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September 15, 1998

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In accordance with API's policy of providing the federal government with information from research designed to determine whether any chemical substance or mixture manufactured, processed or distributed by API member companies may cause a risk of injury to health or the environment, we are enclosing a copy of the following draft final report. This is a partially audited report undergoing review and subject to change.

(Identification no: Not Assigned) Draft Final Title: *A Vapor Inhalation Reproductive Toxicity, Developmental Toxicity, and Developmental Neurotoxicity Study in CrI:CD<sup>®</sup>(SD)BR Rats Exposed to Hydrogen Sulfide*

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**QA/GLP COMPLIANCE STATEMENT**  
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CIIT PROTOCOL 97042

## ABSTRACT

The purpose of this study was to evaluate whether repeated 6-hour daily exposure of male and female CD<sup>1</sup> rats ( $n = 12$  rats/sex/concentration) to hydrogen sulfide (H<sub>2</sub>S) atmospheres at 0, 10, 30, or 80 ppm would result in reproductive toxicity, developmental toxicity, or developmental neurotoxicity. Reproductive and developmental toxicity were assessed following the 2-week prebreed, 2-week mating (evidence of copulation = gestation day 0 = gd 0), and 3-week gestational (gd 0 until gd 19) exposure periods. All F<sub>0</sub> males were exposed to H<sub>2</sub>S for at least 70 consecutive days. Litters were culled (4 pups/sex/litter) on postnatal day (pnd) 4. Exposure of F<sub>0</sub> dams and their pups resumed between pnd 5 -18. Complete necropsies were performed on F<sub>0</sub> animals that included selected organ weights, histopathologic examination of reproductive organs, and andrological assessments. No adult systemic toxicity was observed in F<sub>0</sub> parents. Subchronic exposure of male F<sub>0</sub> rats to 80 ppm H<sub>2</sub>S was associated with sensory neuron loss and basal cell hyperplasia primarily within the olfactory mucosa lining the dorsal medial meatus and the dorsal medial region of the ethmoid recess.

No treatment-related changes in mating index, fertility index, or postimplantation loss per litter were observed. Terminal body weights and feed consumption were statistically equivalent among F<sub>0</sub> treatment groups. There were no statistically significant treatment-related gross or histopathologic findings in reproductive organs from F<sub>0</sub> rats. Teratogenic effects (assessed grossly at birth) including kinked or missing tails (1 litter affected in each H<sub>2</sub>S treatment group), umbilical hernia (1 litter affected in the 10 ppm H<sub>2</sub>S treatment group), cranial defect (1 litter affected in the 10 ppm H<sub>2</sub>S treatment group), and skin lesions (2 litters affected in the 30 ppm H<sub>2</sub>S treatment group) that included webbing of the forelimb skin were only observed in F<sub>1</sub> pups from the H<sub>2</sub>S-exposed groups.

Developmental neurotoxicity in F<sub>1</sub> pups was assessed by: (a) evaluation of developmental landmarks (incisor eruption, negative geotaxis, eyelid separation, vaginal patency, or balanopreputial separation); (b) use of a blinded functional observational battery (FOB) on pnd 60 ± 2, (c) assessment of motor activity (pnd 13, 17, 21, and 60 ± 2), acoustic startle, and passive avoidance (pnd 22 ± 1, 62 ± 3) behaviors, as well as (d) terminal neuropathology (pnd 61 ± 2). Exposure to H<sub>2</sub>S did not affect pup growth, development, or behavior. No gross or microscopic abnormalities were observed in the central nervous system of rats exposed to 80 ppm H<sub>2</sub>S. No statistically significant differences in brain weight or size were observed in H<sub>2</sub>S-exposed rats.

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LABORATORY TITLE PAGE

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VOLUME I OF III

**TITLE:** A Vapor Inhalation Reproductive Toxicity,  
Developmental Toxicity, and Developmental  
Neurotoxicity Study in CrI:CD<sup>®</sup>(SD)BR Rats Exposed to  
Hydrogen Sulfide

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**STUDY INITIATION DATE:** July 31, 1997

**LABORATORY COMPLETION DATE:** Pending

**FINAL REPORT DATE:** Pending

**CIIT PROTOCOL NO.:** 97042

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CIIT PROTOCOL 97042

LABORATORY SIGNATURE PAGE

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	Study Director	Date
Karrie A. Brenneman, DVM, DACVP	_____	_____
	Co-Investigator	Date

LABORATORY INDIVIDUAL SIGNATURE PAGE

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CIIT PROTOCOL 97042

**LABORATORY QA STATEMENT**

**QUALITY ASSURANCE INSPECTION AND REPORTING DATES**

**Study Title:** A Vapor Inhalation Reproductive Toxicity, Developmental Toxicity and Developmental Neurotoxicity Study in Cr:CD,(SD)BR Rats Exposed to Hydrogen Sulfide

**CIIT Protocol No.:** 97042      **Study Director:** David C. Dorman, DVM, PhD, DABVT, DABT

**Sponsor:** American Petroleum Institute  
1220 L Street, NW  
Washington, DC 20005

<u>Phase(s)</u>	<u>Quality Assurance Unit (QAU) Inspection Date(s)</u>	<u>Date of QAU Report to Study Director</u>	<u>Date of QAU Report to Management</u>
Protocol Review	07/18/97	07/18/97	07/18/97
Protocol Review	07/31/97	07/31/97	07/31/97
Pre-Experimental Period	08/04/97	08/04/97	08/04/97
Experimental Period	08/13/97	08/14/97	08/14/97
Experimental Period	08/21/97, 08/25/97 and 08/29/97	09/03/97	09/03/97
Experimental Period	09/03-04/97	09/05/97	09/05/97
Experimental Period	09/16/97	09/17/97	09/17/97
Protocol Amendment Review	09/18/97	09/18/97	09/18/97
Experimental Period	09/24-25/97	09/26/97	09/26/97
Experimental Period	09/30/97	09/30/97	09/30/97
Protocol Amendment Review	10/07/97	10/07/97	10/07/97
Experimental Period	10/17/97 and 10/20/97	10/22/97	10/22/97
Experimental Period	10/29/97	10/30/97	10/30/97
Experimental Period	11/26/97	12/01/97	12/01/97

5/14/98

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LABORATORY QA STATEMENT

QUALITY ASSURANCE INSPECTION AND REPORTING DATES

Study Title: A Vapor Inhalation Reproductive Toxicity, Developmental Toxicity and Developmental Neurotoxicity Study in Cr:CD<sub>1</sub>(SD)BR Rats Exposed to Hydrogen Sulfide

CIIT Protocol No.: 97042 Study Director: David C. Dorman, DVM, PhD, DABVT, DABT

Sponsor: American Petroleum Institute  
1220 L Street, NW  
Washington, DC 20005

<u>Phase(s)</u>	<u>Quality Assurance Unit (QA) Inspection Date(s)</u>	<u>Date of QAU Report to Study Director</u>	<u>Date of QAU Report to Management</u>
Experimental Period	12/01/97	12/01/97	12/01/97
Raw Data/Draft Final Report	05/04/98, 05/06/98, 05/08/98 and 05/11-12/98	05/13/98	05/13/98

Phase inspections, raw data and final report reviews were performed by Quality Assurance Unit personnel in accordance with the U.S. Environmental Protection Agency's Toxic Substances Control Act (TSCA) (40 CFR Part 792). The dates of the QAU inspections and dates results were reported to the Study Director and Management are noted in the above list.

The Quality Assurance Unit at CIIT has reviewed the final report to help assure that the report describes the methods and that the results reflect the raw data.

\_\_\_\_\_  
Patricia O'Brien Pomerleau, M.S. Date  
Quality Assurance Manager, CIIT

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DRAFT FINAL REPORT  
CIIT PROTOCOL 97042**LABORATORY GLP COMPLIANCE STATEMENT**

This study was performed in compliance with the Good Laboratory Practices (GLP) Standards promulgated by the U.S. Environmental Protection Agency, Toxic Substances Control Act (TSCA), Final Rule, *Federal Register* 54, 34034-34050, August 17, 1989, the Good Laboratory Practice regulations as set forth in the Code of Federal Regulations (40 CFR 792). This study was performed, to the extent possible, in compliance with the OECD Guideline for Testing of Chemicals; Guideline 421: Reproduction/Developmental Toxicity Screening Test.

The SOP on external examinations of newborn and neonatal rats was used prior to management approval. Uncertified 5% H<sub>2</sub>S from cylinder EGC-3-6230 was used in pre-study work and for the first 10 days of exposure. No accountability log was maintained for this cylinder during these first few weeks. These deviations did not affect the quality or integrity of the study. There were no other significant deviations from the aforementioned regulations or the signed protocol that would affect the integrity of the study or the interpretation of the test results. The raw data have been reviewed and the evaluation of the test article as presented herein represents an appropriate conclusion within the context of the study design and evaluation criteria.

All test and control results in this report are supported by an experimental data record, and this record has been reviewed. All raw data, documentation, records, protocols, and the Final Report generated as a result of this study will be archived in the CIIT Secure Archives.

\_\_\_\_\_  
David C. Dorman, DVM, PHD, DABVT, DABT  
Study Director  
Chemical Industry Institute of Toxicology

\_\_\_\_\_  
Date

CIIT Protocol No.: 97042

**A Vapor Inhalation Reproductive Toxicity, Developmental Toxicity, and Developmental Neurotoxicity Study in CrI:CD<sup>®</sup>(SD)BR Rats Exposed to Hydrogen Sulfide**

Sponsor: American Petroleum Institute

\*\*\*\*\*

SUMMARY

The purpose of this study was to evaluate whether repeated 6-hour daily exposure of male and female CD<sup>®</sup> rats ( $n = 12$  rats/sex/concentration) to hydrogen sulfide (H<sub>2</sub>S) atmospheres at 0, 10, 30, or 80 ppm would result in reproductive toxicity, developmental toxicity, or developmental neurotoxicity. Reproductive and developmental toxicity were assessed following the 2-week prebreed, 2-week mating (evidence of copulation = gestation day 0 = gd 0), and 3-week gestational (gd 0 until gd 19) exposure periods. All F0 males were exposed to H<sub>2</sub>S for at least 70 consecutive days. Litters were culled (4 pups/sex/litter) on postnatal day (pnd) 4. Exposure of F0 dams and their pups resumed between pnd 5 -18. Complete necropsies were performed on F0 animals that included selected organ weights, histopathologic examination of reproductive organs, and andrological assessments. No adult systemic toxicity was observed in F0 parents. Subchronic exposure of male F0 rats to 80 ppm H<sub>2</sub>S was associated with sensory neuron loss and basal cell hyperplasia primarily within the olfactory mucosa lining the dorsal medial meatus and the dorsal medial region of the ethmoid recess.

No treatment-related changes in mating index, fertility index, or postimplantation loss per litter were observed. Terminal body weights and feed consumption were statistically equivalent among F0 treatment groups. There were no statistically significant treatment-related gross or histopathologic findings in reproductive organs from F0 rats. Teratogenic effects (assessed grossly at birth) including kinked or missing tails (1 litter affected in each H<sub>2</sub>S treatment group), umbilical hernia (1 litter affected in the 10 ppm H<sub>2</sub>S treatment group), cranial defect (1 litter affected in the 10 ppm H<sub>2</sub>S treatment group), and skin lesions (2 litters affected in the 30 ppm H<sub>2</sub>S treatment group) that included webbing of the forelimb skin were only observed in F1 pups from the H<sub>2</sub>S-exposed groups.

Developmental neurotoxicity in F1 pups was assessed by: (a) evaluation of developmental landmarks (incisor eruption, negative geotaxis, eyelid separation, vaginal patency, or balanopreputial separation); (b) use of a blinded functional observational battery (FOB) on pnd 60 ± 2,

(c) assessment of motor activity (pnd 3, 17, 31, and  $60 \pm 2$ ), acoustic startle, and passive avoidance (pnd  $22 \pm 1$ ,  $60 \pm 2$ ) behaviors, as well as (d) terminal neuropathology (pnd  $61 \pm 2$ ). Exposure to H<sub>2</sub>S did not affect pnd growth, development, or behavior. No gross or microscopic abnormalities were observed in the central nervous system of rats exposed to 80 ppm H<sub>2</sub>S. No statistically significant differences in brain weight or size were observed in H<sub>2</sub>S-exposed rats.

Section 1  
INTRODUCTION

The present study was designed to evaluate the potential of inhaled hydrogen sulfide vapor in CD® (Sprague-Dawley) rats to produce alterations in parental fertility, maternal pregnancy and lactation, and growth and development of the offspring.

**Section 2  
MATERIALS AND METHODS****TEST CHEMICAL**

Gas cylinders containing 5% (50,000 ppm) mixtures of H<sub>2</sub>S (CAS No 7783-06-4) with the balance being nitrogen were ordered from Holox Gases (Cary, NC) and manufactured by Praxair Distribution, Inc. (Bethlehem, PA). The chemical and physical properties of H<sub>2</sub>S are listed in Table A1. The first cylinder used in the study was ordered as an unanalyzed mixture and was partially used in an earlier study. After use in this study, this cylinder was returned to Praxair for analysis and certification. The other cylinders of H<sub>2</sub>S needed in this study were ordered as Certified Standard mixtures. Upon receipt at CIIT, each cylinder was stored in monitoring corridor 20CW under ambient temperature and relative humidity conditions. A total of 4 cylinders were used during this study.

Reference gas mixtures for calibration were also ordered as Certified Standards from Holox Gases (Cary, NC) and manufactured by Praxair Distribution, Inc. (Bethlehem, PA). These reference mixtures were further diluted with nitrogen to provide a range of concentrations for the GC calibration curve.

**GENERATION OF THE H<sub>2</sub>S ATMOSPHERE**

Details regarding the conduct of the inhalation exposure are presented in Appendix A. In brief, H<sub>2</sub>S exposure concentrations were generated by metering 5% H<sub>2</sub>S in nitrogen from the gas cylinder through a mass flow controller (MKS Instruments, Andover, MA) into a "T" in the two inch stainless steel air inlet to the inhalation chambers. The "T" was located approximately three feet upstream of the chamber. The H<sub>2</sub>S gas flowed into the "T" and counter-current to the HEPA-filtered chamber air flow of the inhalation chamber which facilitated mixing of the H<sub>2</sub>S vapor with the dilution air. The total chamber air flow through each chamber was maintained at approximately 225-250 L/min. The generation system was operated by the Andover Infinity control system (Andover Controls Corporation, Andover, MA).

F0 animals (both males and females) were exposed in four Hazelton H1000 stainless steel and glass inhalation exposure chambers (Lab Products, Maywood, NJ). One H1000 was used for each target exposure concentration. Each H1000 was contained within CIIT's permanent 8 m<sup>3</sup> Hinners-style stainless steel and glass inhalation exposure chamber as an additional safety measure. Air flow through the 8 m<sup>3</sup> chambers was controlled by a supply fan, an exhaust fan

and two butterfly dampers. Airflow was monitored by measuring the pressure drop across an orifice in the exhaust line and controlled by the damper in the exhaust duct system. Air was pulled through the H1000 and the 8 m<sup>3</sup> chamber into a manifold from the 8 m<sup>3</sup> chamber exhaust duct. A damper at the opening to the manifold from the 8 m<sup>3</sup> chamber was fixed in place to proportion the airflow between the H1000 and the 8 m<sup>3</sup> chamber. Air flow through the H1000 chamber was controlled by the total airflow in the 8 m<sup>3</sup> chamber. An extension connected to the air inlet of the H1000 chamber was positioned near the air inlet at the top of the 8m<sup>3</sup> chamber. The H<sub>2</sub>S vapor flowed into the extension, mixed with incoming air and was distributed to the H1000 chamber.

For the whole body exposure of dams and pups, 4 liter glass exposure cylinders sealed with two anodized aluminum end plates with neoprene gaskets and an aluminum outer face (CH Technologies (USA), Inc., Westwood, NJ) were used. Each unit had an inlet on one endplate and an exhaust port on the other endplate. The exhaust ports were connected to an exhaust manifold system which pulled the inhalation chamber air from the H1000 chamber through the inlet and through the dam/pup exposure chamber. Air flow through the individual glass exposure chambers was controlled by an adjustable stainless steel metering valve (Raleigh Valve and Fitting, Raleigh, NC) and maintained at 2.5 - 3.5 L/min during the exposure times, providing approximately 35 to 50 air changes per hour. Grand mean flow rates for the exposure cylinders were  $2.97 \pm 0.15$ ,  $2.95 \pm 0.12$ ,  $3.07 \pm 0.19$ , and  $2.90 \pm 0.09$  L/min, respectively, for the control, 10, 30, and 80 ppm exposure groups (Tables B1 and B2). The temperature in one exposure cylinder per concentration group was measured using a thermister (PreCon, Memphis, TN) placed in the tube exhaust, and the relative humidity in one exposure cylinder per concentration group was measured by a humidity probe (OMEGA Engineering, Inc., Stamford, CT) located in the inlet end of the glass chamber. Measurements of tube concentration, temperature, and humidity were recorded during each exposure period. The temperature and relative humidity within the exposure cylinders was dependent upon the temperature and relative humidity of the H1000 chamber and the conditions within the individual exposure cylinders. For example, higher temperatures were recorded as the pups grew and the animal load within the chamber increased. Recent urination or breathing near the humidity probe resulted in high humidity readings at various times during the exposures. Approximately 60 g of cellulose fiber chip (ALPHA-dri™, Shepherd Specialty Papers, Kalamazoo, MI) bedding was placed into each exposure cylinder. Approximately 25 g of Transgel® (Charles River Laboratories, Inc. Raleigh, NC) was placed into exposure cylinder prior to the start of the inhalation exposure.

**ANALYSES OF EXPOSURE ATMOSPHERES**

H<sub>2</sub>S exposure atmospheres were measured with a calibrated gas chromatograph (GC) (Hewlett Packard Model 6890, Hewlett Packard Co., Palo Alto, CA) equipped with a flame photometric detector (FPD) and GS-Q (30 meter x 0.53  $\mu$ m) column (Alltech, Deerfield, IL). An eight-position gas sampling valve was used by the GC to sample at a programmed sequence of locations. Prior to animals being placed in the chambers, each chamber was checked for uniformity of distribution of the test compound by measuring the H<sub>2</sub>S concentration at nine positions within the chamber.

Temperature was measured at the top rear of the H1000 chamber by a thermister (PreCon, Memphis, TN) and relative humidity was measured at the top front of the H1000 chamber by a humidity probe (OMEGA Engineering, Inc., Stamford, CT) connected to the Andover Infinity control system. Calibration of the thermisters was checked by comparing the temperature probe to a Certified mercury thermometer. The relative humidity probe was calibrated by immersing the probe in an atmosphere of known humidity generated from saturated salt solutions. Air flow in the H1000 chamber was monitored by measuring the pressure drop across an orifice located at the inlet of the H1000 chamber. Placement of orifices at the inlet and outlet assured that a slight negative static pressure was maintained inside the H1000 chamber. The H1000 temperature, relative humidity, and air flow were recorded by the Andover Infinity control system at the time of gas chromatograph data transfer and a report of the environmental parameters data and the GC data for the 6-hour exposure period was printed every day.

**ANIMALS AND HUSBANDRY**

Eighty (80) virgin female and 80 virgin male outbred albino CD® (Sprague-Dawley) rats (Crj:CD®[SD]BR; birth date June 9, 1997) were received at CIIT from Charles River Laboratories, Raleigh, NC, on August 4, 1997, 56 days old upon arrival. (The actual dates of all major phases of the study are presented in Text Table A.) These animals were acclimated for approximately two weeks in mass air displacement rooms in suspended stainless steel cages with an automatic watering system. During this time, they were weighed, examined by a veterinarian and representative animals were subjected to fecal examination and serum viral antibody analysis. For serum viral antibody analysis, on the day of animal receipt, three (3) rats per sex were randomly chosen from the shipment of animals, euthanized and blood collected for

assessment of viral antibody status. Heat-inactivated serum was sent to Microbiological Associates, Bethesda, MD, for their Level 1 Rat Antibody Screen. The viral screen consisted of evaluation for the presence of antibodies against the following: Toolan H-1 virus (H-1), Sendai virus, Pneumonia virus of mice (PVM), Rat coronavirus/sialodacryoadenitis (RCV/SDA), Kitham rat virus (KRV), CAR Bacillus, and *Mycoplasma pulmonis*. Results of the physical examination, serology and parasitology were negative for signs of infectious disease; the animals were considered to be in good health and suitable for use in this study. One female died on August 12, 1997 prior to the randomization. A necropsy was performed and no significant gross findings were noted. One additional female rat was observed to be dehydrated on August 14, 1997 and she was excluded from the randomization, and was euthanized on September 2, 1997 with all other excess rats.

During a fourteen-day acclimation period, animals were randomly assigned to exposure groups. During acclimation, study males and females were singly housed in stainless steel suspended cages. Study males and females housed in the anterooms were singly housed (except during mating when they were housed 1:1) in polycarbonate cages with stainless steel lids (Laboratory Products, Inc., Rochelle Park, NJ) with Alpha-Dri® cage litter (Shepard Specialty Papers, Kalamazoo, MI). From gestational day (gd) 20 through lactation when dams are housed with their litters, the females were also housed in polycarbonate cages with water bottles, stainless steel wire lids, and Alpha-Dri® cage litter. The polycarbonate cage dimensions were 8" x 19" x 10.5" (height). The dams with litters, on pnd 5-18, were removed from their polycarbonate cages each morning for exposures, group housed in individual glass exposure cylinders during the exposures, and then returned to their polycarbonate cages overnight.

All F0 rats were individually identified by ear tag and transponder after arrival at CIIT. All F1 pups were tattooed on postnatal day (pnd) 4. F1 pups were uniquely identified by eartag on pnd 19. All data generated during the course of this study were tracked by these numbers. The method and numbers for identification were documented in the study records.

Except for animals used for neuropathologic assessments, all weanling and adult animals were euthanized by CO<sub>2</sub> asphyxiation. This includes animals not used in the study and any moribund animal. All pups euthanized during the lactation period were decapitated. This includes pups culled on pnd 4 and any moribund pups. Animals received with the initial shipment, but not used in the study, were euthanized or used as sentinels (12 female rats) prior to the start of the treatment period. Records were kept documenting the fate of all animals received for the study.

Feed (NIH-07, Certified Pelleted Rodent Chow, Zeigler Brothers, Inc., Gardners, PA; Lot Nos. 41070075 [milling dates P-6-11-97-NIH-P, P-7-7-97-NIH-P, P-8-4-97-NIH-P], 41070025 [milling dates P-9-2-97-NIH-P, P-10-6-97-NIH-P, P-11-10-97-NIH-P]) was available *ad libitum*, except during the six-hour exposure periods. Deionized/filtered tap water from the Durham, North Carolina water system was available *ad libitum*, via the automatic watering system for all males on study and females during acclimation and during the six-hour exposures with the exception of lactating females. Approximately 25 g of Transgel<sup>®</sup> (Charles River Laboratories, Inc., Raleigh, NC) was placed in each exposure cylinder prior to the start of the inhalation exposure. When in polycarbonate cages, males and females were watered by plastic water bottles, except during the exposure periods. Water was delivered through Hydro's deionization filtration system (Hydro Systems, RTP, NC). Contaminants were monitored quarterly as per CIIT SOPs. The analysis of the rodent feed for chemical composition and possible chemical contamination and analysis of the drinking water was provided by the suppliers and maintained in the study records.

All contaminant levels were below certified levels for both feed and water and did not affect the design, conduct or conclusions of this study. NIH-07 feed was stored at approximately 60-79°F and the period of use did not exceed four months from the milling date. At all times, the animals were handled, cared for and used in compliance with the NRC Guidelines (1996).

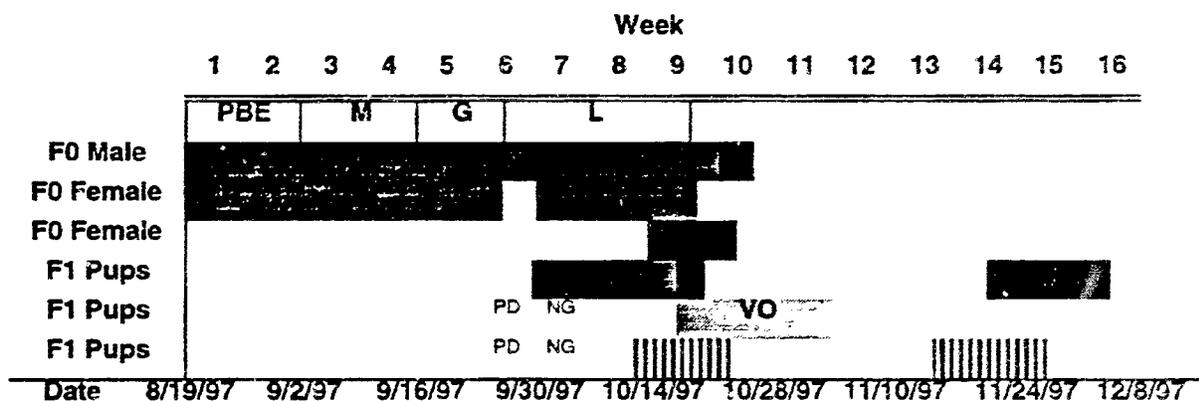
The range of F0 male body weights at the start of the prebreed exposure period was 337.8 – 439.7 g (Tables B3 and B4). The range for F0 females was 184.7 – 276.0 g (Tables B5). All animals assigned to each treatment group were exposed to their respective exposure concentrations, six hours per day, seven days per week during the prebreed exposure periods, and during the postmating holding period (males), and seven days per week during mating (both sexes), gestation (dams) and lactation (dams) throughout the study.

**STUDY DESIGN AND TREATMENT**

Study Design

A graphic representation of the study design is presented in Figure 1 (below). The study began with 12 males/group and 12 females/group to yield at least 8 pregnant females/group at or near term. Exposure began for all F0 animals on August 19, 1997, when they were approximately 10 weeks old (the dates of study are presented in Text Table A). Animals were assigned to the different groups by means of randomization (performed on August 13, 1997) stratified by body weight such that the body weights of all groups were homogeneous by statistical analysis at study initiation.

**Figure 1 – H2S Reproductive Toxicity and Developmental Neurotoxicity Study Design**



**Legend**

H2S Exposure	[Solid black bar]	PBE	Prebreed exposure
Necropsy	[Solid black bar]	M	Mating
		G	Gestation
Behavioral Testing	[Vertical lines]	L	Lactation
		PD	Pinnae detachment
		NG	Negative geotaxis
		VO	Vaginal Opening

TEXT TABLE A  
Study Schedule - H2S

<b>EVENT(s)</b>	<b>DATE(s)</b>
<b><u>Acclimation and Exposure</u></b>	
Animals arrive at CIIT	8/4/97
Pretest health screen	8/4/97
Animal acclimation	8/4/97-8/18/97
Rats weighed, randomized and transported	8/13/97
Prebreed exposure	8/19/97-8/31/97
Breeding exposure	9/1/97-9/14/97
Postbreeding Exposure (males)	9/15/97-10/28/97
Postbreeding Exposure (non-pregnant females)	9/15/97-10/9/97
Pregnant females no exposure: GD 20-PND 4	9/22/97-10/3/97
PND 0 for 2 Litters (0 ppm, 80 ppm)	9/23/97
PND 0 for 7 Litters	9/24/97
((2) 0 ppm, (3) 10 ppm, (1) 30 ppm, (1) 80 ppm)	
PND 0 for 8 Litters	9/25/97
((1) 0 ppm, (2) 10 ppm, (2) 30 ppm, (3) 80 ppm)	
PND 0 for 13 Litters	9/26/97
((4) 0 ppm, (3) 10 ppm, (4) 30 ppm, (2) 80 ppm)	
PND 0 for 4 Litters	9/27/97
((2) 0 ppm, (1) 10 ppm, (4) 30 ppm, (2) 80 ppm)	
PND 0 for 2 Litters (0 ppm, 10 ppm)	9/28/97
PND 0 for 1 Litter (30 ppm)	9/29/97
Dams and pups exposed from PND 5-PND 18	9/28/97-10/17/97
<b><u>Developmental Landmarks</u></b>	
Pinnae detachment (PND 1-7)	9/24/97-10/2/97
Negative geotaxis (PND 7-9)	9/30/97-10/8/97
Incisor eruption (PND 7-14)	9/30/97-10/10/97
Eyelid separation (PND 12-16)	10/5/97-10/13/97
Vaginal opening (PND 27-39)	10/20/97-11/3/97
Balano-preputial separation (PND 35-48)	10/28/97-11/12/97

TEXT TABLE A (CONTINUED)  
Study Schedule - H2S

EVENT(s)	DATE(s)
<b><u>Neurobehavioral Testing</u></b>	
Motor PND 13	10/6/97-10/12/97
Motor PND 17	10/10/97-10/16/97
Motor PND 21, Startle PND 21-23	10/14/97-10/20/97
Passive Avoidance PND 22 ± 1	10/14/97-10/21/97
Motor PND 60 ± 2	11/23/97, 11/24/97, 11/26/97
Passive avoidance PND 62 ± 3	11/24/97, 11/25/97, 11/28/97, 11/29/97
Startle PND 62 ± 3	11/24/97, 11/25/97, 12/1/97
Functional Observational Battery PND 60 ± 2	11/23/97, 11/26/97
<b><u>Necropsy</u></b>	
Adult female	10/10/97, 10/15-10/17/97, 10/19-10/20/97
Adult male	10/28/97, 10/29/97
Neuropathology pups (PND 23 ± 2)	10/15-10/17/97, 10/20/97
Neuropathology pups (PND 61 ± 2)	11/23/97, 11/24/97, 11/26/97, 12/1/97
Complete pups (PND 63 ± 3)	11/24 -11/26, 12/1/97

**Treatment**

The study was conducted with three (3) treatment groups and an air (vehicle control) group, each comprised of 12 rats per sex. The dates of evaluation performance are presented in Text Table A. The target exposure concentrations were 0, 10, 30, and 80 ppm H2S. The rationale for choosing these exposure concentrations was as follows. A previous subchronic H2S toxicity study conducted in Sprague-Dawley rats was performed at CIIT (CIIT Docket Number #32063) and employed H2S exposure concentrations of 0, 10, 30, and 80 ppm. Toxicity (i.e., decreased body weight and decreased terminal brain weight) was observed in male and female rats exposed to 80 ppm for six hours per day, five days per week, for at least 90 days. See Text Table B for summarization of study design and target exposure concentrations.

**TEXT TABLE B**  
**Experimental Exposure Conditions**

Exposure Group	Room	Number of Rats		Target Concentration (ppm)
		Males	Females	
1	308	12	12	0
2	309	12	12	10
3	310	12	12	30
4	311	12	12	80

The decision was also made to simultaneously expose the F1 offspring with their dams between postnatal day (pnd) 5 through 18. The simultaneous exposure of rat dams and pups is often not performed in developmental neurotoxicity inhalation studies. Maternal separation during inhalation exposure results in additional pup stress that may alter behavior development thereby confounding study results. To decrease the impact of pup separation from the dam and increase pup exposure to H<sub>2</sub>S a novel whole-body exposure chamber developed at CIIT was used. Previous research with this exposure system has demonstrated that pups maintained with their dams did not develop significantly delayed development (assessed using surface righting, negative geotaxis, and eye opening) or altered behavior (e.g., assessed using spontaneous motor activity).

F0 animals were exposed to H<sub>2</sub>S vapor six hours per day, seven days per week for two weeks (prebreed exposure periods). During the two week mating period, all F0 male and female rats were exposed to H<sub>2</sub>S for six hours per day, seven days per week. Daily exposure of the F0 males continued until they were exposed for at least 70 consecutive days. Pregnant F0 female rats (evidence of copulation = gestation day 0 = gd 0) were exposed to H<sub>2</sub>S vapor for six hours per day, seven days per week from gd 0 until gd 19. No exposures occurred through the remainder of gestation and during the period of parturition (gd 20 through pnd 4). The date of parturition was designated as postnatal day (pnd) 0. On pnd 4, litters were randomly culled to eight pups with equal sex ratios whenever possible and F0 females (dams) and their pups were concurrently exposed to H<sub>2</sub>S vapor for six hours per day, seven days per week starting on pnd 5 and continuing through pnd 18. No further H<sub>2</sub>S exposures to the dam or pups occurred beyond pnd 18. F0 females without positive evidence of insemination (rat # 71 from the 10 ppm group; rats # 82 and 84 from the 30 ppm group, and rats # 90 and 94 from the 80 ppm group) were exposed to H<sub>2</sub>S vap six hours per day, seven days per week until 23-24 days after the end of the breeding period at which time they were euthanized and gross examination of reproductive tracts performed.

For each daily exposure, F0 male rats were transferred from their home cages in the anteroom (polycarbonate cages) into inhalation cage units, singly housed, and the units were moved into the chamber for exposure. A similar procedure was used for F0 females until parturition. Following parturition, the aforementioned 4.9 liter annealed glass exposure cylinders were used. Lactating F0 female rats and their pups were transferred from their polycarbonate home cages in the anteroom into individual exposure cylinders. Approximately 60 g of cellulose fiber chip (ALPHA-dri™, Shepherd Specialty Papers, Kalamazoo, MI) bedding and approximately 25 g of Transgel<sup>®</sup> (Charles River Laboratories, Inc. Raleigh, NC) was placed into each exposure cylinder prior to the addition of the animals. Following each daily exposure, all animals were transferred back to home cages for residence and access to feed overnight.

#### Clinical Observations, Food Consumption, and Body Weights

Observations for mortality were made twice daily (a.m. and p.m.) and the general condition of all animals was checked daily. Clinical examinations were conducted and recorded daily, prior to and after each exposure period, throughout the course of the study. This record included the time of onset, degree and duration of symptoms. These cage-side observations included, but were not limited to changes in: skin and fur, eyes, mucous membranes, respiratory system, circulatory system, autonomic and central nervous system, somatomotor activity, and behavior pattern.

The body weights of the male rats were determined and recorded initially and weekly throughout the study. The body weights of female rats were recorded in the same manner until confirmation of mating. During gestation, females were weighed on gestational days (gd) 0, 7, 14 and 20. Dams producing litters were weighed on lactational days (postnatal days, pnd) 0, 4, 7, 14, 21 and 28. All live pups were weighed individually at birth (pnd 0) and at pnd 4, 7 and 14 and at weaning (pnd 21). After weaning on pnd 21, pup body weights were collected twice weekly throughout the study. Body weight gains were computed.

Feed consumption for F0 adult males were collected weekly throughout the study except during the period of cohabitation. Feed consumption measurements were recorded weekly for all F0 female rats throughout the pre-breed treatment periods. During pregnancy of F0 females, feed consumption was recorded for gd 0-7, 7-14 and 14-20. During lactation of F1 litters, maternal feed consumption was measured for pnd 0-4, 4-7, 7-14, and 14-21, although maternal feed consumption after pnd 14 was confounded by the contribution from the pups since pups were self-feeding by this time. Feed consumption was not measured during the cohabitation period since two adult animals (breeding pair) were in the same cage.

For nonpregnant F0 females, feed consumption and body weight data were recorded. These data are presented but not analyzed statistically in the Final Report since the small numbers of animals in each group were variable, and the data were therefore not robust for statistical analysis. These data are retained in the study records.

#### Mating Procedures and Pup Exposures

Animals of the F0 generation were approximately ten (10) weeks of age at the commencement of treatment. They were exposed to H<sub>2</sub>S vapor for 6 hours per day, seven days per week at their respective exposure concentrations for at least two weeks prior to mating, *i.e.*, until they were approximately 12 weeks of age. The animals were then mated on the basis of one male to one female selected randomly within each exposure group for a period of 14 days with no change in mating partners, with exposures continuing seven days per week (each female was added to the male's cage in the afternoon after each daily exposure, then removed the next morning for the next exposure day). Vaginal smears were taken daily during the 14-day mating period or until mating was confirmed. Females were examined daily during the cohabitation period for the presence of sperm or copulation plug in the vaginal tract. The observation of vaginal sperm or copulation plug was considered evidence of successful mating (Hafez, 1970). The day vaginal sperm (or plug) was observed was designated gestational day (gd) 0 (Hafez, 1970). Once vaginal sperm were observed, the male and female from that mating pair were individually housed and vaginal smearing for estrous cyclicity ceased. Any female which did not show evidence of successful mating after 14 days of cohabitation, was weighed weekly and feed consumption measured weekly until termination. If a female without a confirmed gd 0 date was, in fact, pregnant and delivered a litter (rat ear tag # 58 in the control group and rat # 74 in the 30 ppm exposure group), her lactational information was collected as described below.

Sperm/plug positive dams were not exposed beginning on gd 20. Beginning on gd 20, each female was observed twice daily (a.m. and p.m.) for evidence of littering. Dams, which littered were not exposed on pnd 0 (date of delivery) through pnd 4. On pnd 4, litters were randomly culled to eight pups with as equal sex ratios as possible and F0 females (dams) and their pups were concurrently exposed to H<sub>2</sub>S vapor for six hours per day, seven days per week starting on pnd 5 and continuing through pnd 18. No further H<sub>2</sub>S exposures to the dam or pups occurred beyond pnd 18. The dams were allowed to rear their young to day 21 postpartum (pnd 21). On pnd 21, each litter was weaned.

All pups (F1 litters) were sexed and examined as soon as possible after birth (date of birth designated pnd 0) to determine the number of viable and stillborn members of each litter. On day 4 after birth, the size of each litter was adjusted by eliminating extra pups by random selection to yield, as nearly as possible, four (4) males and four (4) females per litter and remaining pups were foot tattooed. Culled pups were sacrificed by decapitation and discarded. Survival indices were calculated at 0, 4, 7, 14 days after birth and at weaning (pnd 21). All live pups were counted, sexed, weighed individually, and examined grossly at birth (pnd 0) and at 4, 7, 14 days after birth and at weaning (pnd 21). The body weights and sexes were recorded on an individual basis using their tattoo number and litter number as a unique identifier. All pups were examined for physical abnormalities at birth and throughout the pre-weaning period. Pups dying during lactation were not necropsied.

#### Developmental Landmarks

Developmental landmarks in F1 neonatal animals was assessed (presence or absence) prior to daily exposure. The ability to right from a supine position to a position with all four feet in contact with the flooring (surface righting) was observed for each F1 pup. Each pup was placed on a clean cloth wipe and given a maximum of 30 seconds (s) to right itself. Cloth wipes were changed between litters. All pups were evaluated for presence or absence of surface righting on pnd 4. An approximately 20 X 25 cm inclined plane (30°) was used to test all pups for negative geotaxis. The incline was covered with textured cageboard which was changed between litters. Each pup was placed at the top of the incline with its head facing downward and all four feet in contact with the surface. Each pup was given up to 60 s to turn 180° with the head facing up the incline. Negative geotaxis reflex response was tested in replicate groups of pups on pnd 7, 8, and 9. Other indicators of maturation were examined beginning on the following days and continued until completed: pnd 1 (pinnae detachment), pnd 7 (incisor eruption), and pnd 12 (eyelid separation). Each F1 female was observed beginning on pnd 27 for vaginal patency. The number of females with the vagina patent was recorded daily until all females had this response. In addition, during this approximate time period, each F1 male was observed for cleavage of the balanopreputial gland (preputial separation) beginning on pnd 35. The characteristic is present when the prepuce can be completely retracted to expose the glans penis. The number of males with this separation was recorded daily until all males had this response. All individual animal in-life data are presented in Appendix B.

### Behavior Testing

All random selections on offspring for culling were performed using a CIIT SOP. Whenever possible, one male and one female from each litter was randomly assigned to behavior testing. Whenever possible, neurotoxicology test sessions and motor activity groups were balanced for sex and exposure concentration. Pups with eartag numbers ending in one (1) or five (5) were assigned to motor activity test groups and underwent whole body perfusion on postnatal day (pnd)  $61 \pm 2$ . Pups with eartag numbers ending in two (2) or six (6) were assigned to passive avoidance on pnd 21 and 22 and neuroperfusion on postnatal day  $23 \pm 2$ . Pups with eartag numbers ending in three (3) or seven (7) were assigned to passive avoidance on pnd  $62 \pm 3$ , functional observation battery (FOB) on pnd  $60 \pm 2$ , and underwent complete necropsy on pnd  $63 \pm 3$ . Pups with eartag numbers ending in four (4) or eight (8) were assigned to acoustic startle on pnd 21 and  $62 \pm 3$  with complete necropsy performed on pnd  $63 \pm 3$ . One female rat (eartag # 626) from the 10 ppm group died on pnd 7 and was cannibalized during the six hour H<sub>2</sub>S exposure. One female rat (eartag # 769) from the 30 ppm group was not assigned for any behavior tests. This rat was necropsied on pnd 65.

Motor activity was measured in the same animal (one male and one female from each litter) following the end of the 6-hr H<sub>2</sub>S exposure on pnd 13, 17, 21, and  $60 \pm 2$  days. Spontaneous motor activity was measured during ten 6-min intervals for a total of 60 min using an automated cage rack photobeam activity system (San Diego Instruments, San Diego, CA). Each animal was placed into an individual clear 45.7 x 23.5 x 20.3 cm polycarbonate cage with either seven (neonates) or four (adults) photobeams spaced 5.5 or 11 cm apart on either side of the cage, respectively. Photobeams were positioned approximately 1.7 and 5.7 cm above the cage floor for the neonates and adult rats, respectively. The motor activity trial was initiated by the first activity of the rat and the total number of movements (beams broken) and the number of ambulations (number of times that more than one beam was broken in succession) were recorded. Motor activity testing was completed during the light phase of the animal's diurnal cycle. White noise levels of  $68.8 \pm 0.7$  dBA and room illumination of approximately  $2.7 \pm 0.4$  foot-candles were maintained in the laboratory during motor activity testing (Table T-40). White noise was generated with a Coulbourn Instruments (Allentown, PA) white noise generator.

Passive avoidance with a step-through to darkness paradigm including one training and one retention trial was used to assess learning and memory. Passive avoidance was tested in animals (one male and one female from each litter) on pnd  $22 \pm 1$  and on a separate group of animals (used for FOB) on pnd  $62 \pm 3$ . On the first day of testing, each animal was placed in

the brightly lit side of a two-compartment shuttle box (Coulbourn Instruments, Allentown, PA). After a 20 s acclimation period, the guillotine door opened allowing the animal access to the other darkened compartment. Rats received either a 1 s, 0.5-0.6 mA (neonates) or a 1 s, 1 mA footshock upon entering the dark compartment. The procedure was repeated approximately 24 hr later. Latency to enter the dark compartment was measured for each trial. If the latency reached 240 s, the rat was removed from the chamber and assigned an arbitrary latency (maximum) score of 240. Rats that did not cross over during the first session were excluded from the statistical analysis.

A battery of tests designed to detect gross alterations in nervous system function (functional observation battery; FOB) was performed for one male and one female from each litter. FOB evaluations were performed on pnd  $60 \pm 2$  days. The conduct of the FOB including quantitative assessment of grip strength and foot splay was performed according to standard laboratory SOPs. Room illumination of  $55.7 \pm 2.6$  foot-candles was maintained in the FOB testing laboratory (Table T-39). Prior to FOB testing, animal ear tags were covered with white laboratory tape by the Study Director and a Neurotoxicology Technician not involved in FOB testing to allow examination by a trained Neurotoxicology Technician who was blinded to the animals' exposure assignment. One FOB technician (Struve) performed all evaluations. The FOB order was determined using a random assignment (JMP, SAS Institute, Cary, NC).

**FOB observations were made: 1) while the rat was in the observation cage, 2) during removal of the rat from the observation cage, 3) while the rat was being held and examined for clinical observations, 4) as the animal moved freely about the open field, and 5) during manipulative tests. The animals were observed and data collected for:**

- **Posture**
- **Signs of involuntary muscular movements (tremors, spasms, and convulsions)**
- **Palpebral closure**
- **Handling reactivity**
- **Muscle tone**
- **Fur condition (piloerection, fur appearance, facial crust)**
- **Skin temperature and color**
- **Breathing pattern**
- **Salivation**
- **Lacrimation**
- **Arousal**
- **Activity**
- **Body position**
- **Ataxia and abnormal gait**
- **Excessive vocalization, stereotypy and other unusual behaviors**
- **Number of defecations, urinations, and rears during an open field session**
- **Approach, acoustic, and tail pinch responses**
- **Visual placing**
- **Grip strength**
- **Hind limb splay**
- **Surface righting**
- **Pupillary reflexes**
- **Body weight**
- **Any additional abnormalities**

**Acoustic startle was assessed using a microcomputer-controlled automated test system (SR-LAB, San Diego Instruments, San Diego CA). Rats were individually tested within a sound attenuated room while being enclosed within a 4 cm (pnd 21) or 9 cm (pnd 62±3) diameter Plexiglass cylinder housed inside a ventilated, sound-dampening chamber. A calibrated (in units of the computer's analog-to-digital converter) piezoelectric detector was used to measure the whole-body response following a broad-band acoustic stimulus. Following a 5-min**

acclimation period, 100 trials were administered either with or without prepulse modification. Fifty pulse trials (110 dB, 50 msec stimulus) and fifty prepulse trials (80 dB, 50 msec followed 100 msec later by a 110 dB, 50 msec stimulus) were randomly presented in blocks of two (prepulse and pulse trials). A constant background noise of approximately 70 dB was used. Acoustic startle was assessed for one male and one female from each litter on pnd 21 and 62 ± 3. Grand mean acoustic startle amplitude elicited during prepulse and pulse trials were calculated from individual blocks that represented the mean of five sequential trials.

## NECROPSY AND HISTOLOGY

### Parental (F0) Animals

Animal(s) to be necropsied were transferred to CIIT's necropsy laboratory. At the end of the exposure regimen, adult F0 rats were weighed, euthanized with CO<sub>2</sub>, exsanguinated, and had a complete necropsy performed with special emphasis on the reproductive and associated organs. The non-impregnated adult females (*n* = 11) and adult males (*n* = 48) were necropsied the day after the last day of exposure. The postpartum adult females (*n* = 48) were necropsied the day of or the day after their pups were weaned (PND 21). The order of necropsy was randomized across dose groups in advance using ear tags for animal identification. The necropsy included gross evaluation of the following organs/tissues: skin, salivary glands, lymph nodes (mesenteric and non-mesenteric), nasopharynx, trachea, mainstem bronchi, lungs, heart, aorta, thymic region, thyroids/parathyroids, liver, spleen, kidneys, pancreas, adrenal glands, digestive tract (including esophagus, stomach, small intestines, cecum, and colon), urinary bladder, male reproductive and accessory sex organs (including testes, epididymides, penis with preputial gland, prostate, seminal vesicles, coagulating glands), female reproductive organs (including ovaries with oviducts, uterus, cervix, vagina) brain (including cerebrum, cerebellum, medulla/pons, and pituitary gland), and the eyes.

The following tissues from adult F0 male and female rats were trimmed, blotted, and weighed: brain, liver, kidneys (both), adrenal glands (both), and spleen. The following additional tissues from adult F0 male rats were trimmed, blotted, and weighed: testes (individually), epididymides, total (individually), epididymides, caudae (individually), seminal vesicles/coagulating glands with fluid (both), prostate, heart, and the lungs. Samples of adult male rat brain, heart, liver, kidney, and lung were weighed, frozen in liquid nitrogen, and stored at -70°C for possible future sulfide analysis with concordance of the Sponsor. The following additional tissues from adult F0 female rats were trimmed, blotted, and weighed: ovaries with oviducts and the uterus. Organ weights were reported as absolute and relative to terminal brain weight. Additional details of the methods and results of histologic evaluation are presented in Appendix C.

At the end of the study and following euthanasia with CO<sub>2</sub>, one testis (right) from each F0 parental male was frozen at approximately -20°C for subsequent enumeration of testicular homogenization-resistant spermatid heads. In addition, daily sperm production and cauda spermatid concentration were calculated. (The left testis per male was retained in fixative for subsequent histopathologic examination; see below.) In addition, one cauda epididymis (the right one) was immediately removed, weighed and seminal fluid from the cauda was assessed for sperm number, motility and morphology (percent motile and percent progressively motile). Sperm motility was assessed immediately after necropsy; number and morphology (at least 200 sperm per male, if possible) were evaluated at a later date using fixed sperm appropriately stained. (The left epididymis per male was retained in fixative for possible subsequent histopathologic examination; see below.)

All gross lesions and the forms of animal identification (e.g., ear tag and transponder) were collected from all adult F0 rats and preserved in 10% neutral buffered formalin as appropriate. The ovaries with oviducts, uterus, cervix and vagina were collected from F0 female rats and these tissues were preserved in 10% neutral buffered formalin. Implantation sites were counted in fresh and/or formalin-fixed, potassium ferricyanide-stained uteri from adult F0 female rats. The fixed (buffered neutral 10% formalin) uteri from any F0 females failing to produce a litter were stained with potassium ferricyanide for confirmation of pregnancy status. This staining procedure did not interfere with subsequent histopathologic evaluation. The following tissues from adult F0 male rats were preserved in 10% neutral buffered formalin: seminal vesicles/coagulating glands with fluid (both), prostate, and nasal cavity. The following tissues were collected from adult male rats and preserved in Bouin's fixative, gross trimmed, rinsed in 50% ethanol, and stored in 70% ethanol: the left testis and the head, body, and cauda of the left epididymis. Noses from the F0 males were flushed retrograde and immersion fixed with 10% neutral buffered formalin and then decalcified in 5% formic acid with an ion exchange resin.

There were no unscheduled deaths during the conduct of the study. Histopathologic examination was performed on reproductive and accessory sex organs from F0 rats in the control and high dose groups, that were not reproductively successful, and in the low and middle dose groups with gross findings in any of these organs. Histopathologic examination was not performed on other tissues, because gross observations made in other tissues during necropsy were determined not to be dose-related. Tissues to be examined microscopically

were processed at Experimental Pathology Laboratories, Research Triangle Park, NC).

Specific attention was focused on the examination of the parental reproductive organs from high concentration and control groups, as specified below:

- ovaries with oviducts (2)
- vagina
- uterus
- cervix
- testis (1)
- epididymis (1)
- seminal vesicles/ coagulating glands (2)
- prostate
- any gross lesion/ s

**Testis/ Epididymis:** Besides gross lesions, testicular histopathological examination was conducted in order to identify treatment-related effects such as retained spermatids, missing germ cell layers or types, multinucleated giant cells, or sloughing of spermatogenic cells into the lumen. Examination of the epididymis, including the caput, corpus, and cauda was conducted in order to identify such lesions as sperm granulomas, leukocytic infiltration (inflammation), aberrant cell types within the lumen, or the absence of clear cells in the cauda epididymal epithelium (Russell et al., 1990).

**Ovary:** The postlactational ovary should contain primordial (small), growing, and antral follicles as well as the large corpora lutea of lactation. Histopathological examination should detect depletion of the primordial follicle population.

**Nasal tissues:** At necropsy, male F0 rats were anesthetized with CO<sub>2</sub> and euthanized by exsanguination. Noses were flushed retrograde and immersion fixed with 10% neutral buffered formalin and then decalcified in 5% formic acid with an ion exchange resin. Transverse blocks of the nasal passages were prepared to include six nasal levels (Morgan, 1991). Tissues were processed by standard procedures, embedded in paraffin, sectioned at 4-5 µm, and stained with hematoxylin and eosin. H<sub>2</sub>S-induced histologic changes within the nasal passages were scored subjectively (grade 0 to 4) for the presence or absence of olfactory mucosal lesions.

### Pathological Assessment of F1 Animals

F1 rats were killed by perfusion for neuropathology on PND  $23 \pm 2$  ( $n=72$ , approximately 1 rat/sex/litter) and PND  $61 \pm 2$  ( $n=73$ , approximately 1 rat/sex/litter). Neuropathology techniques followed standard CIIT SOPs. Rats were given heparin (20,000 units USP/kg body weight, ip), anesthetized with sodium pentobarbital (150 mg/kg, ip) and perfused *in situ* by intra-ascending aortic perfusion with a 0.7% sodium nitrite flush solution in 0.05 M sodium phosphate buffer (pH 7.4). Once the effluent leaving the rat was clear, then the perfusion was changed to the fixative consisting of 1.5% glutaraldehyde and 4% formaldehyde in 0.05 M sodium phosphate buffer (pH 7.4). After perfusion, the cranium and vertebral canal were opened, and the peripheral nerves in the hind legs were exposed. The nervous system was examined grossly and stored along with the ear tag in perfusion fixative at 4°C. Brains were weighed and size measured upon removal. The brains were trimmed transversely at 6 levels (forebrain, caudate nucleus, center of the cerebrum, center of the midbrain, cerebellum and pons, and medulla oblongata), processed for paraffin embedding and sectioned at 5-6 microns, stained with hematoxylin and eosin by the CIIT Histology Service Unit, and examined by bright field light microscopy. Histopathologic examination was performed on brains from rats in control and high dose groups. Histopathologic examination was not performed on brains from intermediate dose groups, because histologic lesions were not found in the control and high dose group brains examined.

On PND  $63 \pm 3$  ( $n=144$ ), the remaining F1 rat pups were weighed, euthanized with CO<sub>2</sub>, exsanguinated, and had a complete necropsy performed. A complete necropsy included gross evaluation of the organs/tissues listed above for the parental (F0) generation. The order of necropsy was randomized across dose groups in advance using ear tags for animal identification. The forms of identification were collected from rat pups as appropriate and preserved in 10% neutral buffered formalin. In addition, the following tissues from rat pups were trimmed, blotted, and weighed: brain, liver, both kidneys, both adrenal glands, lungs, heart, spleen, both testes (male), and both ovaries with oviducts (female). Histopathologic examination was not performed on tissues from these animals due to a lack of significant statistical correlation between the incidence of gross changes observed and H<sub>2</sub>S exposure.

### REPRODUCTIVE AND OFFSPRING INDICES

The indices for reproductive performance and gestational and postnatal parameters which were calculated for this study are presented in Text Table C. The indices include those for F0 males

and females to produce F1 litters. The formulas used for calculating standard reproductive toxicity study parameters are presented in Text Table C.

**TEXT TABLE C**  
**Reproductive and Offspring Indices**

Mating index (%)	=	$\frac{\text{No. females sperm-positive}}{\text{No. females paired}} \times 100$
Fertility index (%)	=	$\frac{\text{No. females pregnant}}{\text{No. females sperm-positive}} \times 100$
Gestational index (%)	=	$\frac{\text{Number of females with live litters}}{\text{Number of females pregnant}} \times 100$

The following endpoints are calculated for each litter (dam) and then the mean is calculated using the litter (dam) values.

$$\text{Postimplantation loss per litter (\%)} = \frac{\text{No. implantation sites} - \text{no. live pups on pnd 0}}{\text{No. implantation sites (nidation scars)}} \times 100$$

**Offspring:**

Live birth index (%)	=	$\frac{\text{Number of live pups at birth}}{\text{Total number of pups born}} \times 100$
4-Day survival index (%)	=	$\frac{\text{Number of pups surviving 4 days (pre-cull)}}{\text{Total number of live pups at birth}} \times 100$
7-Day survival index (%)	=	$\frac{\text{Number of pups surviving 7 days}}{\text{Total number of live pups at 4 days (post-cull)}} \times 100$
14-Day survival index (%)	=	$\frac{\text{Number of pups surviving 14 days}}{\text{Total number of live pups at 4 days (post-cull)}} \times 100$
21-Day survival index (%)	=	$\frac{\text{Number of pups surviving 21 days}}{\text{Total number of live pups at 4 days (post-cull)}} \times 100$

TEXT TABLE C (CONTINUED)  
Reproductive and Offspring Indices

The following endpoints are calculated for each animal and then the mean is calculated using the animal values.

1. Feed Consumption in grams per day

$$\frac{[(\text{feed weight at beginning of measurement period}) - (\text{feed weight at end of measurement period})] / \text{number of days in the measurement period}}$$

2. Relative Organ Weight

$$(\text{organ weight} / \text{sacrifice body weight}) \times 100$$

3. Cauda Spermatid Concentration

$$\text{number of spermatid in cauda} / \text{cauda weight in grams}$$

4. Daily Spermatid Production

$$((\text{number of spermatid in testis}) / \text{testis weight in grams}) / \text{number of days for spermatid cycle 6.1}$$

5. Percent Abnormal Sperm

$$100 \times ((\text{no. abnormal}) / (\text{no. normal} + \text{abnormal}))$$

6. Percent Motile Sperm

$$\frac{\left( \frac{(\text{Total sperm sample 1} - \text{Non-motile sperm sample 1})}{\text{Total sperm sample 1}} \right) + \left( \frac{(\text{Total sperm sample 2} - \text{Non-motile sperm sample 2})}{\text{Total sperm sample 2}} \right)}{2} \times 100$$

STATISTICS

The unit of comparison was the male, the female, the pregnant female, or the litter, as appropriate. Statistical analyses were performed using SAS Statistical Software. The probability value of less than 0.05 was used as the critical level of significance within each statistical test. Tests of homogeneity used a significance level of 0.01.

The data for quantitative, continuous variables (e.g., parental and pup body weights, organ weights, feed consumption, etc.) were intercompared for the exposure and control groups by tests for homogeneity of variance (Levene's test), 2-way fixed effects (dose and sex) analysis of variance (ANOVA), and Dunnett's multiple comparison procedure for significant ANOVAs. When the ANOVA indicated statistical significance among experimental groups, the Dunnett's test was used to delineate which groups differ from the control group. When the assumptions

for a parametric ANOVA were not met, nonparametric procedures were used. Group differences were considered significant if the test statistic type I error was less than 0.05 ( $P \leq 0.05$ ).

Mean and standard deviation values were calculated for total motor activity for each six minute interval during the 60-min measurement period. A nested analysis of total motor activity data was performed using a repeated-measures analysis with exposure as a grouping factor and test period as within-subject factors (MANOVA) ( $p \leq 0.05$ ). Levene's test for homogeneity ( $p \leq 0.01$ ) followed by one-way analysis of variance (ANOVA) ( $p \leq 0.05$ ) and Dunnett's t-test ( $p \leq 0.05$ ) were performed for homogeneous data.

A natural log (ln) transformation of the data was used when the Levene's test for homogeneity indicated the data to be non-homogenous. A Levene's test followed by a one-way analysis of variance (ANOVA) ( $p \leq 0.05$ ) and Dunnett's t-test ( $p \leq 0.05$ ) was performed on the transformed data. When the Levene's test on the transformed data indicated non-homogenous data, a Kruskal-Wallis test ( $p \leq 0.05$ ) and Wilcoxon 2-sample Rank-Sum test ( $p \leq 0.05$ ) were used. For developmental landmarks (e.g., vaginal patency and preputial separation), each treatment percent or mean were compared to the control percent or mean by the Kruskal-Wallis test.

Incidence data were compared using the appropriate statistical test, generally Fisher's Exact Test. Incidence data for select FOB endpoints with ordered severity scores were analyzed for group differences using appropriate measures of association.

#### PERSONNEL

The evaluation of H<sub>2</sub>S inhalation exposure for reproductive and developmental neurotoxicity in CD<sup>®</sup> rats was conducted at The Chemical Industry Institute of Toxicology (CIIT), Research Triangle Park, NC, under contract to The American Petroleum Institute (API), Washington, DC. Dr. Robert Barter, of API, was the Sponsor's Representative.

The CIIT personnel indicated below contributed to the completion of this study.

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Georgianna L. Bridges, ALAT, Senior Animal Care Technician  
Steven Butler, ALAT, Senior Animal Care Technician  
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Neurotoxicology

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Endocrine, Reproductive, and Developmental Toxicity

Paul Foster, PhD, Program Manager  
Donald Stedman, BS, Senior Research Associate

Biostatistics

Derek Janszen, Ph.D.

The final report was prepared by Dr. Dorman with assistance from Ms. Melanie Struve, Ms. Kristin Miller, Ms. Marianne Marshall, Mr. Arden James, and Dr. Karrie Brenneman. The individual scientist reports were prepared and signed by the author(s). The protocol and two amendments detailing the design and conduct of the study are presented in Appendix D. The protocol was signed by the Study Director on July 31, 1997.

#### STORAGE OF RECORDS

All original data sheets for the present study are stored in the CIIT archives, along with all biological samples collected during the course of the study which remain the responsibility of CIIT. Work sheets and computer printouts which were generated in the statistical analysis of data are stored in the CIIT Archives. Copies of this report are filed with CIIT as well as with The American Petroleum Institute, Washington, DC.

#### COMPLIANCE

This study was performed in compliance with the Good Laboratory Practices (GLP) Standards promulgated by the U.S. Environmental Protection Agency, Toxic Substances Control Act (TSCA), Final Rule, *Federal Register* 54, 34034-34050, August 17, 1989, the Good Laboratory Practice regulations as set forth in the Code of Federal Regulations (40 CFR 792). This study was performed, to the extent possible, in compliance with the OECD Guideline for Testing of Chemicals; Guideline 421: Reproduction/Developmental Toxicity Screening Test. All records, data and reports will be maintained in storage as specified in the TSCA GLPs (U.S. EPA, 1989) or for as long as the quality of the preparation affords evaluation, whichever is less. GLP compliance for test chemical analyses was the responsibility of the Supplier and CIIT. The CIIT Animal Research Facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), International. This study was conducted in compliance with the TSCA GLP regulations and AAALAC accreditation standards.

## Section 3

**RESULTS AND DISCUSSION/CONCLUSIONS****TEST ATMOSPHERES**

Full results of the exposure phase of the study are presented in Appendix A. The target H<sub>2</sub>S concentrations were 0, 10, 30 and 80 ppm. The grand means for the actual chamber concentrations ( $\pm$  SD) were  $10.0 \pm 0.6$ ,  $30.1 \pm 0.8$ , and  $79.5 \pm 2.4$  ppm for the target concentrations 10, 30 and 80 ppm, respectively, calculated as a grand mean from individual daily mean values. No H<sub>2</sub>S was detected in the control chamber. The grand means for the nominal chamber concentrations ( $\pm$  SD) were  $10.0 \pm 1.0$ ,  $32.3 \pm 2.4$ , and  $78.0 \pm 4.0$  ppm for the target concentrations 10, 30 and 80 ppm, respectively. The grand means ( $\pm$  SD) for air flow were  $241 \pm 3$ ,  $237 \pm 6$ ,  $225 \pm 4$ , and  $232 \pm 5$  L/min. for the target concentrations of 0, 10, 30 and 80 ppm, respectively. The grand means ( $\pm$  SD) for H<sub>2</sub>S flow were  $47.1 \pm 4.0$ ,  $145.7 \pm 10.1$ , and  $361.6 \pm 14.5$  mL/min for the target concentrations of 10, 30 and 80 ppm, respectively. The grand means ( $\pm$  SD) for ratio of actual to nominal concentration were  $102 \pm 13$ ,  $93 \pm 7$ , and  $102 \pm 6$  % for the target concentrations of 10, 30 and 80 ppm, respectively. The grand means ( $\pm$  SD) for ratio of actual to target concentration were  $100 \pm 6$ ,  $100 \pm 3$ , and  $99 \pm 3$  % for the target concentrations of 10, 30 and 80 ppm respectively. (See Appendix A for details.)

A daily mean concentration which was more than 10% above or below the target concentration was identified as a protocol deviation. There were no deviations in the daily average concentration in the 30 and 80 ppm exposure groups. Daily average concentration deviations for the 10 ppm exposure group occurred on study days 1, 2, 3, 4, and 51 when average daily mean concentrations of 8.7, 8.8, 8.7, 8.9, and 11.1 ppm, respectively were observed. These relatively minor excursions outside the protocol-mandated range for target daily average H<sub>2</sub>S concentration did not affect the design, conduct or conclusions of this study.

**ENVIRONMENTAL CONDITIONS**

The temperature and relative humidity of the 1 m<sup>3</sup> exposure chambers were monitored continuously by the Andover Infinity System by recording the electronic signal from a temperature/relative humidity combination probe (Omega Engineering Inc., Stamford, CT) and recorded at least six times during each 6-hour exposure period. The temperature and relative humidity of the anteroom were monitored continuously and recorded approximately twice an hour. Environmental conditions were continuously monitored during the course of the study by the use of the Andover Infinity Direct Digital Control System (DDC; Andover Controls Corp., Andover, MA). Target conditions for temperature and relative humidity in the anterooms and

exposure chambers were 64-79°F and 30-70%, respectively, with a 12-hour light cycle per day. The chamber rooms (anteroom plus exposure chamber) were numbers 308 (control), 309 (10 ppm), 310 (30 ppm) and 311 (80 ppm). The temperature and relative humidity results for the four anterooms and exposure chambers (see Text Table D below) were as follows.

<b>TEXT TABLE D</b>				
<b>Temperature and Relative Humidity Results</b>				
	<b>1. Anterooms (Exposure Concentrations)</b>			
	<b>308 (control)</b>	<b>309 (10 ppm)</b>	<b>310 (30 ppm)</b>	<b>311 (80 ppm)</b>
<b>Grand Mean (± SD) Dry Bulb Temperature (°F)</b>	<b>66.8 ± 0.2<sup>a</sup></b>	<b>66.8 ± 0.2<sup>a</sup></b>	<b>66.2 ± 0.2<sup>a</sup></b>	<b>65.5 ± 0.2</b>
<b>Minimum Reading<sup>b</sup></b>	<b>66.5</b>	<b>66.5</b>	<b>65.7</b>	<b>65.1</b>
<b>Maximum Reading<sup>b</sup></b>	<b>67.1</b>	<b>67.3</b>	<b>66.8</b>	<b>66.2</b>
<b>Grand Mean (± SD) Relative Humidity, %</b>	<b>49 ± 2</b>	<b>50 ± 2</b>	<b>48 ± 2</b>	<b>48 ± 2</b>
<b>Minimum Reading<sup>b</sup></b>	<b>42</b>	<b>44</b>	<b>41</b>	<b>39</b>
<b>Maximum Reading<sup>b</sup></b>	<b>53</b>	<b>53</b>	<b>50</b>	<b>50</b>
	<b>2. H1000 Chambers (Exposure Concentrations)</b>			
	<b>308 (control)</b>	<b>309 (10 ppm)</b>	<b>310 (30 ppm)</b>	<b>311 (80 ppm)</b>
<b>Grand Mean (± SD) Dry Bulb Temperature, °F</b>	<b>73.0 ± 2.0<sup>a</sup></b>	<b>73.7 ± 1.0</b>	<b>71.6 ± 2.2</b>	<b>74.4 ± 0.9</b>
<b>Minimum Reading<sup>c</sup></b>	<b>69.0</b>	<b>60.9</b>	<b>68.4</b>	<b>72.2</b>
<b>Maximum Reading<sup>c</sup></b>	<b>75.9</b>	<b>75.3</b>	<b>75.1</b>	<b>75.7</b>
<b>Grand Mean (± SD) Relative Humidity, %</b>	<b>42 ± 1</b>	<b>46 ± 2</b>	<b>44 ± 5</b>	<b>41 ± 2</b>
<b>Minimum Reading<sup>c</sup></b>	<b>42</b>	<b>41</b>	<b>40</b>	<b>36</b>
<b>Maximum Reading<sup>c</sup></b>	<b>53</b>	<b>55</b>	<b>66</b>	<b>48</b>

<sup>a</sup> Grand mean of the daily means ± SD.  
<sup>b</sup> Minimum and maximum for any 30 minute reading during the study.  
<sup>c</sup> Minimum and maximum for any individual reading during the study.

The grand mean temperatures and grand mean relative humidities for exposure chambers and anterooms were maintained within the limits specified by the protocol. The temperature data for the control chamber (0 ppm) on days 23, 35, and 36 was lost when the thermister failed at the beginning of the exposure time. There were no excursions outside the protocol-mandated

range for temperature (65-79°F) and relative humidity (30-70%) identified in the 0, 10, and 80 ppm H1000 exposure chambers. No excursions outside the protocol-mandated range for temperature were observed during the 30 minute intervals for a 24-hour period in any of the anterooms. There was one brief (0.75 hour) excursion in relative humidity within the 30 ppm H1000 exposure chamber on exposure day 66. One brief (0.5 hour) excursion in relative humidity (to 75%) occurred for Anteroom 308 on exposure day 69. These brief and minor excursions outside the protocol-mandated range for relative humidity in the anterooms or exposure chambers did not affect the design, conduct or conclusions of this study.

#### **F0 BODY WEIGHT AND FEED CONSUMPTION**

All individual in-life and necropsy data are presented in Appendix B; all individual histopathology data are presented in Appendix C. Body weight data for all animals are presented in Tables B3 through B17. Food consumption data are presented in Tables B18 through B25.

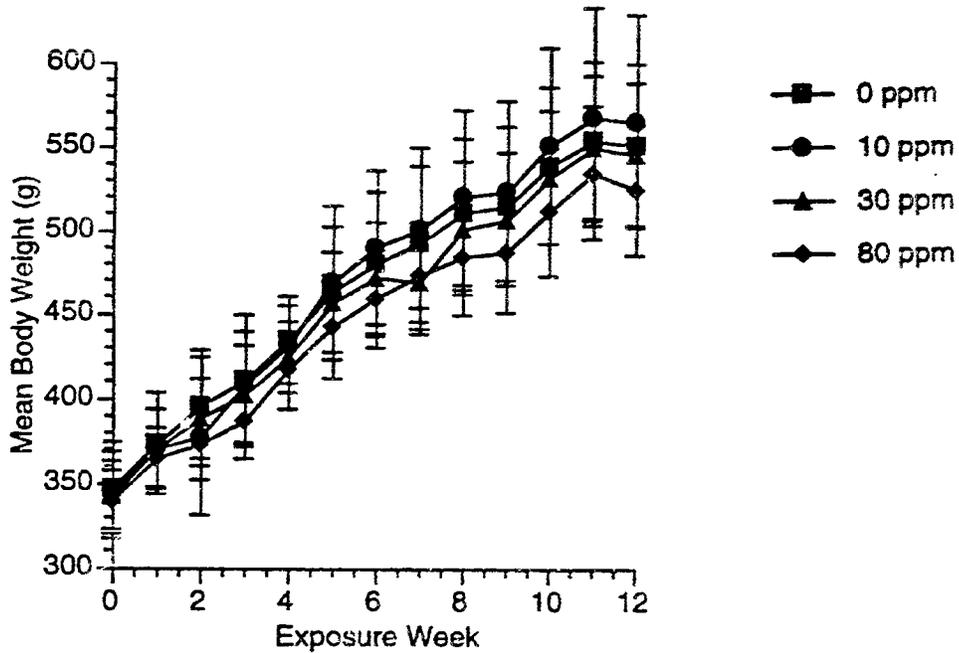
F0 males died prior to or during the conduct of the study. All F0 males in all groups survived to scheduled necropsy. One F0 females died pre-randomization. All remaining F0 females in all groups survived to scheduled necropsy.

No statistically significant differences in overall feed consumption (expressed as g/day) were observed in male F0 rats throughout the study period (Tables B18 and B19). Male rats in the 80 ppm H2S exposure group did, however, demonstrate a statistically significant decrease in feed consumption during the first week of exposure. Females in the 30 and 80 ppm H2S treatment groups also had lower feed consumption during the first week of exposure (pre-breed), however, this difference was not statistically significant (Tables B20). No treatment-related difference in feed consumption was observed during gestation (GD0 through GD20) in any confirmed pregnant rats exposed to H2S (Tables B22 and B23). No treatment-related difference in feed consumption was observed during lactation (PND0 through PND21) in female parental rats exposed to H2S (Tables B24 and B25). No treatment-related difference in feed consumption were observed in sperm-negative female F0 rats exposed to H2S (Table B21).

F0 male (Tables B3 and B4) body weights measured in grams were statistically equivalent among treatment groups at the start of the study (prebreed study day [sd] 0). The mean ( $\pm$  SD) initial body weights of F0 males were  $346.3 \pm 28.4$ ,  $344.9 \pm 24.6$ ,  $342.8 \pm 20.0$ , and  $340.1 \pm 17.9$  grams, respectively for the control, 10, 30 and 80 ppm treatment groups. F0 male rat body weights, recorded weekly were statistically equivalent among treatment groups throughout the

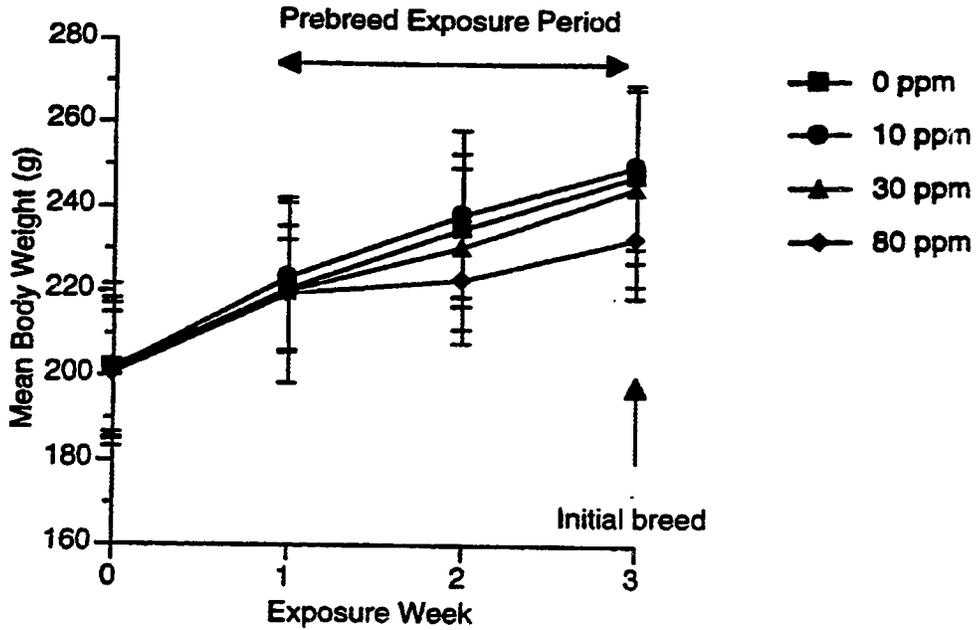
remainder of the study (Tables B3 and B4). Mean terminal body weights (g) were statistically equivalent among treatment groups. The mean ( $\pm$  SD) terminal body weights of F0 males were  $551.4 \pm 47.5$ ,  $565.3 \pm 63.0$ ,  $545.5 \pm 43.1$ , and  $524.6 \pm 38.9$  grams, respectively for the control, 10, 30 and 80 ppm treatment groups. Overall body weight gain as determined by the difference between the terminal and initial (prestudy) body weights were also equivalent among groups. The overall mean ( $\pm$  SD) body weight gain of F0 males were  $208.0 \pm 30.4$ ,  $223.0 \pm 44.1$ ,  $206.2 \pm 32.5$ , and  $194.5 \pm 43.6$  grams, respectively for the control, 10, 30 and 80 ppm treatment groups.

**Figure 2. Mean F0 male body weight (g) following H2S exposure**



F0 female (Tables B5 and B6) body weights were statistically equivalent among treatment groups at the start of the study (prebreed study day [sd] 0). The mean ( $\pm$  SD) initial body weights of F0 females were  $201.43 \pm 15.8$ ,  $201.6 \pm 16.6$ ,  $202.1 \pm 19.2$ , and  $200.6 \pm 14.0$  grams, respectively for the control, 10, 30 and 80 ppm treatment groups.

Figure 3. Mean F0 female prebreed body weight (g) following H2S exposure



No statistically significant differences in body weight gain were observed during gestation (GD0 through GD20) or lactation (PND0 through PND21) in any confirmed pregnant or lactating F0 rats exposed to H2S (Tables B8, B9, B10, and B11).

Figure 4. Mean gestational body weight (g) in sperm-positive pregnant female F0 rats following H2S exposure

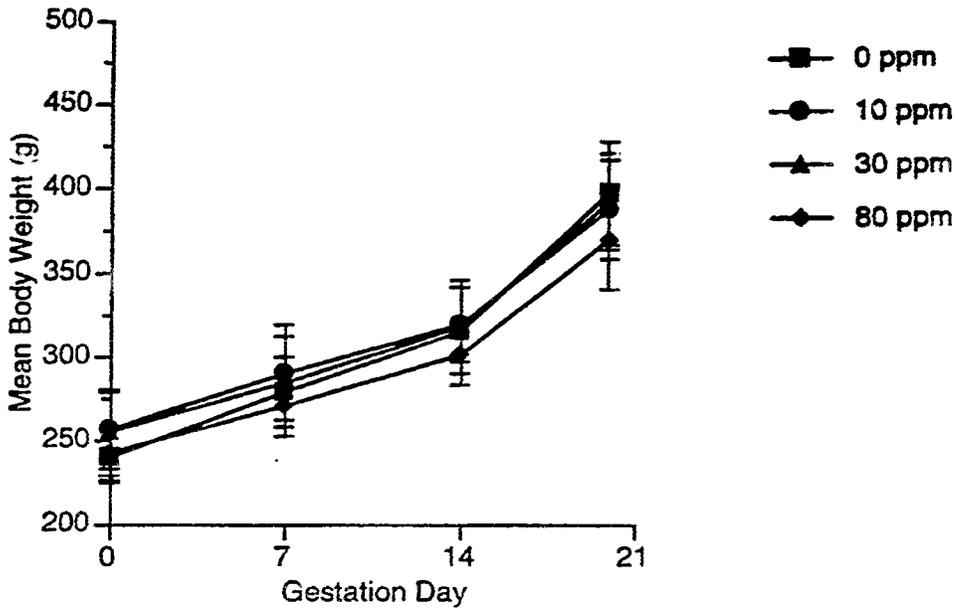
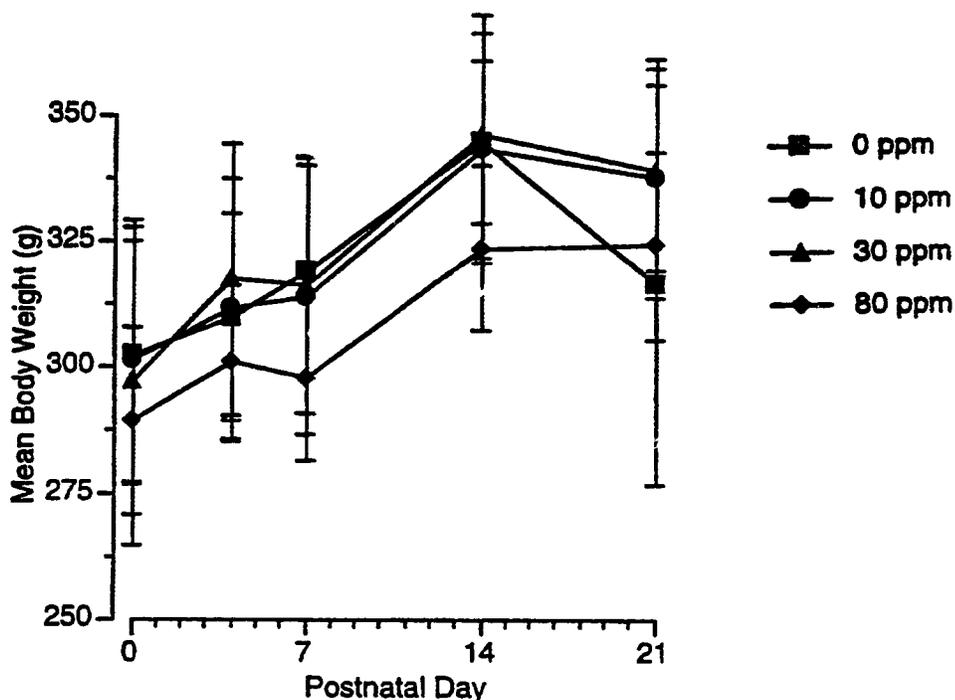


Figure 5. Mean lactational body weight (g) of female F0 rats following H2S exposure



No treatment-related difference in body weight was observed in sperm-negative female F0i rats exposed to H<sub>2</sub>S (Table B7). No statistically significant differences in terminal body weight were observed in female parental rats (Table B5 and B6).

#### F0 REPRODUCTIVE TOXICITY

Reproductive indices for F0 parents (Text Table E, Table B62) exhibited no treatment-related changes in mating index, fertility index, or postimplantation loss per litter. Mean gestational length in days was not significantly affected by H<sub>2</sub>S exposure. Mean ( $\pm$  SEM) gestational length in days were  $21.9 \pm 0.23$ ,  $21.2 \pm 0.50$ ,  $22.3 \pm 0.16$ , and  $21.8 \pm 0.25$  days, respectively for the control, 10 ppm, 30 ppm, and 80 ppm treatment groups. The numbers of implantation sites per litter and the litter size on pnd 0 were equivalent across all groups. Mean ( $\pm$  SEM) numbers of implantation sites per litter were  $12.5 \pm 2.2$ ,  $14.8 \pm 1.46$ ,  $11.9 \pm 2.12$ , and  $9.7 \pm 2.29$  implantation sites per litter, respectively, for the control, 10 ppm, 30 ppm, and 80 ppm treatment groups.

Mean ( $\pm$ SEM) litter size were  $16.3 \pm 0.52$ ,  $14.6 \pm 0.79$ ,  $14.8 \pm 0.43$ , and  $13.9 \pm 1.64$  pups, respectively, for the control, 10 ppm, 30 ppm, and 80 ppm treatment groups. The F1 litter sex ratio (% male pups per litter) was equivalent across groups for pnd 0. The percent male pups on pnd 0 were 45.5, 51.3, 44.7, and 45.5%, respectively, for the control, 10 ppm, 30 ppm, and 80 ppm treatment groups. Survival indices for pnd 4, 7, 14, and 21 were all equivalent across all groups (Tables B63 and B64).

**Text Table E**  
**Reproductive and Developmental Toxicity Indices**

Endpoint	Exposure Group			
	0	10	30	80
Mating Index	92 (11/12)	92 (11/12)	75 (9/12)	83 (10/12)
Fertility Index	82 (9/11)	100 (11/11)	100 (9/9)	80 (8/10)
Postimplantation Loss per Litter %	2.5	11.0	10.2	7.0

A summary of the F0 male andrological assessments is presented in Text Table F. F0 absolute paired testes, paired epididymides and cauda weights were equivalent across all groups (see Text Table J). Percent motile sperm, percent normal sperm, daily sperm production, and cauda sperm count were statistically equivalent across all groups.

**Text Table F**  
**F0 Male Sperm Analysis Following H2S Exposure**

		Exposure Group			
		0 ppm	10 ppm	30 ppm	80 ppm
% Abnormal Sperm	Mean	0.33	1.46	3.33	7.59
	S.D.	0.44	1.89	8.11	21.71
	n	12	12	12	11 <sup>a</sup>
% Motile Sperm	Mean	81.58	74.91	75.91	76.09
	S.D.	6.16	24.58	13.47	11.06
	n	12	12	12	11 <sup>a</sup>
Cauda Sperm Count <sup>b</sup>	Mean	1.017	0.988	1.046	0.822
	S.D.	0.152	0.174	0.225	0.401
	n	12	12	12	12
Daily Sperm Production <sup>c</sup>	Mean	17.92	18.14	17.26	17.30
	S.D.	2.17	1.58	1.89	5.63
	n	12	12	12	12

<sup>a</sup> There were no sperm in the sperm motility and morphology sample from one animal.

<sup>b</sup> Cauda sperm count means and standard deviations are expressed as  $\times 10^9$ .

<sup>c</sup> Daily sperm production means and standard deviations are expressed as  $\times 10^6$ .

#### F0 CLINICAL OBSERVATIONS

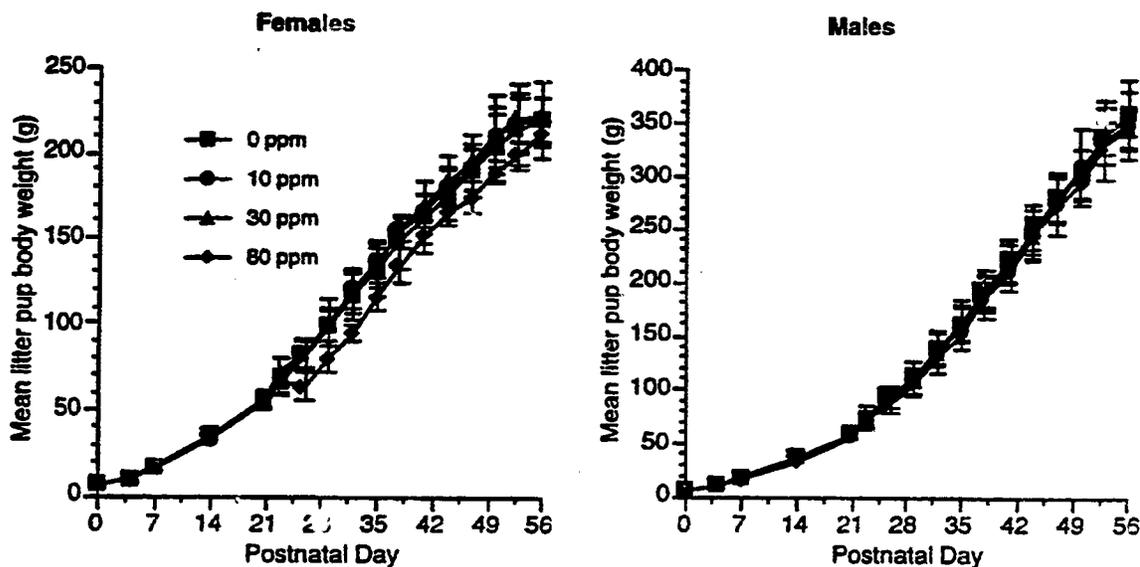
No treatment-related clinical signs of toxicity occurred in F0 animals following H2S exposure. Clinical observations during the approximately 70 day exposure period for F0 males are summarized in Table B26 and B28. Alopecia was observed in 3, 3, 4, and 3 F0 males at 0, 10, 30 and 80 ppm, respectively. Foot lacerations were also observed in 3, 0, 1, and 2 F0 males at 0, 10, 30 and 80 ppm, respectively. Several F0 males also demonstrated aggressive behavior. Aggression was observed in 1, 1, 1, and 1 F0 males at 0, 10, 30 and 80 ppm, respectively.

Clinical observations for F0 females are summarized in Tables B27 and B28. Alopecia was observed in 2, 2, 3, and 1 F0 females at 0, 10, 30 and 80 ppm, respectively. One rat (ear tag # 67) from the 10 ppm treatment group was noted to have a discolored ocular discharge on the 36th day of exposure. Three females (rat # 58 from the control group; rat # 61 from the 10 ppm exposure group; and rat # 74 from the 30 ppm treatment group) began to litter during the six hour H<sub>2</sub>S exposure. One female rat (ear tag # 85) from the 80 ppm treatment group was observed to have a tail laceration that was healed by the 21st exposure day. The observed foot pad and paw lacerations and swelling are not uncommon in young rats housed in caging with stainless steel wire floors. The severity of the injuries were unlikely to result in any detrimental effect to the animal.

#### F1 PUP GROWTH AND DEVELOPMENT

No statistically significant difference in pup body weight gain was observed in H<sub>2</sub>S-exposed pups of either sex either during lactation or the remainder of the postnatal period (PND 22 through PND 60-65) (Tables B12 through B17). No statistically significant difference in terminal body weight were observed in postweanling male or female rat pups. Terminal mean body weights ( $\pm$  SD) for male pups on pnd 59-66 were  $388.9 \pm 39.8$ ,  $401.5 \pm 38.6$ ,  $395.2 \pm 18.2$ , and  $382.3 \pm 41.5$  g, respectively, for the control, 10 ppm, 30 ppm, and 80 ppm treatment groups. Terminal mean body weights ( $\pm$  SD) for female pups on pnd 59-66 were  $236.0 \pm 21.2$ ,  $243.9 \pm 20.2$ ,  $248.3 \pm 25.2$ , and  $233.6 \pm 5.1$  g, respectively, for the control, 10 ppm, 30 ppm, and 80 ppm treatment groups.

**Figure 6. Mean ( $\pm$  SD) litter body weights (g) of H<sub>2</sub>S-exposed male and female pups from postnatal day 0 through 57.**



The ability to surface right on postnatal day 4 was equivalent across all treatment groups (Tables B31 and B37). The mean percent of pups ( $\pm$  SD) that were able to surface right were  $82.1 \pm 11.9$ ,  $81.1 \pm 11.3$ ,  $78.8 \pm 14.2$ , and  $76.36 \pm 15.5$  %, respectively for the control, 10 ppm, 30 ppm, and 80 ppm treatment groups. The mean date on which other landmarks of pup development were acquired by all pups are summarized in Text Table G. Individual pup data are presented in Tables B30, B32 through B36 while summary data for daily landmark acquisition are presented in Table B37. There were no statistically significant differences among groups for any developmental landmark, including incisor eruption, negative geotaxis, eyelid separation, vaginal patency, or balano-preputial separation.

**Text Table G**  
Acquisition of Developmental Landmarks in Neonatal Pups Exposed to H<sub>2</sub>S.

Developmental Landmark <sup>1</sup>		H <sub>2</sub> S Exposure Group			
		0 ppm	10 ppm	30 ppm	80 ppm
Pinnae Detachment	Mean	3.4	3.6	3.1	3.3
	SD	1.1	1.2	0.6	1.0
	n	9	11	9	8
Negative Geotaxis	Mean	8.0	8.6	8.3	7.8
	SD	1.0	0.5	0.8	0.8
	n	7	5	6	6
Incisor Eruption	Mean	10.9	11.5	11.9	11.3
	SD	1.3	0.5	1.6	1.4
	n	9	11	9	8
Eyelid Separation	Mean	14.6	15.2	14.8	15.0
	SD	0.9	0.4	0.8	0.8
	n	8	11	9	8
Vaginal Patency	Mean	35.2	33.3	32.7	33.5
	SD	1.9	1.2	2.6	1.5
	n	9	11	9	8
Balano-Preputial Separation	Mean	42.3	43.9	42.9	43.1
	SD	2.1	2.3	2.3	1.6
	n	9	11	9	7

1. Data Presented Represent Postnatal Age at Which the Entire Litter Has Met Criterion

#### F1 PUP CLINICAL OBSERVATIONS

Clinical observations of F1 pups during the lactation period (Table B29) indicated no treatment-related incidence of found dead, euthanized moribund, or missing and presumed dead pups following H<sub>2</sub>S exposure. Observations consistent with teratogenic effects which were only noted in the H<sub>2</sub>S-exposed groups included kinked tail (1 litter affected in the 10 ppm H<sub>2</sub>S treatment group), umbilical hernia (1 litter affected in the 10 ppm H<sub>2</sub>S treatment group), cranial defect (1 litter affected in the 10 ppm H<sub>2</sub>S treatment group), and skin lesions (2 litters affected in the 30 ppm H<sub>2</sub>S treatment group) that included webbing of the forelimb skin and kinked tail (1 litter affected in the 30 ppm H<sub>2</sub>S treatment group and 1 litter affected in the 80 ppm H<sub>2</sub>S treatment group - no tail) (Text Table H). Hematomas were observed in all groups). The total numbers of pups found dead, euthanized and moribund, or missing and presumed dead through pnd 21 were two from the control group (litters from dam# 54 and 50), six from the 10 ppm

group (litters from dam# 62, 63, 67 and 70), eight from the 30 ppm group (litters from dam# 73 and 76), and two from the 80 ppm group (litter from dam# 92). These pups were not necropsied. Observations present in all groups included skin lesions resulting from suspected trauma, and hematomas in various locations (Table B29). No clinical observations were observed in postnatal day 14 or 21 rat pups from either the control or H<sub>2</sub>S-exposed treatment groups.

Text Table H

Summary of Clinical Observations in Male and Female F1 Rat Pups Exposed to H<sub>2</sub>S.

Postnatal Day	Observation <sup>1</sup>	H <sub>2</sub> S Exposure Concentration (ppm)			
		0	10	30	80
<b>Possible Teratogenic Effects</b>					
0	Cranial defect - indentation on skull		1 (1)		
	Kinked or missing tail		2 (1)	1 (1)	1 (1)
	Late resorption or stillbirth	1 (1)	3 (3)	5 (3)	1 (1)
	Possible missing eye			1 (1)	
	Rear legs and body small		1 (1)		
	Skin lesion, webbing, reddened limbs or rest of body			7 (1)	
	Umbilical hernia		1 (1)		
<b>Possible Traumatic Effects</b>					
	Hematomas	11 (5)	11 (4)	9 (6)	5 (3)
	Left ear with red ring			1 (1)	
	Missing hind left leg, possibly caused by dam			1 (1)	
	Pup euthanized			1 (1)	
	Scratches/cuts/scabs	1 (1)		1 (1)	1 (1)
	Skin defect or bite mark on face			1 (1)	
	Swelling	2 (1)			
4	Hematomas		2 (2)	1 (1)	
	Kinked tail	2 (2)	1 (1)	1 (1)	2 (1)
	Pale		1 (1)		
	Umbilical hernia		1 (1)		
7	Runt - missing at end of exposure		1 (1)		
	Scratches/cuts/scabs		1 (1)		

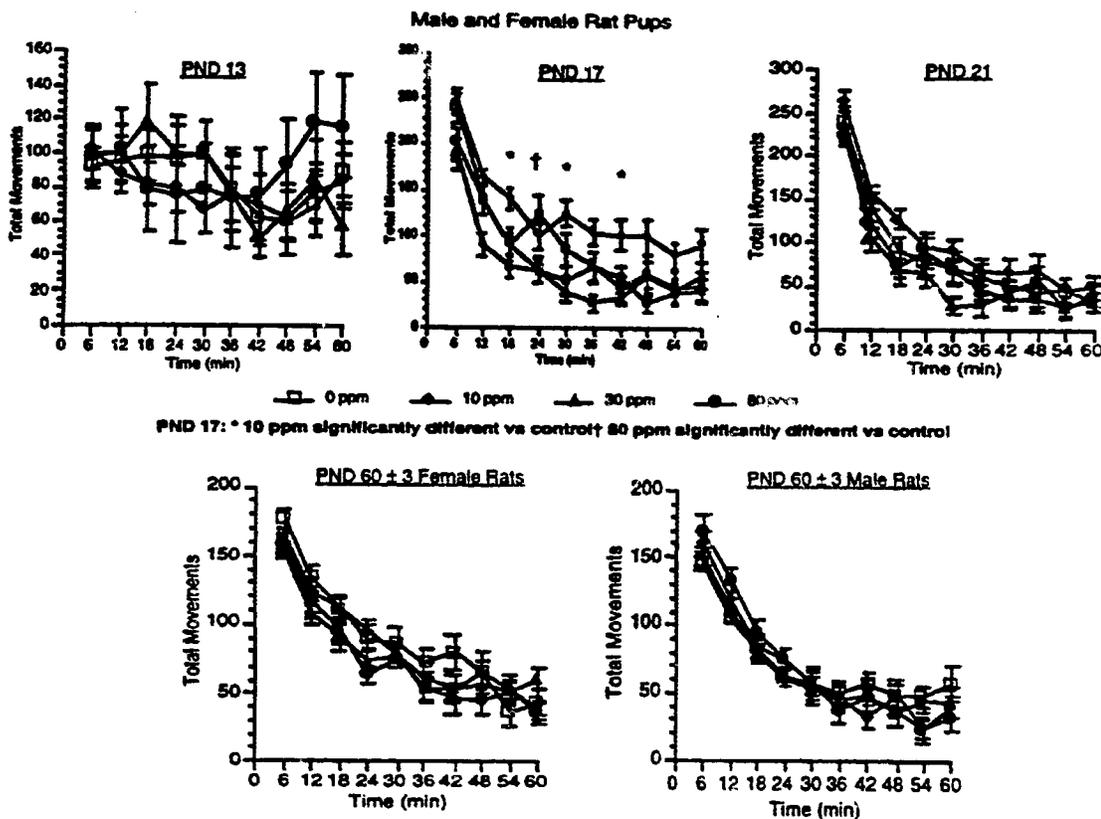
<sup>1</sup> Positive notation indicates that an abnormal clinical observation was demonstrated on any individual on that day. Data presented are for individual animals and in parenthesis number of litters affected.

**F1 PUP NEUROBEHAVIOR**

Mean ( $\pm$  SD) white noise level maintained in the motor laboratory was  $68.8 \pm 0.67$  dB (Table B38). The mean ( $\pm$  SD) illumination level in the motor activity laboratory was  $2.7 \pm 0.41$  ft-candles (Table B38). Motor activity data is presented for all pups in Tables B39 through B42 and graphically in Figure 7. Increased overall motor activity (hyperactivity) was observed in pnd 17 rat pups exposed to 10 ppm H<sub>2</sub>S. Exposure to H<sub>2</sub>S did not affect the ontogeny of motor activity in rats when compared to control animals since no statistically significant treatment-related effects on overall motor activity were observed during the pnd 13, pnd 21, or pnd  $60 \pm 2$  motor activity test sessions. A statistically significant gender-related effect on overall motor activity was observed during the pnd  $60 \pm 2$  test session. Female rats displayed a higher amount of motor activity during this pnd  $60 \pm 2$  test session.

Passive avoidance with a step-through to darkness paradigm, including one training and one retention trial repeated approximately 24 hr later, was used to assess learning and memory. Passive avoidance was tested in separate F1 pups (one male and one female from each litter) on pnd  $22 \pm 1$  and on pnd  $62 \pm 3$ . The latency to enter the dark compartment was measured for each trial. If the latency reached 240 s, the rat was removed from the chamber and assigned an arbitrary latency (maximum) score of 240. Rats that did not cross over during the first session were excluded from the statistical analysis. The difference between the day one and day two crossover latency times were analyzed.

Figure 7  
Mean ( $\pm$  SEM) Motor Activity in F1 Pups Following H2S Exposure



The results of the passive avoidance testing are summarized in Text Table I. Individual pup data are presented in Tables B43 and B44. There were no statistically significant differences between rats of either sex among treatment groups in initial cross over time measured on the first day of testing on either pnd 21 or 22 or on pnd 62  $\pm$  3. There were also no statistically significant difference among groups for the ability to complete the passive avoidance task. Male rats tested on pnd 22  $\pm$  1 had a lower, albeit not statistically significant, decrease in the step through latency time on the second day of testing. Historical control data generated using similar exposure conditions (i.e., air-control individual glass cylinder exposures) and with the same age and strain of rat demonstrate that control male pnd 21 rats had a longer mean ( $\pm$  SEM) retention trial step through latency time (110.8  $\pm$  35 sec) than those observed in the

current study. It is unknown why the male pnd 21 rats had a shorter retention trial step through latency time. The female pnd 22 ± 1 rats in the present study had mean (± SEM) retention trial step through latency times that were similar to historical control values. Both treated male and female pnd 21 ± 1 rats had statistically equivalent mean (± SEM) retention trial step through latency times, thus it is unlikely that the shorter mean (± SEM) retention trial step through latency times observed in control male pnd 21 ± 1 rats had any impact on the study.

Text Table I

Effect of H<sub>2</sub>S Exposure on Passive Avoidance

## Post Natal Day 21-22

Sex	Exposure Group	Mean Difference in Step-through Latency (sec) <sup>1</sup>	Sample size	SEM
F	0 ppm	95.04	9	29.32
F	10 ppm	81.18	10	28.46
F	30 ppm	75.14	9	22.75
F	80 ppm	58.22	8	28.22
M	0 ppm	11.22	8	23.82
M	10 ppm	37.55	10	44.13
M	30 ppm	62.90	7	31.99
M	80 ppm	65.08	7	32.24

## Post Natal Day 62 ± 3

Sex	Exposure Group	Mean Difference in Step-through Latency (sec)	Sample size	SEM
F	0 ppm	160.73	9	22.91
F	10 ppm	203.90	10	8.43
F	30 ppm	179.94	9	20.77
F	80 ppm	153.40	8	38.48
M	0 ppm	177.30	9	22.12
M	10 ppm	189.91	8	14.34
M	30 ppm	219.94	5	4.70
M	80 ppm	197.16	6	18.55

<sup>1</sup> Calculated as the difference between the step through latency time observed during the training and retention trials. All rats that failed to step through during the training trial were excluded from analysis.

A battery of tests (functional observation battery; FOB) designed to detect gross alterations in nervous system function was performed for one male and one female from each litter. FOB evaluations were performed on pnd 60 ( $\pm 2$  days). The animals were examined by a trained technician who was blinded to the animals' exposure assignment. Individual animal and summary FOB data are presented in Tables B54 through B61. No evidence of sensorimotor dysfunction (i.e., altered acoustic response, tail pinch response, approach response, or visual placing response) was observed in any control or H<sub>2</sub>S-exposed F1 rats. Likewise, no evidence of neuromuscular dysfunction (e.g., abnormal muscle tone) was observed in any control or H<sub>2</sub>S-exposed rats. No clinical signs of ataxia, piloerection, excessive vocalization, muscle tremors or spasms, clonic or tonic seizures, increased salivation, abnormal respiration, or abnormal pupil reflex were observed in any H<sub>2</sub>S-exposed rats. No statistically significant effect on body appearance (i.e., altered skin temperature or color, facial crust, or altered fur appearance) was observed in any H<sub>2</sub>S-exposed rats.

Acoustic startle was assessed using a microcomputer-controlled automated test system (SR-LAB, San Diego Instruments, San Diego CA). The magnitude of the acoustic startle amplitude elicited during prepulse and pulse trials were calculated using a calibrated (in units of the computer's analog-to-digital converter) piezoelectric detector. The units of acoustic startle amplitude measurement are proportional to force, however, actual units are in arbitrary units. Different sized Plexiglass cylinders were used for testing different aged animals (e.g., a 4 cm chamber was used for pnd 21 rats while a 9 cm chamber was used for pnd 62  $\pm 3$  rats). Since the magnitude of the acoustic startle amplitude response is influenced by chamber size, no direct comparisons between different age groups were made. Fifty pulse trials (110 dB, 50 msec stimulus) and fifty prepulse trials (80 dB, 50 msec followed 100 msec later by a 110 dB, 50 msec stimulus) were randomly presented in blocks of two (prepulse- and pulse-elicited trials). Individual animal data (Table B45 through B52) and summary tables (Table B53) are presented in Appendix B. Hydrogen sulfide treatment was not associated with any statistically significant alteration in acoustic startle reflex.

Figure 8. Effects of H2S Exposure on Acoustic Startle Reflexes

Prepulse

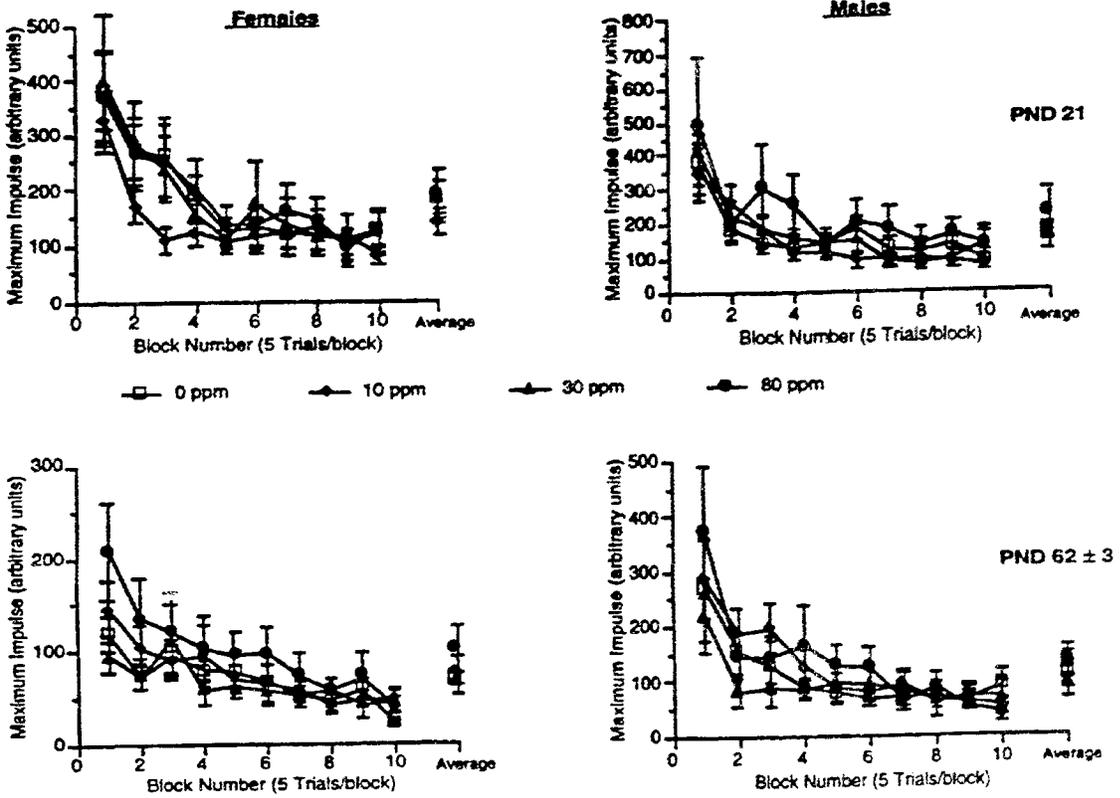


Figure 8. Effects of H2S Exposure on Acoustic Startle Reflexes

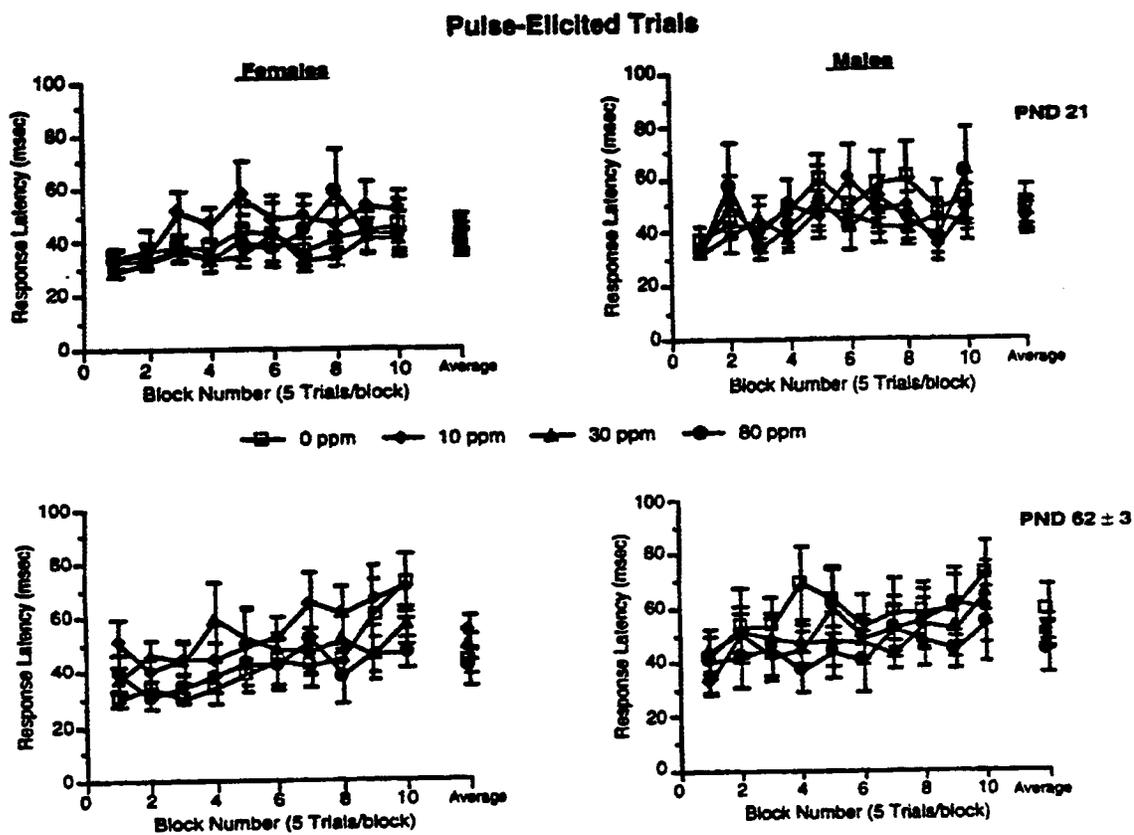


Figure 8. Effects of H2S Exposure on Acoustic Startle Reflexes

Pulse

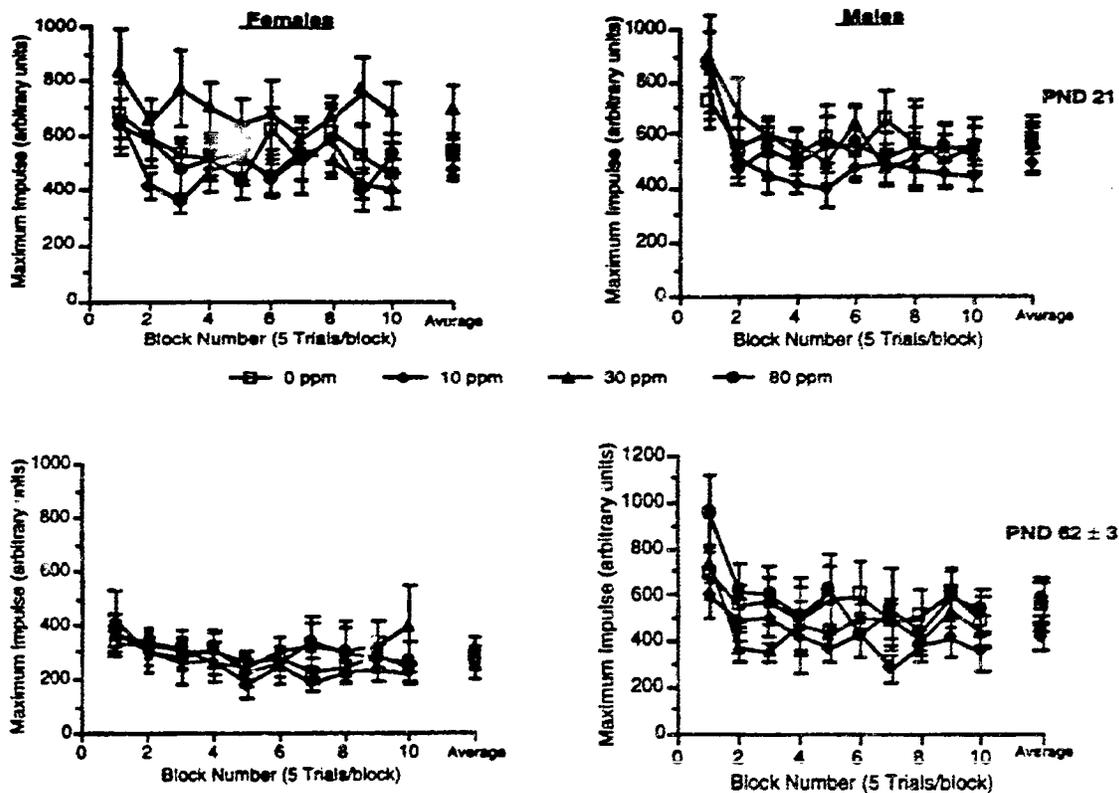
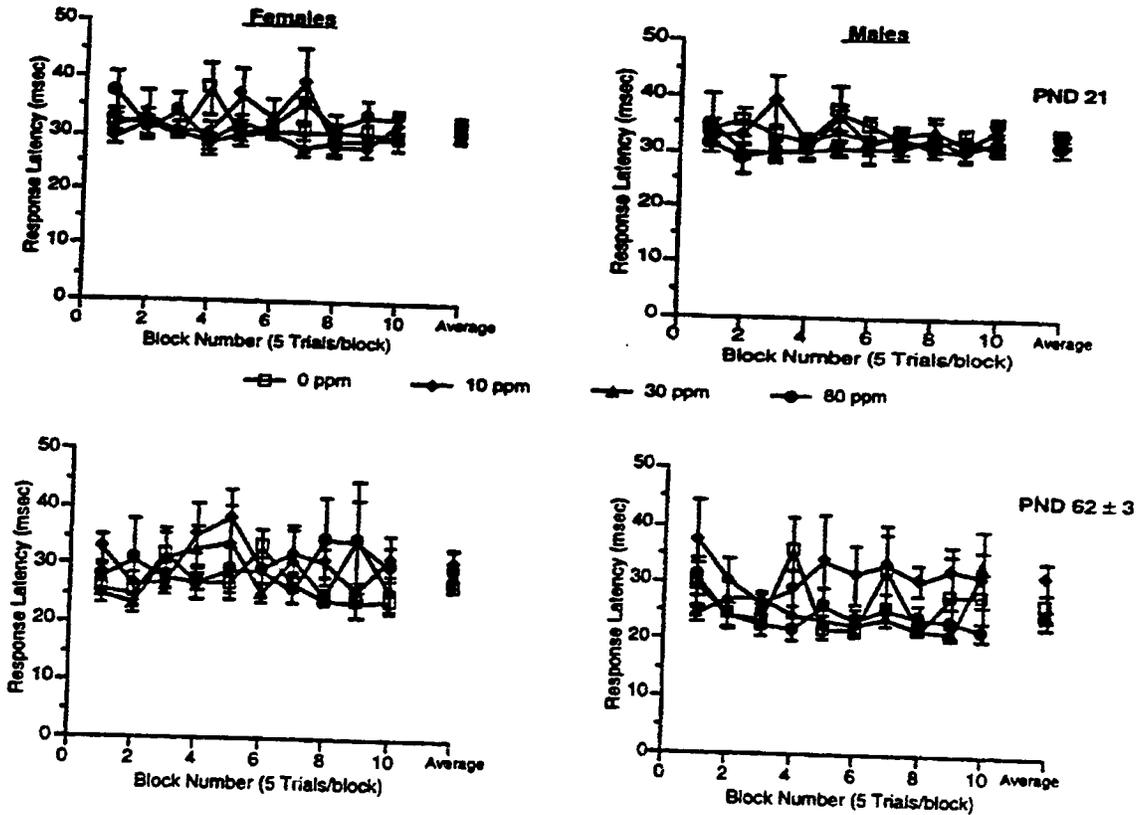


Figure 8. Effects of H2S Exposure on Acoustic Startle Reflexes



NECROPSY AND HISTOLOGY – F0 RATS

At the end of the exposure regimen, adult rats of the parental generation were weighed, euthanized with CO<sub>2</sub>, exsanguinated, and had a complete necropsy performed with special emphasis on the reproductive and associated organs. The non-impregnated adult females (*n* = 11) and adult males (*n* = 48) were necropsied the day after the last day of exposure. The postpartum adult females (*n* = 37) were necropsied the day of or the day after their pups were weaned (PND 21). Full details of the results of gross and histologic evaluation of F0 animals is presented in Appendix C.

**Terminal Body and Absolute Organ Weights**

Terminal body and absolute organ weights for adult F0I males are summarized in Text Table J (see also Table B68). The adult male rats had no H<sub>2</sub>S exposure-related alteration in terminal body weights. The only statistically significant difference from control in either absolute or relative organ weights was a decrease in the absolute and relative weight of the adrenal glands in the 10 and 80 ppm dose groups.

Text Table J

Absolute Mean ( $\pm$  SD) Terminal Body or Organ Weights (g) for Male F0 Rats Exposed to H<sub>2</sub>S

	H <sub>2</sub> S Exposure Concentration (ppm)			
	0	10	30	80
Terminal Weight	551.4 $\pm$ 47.5	565.3 $\pm$ 63.0	545.5 $\pm$ 43.1	524.6 $\pm$ 38.9
Adrenal Glands	0.07 $\pm$ 0.01	0.06 $\pm$ 0.01	0.07 $\pm$ 0.01	0.06 $\pm$ 0.01
Brain	2.12 $\pm$ 0.13	2.14 $\pm$ 0.09	2.12 $\pm$ 0.09	2.10 $\pm$ 0.05
Heart	1.54 $\pm$ 0.13	1.56 $\pm$ 0.12	1.55 $\pm$ 0.17	1.45 $\pm$ 0.09
Kidneys	3.88 $\pm$ 0.43	3.95 $\pm$ 0.34	3.98 $\pm$ 0.40	3.77 $\pm$ 0.33
Liver	19.65 $\pm$ 1.84	20.86 $\pm$ 3.12	19.89 $\pm$ 2.78	19.09 $\pm$ 2.16
Lungs	1.47 $\pm$ 0.14	1.48 $\pm$ 0.11	1.47 $\pm$ 0.18	1.53 $\pm$ 0.14
Spleen	0.85 $\pm$ 0.13	0.78 $\pm$ 0.09	0.83 $\pm$ 0.11	0.82 $\pm$ 0.11
Epididymides Caudae	0.68 $\pm$ 0.09	0.65 $\pm$ 0.09	0.63 $\pm$ 0.05	0.71 $\pm$ 0.37
Epididymides Whole	1.52 $\pm$ 0.17	1.47 $\pm$ 0.15	1.42 $\pm$ 0.06	1.52 $\pm$ 0.52
Prostate	1.37 $\pm$ 0.25	1.29 $\pm$ 0.34	1.35 $\pm$ 0.26	1.33 $\pm$ 0.30
Seminal vesicles and coagulating glands	2.00 $\pm$ 0.37	1.90 $\pm$ 0.29	2.00 $\pm$ 0.28	1.96 $\pm$ 0.50
Testes	3.52 $\pm$ 0.30	3.47 $\pm$ 0.26	3.47 $\pm$ 0.18	3.27 $\pm$ 0.84

Terminal body and absolute organ weights for F0 females are summarized in Text Table K (see also Table B69). The adult female rats had no H<sub>2</sub>S exposure-related alteration in terminal body weights. The only statistically significant difference from control in either absolute or relative organ weights was a decrease in the relative weight (normalized to brain weight) of the ovaries with oviducts in the 10 ppm dose group. The number of implantation sites per rat uterus was not significantly altered by H<sub>2</sub>S exposure. All non-impregnated adult females had no detectable implantation sites at necropsy. All postpartum females had multiple implantation sites present.

Text Table K

Absolute Mean ( $\pm$  SD) Body or Organ Weights (g) for Female F0 Rats Exposed to H<sub>2</sub>S

	Exposure Group (ppm)			
	0	10	30	80
Terminal Body Weight	313.8 $\pm$ 32.7	327.0 $\pm$ 26.0	322.5 $\pm$ 26.7	303.3 $\pm$ 26.8
Adrenal Glands	0.08 $\pm$ 0.01	0.08 $\pm$ 0.01	0.08 $\pm$ 0.01	0.08 $\pm$ 0.01
Brain	1.88 $\pm$ 0.11	1.90 $\pm$ 0.12	1.84 $\pm$ 0.07	1.89 $\pm$ 0.06
Kidneys	2.40 $\pm$ 0.20	2.60 $\pm$ 0.31	2.46 $\pm$ 0.43	2.37 $\pm$ 0.33
Liver	15.10 $\pm$ 3.51	17.47 $\pm$ 3.11	15.66 $\pm$ 3.59	14.79 $\pm$ 3.17
Spleen	0.61 $\pm$ 0.10	0.58 $\pm$ 0.06	0.61 $\pm$ 0.08	0.58 $\pm$ 0.06
Ovaries	0.14 $\pm$ 0.02	0.13 $\pm$ 0.02	0.14 $\pm$ 0.02	0.13 $\pm$ 0.02
Uterus	0.44 $\pm$ 0.17	0.41 $\pm$ 0.12	0.44 $\pm$ 0.09	0.43 $\pm$ 0.12
Implantation sites	12.50 $\pm$ 7.69	14.83 $\pm$ 5.04	11.92 $\pm$ 7.33	9.67 $\pm$ 7.94

Gross Necropsy Observations

The incidences of gross observations made at necropsy of the F0 male rats are summarized in Text Table L (see Table C3). Gross observations made during complete necropsies of the F0 male rats had very low incidences that were not treatment-related. Due to the lack of toxicologically relevant lesions in other body systems, microscopic examination was limited to the reproductive tracts of male rats in the control and high dose groups that were not reproductively successful and that had gross observations made in the reproductive tract.

Text Table L.

Incidence<sup>1</sup> of Gross Lesions in F0 Male Rats Exposed for at Least 70 Consecutive Days to H2S

Organ	Gross Observation <sup>2</sup>	Exposure Group			
		0 ppm	10 ppm	30 ppm	80 ppm
Heart	hypertrophy <sup>3</sup>	2/12	0/12	0/12	1/12
Kidneys	hydronephrosis <sup>4</sup>	0/12	1/12	0/12	0/12
Liver	tan focus	1/12	4/12	0/12	0/12
Testes	small	0/12	0/12	0/12	1/12
Testes	small and soft	0/12	0/12	0/12	1/12
Epididymides	small	0/12	0/12	0/12	1/12
Epididymides	right tail: yellow-green enlarged soft	0/12	0/12	0/12	1/12
Seminal vesicles/ coagulating glands	right small	0/12	1/12	0/12	0/12
Prostate	brown/tan focus	1/12	0/12	0/12	1/12
Adrenal Glands	irregular small	0/12	1/12	0/12	0/12
Lymph nodes	enlarged	1/12	0/12	0/12	0/12
Spleen	bisected	1/12	0/12	0/12	0/12
Nose	red exudate	0/12	0/12	1/12	0/12
Other Tissues <sup>5</sup>	N/A <sup>6</sup>	N/A	N/A	N/A	N/A

<sup>1</sup> = # of rats with gross observation/# of rats grossly examined in dose group.

<sup>2</sup> = does not include the incidental findings of red or dark foci in the kidneys or lungs associated with CO<sub>2</sub> euthanasia.

<sup>3</sup> = firm with small left ventricular lumen

<sup>4</sup> = dilated renal pelvis/es

<sup>5</sup> = includes all tissues examined in which no gross observations were made in any adult male rats: skin, salivary glands, nasopharynx, trachea, mainstem bronchi, aorta, thymic region, thyroids/parathyroids, pancreas, esophagus, stomach, small intestines, colon, urinary bladder, penis w/ preputial gland, brain, pituitary gland and eyes.

<sup>6</sup> = not applicable

The incidences of gross observations made at necropsy of the F0 female rats are summarized in Text Table M (see Table C1). Gross observations made during complete necropsies of the F0 female rats had very low incidences that were not treatment-related. Due to the lack of toxicologically relevant lesions in other body systems, microscopic examination was limited to the reproductive tracts of female rats in the control and high dose groups, that were not reproductively successful, and that had gross observations made in the reproductive tract.

Text Table M.

Incidence<sup>1</sup> of Gross Lesions in F0 Female Rats Exposed to H2S

Organ/tissue	Gross Observation <sup>2</sup>	Exposure Group			
		0 ppm	10 ppm	30 ppm	80 ppm
Uterus	mucometra <sup>3</sup>	1/12	0/12	0/12	0/12
Uterus	mesometrial cyst	0/12	1/12	0/12	0/12
Cervix/Vagina	cystic malformation	1/12	0/12	0/12	0/12
Mammary gland	mass	1/12	0/12	0/12	0/12
Eye	brown exudate	0/12	1/12	0/12	0/12
Other tissues <sup>4</sup>	N/A <sup>5</sup>	N/A	N/A	N/A	N/A

<sup>1</sup> = # of rats with gross observation/# of rats grossly examined in dose group.

<sup>2</sup> = does not include the incidental gross observations of red and dark foci in the lungs and kidneys associated with CO<sub>2</sub> euthanasia and uterine luminal distention by clear fluid which is a normal finding in reproductively cycling rats in proestrus or estrus.

<sup>3</sup> = uterine horn distended with tan opaque fluid (secondary to outflow obstruction by vaginal cyst)

<sup>4</sup> = includes all tissues examined in which no gross observations were made in any adult female rats: skin, salivary glands, lymph nodes (mesenteric and nonmesenteric), nasopharynx, trachea, mainstem bronchi, heart, aorta, thymic region, thyroids/parathyroids, liver, spleen, pancreas, adrenal glands, esophagus, stomach, small intestines, colon, urinary bladder, ovaries and oviducts, brain (cerebrum, cerebellum, medulla/pons), and pituitary gland.

<sup>5</sup> = not applicable

**Histopathological Observations**

Due to the lack of toxicologically relevant lesions in other body systems, microscopic examination of F0 male rats was limited to the reproductive tracts in the control and high dose groups (see Table C4). In addition, F0 males that were not reproductively successful or that had gross observations made in the reproductive tract were also evaluated histologically. The incidences of histologic diagnoses in the male reproductive tracts are summarized in Text Table N. Statistical comparison of the control and high dose groups showed no significant difference from control in the incidences of the gross observations and histologic diagnoses found. However, there were a few histologic diagnoses with a higher incidence in the 80 ppm treatment group when compared to control rats. Most of these diagnoses are related to seminiferous tubular degeneration (including intratubular sperm stasis, tubular mineralization, sperm granulomas, and multinucleated giant cells) and its corresponding changes in the epididymis (degenerate sperm forms in lumen, aspermia, and oligospermia). One case each of epididymal sperm granulomas (rat ear tag #40) and unilateral necrosis of the cauda (rat ear tag # 46) were detected only in the high dose group at very low incidence (8.3%). Also, there was a slightly higher incidence of lymphoid interstitial infiltrate in the ventral prostate in the 80 ppm treatment group when compared to control animals.

Due to the lack of toxicologically relevant lesions in other body systems, microscopic examination was limited to the reproductive tracts of F0 female rats in the control and high dose groups, that were not reproductively successful, and that had gross observations made in the reproductive tract (see Table C2). The incidences of histologic diagnoses in the female reproductive tracts are summarized in Text Table O. Statistical comparison of the control and high dose groups showed no significant difference from control the incidences of the gross observations and histologic diagnoses found. However, there were a few possibly relevant histologic lesions that occurred in exposed groups but not in unexposed rats. One rat each in both the 10 ppm and the 30 ppm dose groups had ovarian cysts. Ovaries of one rat in the 80 ppm dose contained only a few corpora lutea, which were regressing, and a relatively large number of tertiary follicles. This rat, as well as one other in the 30 ppm dose group, also had squamous metaplasia of the endometrium localized to the uterine body.

Text Table N.

Incidence<sup>1</sup> of Histologic Lesions in F0 Male Rats Exposed to H2S

Reproductive Organ <sup>2</sup> and Final Diagnoses <sup>3</sup>	0 ppm	Exposure Group		
		10 ppm <sup>4</sup>	30 ppm <sup>4</sup>	80 ppm
<b>Testis</b>				
tubular degeneration	2/12	1/1	1/3	5/12
intratubular sperm stasis	0/12	0/1	1/3	1/12
tubular mineralization	0/12	0/1	1/3	0/12
sperm granulomas	0/12	0/1	0/3	1/12
multinucleated giant cells	0/12	1/1	0/3	1/12
<b>Epididymis</b>				
degenerate sperm forms in lumen	1/12	1/1	0/3	3/12
sperm granulomas	0/12	0/1	0/3	1/12
aspermia w/ luminal collapse and intraluminal cell debris	0/12	0/1	0/3	1/12
oligospermia	0/12	1/1	0/3	1/12
lymphoid infiltrate w/ pigment-laden macrophages	3/12	0/1	0/3	1/12
cauda: severe unilateral necrosis with chronic inflammation	0/12	0/1	0/3	1/12
<b>Prostate-dorsolateral</b>				
chronic inflammation	6/12	1/2	0/3	2/12
<b>Prostate-ventral</b>				
lymphoid infiltrate	3/12	2/2	0/3	5/12

<sup>1</sup> = # of rats with diagnosis in dose group/# of rats histologically examined in dose group.

<sup>2</sup> = does not include seminal vesicles and coagulating glands, which had no histologic diagnoses.

<sup>3</sup> = includes histologically confirmed diagnoses.

<sup>4</sup> = histologic examination was not performed on reproductive tissues of adult male rats in the 10 and 30 ppm dose groups, unless they were not reproductively successful or had gross observations made at necropsy. The incidence of the 10 and 30 ppm dose groups were not statistically compared with the control and high dose groups due to the small number of animals examined histologically in these dose groups.

Text Table O.  
Incidence<sup>1</sup> of Histologic Lesions in F0 Female Rats Exposed to H2S

Reproductive Organ <sup>2</sup> and Final Diagnoses <sup>3</sup>	Exposure Group			
	0 ppm	10 ppm <sup>4</sup>	30 ppm <sup>4</sup>	80 ppm
<b>Vagina</b>				
cyst - NOS <sup>7</sup>	1/11	0/2	0/3	0/12
Wolffian duct remnant, periurethral	0/11	0/2	1/3	0/12
<b>Cervix</b>				
luminal dilation <sup>8</sup>	1/12	0/2	0/3	0/12
<b>Uterus</b>				
luminal dilation (w/ mucometra <sup>9</sup> and endometrial atrophy) <sup>8</sup>	1/12	0/2	0/3	0/12
squamous metaplasia	0/12	0/2	1/3	1/12
<b>Ovaries</b>				
cyst/s - NOS	0/12	1/2	1/3	0/12
luteal depletion	0/12	0/2	0/3	1/12

<sup>1</sup> = # of rats with diagnosis in dose group/# of number of rats histologically examined in dose group.

<sup>2</sup> = does not include oviducts, which had no histologic diagnoses.

<sup>3</sup> = includes diagnoses histologically confirmed. Does not include the incidental finding of uterine luminal dilation consistent with proestrus or estrus.

<sup>4</sup> = histologic examination was not performed on the cervix and vagina of rat #52 in the 0 ppm dose group.

<sup>5</sup> = histologic examination was not performed on reproductive tissues of adult female rats in the 10 and 30 ppm groups (unless they were not reproductively successful or had gross observations made at necropsy) and the cervix of rat #82 in the 30 ppm group. The incidence of the 10 and 30 ppm dose groups were not statistically compared with the control and high dose groups due to the small number of animals examined histologically in these dose groups.

<sup>6</sup> = histologic examination was not performed on the cervix of rat #89 in the 80 ppm group.

<sup>7</sup> = not otherwise specified

<sup>8</sup> = secondary to vaginal outflow obstruction by cyst

<sup>9</sup> = uterine horn distended with tan opaque fluid

**NECROPSY AND HISTOLOGY – F1 RATS****Terminal Body and Organ Weights**

Terminal body and organ weights of postnatal day 63 ± 3 rat pups that underwent a complete necropsy are summarized in Text Table P. The only statistically significant difference from control in these values is an increase in absolute weight of ovaries with oviducts in the 30 ppm dose group.

**Gross Lesions**

A wide variety of gross observations (Text Table Q, Table C6) occurred at very low incidences that were not treatment-related. Microscopic examination was not performed on tissues from these animals due to a lack of significant statistical correlation between the incidence of gross changes observed and the H2S exposure.

**Neuropathology**

Terminal body weight and brain weight, length, and width of PND 23 ± 2 and PND 61 ± 2 rat pups are summarized in Text Table R, respectively (see also Table B73 and B74). The only statistically significant difference from control in any of these parameters is an increase in brain length in male PND 23 ± 2 rat pups in the 10 ppm dose group. No relevant gross abnormalities were observed at necropsy in the brain, spinal cord, or peripheral nerves of any neuroperfused pup examined. Microscopic examination of H&E stained brain sections at 6 levels from pups in the control and high dose groups failed to demonstrate any relevant histologic abnormalities.

**Text Table P**  
**Absolute Mean ( $\pm$  SD) Body or Organ Weights (g) for Postnatal Day 63  $\pm$  3 Male and Female F1**  
**Rats Exposed to H<sub>2</sub>S**

<u>Maies</u>	Exposure Group			
	0	10	30	80
Terminal Body Weight	393.8 $\pm$ 81.0	411.8 $\pm$ 50.0	409.9 $\pm$ 37.1	404 $\pm$ 26.1
Adrenal Glands	0.07 $\pm$ 0.01	0.06 $\pm$ 0.01	0.07 $\pm$ 0.01	0.06 $\pm$ 0.01
Brain	2.00 $\pm$ 0.10	1.99 $\pm$ 0.15	1.96 $\pm$ 0.12	1.98 $\pm$ 0.07
Heart	1.43 $\pm$ 0.16	1.44 $\pm$ 0.17	1.40 $\pm$ 0.11	1.38 $\pm$ 0.17
Kidneys	3.50 $\pm$ 0.46	3.44 $\pm$ 0.63	3.51 $\pm$ 0.28	3.38 $\pm$ 0.42
Liver	20.63 $\pm$ 2.36	20.05 $\pm$ 3.19	19.60 $\pm$ 2.20	19.83 $\pm$ 2.37
Lungs	1.45 $\pm$ 0.14	1.47 $\pm$ 0.15 <sup>A</sup>	1.50 $\pm$ 0.14	1.56 $\pm$ 0.15
Spleen	0.94 $\pm$ 0.12	0.87 $\pm$ 0.11	0.85 $\pm$ 0.12	0.90 $\pm$ 0.22
Testes	3.23 $\pm$ 0.22	3.07 $\pm$ 0.45	3.19 $\pm$ 0.29	3.07 $\pm$ 0.14
Sample size	18	22	16	13

<sup>A</sup> n = 21, Rat # 674 was excluded because lungs were inflated with formalin prior to weighing.

<u>Females</u>	Exposure Group			
	0	10	30	80
Terminal Body Weight	239.3 $\pm$ 23.8	248.34 $\pm$ 27.7	253.5 $\pm$ 33.6	235.4 $\pm$ 12.3
Adrenal Glands	0.07 $\pm$ 0.01	0.07 $\pm$ 0.01	0.07 $\pm$ 0.01	0.07 $\pm$ 0.01
Brain	1.79 $\pm$ 0.10	1.84 $\pm$ 0.11	1.81 $\pm$ 0.10	1.81 $\pm$ 0.09
Heart	0.92 $\pm$ 0.09	0.95 $\pm$ 0.12	0.97 $\pm$ 0.10	0.92 $\pm$ 0.10
Kidneys	2.05 $\pm$ 0.25	2.11 $\pm$ 0.25	2.18 $\pm$ 0.21	1.98 $\pm$ 0.14
Liver	10.67 $\pm$ 1.32	11.10 $\pm$ 1.31	11.38 $\pm$ 1.84	10.65 $\pm$ 1.03
Lungs	1.14 $\pm$ 0.26 <sup>B</sup>	1.10 $\pm$ 0.17	1.13 $\pm$ 0.13	1.11 $\pm$ 0.13
Spleen	0.57 $\pm$ 0.09	0.55 $\pm$ 0.11	0.61 $\pm$ 0.09	0.58 $\pm$ 0.07
Ovaries and Oviducts	0.12 $\pm$ 0.02	0.13 $\pm$ 0.02	0.14 $\pm$ 0.02*	0.13 $\pm$ 0.02
Sample Size	18	22	16	16

<sup>B</sup> n = 17, Rat # 558 was excluded because lungs were inflated with formalin prior to weighing.

\* p  $\leq$  0.05

Text Table Q.  
Incidence<sup>1</sup> of Gross Lesions in F1 Male and Female Rats Exposed to H2S

Organ	Gross Observation <sup>2</sup>	0 ppm	Exposure Group		
			10 ppm	30 ppm	80 ppm
Heart	enlarged rounded apex	1/36	0/44	0/35	1/29
	RV <sup>3</sup> wall thin	0/36	0/44	0/35	1/29
Kidneys	small	1/36	0/44	0/35	0/29
	cyst/s	1/36	2/44	1/35	2/29
	hydronephrosis <sup>4</sup>	0/36	1/44	3/35	2/29
Liver	enlarged, firm, nodular	0/36	0/44	0/35	1/29
SV/CG <sup>5</sup> (male)	rt SV small	0/18	1/22	0/16	0/13
	CG small	0/18	1/22	0/16	0/13
	small	0/18	1/22	0/16	0/13
Small Intestine	Meckel's diverticulum	1/36	0/44	0/35	0/29
	thickened	0/36	0/44	0/35	1/29
Testes (male)	small	0/18	2/22	0/16	0/13
Stomach	expanded with food	0/36	0/44	0/35	1/29
Adrenal Glands	red-black focus	0/36	1/44	0/35	0/29
Lymph nodes	enlarged	1/36	0/44	0/35	0/29
Spleen	enlarged	0/36	0/44	0/35	1/29
Meninges	hemorrhage	1/36	0/44	0/35	0/29
Pituitary Gland	pale	0/36	1/44	0/35	0/29
Digit	claw absent	1/36	0/44	0/35	0/29
Other Tissues <sup>6</sup>	N/A <sup>7</sup>	N/A	N/A	N/A	N/A

<sup>1</sup> = # of rats with gross observation/# of rats grossly examined in dose group.

<sup>2</sup> = does not include the incidental findings of red foci in kidneys associated with CO<sub>2</sub> euthanasia and uterine swelling and intraluminal fluid and parovarian fluid in cycling female rat pups.

<sup>3</sup> = right ventricular

<sup>4</sup> = dilated renal pelvis/es

<sup>5</sup> = seminal vesicle/s/coagulating gland/s

<sup>6</sup> = includes all tissues examined in which no gross observations were made in any pups: skin, salivary glands, nasopharynx, trachea, mainstem bronchi, lungs, aorta, thymic region, thyroids/parathyroids, pancreas, esophagus, colon, urinary bladder, epididymides, penis w/ preputial gland, prostate, cervix, vagina, and brain (cerebrum, cerebellum, pons), and eyes.

<sup>7</sup> = not applicable

Text Table R

Absolute Mean ( $\pm$  SD) Body and Brain Weights (g) and Brain Size (in mm) for Male and Female  
F1 Rats Exposed to H2S

Postnatal Day $23 \pm 2$	Exposure Group			
	0	10	30	80
<b>Males</b>				
Terminal Body Weight (g)	70.1 $\pm$ 10.8	68.5 $\pm$ 12.0	72.3 $\pm$ 9.6	67.16 $\pm$ 10.5
Brain weight (g)	1.64 $\pm$ 0.10	1.65 $\pm$ 0.08	1.65 $\pm$ 0.07	1.61 $\pm$ 0.08
Brain length (mm)	18.44 $\pm$ 0.53	19.18 $\pm$ 0.40*	18.67 $\pm$ 0.50	18.57 $\pm$ 0.53
Brain width (mm)	14.78 $\pm$ 0.44	14.73 $\pm$ 0.47	14.89 $\pm$ 0.33	14.71 $\pm$ 0.49
Sample Size	9	11	9	7
<b>Females</b>				
Terminal Body Weight (g)	64.7 $\pm$ 9.6	64.6 $\pm$ 12.0	63.6 $\pm$ 7.6	63.2 $\pm$ 8.2
Brain weight (g)	1.54 $\pm$ 0.09	1.58 $\pm$ 0.10	1.57 $\pm$ 0.10	1.53 $\pm$ 0.06
Brain length (mm)	18.56 $\pm$ 0.53	18.80 $\pm$ 0.42	18.60 $\pm$ 0.52	18.25 $\pm$ 0.46
Brain width (mm)	14.56 $\pm$ 0.53	14.70 $\pm$ 0.48	14.30 $\pm$ 0.48	14.50 $\pm$ 0.53
Sample Size	9	10	10	8
<b>Postnatal Day <math>61 \pm 2</math></b>				
<b>Males</b>				
Terminal Body Weight (g)	379.0 $\pm$ 37.3	380.9 $\pm$ 48.3	371.8 $\pm$ 29.1	379.0 $\pm$ 17.0
Brain weight (g)	2.00 $\pm$ 0.10	2.00 $\pm$ 0.15	1.99 $\pm$ 0.05	2.07 $\pm$ 0.10
Brain length (mm)	21.11 $\pm$ 0.93	21.09 $\pm$ 0.49	21.11 $\pm$ 0.65	21.29 $\pm$ 0.76
Brain width (mm)	15.33 $\pm$ 0.50	15.32 $\pm$ 0.60	15.28 $\pm$ 0.51	15.29 $\pm$ 0.49
Sample Size	9	11	9	7
<b>Females</b>				
Terminal Body Weight (g)	233.2 $\pm$ 24.2	235.1 $\pm$ 21.5	241.2 $\pm$ 23.8	230.3 $\pm$ 15.5
Brain weight (g)	1.83 $\pm$ 0.12	1.84 $\pm$ 0.13	1.86 $\pm$ 0.08	1.86 $\pm$ 0.07
Brain length (mm)	20.22 $\pm$ 0.67	20.59 $\pm$ 0.63	20.39 $\pm$ 0.60	20.44 $\pm$ 0.50
Brain width (mm)	14.78 $\pm$ 0.62	14.77 $\pm$ 0.65	14.89 $\pm$ 0.22	14.94 $\pm$ 0.18
Sample Size	9	11	9	8

\*  $p \leq 0.05$

## DISCUSSION/CONCLUSIONS

The purpose of this study was to evaluate whether repeated 6-hour daily exposure of male and female CD<sup>1</sup> rats ( $n = 12$  rats/sex/concentration) to hydrogen sulfide (H<sub>2</sub>S) atmospheres at 0, 10, 30, or 80 ppm would result in reproductive toxicity, developmental toxicity, or developmental neurotoxicity. F0 animals were exposed to H<sub>2</sub>S vapor six hours per day, seven days per week for a two week prebreed exposure period, two week mating period, and through the majority of gestation (gd 0 until gd 19) and lactation (pnd 5-18). F0 males were exposed for at least 70 consecutive days. Pups were exposed with their dams during the lactation period using individual animal (litter) exposure cylinder. The actual chamber concentrations (grand means  $\pm$  SD) were 0 ( $< XX$  ppm),  $10.0 \pm 0.6$ ,  $30.1 \pm 0.8$ , and  $79.5 \pm 2.4$  ppm for the target concentrations 0, 10, 30 and 80 ppm, respectively. The exposure concentrations used in our study are relevant for assessing the hazard associated with environmental (ambient) and occupational exposures to H<sub>2</sub>S. The current threshold limit value-time weighted average (TLV-TWA) for H<sub>2</sub>S is 10 ppm.

Increased mortality or clinical evidence of overt toxicity was not observed in any treated F0 rats. Exposure of F0 male rats to 80 ppm H<sub>2</sub>S was associated with a statistically significant decrease in food consumption during only the first week of the two-week prebreed exposure. Female rats in the 30 and 80 ppm H<sub>2</sub>S treatment groups also had lower feed consumption during the first week of exposure, however, this difference was not statistically significant. Although not statistically significant, both male and female rats exposed to 80 ppm H<sub>2</sub>S also demonstrated a small (approximately 5-6%) decrease in body weight following H<sub>2</sub>S exposure. For example, F0 male rat body weights (mean  $\pm$  SD) near after the pre-breed exposures were  $395.0 \pm 34.2$  and  $373.0 \pm 20.7$  g, for the control and 80 ppm exposure groups, respectively. This approximately 5% decline in F0 male body weight was persistent throughout the course of the 10-week exposure period. The magnitude of the body weight decrease observed in the present study is qualitatively similar to that observed in male and female Sprague-Dawley rats exposed to 80 ppm for six hours per day for five days per week for 13 weeks (CIIT Docket Number #32063). In this latter study an approximate 8% decrease in body weight was observed following an 80 ppm H<sub>2</sub>S exposure. Terminal body weights were statistically equivalent among treatment groups suggesting that a maximal tolerated dose (MTD) was not used in the study. The only statistically significant difference from control in either absolute or relative organ weights was a decrease in the absolute and relative weight of the adrenal glands in the 10 and 80 ppm dose groups.

Nasal pathology was determined in F0 male rats following inhalation exposure to H<sub>2</sub>S for six hours per day seven days per week for at least 70 consecutive days. Subchronic exposure of male F0 rats to 80 ppm H<sub>2</sub>S was associated with mild to marked sensory neuron loss and basal cell hyperplasia in the olfactory mucosa lining the dorsal medial meatus and the dorsal medial region of the ethmoid recess. This lesion was found uniformly in the 80 ppm treatment group and extended caudally from a point approximately midway between the first incisor and the first palatal ridge (level 6, Méry et al., 1994) to the posterior ethmoid region (level 23, Méry et al., 1994). The severity and extent of the olfactory mucosal lesion was markedly decreased in rats exposed to 10 or 30 ppm H<sub>2</sub>S. Treatment-related effects were not observed in either the squamous or respiratory epithelium. Treatment-related nasal lesions were also absent in control animals. Nasal lesions have also been reported by Lopez and coworkers (1988) in male F344 rats following short-term (4 hours) exposure to high concentrations of H<sub>2</sub>S (up to 400 ppm). These investigators reported that inhalation of 400 ppm H<sub>2</sub>S for four hours resulted in olfactory and respiratory mucosal cell necrosis. The no observed adverse effect level (NOAEL) for nasal pathology observed in the present study was 10 ppm.

Hydrogen sulfide exposure did not result in female reproductive toxicity. Exposure of F0 female rats to H<sub>2</sub>S did not result in any changes in mating index, fertility index, postimplantation loss per litter, gestation length, numbers of implantation sites per litter, litter size, or F1 litter sex ratio (Text Table S). Reproductive indices observed in F0 female rats from the present study are qualitatively similar to published historical controls in a similar strain of rat. No statistically significant evidence of female reproductive tract pathology was observed in our study. The only statistically significant difference from control in either absolute or relative organ weights was a decrease in the relative weight of the ovaries with oviducts in the 10 ppm dose group.

Our results are similar to those reported by Sailenfait and coworkers (1989). These investigators exposed pregnant Sprague Dawley rats to 0, 50, 100, or 150 ppm H<sub>2</sub>S for six hours per day during gestation days 6 through 20. Maternal body weight gain was significantly reduced in the 150 ppm exposure group only. Exposure of pregnant rats to H<sub>2</sub>S was not associated with any change in number of implantation sites per litter, litter size, or number of dead fetuses per litter. No maternal toxicity or adverse effects on the developing embryo or fetus was observed in a follow-up experiment in which twenty-three (23) pregnant Sprague

Dawley rats were exposed to 100 ppm H<sub>2</sub>S for six hours per day during gestation days 6 through 20 (Sailenfait et al., 1989).

**Text Table S**  
**Reproductive Toxicity Indices**

Endpoint	Treatment Group	
	H <sub>2</sub> S Control	80 ppm H <sub>2</sub> S
Females that mated (%)	92 (11/12)	83 (10/12)
Gestation length (days)	21.9 ± 0.23	21.8 ± 0.25
Live pups/litter	16.3 ± 0.52	13.9 ± 1.64
% Male pups per litter	45.5	45.5
Implantation sites/female	12.5 ± 2.2	9.7 ± 2.29
Postimplantation loss (%)	2.5	7.0
Male pup birth weight (g)	6.6 ± 0.6	6.1 ± 0.4
Female pup birth weight (g)	6.1 ± 0.4	6.1 ± 1.0

Male F<sub>0</sub> rats in our study did not demonstrate reproductive toxicity following H<sub>2</sub>S exposure. Hydrogen sulfide exposure did not affect percent motile sperm, percent normal sperm, daily sperm production, cauda sperm count, as well as reproductive organ tissue weight. No statistically significant increase in either gross or histopathological lesion incidence were observed following H<sub>2</sub>S exposure of F<sub>0</sub> male rats. Although not statistically significant, there was a higher incidence of testicular tubular degeneration in male F<sub>0</sub> rats from the 80 ppm treatment group (42%) when compared to control animals (17%).

The data obtained from our study may suggest that H<sub>2</sub>S may be teratogenic in rats. Two observations support this conclusion. First, a low incidence of malformations was observed only in H<sub>2</sub>S-exposed animals. Structural malformations observed in H<sub>2</sub>S-exposed animals included kinked tail, agenesis of the tail, anophthalmia, umbilical hernia, frontal bone holes, and skin lesions that included webbing of the forelimb skin. The predominant histologic dermatologic finding is localized to regionally extensive separation of the epidermis from the underlying dermis. This histological finding is most consistent with epidermolysis bullosa, a family of inherited mechanobullous diseases in which minor trauma causes the formation of cutaneous

blisters. Each of these malformations occur with a low incidence in control animals (Text Table T) thus the presence of multiple malformations is suggestive of a chemical-induced effect. Second, the observed malformations occurred in the absence of any apparent maternal toxicity. It should be noted, however, that no dose-response relationship was observed for any external alteration since malformations were observed in litters from all treatment groups. Andrews and coworkers (1980) investigated the effect of H<sub>2</sub>S exposure on prenatal development in Wistar rats. These investigators reported a high incidence (23%) of wavy ribs in fetuses from dams exposed to 220 ppm H<sub>2</sub>S for three hours per day throughout gestation (gd 1 to 18). No evidence of maternal toxicity or embryotoxicity was observed.

**Text Table T**  
**Litter Incidence (%) of External Alterations in H<sub>2</sub>S Exposed and Control Rats**

External Alteration	Treatment Group	
	H <sub>2</sub> S Control <sup>1</sup>	H <sub>2</sub> S Exposed <sup>2</sup>
Anophthalmia	0	4
Tail malformations	0	11
Umbilical hernia	0	4
Frontal bone holes	0	4

1. Nine (9) control litters evaluated.
2. Pooled litter incidence from 11, 9, and 8 litters from the 10, 30, and 80 ppm treatment groups, respectively. No dose-response relationship was observed for any external alteration.

Another goal of the present study was to determine whether repeated exposure to H<sub>2</sub>S result in persistent neurological effects in neonatal pups. Conventional developmental neurotoxicity studies separate the dam from their pups during the postnatal exposure period (Kimmel and Francis, 1990; U.S. EPA, 1991). In most developmental neurotoxicity studies, pups are maintained in their domiciliary caging while only the dam is exposed to test chemical during the postnatal period (from birth until weaning on approximately pnd 21). Exposure of the pups to test chemical thus occurs indirectly to chemical residues that remain on the dam's hair coat and

through lactational transfer. In the case of hydrogen sulfide, little is known about the excretion of sulfides into milk following H<sub>2</sub>S exposure. Separation of the pups from their dams during critical perinatal exposure periods may result in an undesirable underestimate of the neurotoxic risks associated with H<sub>2</sub>S inhalation. Thus, the decision was made to use a whole-body exposure cylinder to simultaneously expose F1 offspring and their dams to H<sub>2</sub>S vapor between postnatal day (pnd) 5 through 18. Previous research with this exposure system indicates that the use of these cylinders was not associated with altered pup growth, development, or behavior (Vitarella et al., 1998).

Other investigators (Skrajny et al., 1992; Hayden et al., 1990) have also simultaneously exposed Sprague-Dawley dam and neonatal rat pups to up to 75 ppm H<sub>2</sub>S for seven hours per day during the neonatal period (i.e., from birth to weaning). The results of our study as well as those of Skrajny and coworkers (1992) and Hayden and colleagues (1990) suggest that daily six hour exposure of neonatal rat pups to 80 ppm H<sub>2</sub>S is not associated with increased pup mortality or altered weight gain. Terminal body weight of pnd 23 ± 2 and pnd 61 ± 2 rat pups was not affected by H<sub>2</sub>S exposure. In addition, we observed no treatment related effect on any developmental landmark, including surface righting, pinnae detachment, incisor eruption, negative geotaxis, eyelid separation, vaginal patency, or balano-preputial separation.

Several investigators have reported changes in steady state brain neurotransmitter levels following acute or chronic H<sub>2</sub>S exposure (Hannah et al., 1991; Kombian et al., 1988; Skrajny et al., 1996; Warenycia et al., 1989). Few studies, however, have focused on the behavioral effects of H<sub>2</sub>S exposure. The functional observation battery (FOB), motor activity, passive avoidance, and acoustic startle assessments used in this study provide useful screens of neurologic function. The neurobehavioral evaluations conducted during our study indicate that no consistent or dose-related pattern of neurologic dysfunction occurred. The results of our study suggest that H<sub>2</sub>S is not a behavioral developmental neurotoxicant.

In addition, exposure to H<sub>2</sub>S did not affect pup motor activity, passive avoidance behavior, or acoustic startle reflex behavior. Terminal brain weight, length, and width were determined on pnd 23 ± 2 and pnd 61 ± 2. The only statistically significant difference from control in any of these parameters was an increase in brain length in male PND 23 ± 2 rat pups in the 10 ppm dose group. Furthermore, no evidence of gross or histologic brain pathology was observed in any H<sub>2</sub>S exposed pups. Brain regions evaluated by light microscopy included forebrain, caudate nucleus, center of the cerebrum, center of the midbrain, cerebellum and pons, and

medulla oblongata. In contrast, Hannah and Roth (1991) have reported that perinatal H<sub>2</sub>S exposure alters dendrite arborization of the rat cerebellar Purkinje cell. These investigators exposed pregnant Sprague-Dawley rats to 20 or 50 ppm H<sub>2</sub>S for seven hours per day from gestation day 7 through postnatal day 21. Arborization of cerebellar Purkinje cell dendrites was not evaluated in our study.

In conclusion, our study suggests that H<sub>2</sub>S exposure is unlikely to result in significant reproductive toxicity or developmental neurotoxicity following exposures at which nasal pathology occurs. The results obtained in this study cautiously suggest that H<sub>2</sub>S may be a weak teratogen in rats exposed throughout the majority of gestation.

## Section 4

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**Appendix Title**

A Vapor Inhalation Reproductive Toxicity, Developmental Toxicity, and Developmental Neurotoxicity Study in Cr:CD<sup>®</sup> (SD)BR Rats Exposed to Hydrogen Sulfide

**Study Protocol**

CIIT: 97042

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## Introduction

The purpose of this study was to evaluate the toxic effects in Crj:CD® (SD)BR rats resulting from repeated (6 hours per day) vapor inhalation exposure to hydrogen sulfide (H<sub>2</sub>S) at concentrations of 0, 10, 30, or 80 ppm before mating and during pregnancy and lactation. A 2-week pre-breeding exposure period was followed by a 2-week breeding exposure period. Pregnant F<sub>0</sub> females were exposed until gestation day (gd) 19. Pregnant females were not exposed from gd 19 through post-natal day (pnd) 4. Beginning on pnd 5, dams and pups were exposed together in 4.9 liter individual glass exposure cylinders (see Exposure Chamber section). The glass cylinders were located on a shelf within the appropriate H-1000 chamber and the test atmosphere was pulled through the cylinders into an exhaust manifold by a controlled vacuum; thus, the dams and pups were exposed to the same test atmosphere as the F<sub>0</sub> males in the H-1000 chamber. The males were exposed for at least 70 consecutive days. After the exposure period, all animals were returned to domiciliary caging and housed in the Anteroom of the appropriate exposure chamber.

## Summary

The concentration of H<sub>2</sub>S in the exposure chamber was monitored using a gas chromatograph equipped with a flame photometric detector (GC-FPD). The concentration in each chamber was determined at least 6 times during the 6-hour exposure period. The overall means (± standard deviation) were 10.0 ± 0.6, 30.1 ± 0.8 and 79.5 ± 2.4 ppm for the target concentrations 10, 30 and 80 ppm, respectively. No H<sub>2</sub>S was detected in the control chamber (limit of detection: 0.06 ppm).

The environmental parameters (temperature, relative humidity and air flow) were maintained at or near the target set points inside the exposure chambers and the chamber anterooms throughout the entire study. Exposures were conducted for 71 consecutive days from August 19, 1997 through October 28, 1997.

## Materials and Methods

### Chemical

Test gas cylinders used to generate the test atmospheres for this study were purchased from HoloX Gases (Cary, NC) and manufactured by Praxair Distribution, Inc. (Bethlehem, PA). They contained 5% (50,000 ppm) mixtures of H<sub>2</sub>S (CAS No 7783-06-4) with the balance being nitrogen. The chemical and physical properties of H<sub>2</sub>S are listed in Table 1. The first cylinder used in the study was purchased as an unanalyzed mixture and was partially used in an earlier study. After use in this study, this cylinder was returned to Praxair for analysis and certification. The analysis results were received on 12/11/97. The other cylinders of H<sub>2</sub>S were ordered as Certified Standard mixtures. Upon receipt at CIRT, each cylinder was stored in monitoring corridor 200W under ambient temperature and relative humidity conditions. A total of 4 cylinders were used to generate the test atmospheres during this study.

Reference gas mixtures for calibration were also ordered as Certified Standards from HoloX Gases (Cary, NC) and manufactured by Praxair Distribution, Inc. (Bethlehem, PA). These reference mixtures were further diluted with nitrogen to provide a range of concentrations for the GC calibration curve.

The gas cylinders used for these exposures are identified in Table 3.

### Generation and Exposure System

H<sub>2</sub>S exposure atmospheres were generated by metering 5% H<sub>2</sub>S in nitrogen from the gas cylinder through a mass flow controller (MFC) (MKS Instruments, Andover, MA) into a mixing "T" in the two inch stainless steel air inlet to the inhalation chambers. The mixing "T" was located approximately three feet upstream of the chamber. The H<sub>2</sub>S gas flowed into the mixing "T" and counter-current to the HEPA-filtered chamber air flow of the inhalation chamber which facilitated mixing of the H<sub>2</sub>S vapor with the dilution air. The total chamber air flow through each chamber was maintained at approximately 225-250 L/min. Figure 1 is a diagrammatic representation of the exposure system setup. All components of the generation system were composed of stainless steel and the delivery lines were Teflon®. The generation system was operated by the Andover Infinity control system (Andover Controls Corporation, Andover, MA).

Animals (both males and females) were initially exposed in four Hazton H-1000 stainless steel and glass inhalation exposure chambers (Lab Products, Maywood, NJ). One H-1000 was used for each target

exposure concentration. Each H-1000 was contained within CIIT's permanent 8m<sup>3</sup> Hinners-style stainless steel and glass inhalation exposure chamber as an additional safety measure. Air flow through the 8m<sup>3</sup> chambers was controlled by a supply fan, an exhaust fan and two butterfly dampers. Airflow was monitored by measuring the pressure drop across an orifice in the exhaust line and controlled by the damper in the exhaust duct system. Air was pulled through the H-1000 and the 8m<sup>3</sup> chamber into a manifold from the 8m<sup>3</sup> chamber exhaust duct. A damper at the opening to the manifold from the 8m<sup>3</sup> was fixed in place to proportion the airflow between the H-1000 and the 8m<sup>3</sup> chamber. Air flow through the H-1000 chamber was controlled by the total airflow in the 8m<sup>3</sup> chamber. An extension connected to the air inlet of the H-1000 chamber was positioned near the air inlet at the top of the 8m<sup>3</sup> chamber. The H<sub>2</sub>S vapor flowed into the extension, mixed with incoming air and was distributed to the H-1000 chamber.

For the whole body exposure of dams and pups, 4.9 liter glass exposure cylinders sealed with two anodized aluminum end plates with neoprene gaskets and an aluminum outer face (CH Technologies (USA), Inc., Westwood, NJ) were used. Each unit had an inlet on one endplate and an exhaust port on the other endplate. The exhaust ports were connected to an exhaust manifold system which pulled air from the H-1000 exposure chamber through the inlet and through the dam/pup exposure cylinder. Air flow through each individual glass exposure cylinder was controlled by an adjustable stainless steel metering valve (Raleigh Valve and Fitting, Raleigh, NC) and maintained at 2.5 - 3.5 L/min during the exposure times, providing approximately 35 to 50 air changes per hour. The temperature in one exposure cylinder per concentration group was measured using a thermister (PreCon, Memphis, TN) placed in the tube exhaust, and the relative humidity in one exposure cylinder per concentration group was measured by a humidity probe (OMEGA Engineering, Inc., Stamford, CT) located in the inlet end of the glass exposure cylinder. Measurements of tube concentration, temperature, and humidity were recorded during each exposure period. The temperature and relative humidity within the glass exposure cylinders was dependent upon the temperature and relative humidity of the H-1000 chamber and the conditions within the individual exposure cylinders. For example, higher temperatures were recorded as the pups grew and the animal load within the exposure cylinder increased. Recent urination or breathing near the humidity probe resulted in high humidity readings at various times during the exposures.

#### Chamber Distribution

Prior to animals being placed in the chambers, each chamber was checked once during pre-study trials for uniformity of distribution of the test compound by measuring the concentration at nine positions within the chamber.

#### Chamber Concentration Analysis

H<sub>2</sub>S exposure atmospheres were measured with a calibrated gas chromatograph (GC) (Model 6890 Hewlett Packard Co., Palo Alto, CA) equipped with a flame photometric detector (FPD) and GS-Q (30 meter x 0.53 µm) column (Alltech, Deerfield, IL). An eight-position gas sampling valve was used by the GC to sample at a programmed sequence of locations. The operating conditions of the GC are listed in Table 2.

#### Calibration

The GC was calibrated using Certified Standard reference mixtures of H<sub>2</sub>S in nitrogen (Praxair, Bethlehem, PA). Bag standards were prepared in Tedlar bags (SKC, Inc., Eighty-Four, PA) by delivering a measured volume of an appropriate reference gas from the Certified Standard cylinder (83.5 ppm or 97.4 µpm) and further dilution with nitrogen if necessary to attain the desired calibration concentrations, using the following formula:

$$C = \frac{C_r \cdot T_t}{T_d + T_r} \quad T_d = T_t - T_r$$

Where:

- C = Desired concentration, (ppm)
- C<sub>r</sub> = Known concentration of reference gas
- T<sub>t</sub> = Total delivery time (minutes)
- T<sub>r</sub> = Delivery time of reference gas (minutes)
- T<sub>d</sub> = Delivery time of diluent gas (minutes)

The flow rate for the reference and diluent gases must be the same when using the formulas above.

### Estimated Limit of Detection

The limit of detection of the GC method for H<sub>2</sub>S can be calculated from the following information: The Response Factor (ppmv/area count) for the GC at the lowest calibration point (8.35 ppm) is  $6.34e^{-3}$ . The Area Reject for the GC was set to 100 when the method for this study was developed. (Area Reject is the minimum area count within the retention time window needed to be considered as a peak above the background noise level.) Therefore, the limit of detection is 100 area counts \*  $6.34e^{-3}$  (ppmv/area count) or about 0.06 ppm.

### **Study Day**

A study day for these exposures was defined as a 6-hour exposure, generally from 8:00 am until 2:00 p.m. Each exposure was followed by a 30-minute clearance period to ensure that the chemical had cleared the exposure chamber prior to opening of the chamber for animal care procedures. The lighting cycle for the animals was 12 hours of light (7:00 am until 7:00 p.m.) followed by 12 hours of darkness and was controlled by the Andover Infinity control system (Andover Controls Corporation, Andover, MA). The study days were numbered consecutively from 1 to 71 (Aug. 19 to Oct. 28, 1997).

### **Environmental Parameters**

#### Exposure Chamber

Temperature was measured at the top rear of the H-1000 chamber by a thermister (PreCon, Memphis, TN) and relative humidity was measured at the top front of the H-1000 chamber by a humidity probe (OMEGA Engineering, Inc., Stamford, CT) connected to the Andover Infinity control system. Calibration of the thermisters was checked by comparing the temperature probe to a Certified mercury thermometer. The relative humidity probe was calibrated by immersing the probe in an atmosphere of known humidity generated from saturated salt solutions.

Air flow in the H-1000 chamber was monitored by measuring the pressure drop across an orifice located at the inlet of the H-1000 chamber. Placement of orifices at the inlet and outlet assured that a slight negative static pressure was maintained inside the H-1000 chamber.

The H-1000 temperature, relative humidity, and air flow were recorded by the Andover Infinity control system at the time of GC data transfer and a report of the environmental parameters data for the 6-hour exposure period was printed every day.

#### Domiciliary Area

Details regarding animal husbandry are provided in the body of the main report. Briefly, animals were housed during non-exposure periods in the exposure chamber anterooms in polycarbonate shoe box cages with stainless steel wire lids with water bottles and Alpha-Dri<sup>®</sup> cage litter (Shepard Specialty Papers, Kalamazoo, MI). The animals were transferred to the stainless steel caging or glass exposure cylinders prior to the start of the exposure. All animals were returned to their domiciliary cages after the 30-minute clearance period at the end of the exposure. The temperature and relative humidity in the anterooms was recorded twice per hour by the Andover Infinity control system (Andover Controls Corporation, Andover, MA). A report of the environmental parameters data for the 24-hour period was printed every day.

The potential for absorption of H<sub>2</sub>S onto the Alpha-Dri<sup>®</sup> cage litter was investigated. There was no detectable absorption of H<sub>2</sub>S onto the bedding material.

### **Statistical Procedures**

At the end of each exposure, a report was printed that listed the sample time, measured concentration, nominal concentration, H-1000 air flow and H<sub>2</sub>S flow rate (also called mass flow controller (MFC) flow rate). The mean, standard deviation, maximum and minimum values of the exposure chamber environmental parameters were printed daily. The reported summary data (mean, standard deviation, maximum and minimum) for these parameters were determined from the average daily mean values.

For the environmental parameters for the anterooms, the temperature and relative humidity were printed each day for 30 minute average readings for a 24-hour period. The mean, standard deviation, maximum and minimum values were determined. The reported summary data (mean, standard deviation, maximum and minimum) for these parameters were determined from the average daily mean values.

#### Nominal Concentration Calculation

The nominal chamber concentration (NCC or Nominal) was determined using the following formula:

$$NCC = \frac{CFR}{AF} \times [C_c \times 10^{-3}]$$

Where:

NCC = Concentration, (ppm)  
CFR = Mass Flow Controller flow rate, (mL/min)  
AF = Air flow rate through H-1000 chamber, (L/min)  
C<sub>c</sub> = Concentration of cylinder (ppm)

For purposes of calculating the nominal chamber concentration, the concentration of the cylinder is assumed to be 50,000 ppm (5%).

#### **Project Inhalation Personnel**

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	Brian A. Wong, Ph. D.

#### **Results**

##### Chemical

The gas cylinders used for these exposures are identified in Table 3. The cylinders were certified by the supplier, PraxAir Distribution, Inc.

##### Chamber Distribution

Results of the chamber distribution measurements are shown in Table 4. The relative standard deviation was less than 3.6% for all of the chambers.

##### Generation and Chamber Concentration

Table 5 shows the summary data for H<sub>2</sub>S generation and characterization for each of the exposure chambers. The mean and standard deviation of the average daily mean values for actual chamber concentration (ACC), nominal chamber concentration (NCC), air flow, mass flow controller (MFC) flow, ratio of actual to nominal chamber concentration (RAN) and the ratio of actual to target chamber concentration (RAT) are shown. The smallest minimum daily mean and the largest maximum daily mean are also shown. The Grand means for the actual chamber concentrations (± standard deviations) were 10.0 ±0.6, 30.1 ±0.8, and 79.5 ±2.4 ppm for the target concentrations 10, 30 and 80 ppm, respectively. The Grand means for the nominal chamber concentrations (± standard deviations) were 10.0 ±1.0, 32.3 ±2.4, and 78.0 ±4.0 ppm for the target concentrations 10, 30 and 80 ppm, respectively. The Grand means for Air Flow were 241 ±3, 237 ±6, 225 ±4, and 232 ± 5 L/min for the target concentrations of 0, 10, 30 and 80 ppm, respectively. The Grand means for H<sub>2</sub>S Flow were 47.1 ±4.0, 145.7 ±10.1, and 361.6 ±14.5 mL/min for the target concentrations of 10, 30 and 80 ppm, respectively. The Grand means for Ratio of Actual to Nominal Concentration were 102 ±13, 93 ±7, and 102 ±6 % for the target concentrations of 10, 30 and 80 ppm, respectively. The Grand means for Ratio of Actual to Target Concentration were 100 ±6, 100 ±3, and 99 ±3 % for the target concentrations of 10, 30, and 80 ppm, respectively.

Table 6 shows the summary data for the exposure chamber and the anteroom environmental conditions. These data are the Grand mean and standard deviation for the average daily mean values for temperature and relative humidity. The smallest minimum daily mean and the largest maximum daily mean are also shown. The Grand mean temperatures and Grand mean relative humidities for exposure chambers were maintained within the limits specified by the protocol. The daily mean temperatures ranged from 69.0 to 75.9, 70.8 to 75.3, 68.4 to 75.1 and 72.2 to 75.7 °F for the 0, 10, 30 and 80 ppm chambers, respectively. The temperature data for the control chamber (0 ppm) on days 23, 35, and 36 was lost when the thermister failed at the beginning of the exposure time. The daily mean relative humidities ranged from 39 to 44, 41 to 55, 40 to 66 and 36 to 48% for the 0, 10, 30 and 80 ppm chambers, respectively. The Grand mean temperatures and relative humidities for the anterooms were maintained within the limits specified by the protocol. The daily mean temperatures ranged from 66.5 to 67.1, 66.5 to 67.3, 65.7 to 66.8 and 65.1 to 66.2 °F for the 0, 10, 30 and 80 ppm chambers, respectively. The daily mean relative humidities ranged from 42 to 53, 44 to 53, 41 to 50, and 39 to 50% for the 0, 10, 30 and 80 ppm chambers, respectively.

The daily mean values for actual chamber concentration, nominal chamber concentration, ratio of actual to nominal concentration, ratio of actual to target concentration, H-1000 temperature, H-1000 relative humidity, Mass flow controller flow rate and H-1000 air flow rate are shown in Tables 7a, 7b, 7c and 7d for chambers 308 (0 ppm), 309 (10 ppm), 310 (30 ppm) and 311 (80 ppm), respectively. The Grand mean (with standard deviation) represents the average of the reported daily mean values. In addition, the minimum daily mean and the maximum daily mean for the reported daily means are also shown. Tables 7e, 7f, 7g, and 7h show the daily mean values for H<sub>2</sub>S concentration, temperature and relative humidity as measured in a single animal exposure tube during the days when these exposures occurred. The Grand mean (with standard deviation) represents the average of the reported daily mean values. In addition, the minimum daily mean and the maximum daily mean for the reported daily means are also shown.

The daily mean values for anteroom temperature and relative humidity are listed in Tables 8a, 8b, 8c and 8d for chambers 308 (0 ppm), 309 (10 ppm), 310 (30 ppm) and 311 (80 ppm), respectively. The Grand mean (with standard deviation) represents the average of the reported daily mean values. In addition, the minimum daily mean and the maximum daily mean are also shown.

## Deviations

### Chemical Accountability

An Accountability Log for the H<sub>2</sub>S cylinders was not used during the pre-study work and the first 10 days of the exposures. Thereafter, a running Accountability Log of cylinder weight was kept.

### Exposure chambers

A daily mean concentration which was more than 10% above or below the target concentration was identified as a protocol deviation. These deviations for the 10 ppm exposure group are shown in Table 9. There were no deviations in the daily average concentration in the 30 and 80 ppm exposure group. Individual H<sub>2</sub>S concentration readings during the exposures which were more than 20% below or above the target concentration have also been identified as deviations. These deviations are listed in Tables 10a, 10b, and 10c for the 10, 30, and 80 ppm exposure groups, respectively. The exposure day, the time of the deviation, the range or value of the deviation, and the deviation's impact on the study are shown.

The protocol states that the temperature in the exposure chambers will be maintained between 64 and 79°F. A temperature that was outside that range was identified as a deviation. The temperatures were recorded at least 6 times during the 6-hour exposure. There were no deviations in the exposure chamber temperatures during the study; however, no temperature data is available for the 0 ppm chamber for days 23, 35, and 36 during the study because the thermister was destroyed by a rat at the beginning of those exposure periods and the thermister could not be changed without interrupting the exposures.

The protocol states that the relative humidity will be maintained between 30 and 70 %. A relative humidity that was outside that range was identified as a deviation. The relative humidities were recorded at least 6 times during the 6-hour exposure. There were no deviations identified in the 0, 10, and 80 ppm chambers; there was one deviation in relative humidity within the 30 ppm exposure chamber, which is identified in Table 11. The exposure day, the duration of the deviation, the range of the deviation and the deviation's impact on the study are shown.

### Anterooms

The protocol states that the temperature in the Anterooms will be maintained between 64 and 79 °F. A temperature that was outside that range would be identified as a deviation. The temperatures were checked and recorded at 30 minute intervals for a 24-hour period. There were no deviations identified.

The protocol states that the relative humidity will be maintained between 30 and 70 %. A relative humidity that was outside that range was identified as a deviation. The relative humidities were checked and recorded at 30 minute intervals for a 24-hour period. There was one deviation identified for Anteroom 308 which is shown in Table 12. The exposure day, the duration of the deviation, the range of the deviation and the deviation's impact on the study are shown.

### **Conclusion**

The concentration of H<sub>2</sub>S in the exposure chamber was monitored using gas chromatography. The concentration in each chamber was determined at least 6 times during the 6-hour exposure period. The Grand means ( $\pm$  standard deviations) were 10.0  $\pm$ 0.6, 30.1  $\pm$ 0.8 and 79.5  $\pm$ 2.4 ppm for the target concentrations 10, 30 and 80 ppm, respectively. The Relative Standard Deviations for concentration (standard deviation / average value) were 6.0, 2.7, and 3.0 %, respectively. These Grand Mean concentrations are within 2% of the target concentrations and therefore the exposures for this study were considered acceptable. No H<sub>2</sub>S was detected in the control chamber.

The environmental parameters, temperature, relative humidity and air flow were maintained at or near the target set points throughout the entire study. The exposures were performed from August 19, 1997, through October 28, 1997.

Table 1. Chemical and Physical Properties of Hydrogen Sulfide.<sup>(1)</sup>

Synonym	H <sub>2</sub> S
Appearance	Flammable, poisonous gas with characteristic odor of rotten eggs.
Molecular formula	H <sub>2</sub> S
CAS No.	7783-06-4
Molecular weight	34.08 g/mole
Specific gravity	1.5392 g/L
Ignition temperature	260° C
Flammability limit	4.3 - 46 % in air
Vapor pressure	18.75 x 10 <sup>5</sup> Pa

<sup>(1)</sup> Source: Merck Manual, 12th Edition

**Table 2. Gas Chromatograph Operating Conditions.**

<b>Instrument</b>	<b>Hewlett Packard 6890</b>
<b>Serial No.</b>	<b>US00001742</b>
<b>Detector:</b>	<b>Flame Photometric (FPD)</b>
<b>Carrier gas:</b>	<b>Helium</b>
<b>Oven temperature</b>	<b>80 deg. C</b>
<b>Detector temperature</b>	<b>225 deg. C</b>
<b>Calibration Range</b>	<b>8.35 to 97.4 (ppm)</b>
<b>Concentrations monitored</b>	<b>0, 10, 30, 80 (ppm)</b>
<b>Column:</b>	<b>GS-Q (J &amp; W Scientific)</b>
<b>Column length</b>	<b>0.53 mm x 30 M</b>
<b>Retention Time:</b>	<b>1.176 minutes <math>\pm</math>5%</b>
<b>Sample loop volume:</b>	<b>250 <math>\mu</math>L</b>

Table 3. H<sub>2</sub>S Cylinders used in study.

	Cylinder ID	CIIT Barcode	Cylinder received	Certified H <sub>2</sub> S Concentration
Reference gas	SA5555	T00036	6/18/97	83.3 ppm
Reference gas	SA 22296	T00037	8/26/97	97.4 ppm
Test gas	EGC-3-6230	T00033	6/18/97	4.98%
Test gas	WOS-179	T00034	8/28/97	5%
Test gas	601704	T00039	9/17/97	5%
Test gas	SA 20509	T00040	10/23/97	5%

Table 4. H<sub>2</sub>S Distribution within H-1000 Chamber.

Sample Position	H <sub>2</sub> S Concentration (ppm)		
	10 ppm Chamber	30 ppm Chamber	80 ppm Chamber
9 <sup>(1)</sup>	10.08	27.91	78.56
1	10.02	30.14	78.37
2	10.09	30.08	78.75
3	10.05	30.78	77.06
4	10.09	30.09	79.40
5	10.20	29.34	79.21
6	10.20	29.62	79.72
7	10.15	28.05	80.03
8	10.21	28.23	80.14
Average	10.12	29.36	79.03
Std. Dev.	0.07	1.05	0.97
Relative Std. Dev. (%)	0.7	3.6	1.2

Position 9 is the center position in the chamber where samples are taken during exposures. It may also be referred to as the Home position for the Distribution.

<sup>(1)</sup> Average of 4 values.



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## INTRODUCTION

The purpose of this study was to evaluate whether repeated 6-hour daily exposure of male and female CD<sup>®</sup> rats ( $n = 12$  rats/sex/concentration) to hydrogen sulfide (H<sub>2</sub>S) atmospheres at 0, 10, 30, or 80 ppm would result in reproductive toxicity, developmental toxicity, or developmental neurotoxicity. Reproductive and developmental toxicity were assessed following the 2-week prebreed, 2-week mating (evidence of copulation = gestation day 0 = gd 0), and 3-week gestational (gd 0 until gd 19) exposure periods. All F<sub>0</sub> males were exposed to H<sub>2</sub>S for at least 70 consecutive days. Litters were culled (4 pups/sex/litter) on postnatal day (pnd) 4. Exposure of F<sub>0</sub> dams and their pups resumed between pnd 5 -18. F<sub>0</sub> animals were necropsied with selected organ weights, histopathologic examination of reproductive organs, and andrological assessments performed. F<sub>1</sub> rats were killed by perfusion for neuropathology on PND 23 ± 2 ( $n = 72$ , approximately 1 rat/sex/litter) and PND 61 ± 2 ( $n = 73$ , approximately 1 rat/sex/litter). On PND 63 ± 3 ( $n = 144$ ), the remaining F<sub>1</sub> rat pups were weighed, euthanized with CO<sub>2</sub>, abdominally exsanguinated, and had a complete necropsy performed.

## SUMMARY

There were no treatment-related gross or histopathologic findings in any reproductive organs examined from F<sub>0</sub> rats. Subchronic exposure of male F<sub>0</sub> rats to 80 ppm H<sub>2</sub>S was associated with moderate to marked sensory neuron loss and basal cell hyperplasia primarily within the olfactory mucosa lining the dorsal medial meatus and the dorsal medial region of the ethmoid recess. In the 80 ppm group, the severity of the olfactory lesion ranges from mild to marked. The lesion extends from the dorsal aspect of the middle portion of the airways (approximately midway between the incisive papilla and the base of the incisor teeth; Mery Level 6) to the caudal regions of the ethmoturbinates (Mery Level 23). In the 30 ppm dose group, the lesion severity ranges from mild to moderate and is almost entirely limited to Mery Levels 6 and 9. At 10 ppm, the olfactory lesion is mild only and limited to Mery Levels 6 and 9. Similar mild changes in the olfactory epithelium are seen at Mery Level 6 in control animals. These changes in control animals are consistent with those described with exposure to dirty cage contaminants (Bolon et al., 1991). Lesions found in nasal epithelia other than olfactory epithelium include goblet cell hyperplasia/epithelial hyperplasia of respiratory epithelium, secretory metaplasia/hyperplasia of transitional epithelium, periodontitis, and localized areas of chronic inflammation with squamous/metaplasia in the anterior nose. Their presence does not appear to be dose-related. Additional histologic grading will be completed, so as to better describe the effect of H<sub>2</sub>S exposure on the character of the olfactory lesion and the severity of the nasal lesions found in epithelia and tissues that are not olfactory.

No gross or microscopic abnormalities were observed in the central nervous systems of rats exposed to 80 ppm H<sub>2</sub>S. No statistically significant differences in brain weight or size were observed in H<sub>2</sub>S-exposed rats. Teratogenic effects (assessed grossly at birth) including kinked or missing tails (1 litter affected in each H<sub>2</sub>S treatment group), umbilical hernia (1 litter affected in the 10 ppm H<sub>2</sub>S treatment group), cranial defect (1 litter affected in the 10 ppm H<sub>2</sub>S treatment group), and skin lesions (2 litters affected in the 30 ppm H<sub>2</sub>S treatment group) that included webbing of the forelimb skin were only observed in F<sub>1</sub> pups from the H<sub>2</sub>S-exposed groups. Five transverse H&E-stained sections of skin from the puppy with thin fragile wrinkled skin (97042-litter 74 pup) were examined. The predominant histologic finding is localized to regionally extensive separation of the epidermis from the underlying dermis. This histological finding is most consistent with epidermolysis bullosa, a family of inherited mechanobullous diseases in which minor trauma causes the formation of cutaneous blisters.

MATERIALS AND METHODSF0 Rat Reproductive Necropsy Procedures

At the end of the exposure regimen, adult rats of the parental generation were weighed, euthanized with CO<sub>2</sub>, abdominally exsanguinated, and had a complete necropsy performed with special emphasis on the reproductive and associated organs. The non-impregnated adult females ( $n=11$ ) and adult males ( $n=48$ ) were necropsied the day after the last day of exposure. The postpartum adult females ( $n=48$ ) were necropsied the day of or the day after their pups were weaned (PND 21). The order of necropsy was randomized across dose groups in advance using ear tags for animal identification. Implantation sites were counted in fresh and/or formalin-fixed, potassium ferricyanide-stained uteri from adult female rats. The right testis and epididymis from adult male rats were frozen and used fresh, respectively, for sperm assessment. A complete necropsy included gross evaluation of the following organs/tissues:

- skin
- salivary glands
- lymph nodes (mesenteric and non-mesenteric)
- nasopharynx
- trachea
- mainstem bronchi
- lungs
- heart
- aorta
- thymic region
- thyroids/parathyroids
- liver
- spleen
- kidneys
- pancreas
- adrenal glands
- digestive tract: esophagus, stomach, small intestines, cecum, colon
- urinary bladder
- male reproductive and accessory sex organs: testes, epididymides, penis with preputial gland, prostate, seminal vesicles, coagulating glands
- female reproductive organs: ovaries with oviducts, uterus, cervix, vagina
- brain: cerebrum, cerebellum, medulla/pons
- pituitary gland
- eyes

The following tissues from F0 rats were trimmed, blotted, and weighed:

Both males and females

brain  
liver  
kidneys (both)  
adrenal glands (both)  
spleen

Males only

testes (individually)  
epididymides, total (individually)  
epididymides, caudae (individually)  
seminal vesicles/coagulating glands with fluid (both)  
prostate  
  
heart  
lungs

Females only

ovaries with oviducts  
uterus

The following tissues and forms of identification were collected from adult rats as appropriate and preserved in 10% neutral buffered formalin:

Both males and females

any gross lesion/s  
ear tag  
transponder

Females only

ovaries with oviducts  
uterus  
cervix and vagina

Males only

seminal vesicles/coagulating glands with fluid (both)  
prostate  
nasal cavity

The following tissues were collected from adult male rats and preserved in Bouin's fixative, gross trimmed, rinsed in 50% ethanol, and stored in 70% ethanol:

testis, left  
epididymis, left, head and body  
epididymis, left, cauda

The following tissues were collected from adult male rats, weighed, frozen in liquid nitrogen, and stored at -70°C for possible future sulfide analysis:

brain, heart, liver, kidney, and lung

The following additional tissues were collected from adult rats as appropriate and preserved in 10% neutral buffered formalin:

Noses were flushed retrograde and immersion fixed with 10% neutral buffered formalin and then decalcified in 5% formic acid with an ion exchange resin.

F0 Rat Histopathologic Examination

Histopathologic examination was performed on reproductive and accessory sex organs from rats in the control and high dose groups, that were not reproductively successful, and in the low and middle dose groups with gross findings in any of these organs. Except for F0 male rat noses, histopathologic examination was not performed on other tissues, because gross observations made in other tissues during necropsy were determined not to be dose-related. Tissues to be examined microscopically were processed at Experimental Pathology Laboratories Research Triangle Park, NC) and included the following:

Males

- testis, left
- epididymis, left
- prostate
- seminal vesicles/coagulating glands
- any gross lesion/s in a reproductive or accessory sex organ
- nasal tissues

Females

- ovaries with oviducts
- vagina
- cervix
- uterus
- any gross lesion/s in a reproductive organ

F1 Rat Pup Neuroperfusion Necropsy Procedure

On PND  $23 \pm 2$  ( $n = 72$ ) and PND  $61 \pm 2$  ( $n = 73$ ), rat pups were weighed, anesthetized with sodium pentobarbital, and perfused *in situ* by intracardiac perfusion with 1.5% glutaraldehyde/4.0% formaldehyde buffered fixative. The order of necropsy was randomized across dose groups in advance using ear tags for animal identification. After perfusion, the cranium and vertebral arches or bodies were removed, and the peripheral nerves in the hind legs were exposed. The nervous system was examined grossly and stored along with the ear tag in perfusion fixative at 4°C. The brains were removed from the base of the skull, weighed, and measured (length and width).

F1 Rat Pup Histopathologic Examination

Histopathologic examination was performed on brains from rats in control and high dose groups. The brains were trimmed transversely at 6 levels (forebrain, center of the cerebrum, center of the midbrain, cerebellum and pons, and medulla oblongata), processed routinely, and stained with H&E by the CIIT Histology Service Unit. Histopathologic examination was not performed on brains from intermediate dose groups, because histologic lesions were not found in the control and high dose group brains examined.

F1 Rat Pup Complete Necropsy and Histopathologic Procedures

On PND  $63 \pm 3$  ( $n=144$ ), rat pups were weighed, euthanized with CO<sub>2</sub>, abdominally exsanguinated, and had a complete necropsy performed. A complete necropsy included gross evaluation of the organs/tissues listed above for the parental generation. The order of necropsy was randomized across dose groups in advance using ear tags for animal identification.

The following tissues from rat pups were trimmed, blotted, and weighed:

- brain
- liver
- kidneys (both)
- adrenal glands (both)
- lungs
- heart
- spleen
- testes (both; male)
- ovaries with oviducts (both; female)

The following tissues and forms of identification were collected from rat pups as appropriate and preserved in 10% neutral buffered formalin:

- any gross lesion/s
- ear tag

Histopathologic examination was not performed on tissues from these animals due to a lack of significant statistical correlation between the gross changes observed and the exposure level.

Unscheduled Deaths

There were no unscheduled deaths during the conduct of the study.

Statistical Methods

The Fisher exact test was used to determine the significance of differences between the incidences of dose groups and control groups ( $p \leq 0.05$ ).

## RESULTS

### F0 Female Rats

Terminal body and organ weights for adult F0 female rats are summarized in Tables B5 and B69. The F0 female rats had no H<sub>2</sub>S exposure-related alteration in terminal body weights. The only statistically significant difference from control in either absolute or relative organ weights was a decrease in the relative weight of the ovaries with oviducts in the 10 ppm dose group. The number of implantation sites per rat uterus was not significantly altered by H<sub>2</sub>S exposure (see Table B62). All non-impregnated adult females had no detectable implantation sites at necropsy. All postpartum females had multiple implantation sites present.

The incidences of gross observations made at necropsy of the adult female rats are summarized in Table C1. Gross observations made during complete necropsies of the adult female population had very low incidences that were not dose-related. Due to the lack of toxicologically relevant lesions in other body systems, microscopic examination was limited to the reproductive tracts of female rats in the control and high dose groups, that were not reproductively successful, and that had gross observations made in the reproductive tract. The incidences of histologic diagnoses in the female reproductive tracts are summarized in Table C2. Statistical comparison of the control and high dose groups showed no significant difference between the incidences of the gross observations and histologic diagnoses found.

However, there were a few histologic diagnoses that occurred in exposed groups but not in unexposed rats. One rat each in both the 10 ppm and the 30 ppm dose groups had ovarian cysts. Ovaries of one rat in the 80 ppm dose contained only a few corpora lutea, which were regressing, and a relatively large number of tertiary follicles. This rat, as well as one other in the 30 ppm dose group, also had squamous metaplasia of the endometrium localized to the uterine body.

### F0 Male Rats

Terminal body and organ weights for F0 males are summarized in Tables B3 and B68. The adult male rats had no H<sub>2</sub>S exposure-related alteration in terminal body weights. The only statistically significant difference from control in either absolute or relative organ weights was a decrease in the absolute and relative weight of the adrenal glands in the 10 and 80 ppm dose groups.

The incidences of gross observations made at necropsy of the F0 male rats are summarized in Table C3. Gross observations made during complete necropsies of the adult male population had very low incidences that were not dose-related. Due to the lack of toxicologically relevant lesions in other body systems, microscopic examination was limited to the reproductive tracts of male rats in the control and high dose groups, that were not reproductively successful, and that had gross observations made in the reproductive tract. The incidences of histologic diagnoses in the male reproductive tracts are summarized in Table C4. Statistical comparison of the control and high dose groups showed no significant difference between the incidences of the gross observations and histologic diagnoses found.

However, there were a few histologic diagnoses with a higher incidence in exposed groups than control. Most of these diagnoses are related to seminiferous tubular degeneration (including intratubular sperm stasis, tubular mineralization, sperm granulomas, and multinucleated giant cells) and its corresponding changes in the epididymis (degenerate sperm forms in lumen, aspermia, and oligospermia). One case each of epididymal sperm granulomas and unilateral necrosis of the cauda were detected only in the high dose group at very low incidence (8.3%). Also, there was a slightly

higher incidence of lymphoid interstitial infiltrate in the ventral prostate in the high dose than control.

The major treatment-related histologic diagnosis in the noses of H<sub>2</sub>S exposed rats is bilaterally symmetrical sensory cell loss and basal cell hyperplasia in the olfactory mucosa lining the dorsal medial meatus and the dorsal medial region of the ethmoid recess (Table C5). In the 80 ppm group, the severity of the olfactory lesion ranges from mild to marked. The lesion extends from the dorsal aspect of the middle portion of the airways (approximately midway between the incisive papilla and the base of the incisor teeth; Mery Level 6) to the caudal regions of the ethmoturbinates (Mery Level 23). In the 30 ppm dose group, the lesion severity ranges from mild to moderate and is almost entirely limited to Mery Levels 6 and 9. At 10 ppm, the olfactory lesion is mild only and limited to Mery Levels 6 and 9. Similar mild changes in the olfactory epithelium are seen at Mery Level 6 in control animals. These changes in control animals are consistent with those described with exposure to dirty cage contaminants (Bolon et al., 1991). Lesions found in nasal epithelia other than olfactory epithelium include goblet cell hyperplasia/epithelial hyperplasia of respiratory epithelium, secretory metaplasia/hyperplasia of transitional epithelium, periodontitis, and localized areas of chronic inflammation with squamous/metaplasia in the anterior nose. Their presence does not appear to be dose-related. Additional histologic grading will be completed, so as to better describe the effect of H<sub>2</sub>S exposure on the character of the olfactory lesion and the severity of the nasal lesions found in epithelia and tissues that are not olfactory.

#### F1 Neuroperfused Rat Pups

Terminal body weight and brain weight, length, and width of PND 23 ± 2 and PND 61 ± 2 rat pups are summarized in Tables B15, B73, and B74, respectively. The only statistically significant difference from control in any of these parameters is an increase in brain length in male PND 23 ± 2 rat pups in the 10 ppm dose group. No relevant gross abnormalities were observed at necropsy in the brain, spinal cord, or peripheral nerves of any neuroperfused pup examined. Microscopic examination of H&E stained brain sections at 6 levels from pups in the control and high dose groups failed to demonstrate any relevant histologic abnormalities.

#### F1 Complete Necropsy Rat Pups

Terminal body and organ weights of PND 63 ± 3 rat pups that underwent a complete necropsy are summarized in Table B15 and B75. The only statistically significant difference from control in these values is an increase in absolute weight of ovaries with oviducts in the 30 ppm dose group. A wide variety of gross observations (Table C6) occurred at very low incidences that were not dose-related. Microscopic examination was not performed on tissues from these animals due to a lack of significant statistical correlation between the gross changes observed and the exposure level.

Five transverse H&E-stained sections of skin from one rat pup with thin fragile wrinkled skin (97042-Litter 74 pup) were examined (also see Figure 1). The predominant histologic finding is localized to regionally extensive separation of the epidermis from the underlying dermis. This histological finding is most consistent with epidermolysis bullosa, a family of inherited mechanobullous diseases in which minor trauma causes the formation of cutaneous blisters.

## DISCUSSION/CONCLUSION

As assessed by postmortem examination, the effects of H<sub>2</sub>S exposure on the F0 female rats are subtle and of indeterminate relevance. The incidences of gross observations and histologic diagnoses were not dose-related. The only statistically significant exposure-related change was a decrease in the relative weight of the ovaries in the 10 ppm dose group. This decrease was small and could not be correlated with an increased incidence of reproductive failure or associated histologic abnormalities in this dose group.

The follicular cysts found in F0 female rats in intermediate dose groups were small to moderate in size, not destructive of otherwise normal appearing ovaries, and unlikely to interrupt normal ovarian function. The luteal depletion found in one F0 female rat in the high dose group suggested a disruption of normal estrous cyclicity and was associated with reproductive failure in conjunction with uterine squamous metaplasia. Uterine squamous metaplasia was reported in one rat each in the 30 and 80 ppm dose groups was associated with reproductive failure in both cases, however, the functional relevance of the lesion cannot be determined given that neither of these female rats were ever found to be sperm positive after 14 days of breeding. Uterine squamous metaplasia is reported to occur spontaneously in rats older than those in this study group, with Vitamin A deficiency, and with chronic administration of estrogenic or other toxic substances (Gopinath, 1992).

As assessed by postmortem examination, the effects of H<sub>2</sub>S exposure on the F0 male rats are also subtle and of indeterminate relevance. The incidences of gross observations and histologic diagnoses were not dose-related. The only statistically significant exposure-related change was a decrease in the relative weight of the adrenal glands in the 10 and 80 ppm dose groups. This decrease was small and could not be correlated with an increased incidence of clinical or other pathological abnormalities in these dose groups. The higher incidence and severity of tubular degeneration and related changes in the testes and epididymides of exposed male rats was not associated with a statistically significant increase in reproductive failure in exposed dose groups. Tubular degeneration of minimal to moderate severity was inconsistently associated with reproductive failure. However, the one case of severe tubular degeneration with aspermia in the high dose group could clearly be implicated as a likely cause of reproductive failure in its mating efforts with a female rat whose reproductive tract had a "normal" histologic appearance. Tubular degeneration is reported to occur as a spontaneous aging change in rats as well as in response to exposure to toxins (Boorman *et al.*, K, 1990). The functional ramifications of the severe necrosis of cauda of the epididymis in one high dose animal cannot be determined due to the significant reproductive pathology in the female rat with which he was mated.

The major treatment-related histologic diagnosis in the noses of H<sub>2</sub>S exposed rats is bilaterally symmetrical sensory cell loss and basal cell hyperplasia in the olfactory mucosa lining the dorsal medial meatus and the dorsal medial region of the ethmoid recess. In the 80 ppm group, the severity of the olfactory lesion ranges from mild to marked. The lesion extends from the dorsal aspect of the middle portion of the airways (approximately midway between the incisive papilla and the base of the incisor teeth; Mery Level 6) to the caudal regions of the ethmoturbinates (Mery Level 23). In the 30 ppm dose group, the lesion severity ranges from mild to moderate and is almost entirely limited to Mery Levels 6 and 9. At 10 ppm, the olfactory lesion is mild only and limited to Mery Levels 6 and 9. Similar mild changes in the olfactory epithelium are seen at Mery Level 6 in control animals. These changes in control animals are consistent with those described with exposure to dirty cage contaminants (Bolon *et al.*, 1991). Lesions found in nasal epithelia other than olfactory epithelium include goblet cell hyperplasia/epithelial hyperplasia of respiratory epithelium, secretory metaplasia/hyperplasia of transitional epithelium, periodontitis, and localized areas of chronic inflammation with squamous/metaplasia in the anterior nose. Their presence does not appear to be dose-related. Additional histologic grading will be completed, so as to better describe the effect of H<sub>2</sub>S exposure on the

character of the olfactory lesion and the severity of the nasal lesions found in epithelia and tissues that are not olfactory.

Significant H<sub>2</sub>S-exposure related alterations in nervous system development in rat pups were not detected. The statistically significant increase in brain length of the male PND 23 ± 2 rat pups in the low dose group was not associated with changes in brain weight, gross or microscopic appearance, or behavior and is a finding of indeterminate relevance.

Significant H<sub>2</sub>S-exposure related alterations in development were not found in the PND 63 ± 3 rat pups. The statistically significant increase in ovary with oviduct weight in the 30 ppm dose group was small, not associated with clinical abnormalities, and is a finding of indeterminate relevance.

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Table C1. Gross Necropsy Observations for F0 Female Rats

Rat #1	Dose (ppm)	Uterus	Cervix/vagina	Kidneys	Lungs	Mammary gland	Eye	Other Tissues <sup>2</sup>
51	0	intraluminal fluid <sup>3</sup>	NL	NL	NL	NL	NL	NL
53	0	NL <sup>4</sup>	NL	red foci <sup>5,6</sup>	NL	NL	NL	NL
54	0	NL	NL	NL	red-black foci <sup>5</sup>	NL	NL	NL
59	0	mucometra <sup>7</sup>	cystic malformation	NL	NL	mass	NL	NL
61	10	NL	NL	red foci	NL	NL	NL	NL
62	10	NL	NL	red foci	NL	NL	NL	NL
63	10	intraluminal fluid mesometrial cyst	NL	red foci	NL	NL	NL	NL
65	10	NL	NL	red foci	NL	NL	brown exudate	NL
69	10	NL	NL	red foci	NL	NL	NL	NL