasm incidences observed in males, and female B6C3F<sub>1</sub> mice in affected studies do not have significant hepatitis at 2 years. The lack of hepatitis in females, however, is based on an analysis in which only late time points were evaluated histologically. Therefore, it is conceivable that hepatitis along with increased cell proliferation could have occurred earlier and resolved by 18 months to 2 years. Data collected to date, however, suggest that *H. hepaticus*-associated hepatitis is a late-developing and persistent disease in the B6C3F<sub>1</sub> mouse. *H. hepaticus*-associated hepatitis has never been observed in any NTP 13-week studies, including five begun during the same 6-month time span as eight of the nine affected 2-year studies. Also, within affected 2-year studies, more males (51%) that were 18 to 24 months of age had hepatitis than those (34%) that were 12 to 18 months of age. This is consistent with a report by Ward *et al.* (1994b) that *H. hepaticus*-associated liver lesions are not observed at early time points in the B6C3F<sub>1</sub> mouse.

Nonetheless, within affected studies, female control mice did have a slightly elevated incidence of liver neoplasms when compared to control mice from unaffected studies, and the data derived from the *H-ras* mutation frequency analysis were inconclusive. The possibility that *H. hepaticus*-infected female mice from affected studies may respond differently to a liver carcinogen than mice from unaffected studies cannot be eliminated at this time. However, because within an affected study hepatitis is observed only rarely in females, until definitive data suggest otherwise, it is concluded that the interpretation of an apparent chemical-induced neoplastic effect in the liver of female mice is not confounded. To censor the few females with *H. hepaticus*-associated hepatitis from any statistical analyses of hepatocellular neoplasms would be prudent. Studies in the ostensibly more sensitive A/JCr mouse (Fox *et al.*, 1996) also showed significant increases in neoplasm incidences and cell proliferation rates in the liver of *H. hepaticus*-infected males, but not females.

Another concern is how to interpret possible chemical-related effects in a study in which the status of *H. hepaticus* infection cannot be determined by PCR-RFLP because only tissues fixed in formalin for more than 48 hours are available. While histologic evaluation is inadequate to identify infection, it appears adequate for identifying hepatitis severe enough to alter the outcome of the study. Therefore, in the absence of significant histologic evidence of *H. hepaticus*-associated hepatitis, the outcome of a 2-year study should not be considered potentially compromised.

The causality between *H. hepaticus* infection and neoplasia has not been proven in the B6C3F<sub>1</sub> mouse in these studies, nor has the mechanism of this association been determined; further studies are needed. However, sufficient information exists to make reasonable scientific judgments relative to the interpretation of data from the nine 2-year carcinogenicity studies in the B6C3F<sub>1</sub> mouse. Refinements to the above interpretive positions may occur if warranted by future information.

## REFERENCES

Craanen, M.E., Blok, P., Top, B., Boerrigter, L., Dekker, W., Offerhaus, G.J.A., Tytgat, G.N.J., and Rodenhuis, S. (1995). Absence of ras gene mutations in early gastric carcinomas. *Gut* **37**, 758-762.

- Dew, J.A., Clifton, L.G., Sanders, B.L., and Reynolds, R.P. (1997). Comparison of results of *Helicobacter* tests performed by commercial laboratories. *Contemp. Top. (AALAS)* **36**, 60. (Abstr.)
- Eldridge, S.R., and Goldsworthy, S.M. (1996). Cell proliferation rates in common cancer target tissues of B6C3F1 mice and F344 rats: Effects of age, gender, and choice of marker. *Fundam. Appl. Toxicol.* **32**, 159-167.
- Fox, J.G., Correa, P., Taylor, N.S., Zavala, D., Fontham, E., Janney, F., Rodriguez, E., Hunter, F., and Diavalitsis, S. (1989). *Campylobacter pylori*-associated gastritis and immune response in a population at increased risk of gastric carcinoma. *Am. J. Gastroenterol.* **84**, 775-781.
- Fox, J.G., Dewhirst, F.E., Tully, J.G., Paster, B.J., Yan, L., Taylor, N.S., Collins, M.J., Jr., Gorelick, P.L., and Ward, J.M. (1994). *Helicobacter hepaticus* sp. nov., a microaerophilic bacterium isolated from livers and intestinal mucosal scrapings from mice. *J. Clin. Microbiol.* **32**, 1238-1245.
- Fox, J.G., Yan, L.L., Dewhirst, F.E., Paster, B.J., Shames, B., Murphy, J.C., Hayward, A., Belcher, J.C., and Mendes, E.N. (1995). *Helicobacter bilis* sp. nov., a novel *Helicobacter* species isolated from bile, livers, and intestines of aged, inbred mice. *J. Clin. Microbiol.* **33**, 445-454.
- Fox, J.G., Li, X., Yan, L., Cahill, R.J., Hurley, R., Lewis, R., and Murphy, J.C. (1996). Chronic proliferative hepatitis in A/JCr mice associated with persistent *Helicobacter hepaticus* infection: A model of *Helicobacter*-induced carcinogenesis. *Infect. Immun.* **64**, 1548-1558.
- Fox, J.G., MacGregor, J., Shen, Z., Li, X., Lewis, R., and Dangler, C.A. (1998). Role of *Helicobacter hepaticus* in confounding results of a triethanolamine carcinogenesis study in mice. *J. Clin. Microbiol.* (in press)
- Garewal, H., Bernstein, H., Bernstein, C., Sampliner, R., and Payne, C. (1996). Reduced bile acid-induced apoptosis in "normal" colorectal mucosa: A potential biological marker for cancer risk. *Cancer Res.* **56**, 1480-1483.
- Garvey, W., Fathi, A., and Bigelow, F. (1985). Modified Steiner for the demonstration of spirochetes. *J. Histotechnol.* **8**, 15-17.
- Gavrieli, Y., Sherman, Y., and Ben-Sasson, S.A. (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* **119**, 493-501.
- Goldsworthy, T.L., Fransson-Steen, R., and Maronpot, R.R. (1996). Importance of and approaches to quantification of hepatocyte apoptosis. *Toxicol. Pathol.* **24**, 24-35.
- Graham, D.Y. (1989). *Campylobacter pylori* and peptic ulcer disease. *Gastroenterology* **96**, 615-625.
- Haseman, J.K. (1992). Value of historical controls in the interpretation of rodent tumor data. *Drug Inf. J.* **26**, 191-200.
- International Agency for Research on Cancer (IARC) (1994). *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: Schistosomes, Liver Flukes and Helicobacter pylori*, Vol. 61. IARC, Lyon, France.
- Lee, A., Fox, J., and Hazell, S. (1993). Pathogenicity of *Helicobacter pylori*: A perspective. *Infect. Immun.* **61**, 1601-1610.

- Lee, G.-H., and Drinkwater, N.R. (1995). Hepatocarcinogenesis in BXH recombinant inbred strains of mice: Analysis of diverse phenotypic effects of the hepatocarcinogen sensitivity loci. *Mol. Carcinog.* **14**, 190-197.
- Malarkey, D.E., Ton, T.-V., Hailey, J.R., and Devereaux, T.R. (1997). A PCR-RFLP method for the detection of *Helicobacter hepaticus* in frozen or fixed liver from B6C3F<sub>1</sub> mice. *Toxicol. Pathol.* **25**, 606-612.
- Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J. Mol. Biol.* **3**, 208-218.
- Maronpot, R.R., Fox, T., Malarkey, D.E., and Goldsworthy, T.L. (1995). Mutations in the *ras* proto-oncogene: Clues to etiology and molecular pathogenesis of mouse liver tumors. *Toxicology* **101**, 125-156.
- Marshall, B.J., and Warren, J.R. (1984). Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* **1**, 1311-1314.
- National Toxicology Program (NTP) (1993). Toxicology and Carcinogenesis Studies of Oxazepam (CAS No. 604-75-1) in Swiss Webster and B6C3F<sub>1</sub> Mice (Feed Studies). Technical Report Series No. 443. NIH Publication No. 93-3359. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.
- National Toxicology Program (NTP) (1996). Toxicology and Carcinogenesis Studies of 1-Trans-delta<sup>9</sup>-tetrahydrocannabinol (CAS No. 1972-08-3) in F344/N Rats and B6C3F<sub>1</sub> Mice (Gavage Studies). Technical Report Series No. 446. NIH Publication No. 97-3362. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.
- National Toxicology Program (NTP) (1997). Toxicology and Carcinogenesis Studies of Scopolamine Hydrobromide Trihydrate (CAS No. 6533-68-2) in F344/N Rats and B6C3F<sub>1</sub> Mice (Gavage Studies). Technical Report Series No. 445. NIH Publication No. 97-3361. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.
- National Toxicology Program (NTP) (1998a). Toxicology and Carcinogenesis Studies of Triethanolamine (CAS No. 102-71-6) in F344/N Rats and B6C3F<sub>1</sub> Mice (Dermal Studies). Technical Report Series No. 449. NIH Publication No. 98-3365. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC. (in preparation)
- National Toxicology Program (NTP) (1998b). Toxicology and Carcinogenesis Studies of Cobalt Sulfate Heptahydrate (CAS No. 10026-24-1) in F344/N Rats and B6C3F<sub>1</sub> Mice (Inhalation Studies). Technical Report Series No. 471. NIH Publication No. 98-3961. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC. (in press)
- National Toxicology Program (NTP) (1998c). Toxicology and Carcinogenesis Studies of Chloroprene (CAS No. 126-99-8) in F344/N Rats and B6C3F<sub>1</sub> Mice (Inhalation Studies). Technical Report Series No. 467. NIH Publication No. 98-3957. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC. (in press)
- National Toxicology Program (NTP) (1998d). Toxicology and Carcinogenesis Studies of Technical Grade Sodium Xylenesulfonate (CAS No. 1300-72-7) in F344/N Rats and B6C3F<sub>1</sub> Mice (Dermal Studies). Technical Report Series No. 464. NIH Publication No. 98-3380. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC. (in press)

National Toxicology Program (NTP) (1998e). Toxicology and Carcinogenesis Studies of AZT (CAS No. 30516-87-1) and AZT/ $\alpha$ -Interferon A/D in B6C3F<sub>1</sub> Mice (Gavage Studies). Technical Report Series No. 469. NIH Publication No. 98-3959. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC. (in press)

National Toxicology Program (NTP) (1998f). Toxicology and Carcinogenesis Studies of Theophylline (CAS No. 58-55-9) in F344/N Rats and B6C3F<sub>1</sub> Mice (Feed and Gavage Studies). Technical Report Series No. 473. NIH Publication No. 98-3963. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC. (in press)

National Toxicology Program (NTP) (1998g). Toxicology and Carcinogenesis Studies of Methyleugenol (CAS No. 93-15-2) in F344/N Rats and B6C3F<sub>1</sub> Mice (Gavage Studies). Technical Report Series No. 491. NIH Publication No. 98-3950. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC. (in preparation)

National Toxicology Program (NTP) (1998h). Toxicology and Carcinogenesis Studies of Diethanolamine (CAS No. 111-42-2) in F344/N Rats and B6C3F<sub>1</sub> Mice (Dermal Studies). Technical Report Series No. 478. NIH Publication No. 98-3968. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC. (in press)

Nomura, A., Stemmermann, G.N., Chyou, P., Kato, I., Perez-Perez, G.I., and Blaser, M.J. (1991). *Helicobacter pylori* infection and gastric carcinoma among Japanese Americans in Hawaii. *N. Engl. J. Med.* **325**, 1132-1136.

Nyska, A., Maronpot, R.R., Eldridge, S.R., Haseman, J.K., and Hailey, J.R. (1997). Alteration in cell kinetics in control B6C3F<sub>1</sub> mice infected with *Helicobacter hepaticus*. *Toxicol. Pathol.* **25**, 591-596.

Parsonnet, J., Friedman, G.D., Vandersteen, D.P., Chang, Y., Vogelman, J.H., Orentreich, N., and Sibley, R.K. (1991). *Helicobacter pylori* infection and the risk of gastric carcinoma. *N. Engl. J. Med.* **325**, 1127-1131.

Rao, G.N., Hickman, R.L., Seilkop, S.K., and Boorman, G.A. (1987). Utero-ovarian infection in aged B6C3F<sub>1</sub> mice. *Lab. Animal Sci.* **37**, 153-158.

Riley, L.K., Franklin, C.L., Hook, R.R., Jr., and Besch-Williford, C. (1996). Identification of murine Helicobacters by PCR and restriction enzyme analyses. *J. Clin. Microbiol.* **34**, 942-946.

Seilkop, S.K. (1995). The effect of body weight on tumor incidence and carcinogenicity testing in B6C3F<sub>1</sub> mice and F344 rats. *Fundam. Appl. Toxicol.* **24**, 247-259.

Shames, B., Fox, J.G., Dewhirst, F., Yan, L., Shen, Z., and Taylor, N.S. (1995). Identification of widespread *Helicobacter hepaticus* infection in feces in commercial mouse colonies by culture and PCR assay. *J. Clin. Microbiol.* **33**, 2968-2972.

Sills, R.C., Hong, H.L., Greenwell, A., Herbert, R.A., Boorman, G.A., and Devereux, T.R. (1995). Increased frequency of K-ras mutations in lung neoplasms from female B6C3F<sub>1</sub> mice exposed to ozone for 24 or 30 months. *Carcinogenesis* **16**, 1623-1628.

Sipowicz, M.A., Weghorst, C.M., Shio, Y.-H., Buzard, G.S., Calvert, R.J., Anver, M.R., Anderson, L.M., and Rice, J.M. (1997). Lack of p53 and ras mutations in *Helicobacter hepaticus*-induced liver tumors in A/JCr mice. *Carcinogenesis* **18**, 233-236.

Taylor, N.S., Fox, J.G., and Yan, L. (1995). In-vitro hepatotoxic factor in *Helicobacter hepaticus*, *H. pylori* and other *Helicobacter* species. *J. Med. Microbiol.* **42**, 48-52.

Ward, J.M., Anver, M.R., Haines, D.C., Tully, J.G., Jr., Collins, M.J., Jr., Gorelick, P.L., Anderson, L., Rice, J.M., and Russell, R.J. (1993). A unique hepatitis in mice associated with a helical bacterium. *Toxicol. Pathol.* **21**, 591. (Abstr.)

Ward, J.M., Fox, J.G., Anver, M.R., Haines, D.C., George, C.V., Collins, M.J., Jr., Gorelick, P.L., Nagashima, K., Gonda, M.A., Gilden, R.V., Tully, J.G., Russell, R.J., Benveniste, R.E., Paster, B.J., Dewhirst, F.E., Donovan, J.C., Anderson, L.M., and Rice, J.M. (1994a). Chronic active hepatitis and associated liver tumors in mice caused by a persistent bacterial infection with a novel *Helicobacter* species. *J. Natl. Cancer Inst.* **86**, 1222-1227.

Ward, J.M., Anver, M.R., Haines, D.C., and Benveniste, R.E. (1994b). Chronic active hepatitis in mice caused by *Helicobacter hepaticus*. *Am. J. Pathol.* **145**, 959-968.

Ward, J.M., Benveniste, R.E., Fox, C.H., Battles, J.K., Gonda, M.A., and Tully, J.G. (1996). Autoimmunity in chronic active *Helicobacter* hepatitis of mice. *Am. J. Pathol.* **148**, 509-517.

Wotherspoon, A.C., Doglioni, C., Diss, T.C., Pan, L., Moschini, A., de Boni, M., and Isaacson, P.G. (1993). Regression of primary low-grade B-cell gastric lymphoma of mucosa-associated lymphoid tissue type after eradication of *Helicobacter pylori*. *Lancet* **342**, 575-577.

**TABLE J1**  
**Incidence of *Helicobacter hepaticus*-Associated Hepatitis in Control B6C3F<sub>1</sub> Mice from Nine NTP 2-Year Studies<sup>a</sup>**

Study	Incidence of Hepatitis (%)	
	Males	Females
Sodium xylenesulfonate	78	4
AZT/5,000 U $\alpha$ -interferon A/D	76	4
Cobalt sulfate heptahydrate	72	8
AZT/500 U $\alpha$ -interferon A/D	66	0
Chloroprene	54	0
Theophylline	32	0
$\alpha$ -Interferon A/D	22	4
Triethanolamine	20	0
AZT	16	2
Average	48	2

<sup>a</sup> Includes regeneration and mild to marked (excludes minimal) chronic inflammation, karyomegaly, oval cell hyperplasia, and bile duct hyperplasia. AZT=3'-azido-3'-deoxythymidine

**TABLE J2**  
**Identification of *Helicobacter hepaticus* with PCR-RFLP-Based Assays in Control B6C3F<sub>1</sub> Mice from 32 NTP 2-Year Studies and Three NTP 13-Week Studies<sup>a</sup>**

Type of Sample	Total Studies	<i>H. hepaticus</i> -Positive Studies <sup>b</sup>	
		Affected Studies	Unaffected Studies
<b>13-Week Studies</b>			
Formalin-fixed liver	3	—	1/3 <sup>c</sup>
<b>2-Year Studies</b>			
Frozen liver	22	3/3	3/19
Formalin-fixed liver	10	1/6 <sup>c</sup>	0/4

<sup>a</sup> PCR-RFLP=polymerase chain reaction-restriction fragment length polymorphism

<sup>b</sup> Number of *H. hepaticus*-positive studies/number of affected or unaffected studies. Affected studies are those in which hepatitis typical of that associated with *H. hepaticus* infection occurred in many male mice.

<sup>c</sup> Only one animal in the positive study was positive for *H. hepaticus*.

**TABLE J3**  
**Comparison of Neoplasm Incidences in Control B6C3F<sub>1</sub> Mice**  
**from *Helicobacter hepaticus*-Affected and Unaffected NTP 2-Year Studies**

	Males		Females	
	Affected Studies <sup>a</sup>	Unaffected Studies	Affected Studies	Unaffected Studies
Number of studies	9	26	9	26
Survival (%)	64	71	68	68
12-Month body wt (g)	48.0	48.3	48.1	47.0
Neoplasm incidence (%)				
Liver	71.3*	54.8	50.3	40.5
Lung	26.6	23.2	7.6	10.3
Pituitary gland	0.4	0.8	14.7	14.3
Harderian gland	5.6	6.1	6.0	4.9
Lymphoma	6.9	6.3	16.2	15.5
Circulatory system	9.8	6.0	5.3	4.7
liver only	7.1*	2.5	—	—
All benign	61.8	57.2	59.1	54.6
All malignant	61.3*	40.9	50.0	44.2
All neoplasms	88.0*	77.4	82.7	75.4

\* Significantly different ( $P \leq 0.05$ ) from the unaffected studies

<sup>a</sup> Affected studies are those in which hepatitis typical of that associated with *H. hepaticus* infection occurred in many male mice.

**TABLE J4**  
**Liver Neoplasm Incidences and Body Weights of Control B6C3F<sub>1</sub> Mice**  
**in Relation to Study Start Dates of *Helicobacter hepaticus*-Affected and Unaffected NTP 2-Year Studies<sup>a</sup>**

Study Start Date	Liver Neoplasm Incidence (%)		Mean Body Weight (g)	
	Affected Studies <sup>a</sup>	Unaffected Studies	Affected Studies	Unaffected Studies
<b>Male</b>				
April to September 1988	—	43.8 (8) <sup>b</sup>	—	46.2 (8)
October 1988	62.0 (1)	—	48.3 (1)	—
November 1988 to September 1989	—	52.6 (7)	—	48.7 (7)
October 1989 to June 1990	—	61.2 (5)	—	48.9 (5)
July 1990 to January 1991	72.5 (8)	66.2 (4)	48.0 (8)	49.0 (4)
February 1991 to April 1992	—	68.0 (2)	—	52.8 (2)
Average	71.3	54.8	48.0	48.3
<b>Female</b>				
April to September 1988	—	31.1 (8)	—	44.8 (8)
October 1988	46.0 (1)	—	46.4 (1)	—
November 1988 to September 1989	—	39.9 (7)	—	47.2 (7)
October 1989 to June 1990	—	38.6 (5)	—	45.9 (5)
July 1990 to January 1991	50.9 (8)	54.2 (4)	48.3 (8)	48.0 (4)
February 1991 to April 1992	—	58.0 (2)	—	55.6 (2)
Average	50.3	40.5	48.1	47.0

<sup>a</sup> Includes nine affected studies (those in which hepatitis typical of that associated with *H. hepaticus* infection occurred in many male mice) and 26 unaffected studies

<sup>b</sup> Number of studies is given in parentheses.

**TABLE J5**  
**Association of Liver Neoplasm Incidence and Severity of *Helicobacter hepaticus*-Associated Hepatitis in Control B6C3F<sub>1</sub> Mice from Nine Affected NTP 2-Year Studies<sup>a</sup>**

Severity of Hepatitis	Liver Neoplasm Incidence	
	Males	Females
Absent	101/175 (58%)	196/396 (49%)
Minimal	44/57 (77%)	23/42 (55%)
Mild/moderate	176/218 (81%)	7/11 (64%)
Significance of association	P < 0.05	NS <sup>b</sup>

<sup>a</sup> Affected studies are those in which hepatitis typical of that associated with *H. hepaticus* infection occurred in many male mice.

<sup>b</sup> NS=not significant

**TABLE J6**  
**H-ras Codon 61 AAA Mutations in Spontaneous Liver Neoplasms in Control B6C3F<sub>1</sub> Mice from *Helicobacter hepaticus*-Affected and Unaffected NTP 2-Year Studies**

Study	Affected <sup>a</sup>	H-ras AAA Mutations
<b>Male</b>		
Cobalt sulfate heptahydrate	+	0/10 (0%)
Chloroprene	+	1/13 (8%)
Triethanolamine	+	1/10 (10%)
Oxazepam	—	7/18 (39%)
Diethanolamine	—	4/16 (25%)
Historical control database		106/333 (32%)
<b>Female</b>		
Chloroprene	+	0/10 (0%)
Triethanolamine	+	1/15 (7%)
Diethanolamine	—	1/11 (9%)
Historical control database		106/333 (32%)

<sup>a</sup> + =affected; — =not affected. Affected studies are those in which hepatitis typical of that associated with *H. hepaticus* infection occurred in many male mice.

**TABLE J7**  
**Proliferating Cell Nuclear Antigen Labeling Indices in the Liver of Control B6C3F<sub>1</sub> Mice<sup>a</sup>**

	Hepatitis	No. of Animals	PCNA Labeling Index <sup>b</sup>	Average PCNA Labeling Index <sup>c</sup>
<b>Male</b>				
Cobalt sulfate heptahydrate <sup>d</sup>	+	15	0.535 ± 0.129	
Chloroprene <sup>d</sup>	+	12	1.452 ± 0.386	
Triethanolamine <sup>d</sup>	+	9	1.215 ± 0.374	1.011
Cobalt sulfate heptahydrate	—	7	0.175 ± 0.117	
Chloroprene	—	10	0.296 ± 0.124	
Triethanolamine	—	12	0.100 ± 0.042	0.186
1-Trans-delta <sup>9</sup> -tetrahydrocannabinol <sup>e</sup>	—	15	0.042 ± 0.011	
Scopolamine hydrobromide trihydrate <sup>f</sup>	—	14	0.043 ± 0.012	
Methyleugenol <sup>f</sup>	—	14	0.077 ± 0.020	
Mouse life-span study <sup>f</sup>	—	15	0.217 ± 0.880	
<b>Female</b>				
Cobalt sulfate heptahydrate	+	5	0.161 ± 0.062	
Cobalt sulfate heptahydrate	—	17	0.055 ± 0.015	
Chloroprene	—	12	0.154 ± 0.050	
Triethanolamine	—	12	0.138 ± 0.053	0.108
1-Trans-delta <sup>9</sup> -tetrahydrocannabinol	—	13	0.156 ± 0.047	
Scopolamine hydrobromide trihydrate	—	15	0.032 ± 0.009	

<sup>a</sup> A portion of these data are presented in Nyska *et al.* (1997). + =hepatitis present; — =no hepatitis present

<sup>b</sup> Mean ± standard error; PCNA=proliferating cell nuclear antigen

<sup>c</sup> Average of the mean labeling indices for animals from all three studies

<sup>d</sup> Affected study (one in which hepatitis typical of that associated with *H. hepaticus* occurred in many male mice)

<sup>e</sup> Unaffected study (one in which the typical hepatitis did not occur in mice)

<sup>f</sup> Unaffected study with no typical hepatitis, but positive for *H. hepaticus* by polymerase chain reaction-restriction fragment length polymorphism-based assay

**TABLE J8**  
**Summary of Target Sites of Carcinogenicity in B6C3F<sub>1</sub> Mice from NTP 2-Year Studies**  
**with *Helicobacter hepaticus*-Associated Hepatitis**

	Males	Females
Chloroprene	Lung Circulatory system <sup>a</sup> Harderian gland Forestomach Kidney	Lung Circulatory system Harderian gland Forestomach Liver Skin Mesentery Zymbal's gland Mammary gland
Cobalt sulfate heptahydrate <sup>b</sup>	Lung	Lung
Triethanolamine	Liver	Liver
AZT <sup>c</sup>	None	Vagina
Sodium xylenesulfonate	None	None
Theophylline	None	None

<sup>a</sup> Hemangioma and hemangiosarcoma of the liver were excluded from the analysis in males.

<sup>b</sup> An apparent treatment-related increase in the incidence of hemangiosarcoma of the liver was discounted in male mice because of the presence of *H. hepaticus*.

<sup>c</sup> AZT=3'-azido-3'-deoxythymidine. Includes four studies: AZT;  $\alpha$ -interferon A/D; AZT/500 U  $\alpha$ -interferon A/D; and AZT/5,000 U  $\alpha$ -interferon A/D

