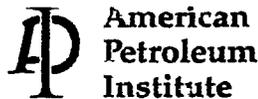


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Carol J. Henry, Ph.D.
Director, Health and
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FYI - 0199 - 1347

January 6, 1999

247 15315

OPPT Document Processing Center (7407)
ATTN: FYI Coordinator
U.S. EPA
401 M Street, S.W.
Washington, D.C. 20460

RE: A Vapor Inhalation Reproductive Toxicity, Developmental Toxicity, and Developmental Neurotoxicity Study in Crl:CD[®](SD)BR Rats Exposed to Hydrogen Sulfide

Dear FYI Coordinator:

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 final report. A draft final report of the same study was submitted on 9/15/98.

A Vapor Inhalation Reproductive Toxicity, Development Toxicity, and Developmental Neurotoxicity Study in Crl:CD[®](SD)BR Rats Exposed to Hydrogen Sulfide

Please note that this information is provided in accordance with the full disclosure policy of API and does not constitute a formal submission as required by a test rule. This document does not contain confidential information. If you have any questions, you may contact me at the above phone numbers or by email.

Sincerely,

David A. Manjillo for
Carol Henry



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FINAL REPORT

A Vapor Inhalation Reproductive Toxicity, Developmental Toxicity, and
Developmental Neurotoxicity Study in CrI:CD[®](SD)CR Rats Exposed to
Hydrogen Sulfide

Conducted by
Chemical Industry Institute of Toxicology

Sponsored by
American Petroleum Institute

Report Submitted
November 19, 1998

Report Accepted
December 29, 1998

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01/19/99

ABSTRACT

The purpose of this study was to evaluate whether repeated 6-hour daily exposure of male and female CD⁰ rats to hydrogen sulfide (H₂S) 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 through gd 19) exposure periods. Adult (F0) females without positive evidence of insemination were exposed to H₂S until 26 days after the end of the breeding period at which time they were euthanized and gross examination of reproductive tracts was performed. All F0 males were exposed to H₂S for at least 70 consecutive days. Litters were culled to 4 pups/sex/litter on postnatal day (pnd) 4. Exposure of F0 dams and their pups resumed between pnd 5 through pnd 18. The postparturient adult females were necropsied the day of or the day after their pups were weaned. Complete necropsies were performed on F0 animals that included a thorough gross examination, selected organ weights, histopathologic examination of reproductive organs, and andrological assessments. No adult systemic toxicity was observed in F0 parents. The nasal cavity was examined histologically in the F0 males only. Subchronic exposure of male F0 rats to 30 and 80 ppm H₂S was associated with sensory neuron loss and basal cell hyperplasia in the olfactory mucosa lining the dorsal medial meatus and the dorsal and medial regions of the ethmoid recess.

No treatment-related changes in mating index, fertility index, or postimplantation loss per litter were observed. Terminal body weights were not statistically different among F0 treatment groups. A statistically significant decrease in body weight gain was observed during gd 0-7 in F0 female rats exposed to 30 or 80 ppm H₂S. Body weight gain throughout gestation (gd 0-20) was also decreased in females exposed to 10 or 80 ppm H₂S. F0 male rats in the 80 ppm H₂S group had significantly decreased feed consumption during the first week of exposure (prebreed). There were no statistically significant treatment-related gross or histopathologic findings in reproductive organs from F0 rats. Structural malformations in newborn (pnd 0) rats, which were only noted in the H₂S-exposed groups, included missing tail (one litter affected in the 10 and 80 ppm H₂S treatment groups), cranial defect (one litter affected in the 10 ppm H₂S treatment group), small lower jaw (one litter affected in the 30 ppm H₂S treatment group), and skin lesions (one litter affected in the 30 ppm H₂S treatment group) that included webbing of the forelimb skin. No dose-response relationship was noted for any structural malformation.

Pup growth, development, and neurotoxicity in F1 pups were assessed by: (a) evaluation of developmental landmarks (pinnae detachment, surface righting, incisor eruption, negative geotaxis, eyelid separation, vaginal patency, or balano-preputial 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 neuropathologic examination (pnd 23 ± 2 , 61 ± 2) and complete gross examination (pnd 63 ± 3). Exposure to H₂S did not affect postnatal growth, development, or behavior. No gross or microscopic abnormalities were observed in the central nervous system of rats exposed to 80 ppm H₂S; therefore, additional histologic evaluations of the 10 and 30 ppm exposure groups were not performed. No statistically significant differences in brain weight or size were observed in H₂S-exposed rats.

The results of our study suggest that exposure to H₂S at occupationally relevant concentrations (≤ 10 ppm) is neither a reproductive toxicant nor a behavioral developmental neurotoxicant in rats.

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FINAL REPORT
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TITLE: A Vapor Inhalation Reproductive Toxicity,
Developmental Toxicity, and Developmental
Neurotoxicity Study in Cri:CD[®](SD)BR Rats Exposed to
Hydrogen Sulfide

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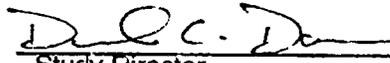
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American Petroleum Institute

STUDY INITIATION DATE: July 31, 1997

FINAL REPORT DATE: November 19, 1998

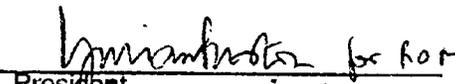
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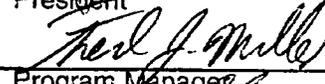
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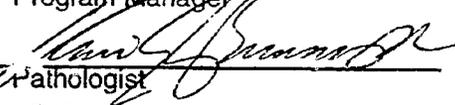
David C. Dorman, DVM, PhD, DABVT, DABT  11/19/98
Study Director Date

LABORATORY INDIVIDUAL SIGNATURE PAGE

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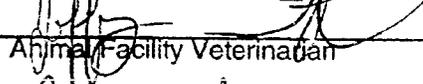
Roger O. McClellan, DVM, DABVT, DABT  11-17-98
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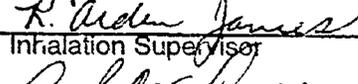
Fred J. Miller, PhD  11/17/98
Program Manager Date

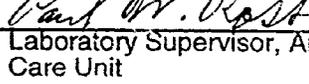
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Research Associate Date

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Jeffrey I. Everitt, DVM, ACLAM, DACVP  11/19/98
Animal Facility Veterinarian Date

R. Arden James, BA  11/16/98
Inhalation Supervisor Date

Paul W. Ross, BS, LATG  11/16/98
Laboratory Supervisor, Animal Care Unit Date

LABORATORY QA STATEMENT

QUALITY ASSURANCE INSPECTION AND REPORTING DATES

Study Title: A Vapor Inhalation Reproductive Toxicity, Developmental Toxicity and Developmental Neurotoxicity Study in Crl: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	08/21,25,29/97	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

LABORATORY QA STATEMENT

QUALITY ASSURANCE INSPECTION AND REPORTING DATES

Study Title: A Vapor Inhalation Reproductive Toxicity, Developmental Toxicity and Developmental Neurotoxicity Study in Crl: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>
Experimental Period	12/01/97	12/01/97	12/01/97
Raw Data/Draft Final Report	05/04,06,08/98, and 05/11-12/98	05/13/98	05/13/98
Raw Data/Draft Final Report	05/19,20,21,23/98	05/27/98	05/27/98
Raw Data/Draft Final Report	05/29/98, 06/01/98, and 06/03/98	06/04/98	06/04/98
Raw Data/Draft Final Report	06/16,17,19/98	06/26/98	06/26/98
Raw Data/Draft Final Report	07/07,09,10/98	07/13/98	07/13/98
Raw Data Review	07/14,15,16,22/98	07/29/98	07/29/98
Raw Data/Draft Final Report	08/27-28/98	09/01/98	09/01/98
Raw Data Review	09/04/98 and 09/09/98	09/18/98	09/18/98
Raw Data Review	09/24/98	09/24/98	09/24/98
Raw Data/Draft Final Report	09/22-25/98	09/29/98	09/29/98
Protocol Amendment Review	09/30/98	09/30/98	09/30/98
Draft Final Report Review	10/29-30/98	11/3/98	11/3/98
Final Report Review	11/17/98	11/17/98	11/17/98

LABORATORY QA STATEMENT

QUALITY ASSURANCE INSPECTION AND REPORTING DATES

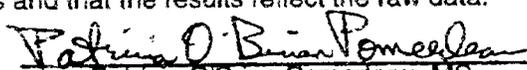
Study Title: **A Vapor Inhalation Reproductive Toxicity, Developmental Toxicity and Developmental Neurotoxicity Study in CrI:CD[®](SD)BR Rats Exposed to Hydrogen Sulfide**

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

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

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.

 11/18/98
Patricia O'Brien Pomerleau, MS Date
Quality Assurance Manager, CIIT

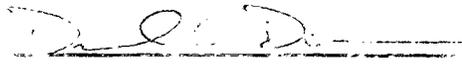
LABORATORY GLP COMPLIANCE STATEMENT

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.

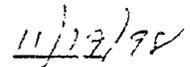
The SOP on external examinations of newborn and neonatal rats was used prior to management approval. It is unknown whether the test substance was fabricated according to GLPs. Batch identification numbers were not available from the supplier. The identity, strength, and purity of the test substance were not determined before the start of the study; however, these analyses were performed prior to the completion of the study. The test substance cylinders were sent to the manufacturer approximately five months after the completion of the in-life phase of the study for additional gas chromatographic analyses. The manufacturer lost the cylinders after completion of these analyses, therefore, the test substance storage containers were not retained until study completion. Uncertified 5% H₂S 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. Cauda sperm samples were discarded following analysis without QAU verification. Computer validation has not been completed on some equipment (e.g. neurobehavioral test equipment). 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 substance as presented herein represents an appropriate conclusion within the context of the study design and evaluation criteria.

LABORATORY GLP COMPLIANCE STATEMENT (CONTINUED)

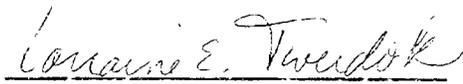
All test and control results in this report are supported by an experimental data record, which has been reviewed. All raw data, documentation, records, protocols, and the Final Report generated as a result of this study will be archived by CIIT.



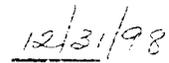
David C. Dorman, DVM, PhD, DABVT, DABT
Study Director
Chemical Industry Institute of Toxicology



Date



Lorraine Twerdok, PhD, DABT
Senior Toxicologist
American Petroleum Institute



Date

A Vapor Inhalation Reproductive Toxicity, Developmental Toxicity, and Developmental Neurotoxicity Study in CrI:CD[®](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[®] rats to hydrogen sulfide (H₂S) 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 through gd 19) exposure periods. Adult (F0) females without positive evidence of insemination were exposed to H₂S until 26 days after the end of the breeding period at which time they were euthanized and gross examination of reproductive tracts was performed. All F0 males were exposed to H₂S for at least 70 consecutive days. Litters were culled to 4 pups/sex/litter on postnatal day (pnd) 4. Exposure of F0 dams and their pups resumed between pnd 5 through pnd 18. The postparturient adult females were necropsied the day of or the day after their pups were weaned. Complete necropsies were performed on F0 animals that included a thorough gross examination, selected organ weights, histopathologic examination of reproductive organs, and andrological assessments. No adult systemic toxicity was observed in F0 parents. The nasal cavity was examined histologically in the F0 males only. Subchronic exposure of male F0 rats to 30 and 80 ppm H₂S was associated with sensory neuron loss and basal cell hyperplasia in the olfactory mucosa lining the dorsal medial meatus and the dorsal and medial regions of the ethmoid recess.

No treatment-related changes in mating index, fertility index, or postimplantation loss per litter were observed. Terminal body weights were not statistically different among F0 treatment groups. A statistically significant decrease in body weight gain was observed during gd 0-7 in F0 female rats exposed to 30 or 80 ppm H₂S. Body weight gain throughout gestation (gd 0-20) was also decreased in females exposed to 10 or 80 ppm H₂S. F0 male rats in the 80 ppm H₂S group had significantly decreased feed consumption during the first week of exposure (prebreed). There were no statistically significant treatment-related gross or histopathologic findings in reproductive organs from F0 rats. Structural malformations in newborn (pnd 0) rats, which were only noted in the H₂S-exposed groups, included missing tail (one litter affected in

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FINAL REPORT
CIIT PROTOCOL 97042

the 10 and 80 ppm H₂S treatment groups), cranial defect (one litter affected in the 10 ppm H₂S treatment group), small lower jaw (one litter affected in the 30 ppm H₂S treatment group), and skin lesions (one litter affected in the 30 ppm H₂S treatment group) that included webbing of the forelimb skin. No dose-response relationship was noted for any structural malformation.

Pup growth, development, and neurotoxicity in F1 pups were assessed by: (a) evaluation of developmental landmarks (pinnae detachment, surface righting, incisor eruption, negative geotaxis, eyelid separation, vaginal patency, or balano-preputial 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 neuropathologic examination (pnd 23 ± 2, 61 ± 2) and complete gross examination (pnd 63 ± 3). Exposure to H₂S did not affect postnatal growth, development, or behavior. No gross or microscopic abnormalities were observed in the central nervous system of rats exposed to 80 ppm H₂S; therefore, additional histologic evaluations of the 10 and 30 ppm exposure groups were not performed. No statistically significant differences in brain weight or size were observed in H₂S-exposed rats.

The results of our study suggest that exposure to H₂S at occupationally relevant concentrations (≤ 10 ppm) is neither a reproductive toxicant nor a behavioral developmental neurotoxicant in rats.

**Section 1
INTRODUCTION**

This report describes the results of a reproductive toxicity, developmental toxicity, and developmental neurotoxicity study with inhaled hydrogen sulfide (H₂S) that was conducted in CD[®] rats. Although the adverse effects arising from acute exposure of adults to high concentrations of H₂S are well recognized, the toxicological sequelae following perinatal exposure have only been partially evaluated. Several studies conducted in rodents suggest that perinatal H₂S exposure can affect brain neurochemistry (Hannah and Roth, 1991; Skrajny et al., 1992); however, few studies have focused on the reproductive or neurobehavioral toxicity of H₂S exposure. The purpose of this study was to evaluate whether repeated 6-hr daily exposure of male and female CD[®] rats (*n* = 12 rats/sex/concentration) to H₂S atmospheres at 0, 10, 30, or 80 ppm would result in reproductive or developmental toxicity. Virgin male and female CD rats were exposed for 2 weeks prior to breeding. Exposures continued during a 2-week mating period (evidence of copulation = gestation day 0 = gd 0) and then from gd 0 through gd 19. Exposure of dams and their pups (8 rats/litter whenever possible) resumed between postnatal day (pnd) 5 through 18. Adult male rats were exposed for at least 70 consecutive days.

This study was performed, to the extent possible, in compliance with the OECD screening test guideline for reproduction and developmental toxicity (Guideline 421). Another goal of the present investigation was to determine whether repeated exposure to H₂S during the perinatal period of development would result in adverse neurobehavioral effects in the offspring. Developmental neurotoxicity in pups was assessed by evaluating the ontogeny of a number of developmental milestones, use of a blinded functional observational battery (FOB), an assessment of spontaneous motor activity until adulthood, and an evaluation of acoustic startle and passive avoidance behaviors.

This report is organized in five sections. The main body of the report provides a description of the study design and methods, results including data summaries, and a discussion of the study findings. Appendix A provides additional details regarding the inhalation exposure system and a report of the raw data from the inhalation portion of the study. Appendix B is a report of the animal raw data from the in-life portion of the study. Appendix C provides additional details about the pathological assessment of the adult animals and their pups. Appendix C also contains the raw data for the gross pathology and all histological evaluations. Appendix D contains the protocol for this study and its amendments.

Section 2
MATERIALS AND METHODS

TEST SUBSTANCE

Gas cylinders containing 5% (50,000 ppm) mixtures of H₂S (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 H₂S are listed in Appendix A, Table 1. 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 H₂S needed in this study were ordered as Certified Standard mixtures. Upon receipt at CIIT, each cylinder was stored in monitoring corridor 200W under ambient temperature and relative humidity conditions. A total of four 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. All cylinders were analyzed by the manufacturer by gravimetric and gas chromatographic analyses. Additional analyses were performed after the study that demonstrated that the test substance was stable.

GENERATION OF THE H₂S ATMOSPHERE

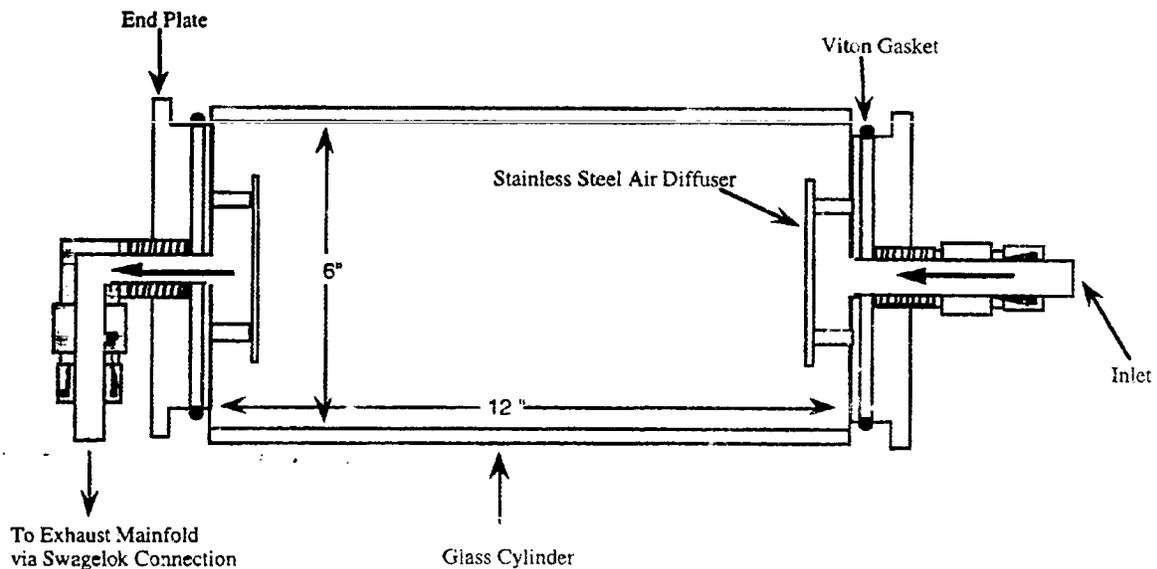
Details regarding the conduct of the inhalation exposure are presented in Appendix A. In brief, H₂S exposure concentrations were generated by metering 5% H₂S in nitrogen from the gas cylinder through a mass flow controller (MKS Instruments, Andover, MA) into a "T" in the two inch stainless steel air inlet to the inhalation chambers. The "T" was located approximately three feet upstream of the chamber. The H₂S gas flowed into the "T" and counter-current to the HEPA-filtered chamber air flow of the inhalation chamber which facilitated mixing of the H₂S vapor with the dilution air. The total chamber air flow through each chamber was maintained at approximately 200-250 L/min. The generation system was operated by the Infinity Building Automation 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 m³ Hinners-style stainless steel and glass inhalation exposure chamber as an additional safety

measure. Air flow through the 8 m³ 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 m³ chamber into a manifold from the 8 m³ chamber exhaust duct. A damper at the opening to the manifold from the 8 m³ chamber was fixed in place to proportion the airflow between the H1000 and the 8 m³ chamber. Air flow through the H1000 chamber was controlled by the total airflow in the 8 m³ chamber. An extension connected to the air inlet of the H1000 chamber was positioned near the air inlet at the top of the 8 m³ chamber. The H₂S 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, 4.9 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 the inhalation chamber atmosphere from the H1000 chamber through the inlet and through the dam/pup exposure cylinder (Figure 1).

Figure 1. Glass exposure cylinder design as described by Vitarella et al., 1998.



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Air flow through the individual glass exposure cylinders 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) inserted in the inlet end cap of the glass cylinder. Measurements of tube concentration, temperature, and humidity were recorded during each exposure period. 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 Transgoc[®] (Chapel's River Laboratories, Inc. Raleigh, NC) was placed into each exposure cylinder prior to the start of the inhalation exposure.

ANALYSES OF EXPOSURE ATMOSPHERES

H₂S 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 mm) column (J & W Scientific, Folsom, CA). 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 H1000 chambers, each H1000 chamber was checked for uniformity of distribution of the test compound by measuring the H₂S 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 Infinity Building Automation 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 Infinity Building Automation 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 (CrI: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 II 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), Kilham rat virus (KRV), CAR Bacillus, Parvovirus, Lymphocytic choriomeningitis virus (LCM), *Mycoplasma pulmonis* and Reovirus 1, 2, or 3 (REO). 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 wire lids (Laboratory Products, Inc., Rochelle Park, NJ) with Alpha-Dri[®] cage litter (Shepard Specialty Papers, Kalamazoo, MI). During lactation dams were housed with their litters 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 were removed from their polycarbonate cages each morning for exposures on pnd 5-18, 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 eartag 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.

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 (Table 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.

Except for animals used for neuropathologic assessments, all weanling and adult animals were euthanized by CO₂ asphyxiation. This includes animals not used in the study. 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 prior to the start of the treatment period or used as sentinels (12 female rats). 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[®] (Charles River Laboratories, Inc., Raleigh, NC) was placed in each glass exposure cylinder prior to the start of the inhalation exposure. When in polycarbonate cages, males and females were watered by plastic water bottles. 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.

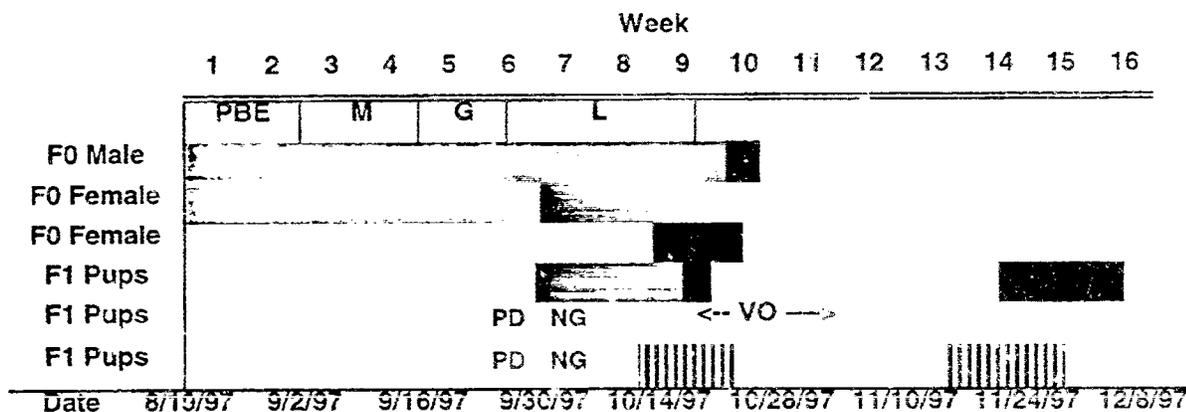
All contaminants were below certified levels for both feed and water and did not affect the design, conduct or conclusions of this study. NIH-07 feed was stored at approximately 60-79°F and the period of use did not exceed four months from the milling date. At all times, the animals were handled, cared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

STUDY DESIGN AND TREATMENT

Study Design:

A graphic representation of the study design is presented in Figure 2 (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 2 – H₂S Reproductive Toxicity and Developmental Neurotoxicity Study Design



Legend

H ₂ S Exposure	[Solid Black Box]	PBE	Prebreed exposure
Necropsy	[Solid Black Box]	M	Mating
Behavioral Testing	[Vertical Lines]	G	Gestation
		L	Lactation
		PD	Pinnae detachment
		NG	Negative geotaxis
		<-- VO -->	Vaginal Opening

TEXT TABLE A
Study Schedule - H₂S

EVENT(s)	DATE(s)
<u>Acclimation and Exposure</u>	
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 transponded	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 ((2) 0 ppm, (3) 10 ppm, (1) 30 ppm, (1) 80 ppm)	9/24/97
pnd 0 for 8 Litters ((1) 0 ppm, (2) 10 ppm, (2) 30 ppm, (3) 80 ppm)	9/25/97
pnd 0 for 13 Litters ((4) 0 ppm, (3) 10 ppm, (4) 30 ppm, (2) 80 ppm)	9/26/97
pnd 0 for 4 Litters ((2) 0 ppm, (1) 10 ppm, (4) 30 ppm, (2) 80 ppm)	9/27/97
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	9/29/97-10/17/97
<u>Developmental Landmarks</u>	
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)
<u>Neurobehavioral Testing</u>	
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
<u>Necropsy</u>	
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 evaluation dates 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 H₂S 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 H₂S 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 H₂S 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 H₂S vapor for six hours per day, seven days per week from gd 0 through 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 H₂S vapor for six hours per day, seven days per week starting on pnd 5 and continuing through pnd 18. No further H₂S 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 H₂S vapor six hours per day, seven days per week until 26 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® (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, Feed 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 and 21. 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 for dams producing litters were computed.

Feed consumption for F0 adult males was 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 weeks of age at the commencement of treatment. They were exposed to H₂S 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. The day vaginal sperm (or plug) was observed was designated gestational day (gd) 0. Once vaginal sperm were observed, the male and female from that mating pair were individually housed. 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 body weight and feed consumption data was collected on pnd 0, 4, 7, 14, and 21.

Sperm/plug positive dams were exposed through gd 19 after which their gestational exposures were terminated. 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 H₂S vapor for six hours per day, seven days per week starting on pnd 5 and continuing through pnd 18. No further H₂S 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 and assess for structural malformations. 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 retained 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 were 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 ± 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 ± 2 . Pups with eartag numbers ending in three (3) or seven (7) were assigned to passive avoidance on pnd 62 ± 3 , functional observation battery (FOB) on pnd 60 ± 2 , and underwent complete necropsy on pnd 63 ± 3 . Pups with eartag numbers ending in four (4) or eight (8) were assigned to acoustic startle on pnd 21 and 62 ± 3 with complete necropsy performed on pnd 63 ± 3 . One female rat (eartag # 626) from the 10 ppm group died on pnd 7 and was cannibalized during the six hour H₂S 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 H₂S exposure on pnd 13, 17, 21, and 60 ± 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 ± 0.7 dBA and room illumination of approximately 2.7 ± 0.4 foot-candles were maintained in the laboratory during motor activity testing (Table B38). 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 ± 1 and on a separate group of animals (used for FOB) on pnd 62 ± 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 1-1 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 ± 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 approximately 55.7 ± 2.6 foot-candles was maintained in the FOB testing laboratory (Table B38). 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 technician (Struve) performed all FOB 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 seizures)
- 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 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 Plexiglas 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 . The overall average acoustic startle amplitude and latency elicited during prepulse and pulse trials were calculated from mean values of ten blocks of five trials each. Each block mean was calculated from five sequential trials values of the appropriate type (pulse or prepulse).

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 CC_2 , exsanguinated, and had a complete necropsy performed with special emphasis on the reproductive and associated organs. The non-pregnant adult females ($n=11$) and adult males ($n=48$) were necropsied the day after their 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). The order of necropsy was randomized across dose groups in advance using ear tags for animal identification. The complete necropsy included gross evaluation of the following organs: 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), whole epididymides (individually), caudae epididymides, (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 CO₂, the right testis from each F0 parental male was weighed and frozen at approximately -20°C for subsequent enumeration of testicular homogenization-resistant spermatid heads. In addition, the right cauda epididymis was immediately removed, weighed and seminal fluid from it was assessed for sperm number, motility and morphology. 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. In addition, daily sperm production and cauda spermatid concentration were calculated. (The left testis and epididymis per male were collected for possible subsequent histopathologic examination; see below.)

All gross lesions and the forms of animal identification (i.e., eartag and transponder) were collected from all adult F0 rats and preserved in 10% neutral buffered formalin as appropriate. The ovaries with oviducts, uterus, and vagina with cervix were collected from F0 female rats and preserved in 10% neutral buffered formalin. Implantation sites were counted in fresh and/or formalin-fixed, potassium ferricyanide-stained uteri from F0 female rats. The formalin-fixed 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 F0 male rats were preserved in 10% neutral buffered formalin: seminal vesicles/coagulating glands with fluid (both), prostate, and nose. The following tissues were collected from F0 male rats and preserved in Bouin's fixative, gross trimmed, rinsed in 50% ethanol, and stored in 70% ethanol: the left testis and the caput, corpus, 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 20% 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 all F0 rats in the control and high concentration exposure groups, and from rats in the low and middle concentration exposure groups that were not reproductively successful or had 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 treatment-related. Tissues to be examined microscopically were processed, paraffin embedded,

sectioned at 5 μ m, and stained with hematoxylin and eosin (H&E) 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) | - testis (1) |
| - vagina | - epididymis (1) |
| - uterus | - seminal vesicles/ coagulating glands (2) |
| - cervix | - prostate |
| | - any gross lesion(s) |

Histologic assessment of male reproductive tissues consisted of a qualitative evaluation of the presence and appearance of normal structures required for normal reproductive function (e.g. sperm maturation in seminiferous tubules). Histologic abnormalities were also identified and graded on a subjective scale as minimal, mild, moderate, marked, or severe, when appropriate. Possible treatment-related effects evaluated in the testes included 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. Histological abnormalities observed were correlated with gross findings or lack of reproductive success, when possible.

Histological assessment of female reproductive tissues was conducted in a manner similar to that of the male. Tissues were first evaluated qualitatively for the presence and appearance of normal stage-appropriate structures (e.g. the postlactational ovary should contain primordial, primary, secondary, and tertiary follicles as well as large corpora lutea). Tissues were then evaluated for histologic abnormalities, which were graded using the same scale described for males and correlated, when possible, with gross findings or lack of reproductive success.

Histologic evaluation of the nose of the F0 male rat included examination of transverse sections taken from six different levels of the nose (Morgan, 1991). Nasal sections were prepared routinely, as described above, by the CIIT Histology Service Unit and stained with H&E. Histopathologic evaluation included confirmation of the presence and normal appearance of structures appropriate for the level of the nose examined and identification of histological abnormalities, whose severity was graded on a scale of 0 to 4 (0 = within normal limits, 1 = mild, 2 = moderate, 3 = marked, and 4 = severe). Non-olfactory nasal lesions were graded

subjectively as appropriate. Olfactory nasal lesions (olfactory neuronal loss and basal cell hyperplasia) were graded according to defined morphologic criteria (see Appendix C).

Pathological Assessment of F1 Animals

F1 rats were killed by perfusion for neuropathology on pnd 23 ± 2 ($n = 72$, approximately one rat/sex/litter) and pnd 61 ± 2 ($n = 73$, approximately one rat/sex/litter). The order of necropsy was randomized across dose groups in advance using ear tags for animal identification. 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 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, sectioned at 5-6 microns, and stained with H&E by the CIIT Histology Service Unit. Histopathologic examination was performed on brains from rats in control and high exposure groups. Histopathologic examination was not performed on brains from intermediate exposure groups, because histologic lesions were not found in the control and high exposure group brains examined.

On pnd 63 ± 3 ($n = 144$), the remaining F1 rat pups were weighed, euthanized with CO₂, exsanguinated, and had a complete necropsy performed. A complete necropsy included gross evaluation of the organs listed above for the parental (F0) generation. The order of necropsy was randomized across exposure groups in advance using ear tags for animal identification. The forms of identification and any gross findings 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 rats, because the gross observations made at necropsy were determined not to be treatment-related.

One pnd 0 F1 rat pup from litter 74 found to have a congenital skin abnormality was examined grossly externally and internally via a ventral midline incision after formalin fixation and had full thickness skin sections taken from five sites. These skin sections were routinely processed, sectioned, and stained with H&E, Periodic acid-Schiff method, Masson's Trichrome method, and Phosphotungstic acid-hematoxylin method at Experimental Pathology Laboratories (Research Triangle Park, NC).

There were no unscheduled deaths during the conduct of the study, therefore, necropsy procedures were not performed on such animals.

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

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 Sperm Concentration

$$\frac{\left(\frac{(\text{spermatids cauda sample 1} + \text{spermatids cauda sample 2})}{2} \right) \cdot 10000 \cdot 200}{\text{cauda weight (g)}}$$

Where, 200 is the sample dilution factor, and 10,000 is the hemocytometer conversion factor

4. Daily Sperm Production

$$\frac{\left(\frac{(\text{sperm sample 1} + \text{sperm sample 2})}{2} \cdot 10000 \cdot 200 \right)}{\text{testis weight (g)}} \cdot 6.1$$

Where, 6.1 = number of days for spermatid cycle; 200 is the sample dilution factor, and 10,000 is the hemocytometer conversion factor

5. Percent Abnormal Sperm

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

6. Percent Motile Sperm

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

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. A 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 ($P < 0.05$).

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) ($p < 0.05$). Levene's test for homogeneity ($p < 0.01$) followed by one-way analysis of variance (ANOVA) ($p < 0.05$) and Dunnett's t-test ($p < 0.05$) 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) ($p < 0.05$) and Dunnett's t-test ($p < 0.05$) was performed on the transformed data. When the Levene's test on the transformed data indicated non-homogenous data, a Kruskal-Wallis test ($p < 0.05$) and Wilcoxon 2-sample Rank-Sum test ($p < 0.05$) 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. The Bonferroni correction

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($p < 0.0167$) for multiple comparisons was used in addition to the Fisher's exact test to aid in interpretation of the incidences of the nasal findings.

PERSONNEL

The evaluation of H₂S inhalation exposure for reproductive and developmental neurotoxicity in CD[®] 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. Drs. Robert Barter and Lorraine Twerdok, of API, were the Sponsor's Representatives.

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

Study Director: Dr. David C. Dorman, DVM, PhD, DABVT, DABT

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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

The protocol and associated amendments, as well as all original data sheets for the present study are stored in the CIIT archives, along with all usable 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. The original report is on file at CIIT and copies are on file 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**TEST SUBSTANCE CHARACTERIZATION**

The test substance was prepared by the supplier, Praxair Distribution, Inc. by dilution of H₂S with nitrogen to the desired cylinder concentration. The concentration of H₂S in the gas cylinders used for these exposures were certified by the supplier, Praxair Distribution, Inc., through gravimetric methods prior to the study start. Purity of the H₂S in the gas cylinders used for these experiments was evaluated at CIIT with a 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 mm) column (J & W Scientific, Folsom, CA). No impurities were noted by GC-FPD in either the reference or test substances. A comparison between nominal and actual concentrations provided a running measure of the test chemical's stability. No degradation in this ratio was observed suggesting that the test material was stable during the course of the study. After the study was over, the gas cylinders were returned to Praxair Distribution, Inc. and additional GC analyses of the reference gas and test substance were performed by the supplier. Hydrogen sulfide trace impurities detected in the gas cylinders included propylene (0.19%), propane (< 0.01%), and water (0.01%). No significant change in the prestudy and poststudy H₂S concentrations were observed (Table A-3) in the reference gases or test substances suggesting that the test material was stable throughout the study. In addition, the difference in the analysis by gravimetric method and the GC analysis were less than 4% and had no impact on the outcome of the study.

GLASS EXPOSURE CYLINDER PERFORMANCE

The potential for adsorption of H₂S onto the Alpha-Dri[®] cage litter was investigated. A glass cylinder was filled with approximately 60 g of Alpha-Dri[®] cage litter; this amount of bedding represented approximately 40% of the cylinder volume. A test atmosphere of 10 ppm H₂S in air was generated in a 1 m³ inhalation chamber. A portion of the test atmosphere was pulled from the 1 m³ inhalation chamber through the glass cylinder for approximately 30 minutes. The average concentration (± SD) at the inlet was 11.6 (± 0.2) ppm as compared to the average concentration (± SD) at the outlet, which was 11.6 (± 0.1) ppm. It was concluded that there was no detectable adsorption of H₂S onto the bedding material. The H₂S concentration was monitored after the H₂S delivery was turned off to determine if there was any residual H₂S absorbed onto the Alpha-Dri[®] cage litter. The concentrations at the sample locations were

below the GC detection limits within two minutes. These results suggest that negligible adsorption of H₂S onto the Alpha-Dri® bedding occurred.

Full results of the exposure phase of the study are presented in Appendix A. Grand mean air flow rates for the exposure cylinders during the study were 2.97 ± 0.15 , 2.95 ± 0.12 , 3.07 ± 0.19 , and 2.90 ± 0.09 L/min, respectively, for the control, 10, 30, and 80 ppm exposure groups (Tables B1 and B2). 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 transient higher humidity readings at various times during the exposures.

TEST ATMOSPHERES

The target H₂S concentrations were 0, 10, 30 and 80 ppm. The grand means for the actual chamber concentrations (\pm SD) 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, calculated as a grand mean from individual daily mean values. No H₂S was detected in the control chamber (limit of detection was 0.6 ppm H₂S in air). The grand means for the nominal chamber concentrations (\pm SD) 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 (\pm SD) 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 (\pm SD) for H₂S 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 (\pm SD) 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 (\pm SD) 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. (See Appendix A for details.)

A daily mean concentration that 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, 8.6, 8.7, 8.9, and 11.1 ppm, respectively were observed. These

relatively minor excursions outside the protocol-mandated range for target daily average H₂S concentration did not affect the design, conduct or conclusions of this study.

ENVIRONMENTAL CONDITIONS

The temperature and relative humidity of the 1 m³ exposure chambers and anterooms were monitored continuously by the Infinity Building Automation System. 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) were as follows.

Text Table D				
Temperature and Relative Humidity Results				
1. Anterooms (Exposure Concentrations)				
	308 (control)	309 (10 ppm)	310 (30 ppm)	311 (80 ppm)
Grand Mean* (± SD) Dry Bulb Temperature (°F)	66.8 ± 0.2	66.8 ± 0.2	66.2 ± 0.2	65.6 ± 0.2
Minimum Daily Mean	66.5	66.5	65.7	65.1
Maximum Daily Mean	67.1	67.3	66.8	66.2
Grand Mean* (± SD) Relative Humidity, %	49 ± 2	50 ± 2	48 ± 2	48 ± 2
Minimum Daily Mean	42	44	41	39
Maximum Daily Mean	53	53	50	50
2. H1000 Chambers (Exposure Concentrations)				
	308 (control)	309 (10 ppm)	310 (30 ppm)	311 (80 ppm)
Grand Mean* (± SD) Dry Bulb Temperature, °F	73.0 ± 2.0	73.7 ± 1.0	71.6 ± 2.2	74.4 ± 1.0
Minimum Daily Mean	69.0	70.8	68.4	71.8
Maximum Daily Mean	75.9	75.3	75.1	75.7
Grand Mean* (± SD) Relative Humidity, %	42 ± 1	46 ± 2	44 ± 5	41 ± 2
Minimum Daily Mean	39	41	40	36
Maximum Daily Mean	44	55	66	48

* Grand mean of the daily means ± SD.

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 prolonged excursions outside the protocol-mandated range for temperature (64-79°F) and relative humidity (30-70%) identified in the 0, 10, and 80 ppm H1000 exposure chambers or in any of the anterooms. There was one brief (0.75 hour) excursion in relative humidity to 71% 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. The relative humidity in anteroom 311 was 29.1 % for 0.5 hour, 29.5 % for 0.5 hr and 29.8% for 0.5 hour on study day 91 (post exposure). 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

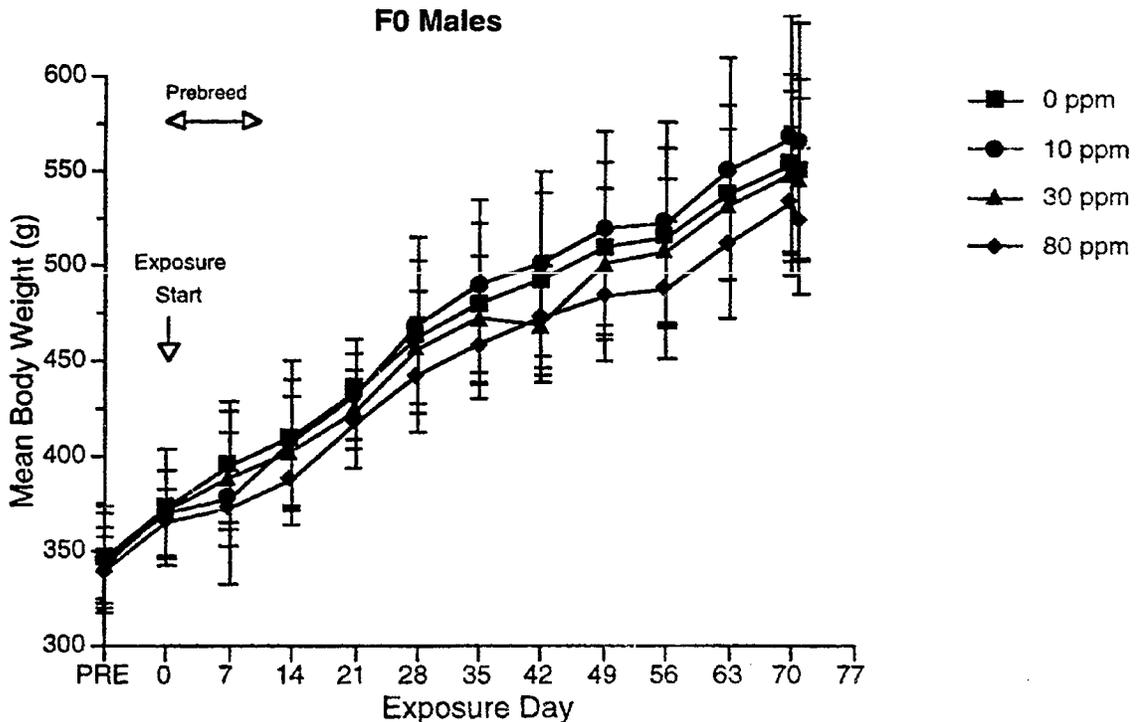
All individual in-life and organ weight data are presented in Appendix B; all individual histopathology data are presented in Appendix C. Body weight data for all animals are presented in Tables B3 through B17. Feed consumption data are presented in Tables B18 through B25.

No F0 males died prior to or during the conduct of the study. All F0 males in all groups survived to scheduled necropsy. One F0 female 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 (Table 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. The mean (\pm SD) initial body weights of F0 males were 346.3 ± 28.4 , 344.9 ± 24.6 , 342.8 ± 20.0 , and 340.1 ± 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 ± 47.5 , 565.3 ± 63.0 , 545.5 ± 43.1 , and 524.6 ± 38.9 grams, respectively for the control, 10, 30 and 80 ppm treatment groups. Body weight gain throughout the study was equivalent among treatment groups (Text Table E).

Figure 3. 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. The mean (\pm SD) initial body weights of F0 females were 201.43 ± 15.8 , 201.6 ± 16.6 , 202.1 ± 19.2 , and 200.6 ± 14.0 grams, respectively for the control, 10, 30 and 80 ppm treatment groups.

Statistically significant decreases in body weight gain were observed in pregnant female rats exposed to 30 or 80 ppm H₂S during early gestation (gd 0-7). Decreased body weight gain was also observed throughout gestation (gd 0-20) in female rats exposed to 10 or 80 ppm H₂S (Text Table E). Altered body weight gains were also observed during the neonatal period. However, no apparent dose-response relationship or consistent pattern of weight gain or loss was observed for any treatment group.

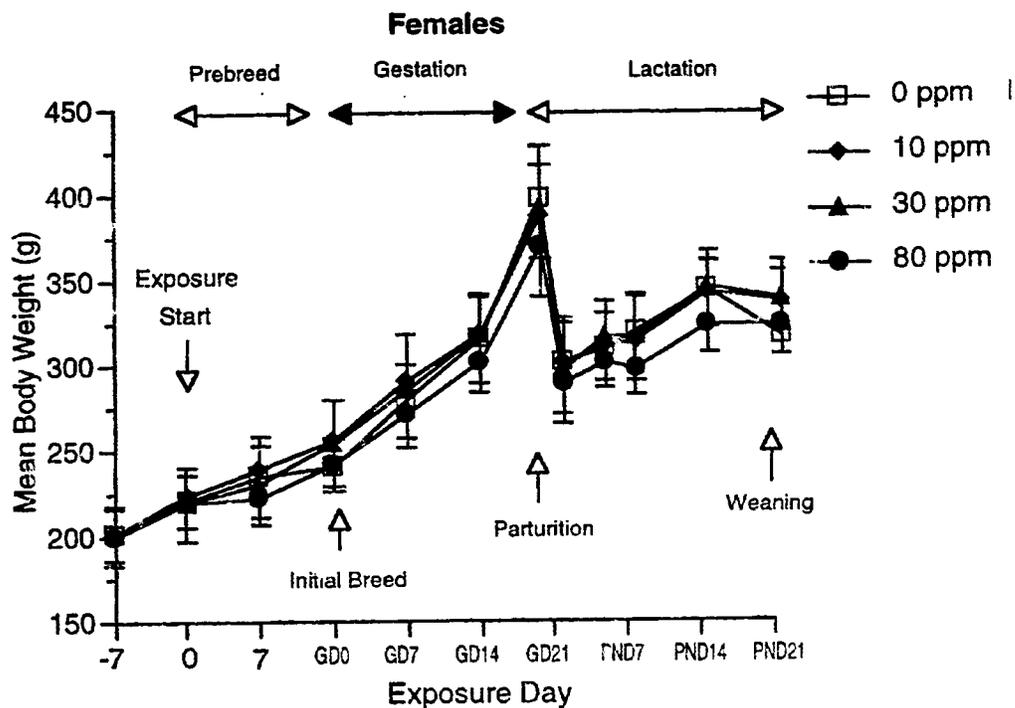
Text Table E
Mean (\pm SD) Body Weight Gain (g) in F0 Rats Exposed to H₂S

Evaluation Interval	H ₂ S Exposure Concentration (ppm)				
	0	10	30	80	
F0 Female Rat Gestation Period					
	n =	8	11	8	8
gd 0-7		38.3 \pm 5.8	34.6 \pm 6.4	30.3 \pm 4.1 ^a	28.7 \pm 4.1 ^a
gd 7-14		36.4 \pm 6.1	34.2 \pm 3.6 ^b	33.0 \pm 4.1	30.7 \pm 7.1
gd 14-20		82.0 \pm 11.7	68.2 \pm 12.1 ^b	73.8 \pm 7.7	68.1 \pm 17.7
gd 0-20		156.9 \pm 16.0	136.1 \pm 16.5 ^{ab}	137.1 \pm 10.8	127.5 \pm 20.5 ^a
F0 Female Rat Lactation Period					
	n =	9	11	9	8
pnd 0-4		7.6 \pm 13.6	10.3 \pm 15.0	20.3 \pm 17.0	11.7 \pm 19.1
pnd 4-7		9.2 \pm 10.4	2.5 \pm 6.5	10.1 \pm 3.4 ^a	-3.3 \pm 6.1 ^a
pnd 7-14		25.9 \pm 6.9	29.7 \pm 10.1	29.8 \pm 8.7	26.1 \pm 9.0
pnd 14-21		-28.4 \pm 33.4	-5.9 \pm 10.3 ^a	-6.5 \pm 14.0	0.6 \pm 9.0 ^a
pnd 0-21		14.2 \pm 31.3	36.6 \pm 20.1	42.5 \pm 15.0 ^a	35.1 \pm 12.2
F0 Male Rat Exposure Days					
	n =	12	12	12	12
0-14		36.8 \pm 14.2	37.1 \pm 14.1	31.2 \pm 16.4	22.6 \pm 12.8
14-42		81.9 \pm 12.7	94.1 \pm 19.5	67.4 \pm 36.3	86.0 \pm 17.3
42-70		61.9 \pm 9.3	66.5 \pm 20.9	79.8 \pm 42.7	61.1 \pm 32.4
0-70		180.6 \pm 26.4	197.7 \pm 47.5	178.4 \pm 34.2	169.7 \pm 40.9

^a Significantly different from control ($p < 0.05$).

^b n=10, Dam #61 was not weighed on GD14.

Figure 4. Mean (\pm SD) F0 female body weight (g) following H₂S exposure



No treatment-related difference in body weight was observed in sperm-negative female F0 rats exposed to H₂S (Table B7). No statistically significant differences in terminal body weight were observed in female parental rats (Tables B5 and B6).

F0 REPRODUCTIVE TOXICITY

Reproductive indices for F0 parents (Text Table F, Table B62) exhibited no treatment-related changes in mating index, fertility index, postimplantation loss per litter, or number of stillbirths or resorptions. Mean gestational length in days was not significantly affected by H₂S 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 16.7 \pm 0.6, 16.2 \pm 0.6, 15.9 \pm 0.6, and 14.5 \pm 1.5 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 0.53, 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 45.6, 51.2, 44.7, and 39.8%, 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 F
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
Late resorption or stillbirth	1 (1)	3 (3)	5 (3)	1 (1)

A summary of the F0 male andrological assessments is presented in Text Table G. F0 absolute paired testes, paired epididymides and cauda weights were equivalent across all groups (see Text Table K). Percent motile sperm, percent normal sperm, daily sperm production, and cauda sperm count were statistically equivalent across all groups. A large percentage of abnormal sperm was observed in two F0 rats, #31 (30 ppm) and #46 (80 ppm). Abnormal sperm accounted for 29 and 73% of all sperm analyzed from rats #31 and #46, respectively (see Table B-66).

Text Table G
F0 Male Sperm Analyses 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 ^a
% 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 ^a
Cauda Sperm Count ^b	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 ^c	Mean	17.92	18.14	17.26	17.30
	S.D.	2.17	1.58	1.89	5.63
	n	12	12	12	12

^a There were no sperm in the sperm motility and morphology sample from one animal.

^b Cauda sperm count means and standard deviations are expressed as X10⁹.

^c Daily sperm production means and standard deviations are expressed as X10⁶.

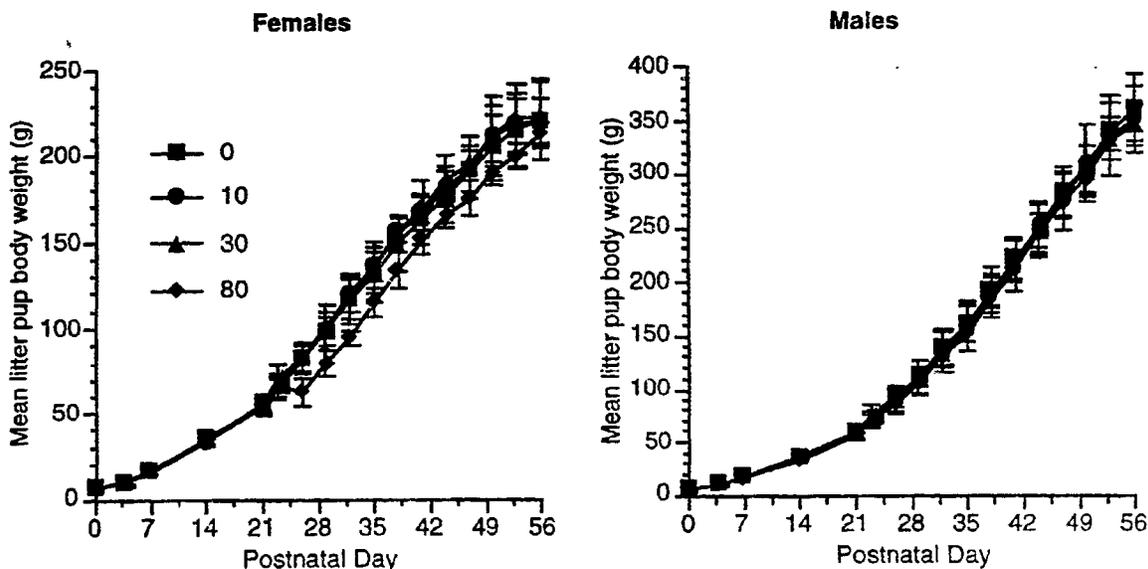
F0 CLINICAL OBSERVATIONS

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 Tables 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 H₂S 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 was unlikely to result in any detrimental effect to the animal.

F1 PUP GROWTH AND DEVELOPMENT

Pup body weight data are presented in Tables B12 through B17. No statistically significant difference in pup body weight gain was observed in H₂S-exposed pups of either sex either during lactation or the remainder of the postnatal period (pnd 22 through pnd 60-65). No statistically significant difference in terminal body weight was 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 5. Mean (\pm SD) litter body weights (g) of H₂S-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 ± 11.9 , 81.1 ± 11.3 , 78.8 ± 14.2 , and 76.36 ± 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 Fi. 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 H
Acquisition of Developmental Landmarks in Neonatal Pups Exposed to H₂S

Developmental Landmark ¹		H ₂ 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	12.0	11.3
	SD	1.3	0.5	1.7	1.4
	n	9	11	9	8
Eyelid Separation	Mean	14.6	15.1	14.8	15.0
	SD	0.9	0.5	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 H₂S exposure. Structural malformations in newborn (pnd 0) pups were only noted in the H₂S-exposed groups. Structural malformations included kinked or missing tails (one litter affected in the 10, 30, and 80 ppm H₂S treatment groups), cranial defect (one litter affected in the 10 ppm H₂S treatment group), anophthalmia (1 litter affected in the 30 ppm H₂S treatment group), small body size (one litter affected in the 10 ppm H₂S treatment group), small lower jaw (one litter affected in the 30 ppm H₂S treatment group), and skin lesions (one litter affected in the 30 ppm H₂S treatment group) that included webbing of the forelimb skin (Text Table I). No dose-response relationship was observed for any structural malformation. Kinked tails were also observed on pnd 4 in pups exposed to H₂S. This observation is not considered treatment-

related, since in no case did this lesion persist to pnd 7 as would have been expected had a true tail malformation existed. Traumatic effects, the most common of which were 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. No clinical observations were observed in postnatal day 14 or 21 rat pups from the control or H₂S-exposed treatment groups.

Text Table I
Summary of Clinical Observations in Male and Female F1 Rat Pups Exposed to H₂S

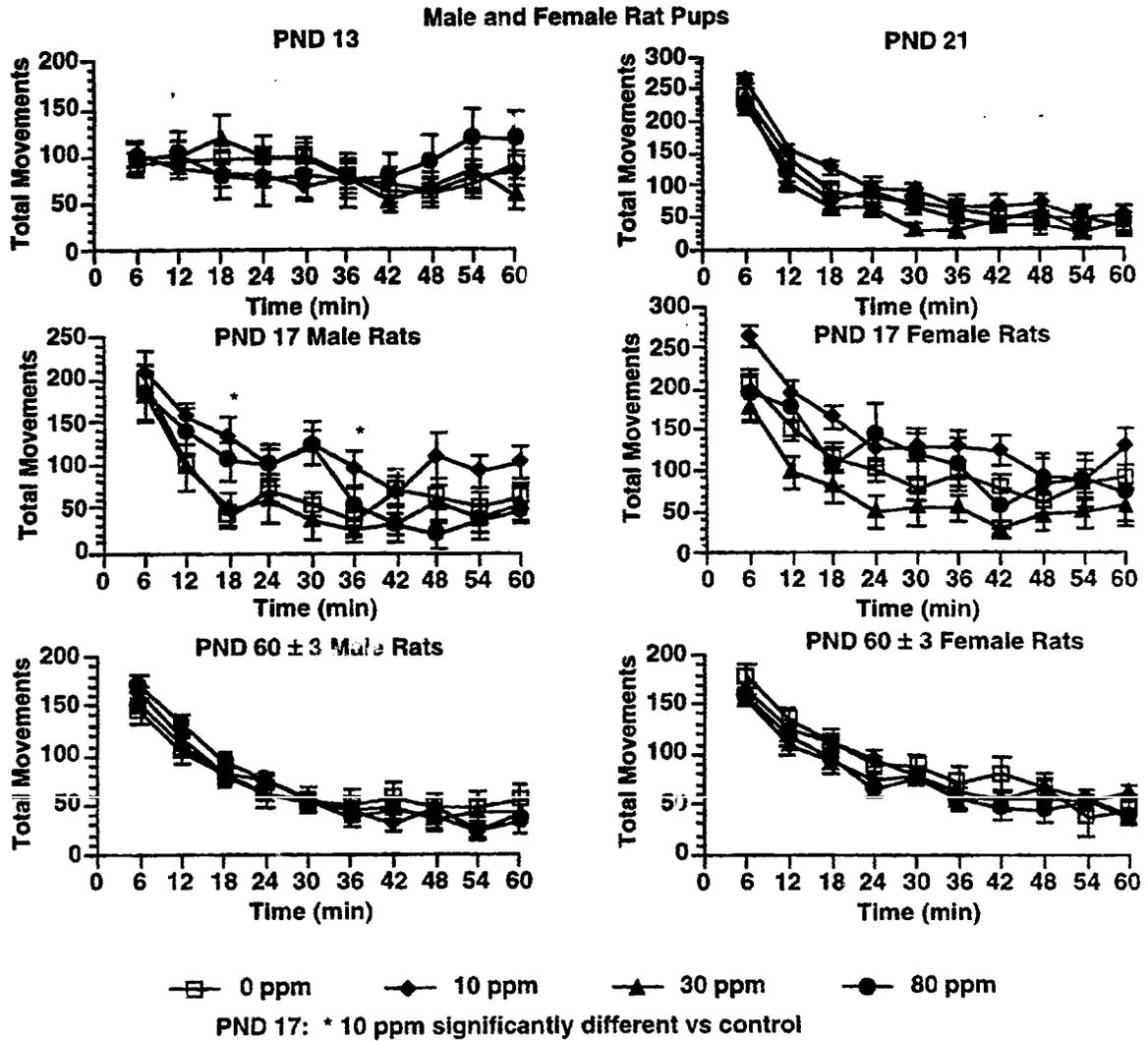
Postnatal Day	Observation ¹	H ₂ S Exposure Concentration (ppm)			
		0	10	30	80
Structural Malformations Observed at Birth					
0	Cranial defect - indentation on skull		1 (1)		
	Kinked tail		1 (1)	1 (1)	
	Missing tail		1 (1)		1 (1)
	Missing eye			1 (1)	
	Rear legs and body small		1 (1)		
	Skin lesion +/- webbing			7 (1)	
	Small lower jaw			2 (1)	
Traumatic Effects Observed in Neonates					
0	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)	
	Pale		1 (1)		
7	Runt - missing at end of exposure		1 (1)		
	Scratches/cuts/scabs		1 (1)		

¹ 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 ± 0.67 dB (Table B38). The mean (\pm SD) illumination level in the motor activity laboratory was 2.7 ± 0.41 ft-candles (Table B38). Motor activity data is presented for all pups in Tables B39 through B42 and graphically in Figure 6. Increased overall motor activity (hyperactivity) was observed in pnd 17 rat pups exposed to 10 ppm H₂S. Exposure to H₂S 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 ± 2 motor activity test sessions. A statistically significant gender-related effect on overall motor activity was observed during the pnd 60 ± 2 test session. Female rats displayed a higher amount of motor activity during this pnd 60 ± 2 test session.

Figure 6. Mean (\pm SEM) Motor Activity in F1 Pups Following H₂S Exposure



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 ± 1 and on pnd 62 ± 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.

The results of the passive avoidance testing are summarized in Text Table J. 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 ± 3 . There were also no statistically significant differences among groups for the ability to complete the passive avoidance task. Male rats tested on pnd 22 ± 1 had a lower, albeit not statistically significant, decrease in the step through latency time on the second day of testing. Historical control data (Vitarella et al., 1998) 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 ± 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 (\pm 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 (\pm SEM) retention trial step through latency times, thus it is unlikely that the shorter mean (\pm SEM) retention trial step through latency times observed in control male pnd 21 ± 1 rats had any impact on the study.

Text Table J

Effect of H₂S Exposure on Passive Avoidance

Post Natal Day 21-22

Sex	Exposure Group	Mean Difference in Step-through Latency (sec) ¹	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

¹ 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 (± 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 H₂S-exposed F1 rats. Likewise, no evidence of neuromuscular dysfunction (e.g., abnormal muscle tone) was observed in any control or H₂S-exposed rats. No clinical signs of ataxia, piloerection, excessive vocalization, muscle tremors or

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spasms, clonic or tonic seizures, increased salivation, abnormal respiration, or abnormal pupil reflex were observed in any H₂S-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 H₂S-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 Plexiglas 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 ± 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 (Tables 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 7a. Effects of H₂S Exposure on Acoustic Startle Reflexes (Prepulse - Maximum Impulse)

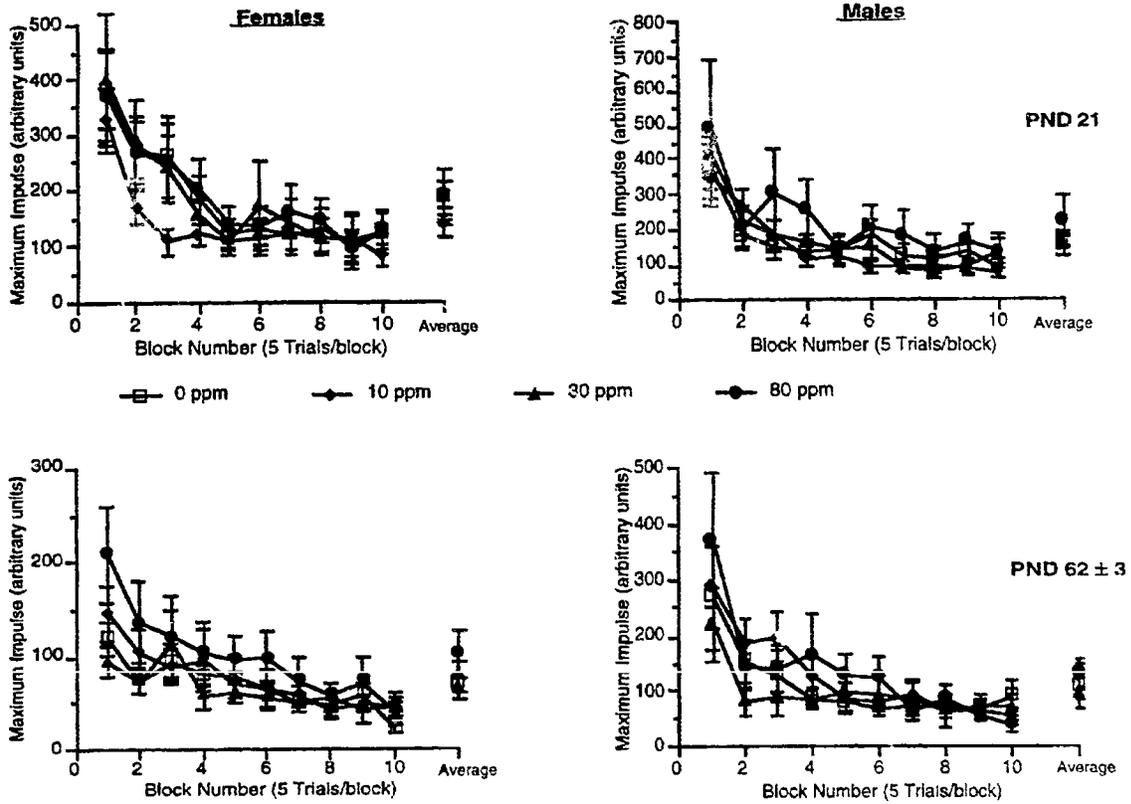


Figure 7b. Effects of H₂S Exposure on Acoustic Startle Reflexes (Prepulse - Latency)

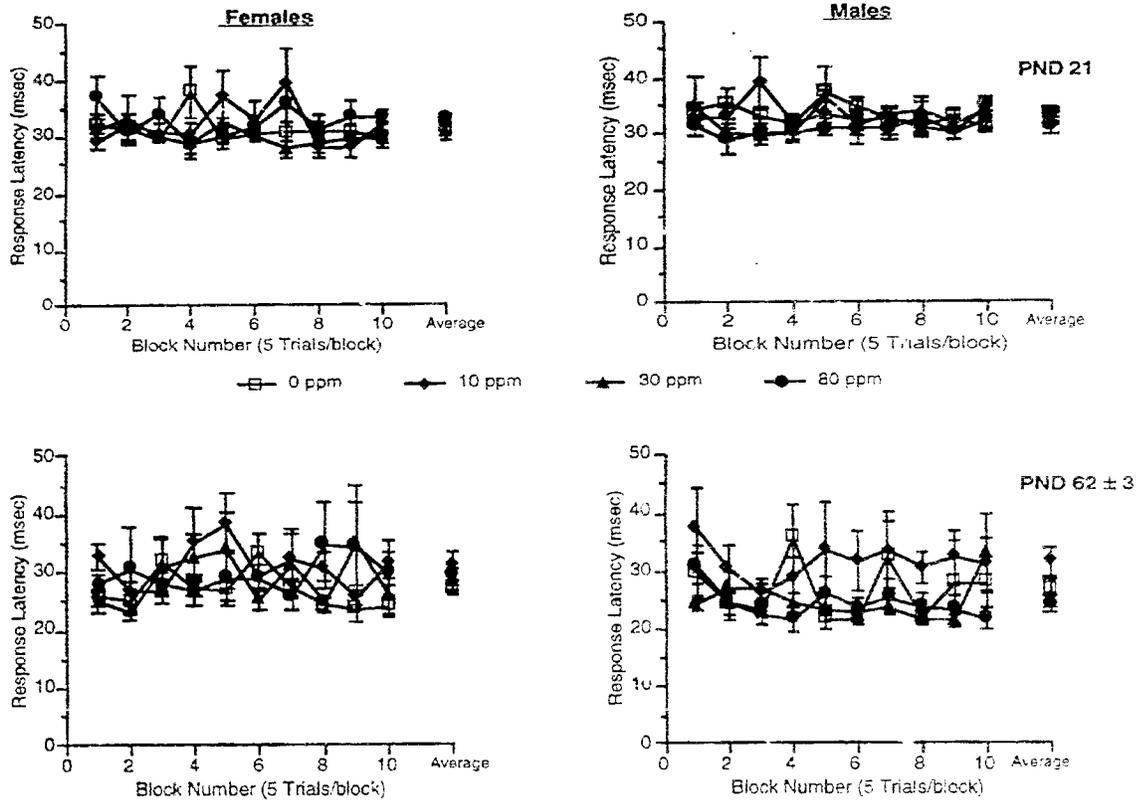


Figure 7c. Effects of H₂S Exposure on Acoustic Startle Reflexes (Pulse - Maximum Impulse)

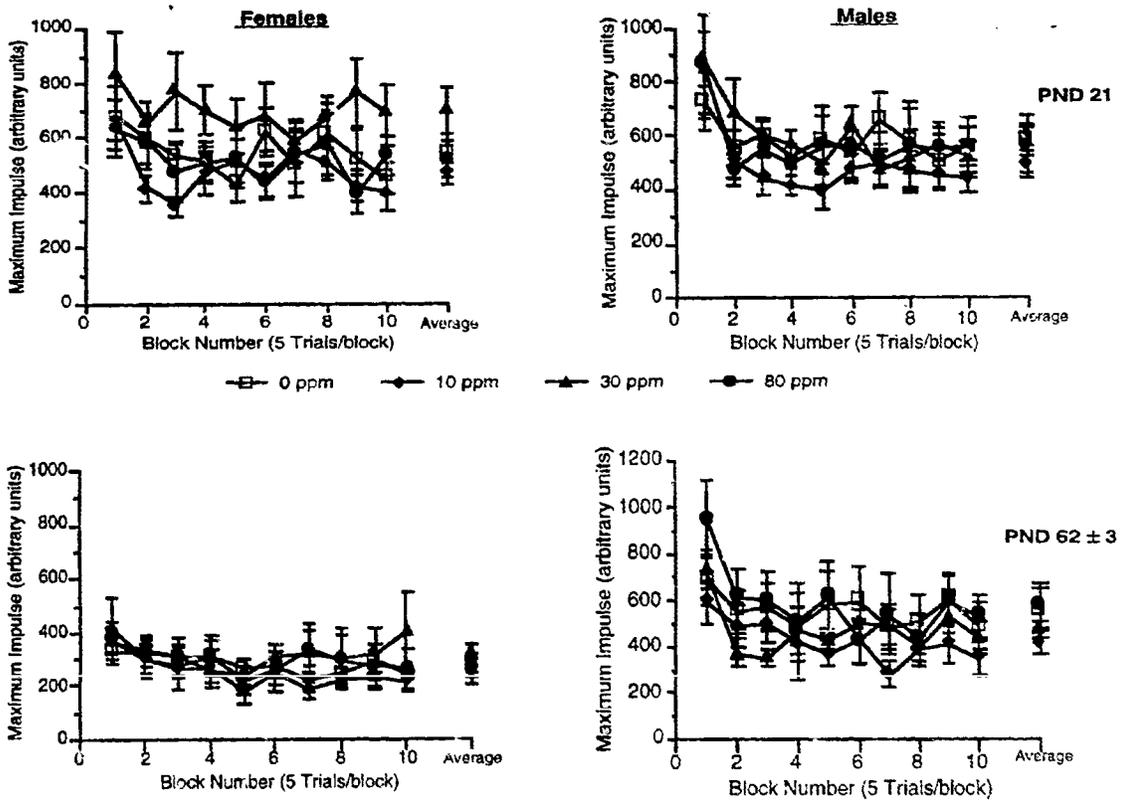
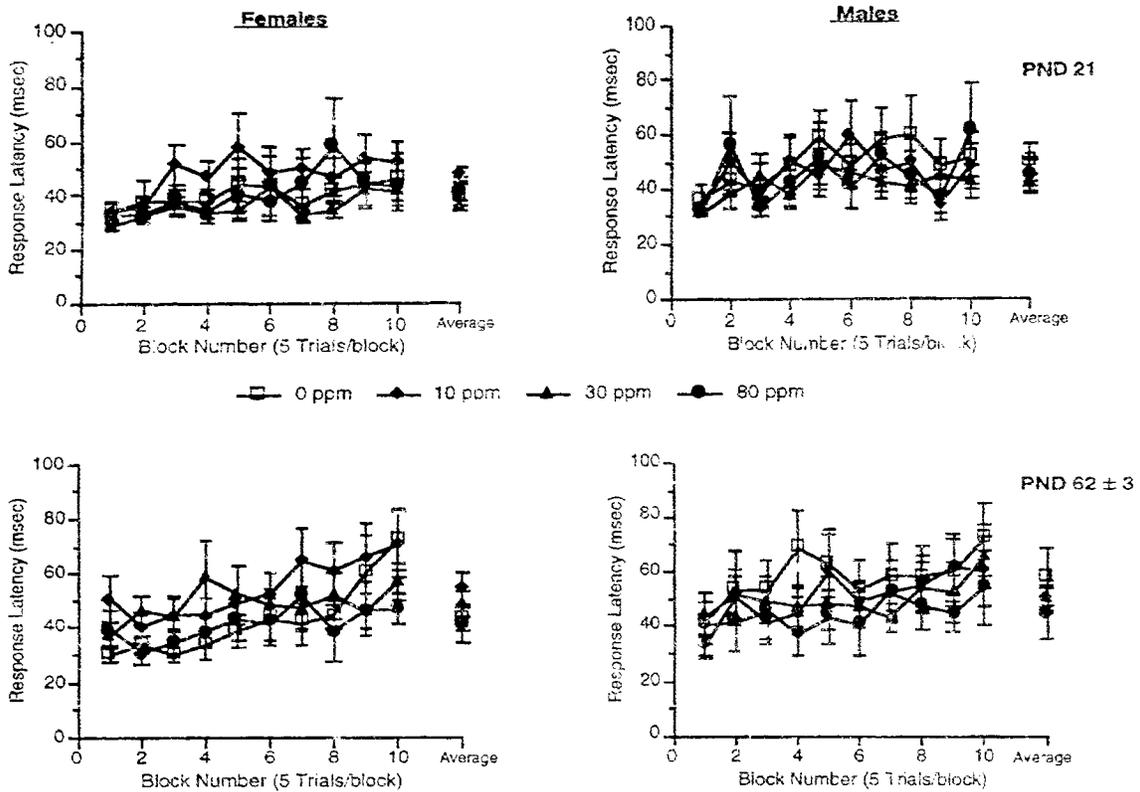


Figure 7d. Effects of H₂S Exposure on Acoustic Startle Reflexes (Pulse - Latency)



NECROPSY AND HISTOLOGY – F0 RATS

At the end of the exposure regimen, adult rats of the parental generation were weighed, euthanized with CO₂, exsanguinated, and had a complete necropsy performed with special emphasis on the reproductive and associated organs. The non-pregnant adult females (*n* = 11) and adult males (*n* = 48) were necropsied the day after their 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 F0 males are summarized in Text Table K (see also Table B68). The adult male rats had no H₂S 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 exposure groups.

Text Table K

Absolute Mean (± SD) Terminal Body or Organ Weights (g) for Male F0 Rats Exposed to H₂S

	H ₂ S Exposure Concentration (ppm)			
	0	10	30	80
Terminal Weight	551.4 ± 47.5	565.3 ± 63.0	545.5 ± 43.1	524.6 ± 35.9
Adrenal Glands	0.07 ± 0.01	0.06 ± 0.01*	0.07 ± 0.01	0.06 ± 0.01*
Brain	2.12 ± 0.13	2.14 ± 0.09	2.12 ± 0.09	2.10 ± 0.05
Heart	1.54 ± 0.13	1.56 ± 0.12	1.55 ± 0.17	1.45 ± 0.09
Kidneys	3.88 ± 0.43	3.95 ± 0.34	3.98 ± 0.40	3.77 ± 0.33
Liver	19.65 ± 1.84	20.86 ± 3.12	19.89 ± 2.78	19.09 ± 2.16
Lungs	1.47 ± 0.14	1.48 ± 0.11	1.47 ± 0.18	1.53 ± 0.14
Spleen	0.85 ± 0.13	0.78 ± 0.09	0.83 ± 0.11	0.82 ± 0.11
Epididymides Caudae	0.68 ± 0.09	0.65 ± 0.09	0.63 ± 0.05	0.71 ± 0.37
Epididymides Whole	1.52 ± 0.17	1.47 ± 0.15	1.42 ± 0.06	1.52 ± 0.52
Prostate	1.37 ± 0.25	1.29 ± 0.34	1.35 ± 0.26	1.33 ± 0.30
Seminal vesicles and coagulating glands	2.00 ± 0.37	1.90 ± 0.29	2.00 ± 0.26	1.96 ± 0.50
Testes	3.52 ± 0.30	3.47 ± 0.26	3.47 ± 0.18	3.27 ± 0.84
Sample size (n)	12	12	12	12

* p < 0.05

Terminal body and absolute organ weights for F0 females are summarized in Text Table L (see also Table B69). The adult female rats had no H₂S 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 exposure group. The number of implantation sites per rat uterus was not significantly altered by H₂S exposure. All non-pregnant adult females had no detectable implantation sites at necropsy. All postpartum females had multiple implantation sites present.

Text Table L

Absolute Mean (\pm SD) Terminal Body or Organ Weights (g) for Female F0 Rats Exposed to H₂S

	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
Sample size (n)	12	12	12	12

Gross Necropsy Observations

The incidences of gross observations made at necropsy of the F0 male rats are summarized in Text Table M (see Table C5 for details). Gross observations made during complete necropsies of the F0 male rats had very low incidences that were not treatment-related.

Text Table M

Incidence¹ of Gross Lesions in F0 Male Rats Exposed for at Least 70 Consecutive Days to H₂S

Organ	Gross Observation ²	Exposure Group			
		0 ppm	10 ppm	30 ppm	80 ppm
Heart	hypertrophy ³	2/12	0/12	0/12	1/12
Kidneys	hydronephrosis ⁴	0/12	1/12	0/12	0/12
Live,	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 ⁵	N/A ⁶	N/A	N/A	N/A	N/A

¹ = # of rats with gross observation/# of rats grossly examined in exposure group.

² = does not include the incidental findings of red or dark foci in the kidneys or lungs associated with CO₂ euthanasia.

³ = firm with small left ventricular lumen

⁴ = dilated renal pelvis/es

⁵ = 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.

⁶ = not applicable

The incidences of gross observations made at necropsy of the F0 female rats are summarized in Text Table N (see Table C1 for details). Gross observations made during complete necropsies of the F0 female rats had very low incidences that were not treatment-related.

Text Table N
Incidence¹ of Gross Lesions in F0 Female Rats Exposed to H₂S

Organ/tissue	Gross Observation ²	Exposure Group			
		0 ppm	10 ppm	30 ppm	80 ppm
Uterus	mucometra ³	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 ⁴	N/A ⁵	N/A	N/A	N/A	N/A

¹ = # of rats with gross observation/# of rats grossly examined in exposure group.

² = does not include the incidental gross observations of red and dark foci in the lungs and kidneys associated with CO₂ euthanasia and uterine luminal dilation by clear fluid which is a normal finding in reproductively cycling rats in proestrus or estrus.

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

⁴ = 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.

⁵ = 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 exposure groups (see Table C7 for details). In addition, F0 males in the 10 and 30 ppm exposure groups 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 O. There was a background incidence of some histologic reproductive diagnoses in F0 male rats; however, the severity and/or incidences of these findings in control rats were not considered to be sufficient to confound interpretation of treatment-related effects in this study. Statistical comparison of the control and high exposure groups showed no significant difference from control in the incidences of the 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 exposure group (n=12 rats). Although not statistically significant, lymphoid interstitial infiltrate in the ventral prostate was observed more frequently in the 80 ppm treatment group (5/12) when compared to control animals (3/12).

Similarly, 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 exposure groups, rats that were not reproductively successful, and those that had gross observations made in the reproductive tract (see Table C3 for details). The incidences of histologic diagnoses in the female reproductive tracts are summarized in Text Table P. There was a background incidence of some histologic reproductive diagnoses in F0 female rats; however, the severity and/or incidences of these findings in control rats were not considered to be sufficient to confound interpretation of treatment-related effects in this study. Statistical comparison of the control and high exposure groups showed no significant difference from control in the incidences of 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 exposure groups had ovarian cysts. Ovaries of one rat in the 80 ppm group 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 exposure group, also had squamous metaplasia of the endometrium localized to the uterine body.

Text Table O
Incidence¹ of Histologically Confirmed Diagnoses in F0 Male Rats Exposed to H₂S

Reproductive Organ and Final Diagnoses	Exposure Group			
	0 ppm	10 ppm ²	30 ppm ²	80 ppm
Testis				
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
Epididymis				
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
Seminal vesicles and coagulating glands				
any diagnoses	0/12	0/2	0/3	0/12
Prostate-dorsolateral				
chronic inflammation	6/12	1/2	0/3	2/12
Prostate-ventral				
lymphoid infiltrate	3/12	2/2	0/3	5/12

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

Text Table P
Incidence¹ of Histologically Confirmed Diagnoses in F0 Female Rats Exposed to H₂S

Reproductive Organ and Final Diagnoses	0 ppm	Exposure Group		
		10 ppm ²	30 ppm ²	80 ppm
Vagina				
cyst , not otherwise specified	1/11 ³	0/2	0/3	0/12
Wolffian duct remnant, periurethral	0/11	0/2	1/3	0/12
Cervix				
luminal dilation secondary to vaginal outflow obstruction by cyst	1/11 ³	0/2	0/2	0/11 ⁴
Uterus⁵				
luminal dilation secondary to vaginal outflow obstruction by cyst (w/ mucometra ⁶ and endometrial atrophy)	1/12	0/2	0/3	0/12
squamous metaplasia	0/12	0/2	1/3	1/12
Oviducts				
any diagnoses	0/12	0/2	0/3	0/12
Ovaries				
cysts , not otherwise specified	0/12	1/2	1/3	0/12
luteal depletion	0/12	0/2	0/3	1/12

1 = # of rats with gross observation/# of rats grossly examined in exposure group.
 2 = 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 exposure groups were not statistically compared with the control and high exposure groups due to the small number of animals examined histologically in these exposure groups.
 3 = histologic examination was not performed on the cervix and vagina of rat #52 in the 0 ppm exposure group.
 4 = histologic examination was not performed on the cervix of rat #89 in the 80 ppm group.
 5 = Does not include the incidental finding of uterine luminal dilation consistent with proestrus or estrus.
 6 = uterine horn distended with tan opaque fluid

NECROPSY AND HISTOLOGY – F1 RATS

Terminal Body and Organ Weights

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

Gross Observations

A wide variety of gross observations in the pnd 63 ± 3 F1 rats (Text Table R, Table C6) occurred at very low incidences that were not treatment-related; therefore, microscopic examination was not performed on tissues from these animals.

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 S (see also Tables 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 exposure 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 six levels from pups in the control and high exposure groups failed to demonstrate any relevant histologic abnormalities.

Text Table Q
Absolute Mean (\pm SD) Body and Organ Weights (g) for Postnatal Day 63 \pm 3 Male and Female
F1 Rats Exposed to H₂S

<u>Males</u>	Exposure Group			
	0 ppm	10 ppm	30 ppm	80 ppm
Terminal Body Weight	410.6 \pm 39.6 ^a	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 ^b	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

^a n=17, Rat # 524 was excluded because of inaccurate terminal body weight.

^b n = 21, Rat # 674 was excluded because lungs were inflated with formalin prior to weighing.

<u>Females</u>	Exposure Group			
	0 ppm	10 ppm	30 ppm	80 ppm
Terminal Body Weight	239.3 \pm 23.8	248.34 \pm 27.7	253.5 \pm 33.6	235.4 \pm 12.2
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	10.87 \pm 1.32	11.10 \pm 1.31	11.38 \pm 1.84	10.65 \pm 1.03
Lungs	1.14 \pm 0.26 ^c	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	19	16

^c n = 17, Rat # 558 was excluded because lungs were inflated with formalin prior to weighing.

* p < 0.05

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Text Table R

Incidence¹ of Gross Lesions in Postnatal Day 63 ± 3 Male and Female F1 Rats Exposed to H₂S

Organ	Gross Observation ²	Exposure Group			
		0 ppm	10 ppm	30 ppm	80 ppm
Heart	enlarged rounded apex	1/36	0/44	0/35	1/29
	right ventricular 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 (dilated renal pelvis/es)	0/36	1/44	3/35	2/29
Liver	enlarged, firm, nodular	0/36	0/44	0/35	1/29
Seminal Vesicles/ Coagulating Glands (male)	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 ³	any diagnoses	0/36	0/44	0/35	0/29

¹ = # of rats with gross observation/# of rats grossly examined in exposure group.

² = does not include the incidental findings of red foci in kidneys associated with CO₂ euthanasia and uterine swelling and intraluminal fluid and parovarian fluid in cycling female rat pups.

³ = 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.

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

Postnatal Day 23 \pm 2	Exposure Group			
	0 ppm	10 ppm	30 ppm	80 ppm
Males				
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
Females				
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
Postnatal Day 61 \pm 2				
	Exposure Group			
	0 ppm	10 ppm	30 ppm	80 ppm
Males				
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
Females				
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 < 0.05

Section 4

DISCUSSION/CONCLUSIONS

The purpose of this study was to evaluate whether repeated 6-hour daily exposure of male and female CD¹ rats to hydrogen sulfide (H₂S) atmospheres at 0, 10, 30, or 80 ppm would result in reproductive toxicity, developmental toxicity, or developmental neurotoxicity. 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. Several studies investigating the developmental neurotoxicity of H₂S have been published (Skrajny et al., 1992; Hayden et al., 1990). Both dams and neonatal Sprague-Dawley pups were exposed 7 hours per day to either 0, 20, 50, or 75 ppm H₂S during the neonatal period from birth to weaning. The only outward clinical effects noted in these studies were delayed parturition in dams and decreased time in the pup's ear detachment and hair growth. No significant changes in pup weight gain occurred following neonatal H₂S exposure. Hannah and Roth (1991) have reported that perinatal exposure of rats to 20 or 50 ppm H₂S for 7 hours per day from gestation day (gd) 5 through postnatal day (pnd) 21 results in longer dendritic branches with reduced arborization of the cerebellar Purkinje cells. Exposure of neonatal rats to H₂S has also been demonstrated to both increase and decrease concentrations of critical brain neurotransmitters. Exposure of neonatal rats to H₂S (75 ppm, 7 hours/day) beginning on pnd 5 and ending on pnd 21 results in decreased cerebellar aspartate and glutamate levels, depressed cerebellar and cerebral gamma aminobutyric acid (GABA) levels, and increases in cerebellar serotonin and norepinephrine concentrations (Hannah and Roth, 1991; Skrajny et al., 1992). Although these results suggest that perinatal H₂S exposure can affect brain neurochemistry, few studies have focused on the reproductive or neurobehavioral toxicity of H₂S exposure.

In the present study, F0 animals were exposed to H₂S 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 through gd 19) and lactation (pnd 5 through pnd 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 cylinders. The actual chamber concentrations (grand means ± SD) were 10.0 ± 0.6, 30.1 ± 0.8, and 79.5 ± 2.4 ppm H₂S in air

for the target concentrations 10, 30 and 80 ppm, respectively. No H₂S was detected in the control chamber. The exposure concentrations used in our study are relevant for assessing the hazard associated with environmental (ambient) and occupational exposures to H₂S. The current threshold limit value-time weighted average (TLV-TWA) for H₂S 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 H₂S 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 H₂S 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 H₂S also demonstrated a small (approximately 5-6%) decrease in body weight following H₂S exposure. For example, F0 male rat body weights (mean \pm SD) 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 H₂S exposure. 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 exposure groups.

Nasal pathology was determined in F0 male rats following inhalation exposure to H₂S for six hours per day seven days per week for at least 70 consecutive days. Subchronic exposure of male F0 rats to 80 ppm H₂S 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 and medial regions 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 to the posterior ethmoid region. The severity and extent of the olfactory mucosal lesion was decreased in rats exposed to 30 ppm H₂S, and difficult to distinguish from control at 10 ppm. Treatment-related effects were not observed in either the squamous or respiratory epithelium. Treatment-related nasal lesions were also absent in control animals. Olfactory and respiratory mucosal necrosis have been reported by Lopez and coworkers (1988)

in male F344 rats following short-term (4 hours) exposure to 400 ppm H₂S. 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 H₂S 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 T). 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 Saillenfait and coworkers (1989). These investigators exposed pregnant Sprague Dawley rats to 0, 50, 100, or 150 ppm H₂S 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 H₂S 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 H₂S for six hours per day during gestation days 6 through 20 (Saillenfait et al., 1989).

Text Table T
Reproductive Toxicity Indices

Endpoint	Treatment Group	
	H ₂ S Control	80 ppm H ₂ S
Females that mated (%)	92 (11/12)	83 (10/12)
Fertility index (%)	82 (9/11)	80 (8/10)
Gestation length (days) ^a	21.9 ± 0.6	21.8 ± 0.7
Live pups/litter ^a	16.2 ± 1.6	14.0 ± 4.6
% Male pups per litter ^a	45.6 ± 14.1	39.8 ± 18.2
Implantation sites/female ^a	16.7 ± 1.8	14.5 ± 4.3
Postimplantation loss (%) ^a	2.5 ± 3.9	7.0 ± 9.7

^aMean ± SD

Male F0 rats in our study did not demonstrate reproductive toxicity following H₂S 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. A large percentage of abnormal sperm was observed in two F0 rats, #31 (30 ppm) and #46 (80 ppm). Abnormal sperm accounted for 29 and 73% of all sperm analyzed from rats #31 and #46, respectively. No statistically significant increase in either gross or histopathological lesion incidence were observed following H₂S exposure of F0 male rats. Although not statistically significant, there was a higher incidence of testicular tubular degeneration in male F0 rats from the 80 ppm treatment group (42%) when compared to control animals (17%).

A low incidence of malformations was observed only in newborn H₂S-exposed animals. Structural malformations observed in H₂S-exposed animals included kinked tail, agenesis of the tail, anophthalmia, frontal bone holes, small lower jaw, and skin lesions that included webbing of the forelimb skin. Histologic examination of the skin lesion showed a localized to regionally extensive non-inflammatory separation of the epidermis from the underlying dermis. This histological finding resembles epidermolysis bullosa, a family of inherited or acquired mechanobullous diseases in which minor trauma causes the formation of cutaneous blisters. This finding has not been previously reported in the rat. Each of these malformations occurs with a low incidence in control animals, thus the presence of multiple malformations is suggestive of a chemical-induced effect. These 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. Andrew and coworkers (1980) investigated the effect of H₂S 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 H₂S for three hours per day throughout gestation (gd 1 to 18). No evidence of maternal toxicity or embryotoxicity was observed by Andrew and coworkers. The low incidence of developmental effects, plus the lack of any dose-response relationship, makes it difficult to draw a firm conclusion on whether H₂S may have weak developmental or teratogenic properties.

Another goal of the present study was to determine whether repeated exposure to H₂S 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 H₂S 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 H₂S inhalation. Thus, the decision was made to use a whole-body exposure cylinder to simultaneously expose F1 offspring and their dams to H₂S 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 H₂S 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 H₂S 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 H₂S 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 H₂S exposure (Hannah and Roth, 1991; Kombian et al., 1988; Skrajny et al., 1996; Werenyia et al., 1989). Few studies, however, have focused on the behavioral effects of H₂S exposure. In the present study, exposure to H₂S did not affect pup motor activity, passive avoidance behavior, or acoustic startle reflex behavior. 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 H₂S is not a behavioral developmental neurotoxicant.

In addition, 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 H₂S 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 H₂S exposure alters dendrite arborization of the rat cerebellar Purkinje cell. These investigators exposed pregnant Sprague-Dawley rats to 20 or 50 ppm H₂S 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 H₂S exposure is unlikely to result in significant reproductive toxicity or developmental neurotoxicity in rats following exposures at which nasal pathology occurs.

REFERENCES

- Andrew, F.D., R.A. Renne and W.C. Cannon. 1980. Reproductive toxicity testing for effects of H₂S in rats. In: Pacific Northwest Laboratory annual report for 1979 to the DOE Assistant Secretary for Environment: part 1, biomedical sciences. Richland WA. Report no. PNL 3300 PTI (Cited in USEPA, Health assessment document for hydrogen sulfide, Office of Research and Development, Research Triangle Park, 1993).
- Hannah, R.S. and S.H. Roth. 1991. Chronic exposure to low concentrations of hydrogen sulfide produces abnormal growth in developing cerebellar Purkinje cells. *Neuroscience Letters*. 122:225-228.
- Hayden, L.J., H. Goeden and S.H. Roth. 1990. Growth and development in the rat during sub-chronic exposure to low levels of hydrogen sulfide. *Toxicology and Industrial Health*. 6:389-401.
- Kimmel, C.A. and E.Z. Francis. 1990. Proceedings of a workshop on the acceptability and interpretation of dermal developmental toxicity studies. *Fundamental and Applied Toxicology*. 14:386-398.
- Kombian, S.B., M.W. Warena, F.G. Mele and R.J. Reiffenstein. 1988. Effects of acute intoxication with hydrogen sulfide on central amino acid transmitter systems. *Neurotoxicology*. 9(4):587-596.
- Lopez, A., M. Prior, S. Yong, L. Lillie and M. Lefebvre. 1988. Nasal lesions in rats exposed to hydrogen sulfide for four hours. *American Journal of Veterinary Research*. 49:1107-1111.
- Mery, S., E.A. Gross, D.R. Joyner, M. Godo and K.T. Morgan. 1994. Nasal diagrams: a tool for recording the distribution of nasal lesions in rats and mice. *Toxicologic Pathology*. 22:353-372.
- National Research Council. 1996. *Guide for the Care and Use of Laboratory Animals*. National Academic Press, Washington, D.C.
- Saillenfait, A.M., P. Bonnet and J. de Ceaurriz. 1989. Effects of inhalation exposure to carbon disulfide and its combination with hydrogen sulfide on embryonal and fetal development in rats. *Toxicology Letters*. 48:57-66.
- Skrajny, B., R.S. Hannah and S.H. Roth. 1992. Low concentrations of hydrogen sulphide alter monoamine levels in the developing rat central nervous system. *Canadian Journal of Physiology and Pharmacology*. 70:1515-1518.
- Skrajny, B., R.J. Reiffenstein, R.S. Sainsbury and S.H. Roth. 1996. Effects of repeated exposures of hydrogen sulphide on rat hippocampal EEG. *Toxicology Letters*. 84:43-53.
- US EPA. 1991. Pesticide assessment guidelines - Subdivision F Hazard Evaluation: Human and domestic animals, Addendum 10 - Neurotoxicity Series 81, 82, and 83. US EPA, Washington, D.C.
- Vitarella, D., B.A. Wong, R.A. James, K.L. Miller, M.F. Struve and D.C. Dornan. (in press). Development of an inhalation system for the conduct of developmental neurotoxicity studies. *Inhalation Toxicology*.

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CIIT PROTOCOL 97042

Warencya, M.W., K.A. Smith, C.S. Blashko, S.B. Kombian and R.J. Reiffenstein. 1989.
Monamine oxidase inhibition as a sequel of hydrogen sulfide intoxication: increases in brain
catecholamine and 5-hydroxytryptamine levels. *Archives of Toxicology*. 63:131-136.

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LABORATORY TITLE PAGE

**FINAL REPORT
VOLUME III OF III**

TITLE: A Vapor Inhalation Reproductive Toxicity,
Developmental Toxicity, and Developmental
Neurotoxicity Study in Crl:CD[®](SD)BR Rats
to Hydrogen Sulfide

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CIIT PROTOCOL NO.: 97042

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Appendix C

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APPENDIX C: ANATOMIC PATHOLOGY EVALUATIONS

Protocol Title

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

Study Protocol

CIIT: 97042

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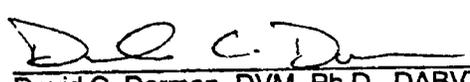

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Appendix C

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INTRODUCTION

The purpose of this study was to evaluate whether repeated 6-hour daily exposures of male and female CD^s rats ($n = 12$ rats/sex/concentration) to hydrogen sulfide (H₂S) atmospheres of 0, 10, 30, or 80 ppm would result in reproductive toxicity, developmental toxicity, or developmental neurotoxicity. Exposures were conducted during the 2-week prebreed, 2-week mating (evidence of copulation = gestation day 0 = gd 0), and 3-week gestational (gd 0 until gd 19) periods. Litters were culled to 4 pups/sex/litter on postnatal day (pnd) 4. Exposure of F0 dams and their pups resumed between pnd 5-18. All F0 male rats were exposed to H₂S for at least 70 consecutive days. After the end of exposure, F0 rats were necropsied, which included a complete gross examination, terminal body and select organ weights, histologic examination of reproductive organs, counting of implantation sites in females, and andrological assessments of males. Noses from F0 male rats were also examined histologically. On pnd 23 ± 2 ($n=72$) and pnd 61 ± 2 ($n = 73$), F1 rats were weighed and euthanized by perfusion for neuropathology, which included gross examination of the nervous system, brain weight and measurements, and histological examination of the brain. On pnd 63 ± 3 ($n=144$), the remaining F1 rats had a complete gross necropsy performed, which included terminal body and select organ weights.

SUMMARY

In F0 rats, there were no significant exposure-related effects found on terminal body or organ weights, gross postmortem observations, or reproductive histologic findings. The only lesion with a statistically significant increase in incidence relative to control was sensory neuron loss and basal cell hyperplasia in the olfactory mucosa of male F0 rat noses, which was observed with subchronic exposure to 30 and 80 ppm H₂S. The olfactory lesion was localized to the dorsal medial meatus and the dorsal and medial regions of the ethmoid recess. Exposure-related lesions were not found in the squamous, transitional, or respiratory mucosae of the nasal cavity. The F1 pnd 23 ± 2 and pnd 61 ± 2 rats, which underwent a postmortem neuropathological examination, had no significant exposure-related alterations in terminal body weight, brain weight, brain size, or the gross appearance of the central nervous system. Histopathologic abnormalities were not observed in H&E-stained sections of brain from F1 rats exposed to 80 ppm of H₂S. The remaining F1 rats (pnd 63 ± 3), which underwent a complete necropsy, had no significant exposure-related changes in terminal body weights, organ weights, or gross observations. The

histologic appearance of a skin lesion found in a pnd 0 F1 rat exposed to 30 ppm H₂S was consistent with epidermolysis bullosa, reports of which could not be found in available literature.

MATERIALS AND METHODS

F0 Rat Necropsy Procedures

At the end of the exposure regimen, rats of the parental generation were weighed, euthanized with CO₂, abdominally exsanguinated, and had a complete necropsy performed with special emphasis on the reproductive and associated organs. The non-impregnated F0 females (*n* = 11) and F0 males (*n* = 48) were necropsied the day after their last day of exposure. The postpartum F0 females (*n* = 37) were necropsied the day of or the day after their pups were weaned (pnd 21). The order of necropsy was randomized across exposure groups in advance using ear tags for animal identification. Implantation sites were counted in fresh and/or formalin-fixed, potassium ferricyanide-stained uteri from F0 female rats. The right testis and epididymis from F0 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

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The following tissues and forms of identification were collected from F0 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
- nose

The following tissues were collected from F0 male rats and preserved in Bouin's fixative, gross trimmed, rinsed in 50% ethanol, and stored in 70% ethanol :

- testis, left
- epididymis, left, caput and corpus
- epididymis, left, cauda

The following tissues were collected from F0 male rats, weighed, frozen in liquid nitrogen, and stored at -70°C for possible future sulfide analysis: brain, heart, liver, kidney, and lung.

Noses were also collected from F0 male rats. The noses were flushed retrograde and immersion fixed with 10% neutral buffered formalin and then decalcified in 20% formic acid with an ion exchange resin.

F0 Rat Histopathologic Examination of Reproductive Tracts

Histopathologic examination was performed on reproductive and accessory sex organs from F0 rats in the control and high exposure groups and from rats in the low and middle exposure groups, which were not reproductively successful or had gross findings in any of these organs. Except for F0 male rat nose, histopathologic examination was not performed on other tissues, because gross observations made in other tissues during necropsy were determined not to be exposure-related. Reproductive tissues to be examined microscopically were processed and sectioned at Experimental Pathology Laboratories (EPL; 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

Females

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

F0 Male Rat Histopathologic Examination of Noses

Histopathologic examination was performed on noses from F0 male rats in the 0, 10, 30, and 80 ppm H₂S exposure groups. The noses were trimmed transversely perpendicular to the bridge of the nose at six levels, processed routinely, sectioned at 4-5 µm thickness, and stained with H&E by the CIIT Histology Service Unit. The tissue blocks for nose levels 2-6 resulted from trimming at the following respective locations: just rostral to the incisor tooth, approximately 2 mm posterior to the free tips of the incisor teeth, through the incisive papilla, through the middle of the second

palatine ridge (which is just anterior to the molar teeth), and through the palatal ridge just caudal to the molar teeth. Nose blocks 2-6 were sectioned from their rostral face. The tissue block for Nose level 1 was the tip of the nose, which was sectioned from its caudal face.

F1 Rat Neuroperfusion Necropsy Procedure

On pnd 23 ± 2 ($n=72$) and pnd 61 ± 2 ($n=73$), F1 rats were weighed, heparinized, 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 exposure 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 Histopathologic Examination of Brain

Histopathologic examination was performed on perfused brains from pnd 23 ± 2 and pnd 61 ± 2 F1 rats in control and high exposure groups. The brains were trimmed transversely at six levels (forebrain, caudate nucleus, center of the cerebrum, center of the midbrain, cerebellum and pons, and medulla oblongata), processed routinely, sectioned at a 4-5 μm thickness, and stained with H&E by the CIIT Histology Service Unit. Histopathologic examination was not performed on brains from intermediate exposure groups, because histologic lesions were not found in the control and high exposure group brains examined.

F1 Rat Complete Necropsy and Histopathologic Procedures

On pnd 63 ± 3 ($n=144$), rat pups were weighed, euthanized with CO_2 , abdominally exsanguinated, and had a complete necropsy performed. A complete necropsy included gross examination of the organs/tissues listed above for the parental generation. The order of necropsy was randomized across exposure groups in advance using ear tags for animal identification.

The following organs from pnd 63 ± 3 F1 rats 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 pnd 63 ± 3 F1 rats and preserved in 10% neutral buffered formalin as appropriate:

- any gross lesion/s
- ear tag

Histopathologic examination was not performed on these tissues, because the incidence of gross observations made at necropsy were determined not to be exposure-related.

F1 Pnd 0 Rat (Litter 76) with Skin Lesion Necropsy Procedure

The pup was fixed intact by immersion in 10% buffered neutral formalin. The formalin-fixed carcass was weighed, measured, and opened by a single ventral midline incision so that its internal viscera could be examined grossly. Full thickness skin sections were trimmed from five sites on the body's surface and processed for sectioning and staining with H&E by CIIT's Histologic Service Unit. Special stains, including Periodic acid-Schiff, Masson's Trichrome, and Phosphotungstic Acid Hematoxylin methods, were performed on skin sections by EPL.

Unscheduled Deaths

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

Statistical Methods

The Fisher's exact test (pair-wise) was used to determine the significance of differences between the incidences of gross and microscopic lesions found in control versus exposed groups ($p \leq 0.05$). The Bonferroni correction ($p \leq 0.0167$) for multiple comparisons was used in addition to the Fisher's exact test in interpretation of the nasal histopathologic findings.

RESULTS

F0 Female Rats

Terminal body and organ weights for F0 female rats are summarized in Tables B5, B69, and Text Table L. The F0 female rats had no H₂S exposure-related alterations 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 exposure group (Table B72). The number of implantation sites per rat uterus was not significantly altered by H₂S exposure (Table B62). All non-impregnated F0 female rats had no detectable implantation sites at necropsy. All postpartum F0 female rats had multiple implantation sites present.

Gross observations made during necropsy of the F0 female rats are summarized in Table C1. The gross observations made had very low incidences that were not exposure-related (Table C2). 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 exposure groups and of female F0 rats in the low and middle exposure groups, which were not reproductively successful or had gross findings in the reproductive tract. The histologic diagnoses of the F0 female reproductive tracts and their incidences are summarized in Tables C3 and C4, respectively. Statistical comparison of the control and high exposure groups showed no significant difference between the incidences of the gross observations and histologic diagnoses found.

However, despite the absence of any clearly exposure-related pathologic changes, it should be noted that there were a few histologic diagnoses that occurred in H₂S-exposed but not

unexposed rats. One rat each in both the 10 ppm and the 30 ppm exposure groups had ovarian cysts. Ovaries of one rat exposed to 80 ppm H₂S 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 exposure 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 and Text Table K. The F0 male rats had no H₂S exposure-related alterations in terminal body weights. The only statistically significant difference from control in organ weights was a decrease in the absolute and relative weights of the adrenal glands in the 10 and 80 ppm exposure groups (Table B 72).

Gross necropsy observations of the F0 male rats and their incidences are summarized in Table C5 and C6, respectively. Gross observations made during complete necropsies of the F0 rats had very low incidences that were not exposure-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 exposure groups and of F0 male rats in the low and middle exposure groups, which were not reproductively successful or had gross observations made in the reproductive tract and/or related organs. Histologic diagnoses in the F0 male reproductive tracts and their incidences are summarized in Tables C7 and C8, respectively. Statistical comparison of the control and high exposure groups showed no significant difference between the incidences of the gross observations made or histologic diagnoses found.

However, despite the absence of any clearly exposure-related pathologic changes, there were a few histologic diagnoses with a higher incidence in exposed groups than control. Most of these diagnoses were 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 exposure group. Also, there was a slightly higher incidence in the high exposure group than control of lymphoid interstitial infiltrate in the ventral prostate.

F0 Male Rat Noses

The major exposure-related histologic diagnoses in the noses of F0 male H₂S-exposed rats were bilaterally symmetrical olfactory neuronal loss and basal cell hyperplasia in the olfactory mucosa lining the dorsal medial meatus and the dorsal and medial regions of the ethmoid recess (Tables C9 and C10). In general, the lesion was detectable from the most rostral portion of the olfactory mucosa (level 3) to the caudal regions of the ethmoturbinates (level 6). The caudal extent of the lesion's distribution and the total surface area of the olfactory mucosa affected increased with exposure concentration.

The diagnosis of olfactory neuronal loss had an increased incidence and severity relative to control in the 30 and 80 ppm exposure groups at nose levels 3, 4, and 5 and in the 80 ppm exposure group only at nose level 6. In the 30 ppm exposure group, the olfactory neuronal loss was typically mild to moderate; in the 80 ppm exposure group, the severity was typically moderate to marked. A statistically significant increase in incidence of olfactory neuronal loss relative to control was only demonstrable at nose level 4 for the 80 ppm exposure group.

There is an increased incidence and severity relative to control of basal cell hyperplasia in the olfactory mucosa in the 10, 30 and 80 ppm exposure groups at nose level 4, in the 30 and 80 ppm exposure groups at nose level 5, and in the 80 ppm exposure group only at nose level 6. Typical severities at all affected nose levels were mild to moderate. A statistically significant increase in incidence of basal cell hyperplasia relative to control was demonstrable at nose level 4 for the 30 and 80 ppm exposure groups and at nose level 5 for the 80 ppm concentration.

Background incidences of mild olfactory neuronal loss at nose levels 3-6 and mild to moderate basal cell hyperplasia at nose level 3 were consistently seen across all exposure groups. The cause of these background lesions is not known, however, fixation artifact, normal histologic variation, exposure to soiled cage contaminants (Bolon *et al.*, 1991), or spontaneous disease may have been contributory factors.

Lesions found in nasal tissues other than olfactory mucosa included goblet cell hyperplasia/epithelial hyperplasia of respiratory epithelium, secretory metaplasia/hyperplasia of

transitional epithelium, periodontitis, periodontal bone and tooth fractures, nasolacrimal duct inflammation, and localized areas of chronic inflammation with squamous metaplasia/hyperplasia in the anterior nose. Their incidences were not exposure-related.

F1 Neuroperfused Rats

Terminal body weight and brain weight, length, and width of pnd 23 ± 2 and pnd 61 ± 2 F1 rats are summarized in Tables B15, B16, B17, B73, and B74 and Text Tables Q and S. The only statistically significant difference from control in any of these parameters is an increase in brain length in male pnd 23 ± 2 F1 rats in the 10 ppm exposure 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 six levels from pups in the control and high exposure groups from both age groups failed to demonstrate any relevant histologic abnormalities (Tables C11 and C12).

F1 Complete Necropsy Rats

Terminal body and organ weights of F1 pnd 63 ± 3 rats that underwent a complete necropsy are summarized in Tables B15 and B75. No statistically significant differences from control were observed in these values. A wide variety of gross observations (Table C13 and C14) occurred at very low incidences that were not exposure-related. Microscopic examination was not performed on tissues from these animals due to a lack of a statistically significant exposure-related increase relative to control in the incidences of gross changes observed.

F1 Pnd 0 Rat (Litter 76) with Skin Lesion

Five transverse H&E-stained sections of skin from a F1 pnd 0 rat (Litter 76, 30 ppm group) with very thin, fragile, wrinkled, and loosely adherent skin were examined (Figure 1). The predominant histologic finding was a localized to regionally-extensive, non-inflammatory separation of the epidermis from the underlying dermis. This histologic description is most consistent with epidermolysis bullosa, a family of inherited or acquired mechanobullous diseases in which minor trauma causes the formation of cutaneous blisters. Gross examination of the formalin-fixed pup showed no other distinct physical abnormalities other than a mildly decreased body size.

DISCUSSION/CONCLUSION

As assessed by postmortem examination, the effects of H₂S exposure on the F0 female rats were few and of indeterminate relevance. Terminal body weights and the incidences of gross observations and reproductive histologic diagnoses were not significantly altered relative to control by H₂S exposure. The only statistically significant exposure-related change was a decrease in the relative weight of the ovaries in the 10 ppm exposure group. This decrease was small and was not associated with an increased incidence of reproductive failure or associated histologic abnormalities in this exposure group.

The few histologic diagnoses that occurred exclusively in H₂S-exposed F0 female rats were unlikely of any toxicological significance. Their functional relevance was difficult to interpret given that both rats in the mating pair were exposed to H₂S and neither rat had an established history of successful reproductive performance prior to participation in the study. The follicular cysts found in F0 female rats (#71 and #81) in intermediate exposure groups were small to moderate in size, not destructive of otherwise normal appearing ovaries, and appeared unlikely to interrupt normal ovarian function. The luteal depletion found in one F0 female rat (#94) in the high exposure group suggested a disruption of normal estrous cyclicity and was associated with reproductive failure in conjunction with uterine squamous metaplasia. Uterine squamous metaplasia, which was reported in one rat each in the 30 and 80 ppm exposure groups (#82 and #94, respectively), was associated with reproductive failure in both cases. However, the functional relevance of the lesion could not 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 H₂S exposure on the F0 male rats were also of indeterminate relevance, with the exception of a distinctly dose-related pathologic response in the olfactory mucosa of the nasal cavity. Terminal body weights and the incidences of gross observations and reproductive histologic diagnoses were not significantly altered relative to control by H₂S exposure. The only statistically significant exposure-related change was a decrease in the relative weight of the adrenal glands in the 10 and 80 ppm exposure groups. This

decrease was small and was not associated with a significantly increased incidence of clinical or other pathological abnormalities in these exposure groups.

The few histologic diagnoses that occurred more frequently in H₂S-exposed F0 male rats were unlikely of any toxicological significance. Their functional relevance again was difficult to interpret given that both rats in the mating pair were H₂S-exposed and had not proven their reproductive capabilities prior to being entered into the study. The higher incidences of seminiferous tubular degeneration and related changes in the testes and epididymides of exposed male rats were not associated with a statistically significant change in reproductive indices in H₂S-exposed groups (Text Table E). Seminiferous tubular degeneration of minimal to moderate severity was not consistently associated with reproductive failure. However, the one case (rat # 42) of severe seminiferous tubular degeneration with aspermia in the high exposure group, assuming that the lesion was bilateral, could 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. Seminiferous tubular degeneration is reported to occur in response to exposure to toxins, but is also a spontaneous aging change in older rats (Boorman *et al.*, 1990). The functional ramifications of the severe necrosis of the cauda of the epididymis in one high exposure group rat (#46) could not be determined due to the significant reproductive pathology in the female rat (#94) with which he was mated.

There was a background incidence of some histologic reproductive diagnoses in F0 male and female rats, however, the severity and/or incidences of these findings in control rats were not considered to be sufficient to confound interpretation of exposure-related effects in this study. Similarly, common tissue artifacts found to varying extents in histologic sections examined for all phases of the study were also considered not to be sufficient to prevent recognition of exposure-related effects.

The olfactory neuronal cell loss and basal cell hyperplasia found in the olfactory mucosa of the dorsal medial meatus and dorsal and medial regions of the ethmoid recess in the F0 male rats were clearly dose-related responses at the 30 and 80 ppm exposure concentrations. The appearance and distribution of the olfactory lesion were similar to those described with exposure to other irritant gases, including dimethylamine and chlorine (Morgan, 1991). Olfactory nasal lesions associated with subchronic inhalation exposure to H₂S have not been previously described in

the literature. However, Lopez *et al.* (1988) describes acute olfactory mucosal necrosis in the dorsal medial meatus and adjacent ethmoid conchae in the middle portion of the nasal cavity of rats with acute inhalation exposure to H₂S. The acute olfactory lesion observed by Lopez *et al.* was accompanied by respiratory mucosal necrosis and had a high incidence with exposure to 400 ppm but not 200 ppm or 10 ppm H₂S.

Neuropathological examination of pnd 23 ± 2 and pnd 61 ± 2 perused F1 rats did not detect any significant H₂S-exposure related alterations in terminal body weight, brain weight, brain width, gross appearance of the nervous system, or histologic appearance of H&E-stained sections of the brain. The only statistically significant exposure-related change was an increase in brain length of the male F1 pnd 23 ± 2 rats in the 10 ppm exposure group, which was not associated with significant changes in behavior or other pathological parameters and is a finding of indeterminate relevance.

Significant H₂S-exposure related alterations in development in general were not found during complete gross examination of the pnd 63 ± 3 F1 rats, with the exception of a statistically significant increase in the weight of the ovary with oviduct in the 30 ppm exposure group. This increase was small, not associated with clinical abnormalities, and was also a finding of indeterminate relevance.

Previous reports on epidermolysis bullosa in rats could not be found. In humans, this disease process is most commonly an inherited defect in a protein integral to the normal adherence of the epidermis to the underlying dermis, although certain subtypes of this disease may also be acquired later in life (Smith *et al.*, 1996). The occurrence of an unreported disease in H₂S-exposed rat pups is considered to be of possible toxicological significance given its extreme rarity.

REFERENCES

- Bolon B, Bonnefoi MS, Roberts KC, Marshall MW, and Morgan KT. Toxic interactions in the rat nose: Pollutants from soiled bedding and methyl bromide. *Toxicol. Pathol.* 19: 571-579, 1991.
- Boorman GA, Chapin RE, Mitsumori, K. Testis and epididymis. In: *Pathology of the Fischer Rat*, Boorman GA, Eustis SL, Eiwel MR, Montgomery CA, MacKenzie WF, eds., San Diego, Academic Press, Inc., 1990, p. 409.
- Gopinath C. Susceptibility of the uterus to toxic substances. In: *Pathobiology of the Aging Rat*, Vol. 1., Mohr U, Dungworth DL, Capen CC, eds., Washington, DC, ILSI Press, 1992, p. 390.
- Lopez A, Prior M, Yong S, Lippert L, and Lefebvre M. Nasal lesions in rats exposed to hydrogen sulfide for four hours. *Am. Res.* 49(7): 1107-1111, 1988.
- Morgan KT. Approaches to the identification and recording of nasal lesions in toxicology studies. *Toxicol. Pathol.* 19(4): 337-351, 1991.
- Smith FJ, Eady RA, Leigh IM, McMillan JR, Rugg EL, Kelsell DP, Bryant SP, Spurr NK, Geddes JF, Kirtschig G, Milana G, de Bono AG, Owaribe K, Wiche G, Pulkkinen L, Uitto J, McLean WH, Lane EB. Plectin deficiency results in muscular dystrophy with epidermolysis bullosa. *Nature Genetics* 13(4): 450-457, 1996.

EPL[®]

EXPERIMENTAL PATHOLOGY LABORATORIES, INC.

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QUALITY ASSURANCE FINAL CERTIFICATION

Study Title: A Vapor Inhalation Reproductive Toxicity, Developmental Toxicity and Developmental Neurotoxicity Study in Cr:CD₀₁(SD)BR Rats Exposed to Hydrogen Sulfide

Client Study: 97042

EPL Project Coordinator: Not Assigned

EPL Project Number: 304-207 #746 & #747

EPL Pathologist: N/A

The following audits of facility compliance were conducted by the Quality Assurance Unit of Experimental Pathology Laboratories, Inc. The results were documented and findings reported to the Project Director and Management are indicated below.

Area Inspected	Dates	
	Inspection	Reporting
Phase/Data Review	November 24, 1997	December 22, 1997

Date reported to Study Director January 28, 1998

Date of last quarterly facility inspection July, 1998

Jane T. Hollingsworth
JANE T. HOLLINGSWORTH

September 9, 1998
Date

EPL Quality Assurance Unit

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Table C1. Gross Necropsy Observations in F0 Female Rats

Flat #1	Exposure Concentrations (ppm)	Uterus	Cervix/Vagina	Kidneys	Lungs	Mammary Gland	Eye	Other Tissues2
51	0	intraluminal fluid; mildly swollen3	NL	NL	NL	NL	NL	NL
53	0	NL4	NL	red foci5,6	NL	NL	NL	NL
54	0	NL	NL	NL	red-black foci6	NL	NL	NL
59	0	mucometra7	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
73	30	NL	NL	red foci	NL	NL	NL	NL
75	30	NL	NL	red foci	NL	NL	NL	NL
76	30	NL	NL	red foci	NL	NL	NL	NL
77	30	NL	NL	red foci	NL	NL	NL	NL
78	30	NL	NL	red foci	NL	NL	NL	NL
79	30	NL	NL	red foci	NL	NL	NL	NL
82	30	intraluminal fluid; mildly swollen	NL	red foci	NL	NL	NL	NL
83	30	NL	NL	red foci	NL	NL	NL	NL
84	80	intraluminal fluid	NL	NL	NL	NL	NL	NL

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Table C1. Gross Necropsy Observations in F0 Female Rats (continued)

Rat #1	Exposure Concentrations (ppm)	Uterus	Cervix/Vagina	Kidneys	Lungs	Mammary Gland	Eye	Other Tissues?
88	80	intraluminal fluid	NL	red foci	NL	NL	NL	NL
93	80	NL	NL	red foci	NL	NL	NL	NL
94	80	NL	NL	red foci	NL	NL	NL	NL

1 = list does not include F0 female rats which had no gross observations at necropsy, including: 49, 50, 52, 55, 56, 57, 58, 59, 60, 64, 66, 67, 68, 70, 71, 72, 74, 80, 81, 85, 86, 87, 89, 90, 91, 92, 95, 96.

2 = includes all tissues examined in which no gross observations were made. F0 female rats: skin, salivary glands, lymph nodes (mesenteric and nonmesenteric), nasopharynx, trachea, mainstem bronchi, heart, aorta, thymic region, thymic parathyroids, liver, spleen, pancreas, adrenal glands, esophagus, stomach, small intestines, colon, urinary bladder, ovaries and oviducts, brain (cerebrum, cerebellum, medulla/pons), and pituitary gland.

3 = clear fluid in the uterine lumen, which commonly has the external appearance of uterine swelling, is consistent with a normal female rat reproductive tract in proestrus or estrus.

4 = no lesion/s

5 = red spots, red speckling, red mottling

6 = red or dark foci in the kidneys or lungs are incidental gross findings associated with CO2 euthanasia.

7 = uterine lumen distended with tan opaque fluid

Table C2. Incidence¹ of Gross Necropsy Observations in F0 Female Rats

Organ/Tissue	Gross Observation ²	Exposure Concentrations (ppm)			
		0	10	30	80
Uterus	mucometra ³	1/12	0/12	0/12	0/12
	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 Tissues⁴	N/A ⁵	N/A	N/A	N/A	N/A

¹ = # of rats with gross observation/# of rats grossly examined in exposure group.

² = does not include the incidental gross observations of red and dark foci in the lungs and kidneys associated with CO₂ euthanasia and uterine luminal dilation by clear fluid which is a normal finding in actively cycling rats in proestrus or estrus.

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

⁴ = includes all tissues examined in which no gross observations were made in any F0 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.

⁵ = not applicable

Table C3. Histopathologic Findings in F0 Female Rat Reproductive Organs

Rat #	Exp. Conc. (ppm)	Repro-ductively Successful ?	Gross Observations: Repro-ductive	Vagina (Slide 2)	Cervix (Slide 2)	Uterus (Slide 1)	Ovaries (Slide 1)	Oviducts (Slide 1)	Histologic Correlates of Reproductive Gross Observations or Lack of Reproductive Success
49	0	Yes	NL ¹	NL	NL	NL	NL	NL	N/A ²
50	0	Yes	NL	NL	NL	one horn: moderate luminal dilation (proestrus)	NL	NL	N/A
51	0	No	uterus: intraluminal fluid; mildly swollen	NL	NL	mild luminal dilation (proestrus)	NL	NL	uterus: luminal dilation correlates with gross finding -cause of reproductive failure not apparent
52	0	Yes	NL	TNP ³ (not saved)	TNP (not saved)	NL	NL	NL	N/A
53	0	Yes	NL	NL	NL	NL	NL	NL	N/A
54	0	Yes	NL	NL	NL	mild to moderate luminal dilation (early estrus)	NL	NL	N/A
55	0	Yes	NL	NL	NL	NL	NL	NL	N/A
56	0	No	NL	NL	NL	NL	NL	NL	cause of reproductive failure not apparent
57	0	Yes	NL	NL	NL	NL	NL	NL	N/A
58	0	Yes	NL	NL	NL	NL	NL	NL	N/A
59	0	No	vagina: cystic malformation uterus: mucometra ⁴	intramural cyst (w/ compression of vaginal lumen)	moderate luminal dilation	moderate luminal dilation with endometrial atrophy	NL	NL	vagina: cyst (obstructive) cervix and uterus: luminal dilation
60	0	Yes	NL	NL	NL	mild luminal dilation (proestrus)	NL	NL	N/A
61	10	Yes	NL	TNT ⁵	TNT	TNT	TNT	TNT	N/A
62	10	Yes	NL	TNT	TNT	TNT	TNT	TNT	N/A
63	10	Yes	uterus: intraluminal fluid mesometrial cyst	NL	NL	one horn: moderate luminal dilation (proestrus - estrus)	NL	NL	uterus: luminal dilation, mesometrial cyst not present histologically ⁶
64	10	Yes	NL	TNT	TNT	TNT	TNT	TNT	N/A
65	10	Yes	NL	TNT	TNT	TNT	TNT	TNT	N/A

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Table C3. Histopathologic Findings in F0 Female Rat Reproductive Organs (continued)

Rat #	Exp. Conc. (ppm)	Repro-ductively Successful ?	Gross Observations: Repro-ductive	Vagina (Slide 2)	Cervix (Slide 2)	Uterus (Slide 1)	Ovaries (Slide 1)	Oviducts (Slide 1)	Histologic Observations or Lack of Reproductive Success
66	10	Yes	NL	TNT	TNT	TNT	TNT	TNT	N/A
67	10	Yes	NL	TNT	TNT	TNT	TNT	TNT	N/A
68	10	Yes	NL	TNT	TNT	TNT	TNT	TNT	N/A
69	10	Yes	NL	TNT	TNT	TNT	TNT	TNT	N/A
70	10	Yes	NL	TNT	TNT	TNT	TNT	TNT	N/A
71	10	No	NL	NL	NL	NL	cyst (x 2; small)	NL	cause of reproductive failure not apparent
72	10	Yes	NL	TNT	TNT	TNT	TNT	TNT	N/A
73	30	Yes	NL	TNT	TNT	TNT	TNT	TNT	N/A
74	30	Yes	NL	TNT	TNT	TNT	TNT	TNT	N/A
75	30	Yes	NL	TNT	TNT	TNT	TNT	TNT	N/A
76	30	Yes	NL	TNT	TNT	TNT	TNT	TNT	N/A
77	30	Yes	NL	TNT	TNT	TNT	TNT	TNT	N/A
78	30	Yes	NL	TNT	TNT	TNT	TNT	TNT	N/A
79	30	Yes	NL	TNT	TNT	TNT	TNT	TNT	N/A
80	30	Yes	NL	TNT	TNT	TNT	TNT	TNT	N/A
81	30	No	NL	Wolfian duct remnant, periurethral	NL	NL	cyst (moderate in size)	NL	cause of reproductive failure not apparent
82	30	No	uterus: intraluminal fluid; mildly swollen	NL	TNP (section too caudal)	-mild luminal dilation -squamous metaplasia, endometrium	NL	NL	uterus: luminal dilation; squamous metaplasia, endometrium
83	30	Yes	NL	TNT	TNT	TNT	TNT	TNT	N/A
84	30	No	uterus: intraluminal fluid	NL	NL	mild luminal dilation (proestrus)	NL	NL	cause of reproductive failure not apparent
85	80	No	NL	NL	NL	NL	NL	NL	cause of reproductive failure not apparent
86	80	Yes	NL	NL	NL	NL	NL	NL	N/A
87	80	Yes	NL	NL	NL	NL	NL	NL	N/A

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Table C3. Histopathologic Findings in F0 Female Rat Reproductive Organs (continued)

Rat #	Exp. Conc. (ppm)	Reproductively Successful?	Gross Observations: Repr. ductive	Vagina (Slide 2)	Cervix (Slide 2)	Uterus (Slide 1)	Ovaries (Slide 1)	Oviducts (Slide 1)	Histologic Correlates of Reproductive Gross Observations or Lack of Reproductive Success
88	80	No	uterus: intraluminal fluid	NL	NL	NL	NL	NL	uterus: lumen not dilated histologically (diestrus - proestrus) - cause of reproductive failure not apparent
89	80	Yes	NL	NL	TNP (section too caudal)	NL	NL	NL	N/A
90	80	No	NL	NL	NL	NL	NL	NL	cause of reproductive failure not apparent
91	80	Yes	NL	NL	NL	NL	NL	NL	N/A
92	80	Yes	NL	NL	NL	NL	NL	NL	N/A
93	80	Yes	NL	NL	NL	NL	NL	NL	N/A
94	80	No	NL	NL	NL	squamous metaplasia ¹ , endometrium	luteal depletion (regressing CL's; many tertiary follicles)	NL	uterus: squamous metaplasia, endometrium ovary: luteal depletion
95	80	Yes	NL	NL	NL	mild luminal dilation (estrus)	NL	NL	N/A
96	80	Yes	NL	NL	NL	NL	NL	NL	N/A

¹ = no lesion/s
² = not applicable
³ = tissue not present
⁴ = uterine horn distended with tan opaque fluid
⁵ = tissue not trimmed, as per Protocol
⁶ = 10 step sections of uterus cut at 25 µm intervals were examined
⁷ = locally-extensive, involves luminal epithelium of body
⁸ = focal, in glandular epithelium of body

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Table C4. Incidence¹ of Histopathologic Findings in F0 Female Rat Reproductive Organs

Reproductive Organ ²	Final Reproductive Diagnosis ³	Exposure Concentration (ppm)			
		0 ⁴	10 ⁵	30 ⁵	80 ⁶
Vagina	cyst - NOS ⁷	1/11	0/2	0/3	0/12
	Wolffian duct remnant, periurethral	0/11	0/2	1/3	0/12
Cervix	luminal dilation ⁸	1/11	0/2	0/2	0/11
Uterus	luminal dilation (w/ mucometra ⁹ and endometrial atrophy) ⁸	1/12	0/2	0/3	0/12
	squamous metaplasia	0/12	0/2	1/3	1/12
Ovaries	cyst/s - NOS	0/12	1/2	1/3	0/12
	luteal depletion	0/12	0/2	0/3	1/12

1 = # of rats with diagnosis/# of rats histologically examined in exposure group.

2 = does not include oviducts, which had no histologic diagnoses.

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

4 = histologic examination was not performed on the cervix and vagina of rat #52 in the 0 ppm exposure group.

5 = histologic examination was not performed on reproductive tissues of F0 female rats in the 10 and 30 ppm exposure 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 incidences of the 10 and 30 ppm exposure groups were not statistically compared with the control and high dose groups due to the small number of animals examined histologically in these groups.

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

7 = not otherwise specified

8 = secondary to vaginal outflow obstruction by cyst

9 = uterine horn distended with tan opaque fluid

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Table C5. Gross Necropsy Observations in F0 Male Rats

0 ppm H₂S Exposure Group

Rat #1	Heart	Kidneys	Liver	Lungs	Testes	Epididymides	Seminal vesicles/ coagulating glands	Prostate	Adrenal Glands	Lymph nodes	Spleen	Nose	Other Tissues ⁶
2	NL ²	red foci ^{4,5}	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL
3	NL	red foci	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL
4	NL	red foci	NL	NL	NL	NL	NL	NL	NL	enlarged	NL	NL	NL
5	NL	red foci	NL	grey focus ⁵	NL	NL	NL	NL	NL	NL	NL	NL	NL
6	NL	red foci	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL
7	NL	red foci	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL
8	hyper-trophy ³	red foci	NL	NL	NL	NL	NL	NL	NL	NL	bisected	NL	NL
9	NL	red foci	NL	red focus ⁵	NL	NL	NL	NL	NL	NL	NL	NL	NL
11	NL	red foci	NL	NL	NL	NL	NL	tan-brown focus ⁷	NL	NL	NL	NL	NL
12	hyper-trophy	red foci	tan focus	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL

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Table C5. Gross Necropsy Observations in F0 Male Rats (continued)

10 ppm H2S Exposure Group

Rat #	Heart	Kidneys	Liver	Lungs	Testes	Epididymides	Seminal vesicles/ coagulating glands	Prostate	Adrenal Glands	Lymph nodes	Spleen	Nose	Other Tissues
13	NL	red foci	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL
14	NL	red foci	NL	red focus	NL	NL	NL	NL	NL	NL	NL	NL	NL
15	NL	red foci	NL	grey focus	NL	NL	NL	NL	NL	NL	NL	NL	NL
16	NL	red foci	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL
17	NL	red foci	NL	NL	NL	NL	NL	NL	irregular small	NL	NL	NL	NL
18	NL	red foci	tan focus	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL
19	NL	dilated p6,vis	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL
20	NL	red foci	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL
21	NL	red foci	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL
22	NL	red foci	tan focus	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL
23	NL	red foci	tan focus	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL
24	NL	red foci	tan focus	NL	NL	NL	rt small	NL	NL	NL	NL	NL	NL

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Table C5. Gross Necropsy Observations in F0 Male Rats (continued)

30 ppm H₂S Exposure Group

Flat #	Heart	Kidneys	Liver	Lungs	Testes	Epididymides	Seminal vesicles/ coagulating glands	Prostate	Adrenal Glands	Lymph nodes	Spleen	Nose	Other Tissues
25	NL	red foci	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL
27	NL	red foci	NL	grey focus	NL	NL	NL	NL	NL	NL	NL	NL	NL
28	NL	red foci	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL
29	NL	red foci	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL
30	NL	red foci	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL
31	NL	red foci	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL
32	NL	red foci	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL
33	NL	red foci	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL
34	NL	red foci	NL	NL	NL	NL	NL	NL	NL	NL	NL	red exudate ⁸	NL
35	NL	red foci	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL
36	NL	red foci	NL	red-grey foci ⁵	NL	NL	NL	NL	NL	NL	NL	NL	NL

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Table C5. Gross Necropsy Observations in F0 Male Rats (continued)

80 ppm H₂S Exposure Group

Rat #	Heart	Kidneys	Liver	Lungs	Testes	Epididymides	Seminal vesicles/ coagulating glands	Prostate	Adrenal Glands	Lymph nodes	Spleen	Nose	Other Tissues
37	NL	red foci	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL
38	NL	red foci	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL
39	NL	red foci	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL
40	NL	red foci	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL
41	NL	red foci	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL
42	NL	red foci	NL	NL	small	small	NL	NL	NL	NL	NL	NL	NL
43	NL	red foci	NL	NL	NL	NL	NL	tan-brown focus	NL	NL	NL	NL	NL
44	NL	red foci	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL
45	NL	red foci	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL
46	NL	red foci	NL	NL	NL	right tail: yellow-green enlarged soft	NL	NL	NL	NL	NL	NL	NL
47	hyper- trophy	red foci	NL	NL	small & soft	NL	NL	NL	NL	NL	NL	NL	NL
48	NL	red foci	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL	NL

1 = list does not include F0 male rats which had no gross necropsy findings in any tissue, including: 1, 10, 26.

2 = no lesions

3 = firm with small left ventricular lumen

4 = red spots, red speckling

5 = red or dark foci in the kidneys or lungs are incidental findings associated with CO₂ euthanasia.

6 = includes all tissues examined in which no gross observations were made in any F0 male rats: skin, salivary glands, nasopharynx, trachea, mainstem bronchi, aorta, thymic region, thyroid/parathyroids, pancreas, esophagus, stomach, small intestine, colon, urinary bladder, penis w/ preputial gland, brain, pituitary gland and eyes.

7 = 2 mm diameter tan-brown area

8 = small amount of dark red stain around nares

Table C6. Incidence¹ of Gross Necropsy Observations in F0 Male Rats

Organ	Gross Observation ²	Exposure Concentration (ppm)			
		0	10	30	80
Heart	hypertrophy ³	2/12	0/12	0/12	1/12
Kidneys	hydronephrosis ⁴	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
	small and soft	0/12	0/12	0/12	1/12
Epididymides	small	0/12	0/12	0/12	1/12
	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	tan-brown focus ⁷	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 ⁸	0/12	0/12	1/12	0/12
Other Tissues ⁵	N/A ⁶	N/A	N/A	N/A	N/A

1 = # of rats with gross observation / # of rats grossly examined in exposure group.

2 = does not include the incidental findings of red or dark foci in the kidneys or lungs associated with CO₂ euthanasia.

3 = firm with small left ventricular lumen

4 = dilated renal pelvis/es

5 = includes all tissues examined in which no gross observations were made in any F0 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.

6 = not applicable

7 = 2 mm diameter tan-brown area

8 = small amount of dark red stain around nares