CHRONIC INHALATION TOXICITY OF 1,2-DIBROMOETHANE IN RATS

With and Without Dietary Disulfiram

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16. Abstracts

The carcinogenicity of chromic inhalation of ethylene-dibromide (106934) (EDB) vapor with ingestion of disulfiram (97778) was studied in Sprague-Dawley rats. Four groups of animals received either control air and control diet; control air and 0.05 percent disulfiram; 20 parts per million (ppm) EDB and control diet; or 20ppm EDB and 0.05 percent disulfiram. Dying rats that received EDB and disulfiram were pale anemic, however, dying rats from other treatment groups were not. Rats receiving EDB alone and those receiving EDB and disulfiram had high mortality rates compared with control disulfiram treated rats. The mortality rate for EDB and disulfiram treated rats was 75 percent for males and 94 percent for females. Rats administered EDB alone had a mortality rate of 9 percent at the end of 12 months. At the end of 18 months, the mortality rate was 90 percent for male rats and 77 percent for female rats. The control air and control diet group and the disulfiram group had mortality rates of 11.5 and 9.3 percent at the end of 18 months. After 18 months rats receiving conrol diet and 0.05 percent disulfiram had less body weight gain than those receiving control air and control diet and those exposed to 20ppm EDB and control diet. By the end of the 13 months period of EDB and disulfiram treatment, all animals either died or were terminated because of their moribund state. Rats receiving EDB and disulfiram treatment had low hematocrit, hemoglobin and red blood cell counts, and females were more affected than males. EDB treatment alone caused increased liver and kidney weights. Ninety percent of male rats receiving EDB and disulfiram suffered from testicular atrophy and had higher incidences of tumors than rats receiving only EDB. The authors suggest that the high mortality rates in rats receiving EDB and disulfiram are a result of earlier development of tumors in these rats or of the toxicity of the combined treatment.

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PREFACE

The purpose of this study was to assess the chronic toxicity of inhaled 1,2-dibromoethane in rats exposed to a level of 20 ppm under simulated occupational exposure conditions. The study also included an evaluation of the effects of the addition of disulfiram to the diet upon the chronic toxicity of inhaled 1,2-dibromoethane.

The Biological Sciences Division of Midwest Research Institute (MRI), Kansas City, Missouri, performed the study under Contract No. 210-76-0131 with the National Institute for Occupational Safety and Health (NIOSH). Dr. Paul Peters was the principal MRI investigator from January 1977 to August 1977. Dr. Laurence C. K. Wong assumed those duties in August 1977.

The NIOSH project officer for this study was Dr. Harry B. Plotnick, Chief of the Toxicologic Mechanisms Section, Experimental Toxicology Branch, Division of Biomedical and Behavioral Science.

The contractor's final report was revised extensively by the NIOSH project officer, and this report incorporates those revisions. Additional studies of the purity and stability of the 1,2-dibromoethane used in the inhalation exposures were conducted in the NIOSH laboratories by Harry B. Plotnick, Ph.D., Lloyd E. Stettler, Ph.D. and Donald E. Richards, M.S. Duplicate sets of histopathology slides prepared by MRI were reviewed by David H. Groth, M.D., and Choudari Kommineni, D.V.M., Ph.D., of NIOSH. Some minor changes in the histopathology data supplied by MRI were made following this review.

In addition, the formalin preserved heads of 315 of the rats from this study were sent to Experimental Pathology Laboratories, Incorporated (EPL), Herndon, Virginia, for the preparation of histologic sections and microscopic evaluation of the nasal cavities. Histopathologic evaluations of these sections were performed at EPL by Melvin H. Hamlin, II, D.V.M., and was supported by the National Cancer Institute under Contract No. ICP95618.

ABSTRACT

A study of the effects of inhalation of 1,2-dibromoethane at the current U.S. occupational standard of 20 ppm, with and without disulfiram in the diet, was conducted in Sprague-Dawley rats. Each of the four groups of animals utilized in the study contained 48 male and 48 female rats. The group designations were:

A. Control/Control--exposed to filtered air, fed standard rat diet

B. Control/Disulfiram--exposed to filtered air, fed diet containing 0.05% disulfiram by weight

C. EDB/Control--exposed to 20 ppm 1,2-dibromoethane, fed standard rat diet

D. EDB/Disulfiram--exposed to 20 ppm 1,2-dibromoethane, fed diet containing 0.05% disulfiram by weight.

Inhalation exposures took place 7 hours/day, 5 days/week, for 18 months, to simulate occupational exposure to 1,2-dibromoethane. The EDB/Control group exhibited a high mortality rate and a significant increase in the incidence of tumors of the spleen, mammary gland and nasal cavity. The EDB/Disulfiram group exhibited a higher mortality rate than those exposed only to the 1,2-dibromoethane. The combined regimen also resulted in decreased body-weight gain and food consumption as well as an earlier development and a significantly increased incidence of tumors of the liver and mesentery compared with those animals exposed to 1,2-dibromoethane alone. In addition, 88% of the male rats receiving the combined treatment had testicular atrophy.

The data demonstrate that 1,2-dibromoethane is a carcinogen in the rat at the current permissible exposure limit of 20 ppm under simulated occupational exposure conditions. The data also show that the addition of disulfiram to the diet enhances the carcinogenic and toxic effects of 1,2-dibromoethane, which indicates that a toxic interaction exists between the industrial chemical and the pharmacologic agent.

INTRODUCTION

Used primarily as a gasoline additive, 1,2-dibromoethane (ethylene dibromide, ethylene bromide, EDB) serves as a lead scavenger in leaded gasoline. In 1976, the annual U.S. production of this synthetic organic chemical was estimated to be 201.1 million pounds. Although its use as a fuel additive accounts for approximately 90% of EDB's annual consumption, some 7 million pounds a year are also used for soil fumigation and other pesticidal applications (Stanford Research Institute 1978).

The literature describes the adverse effects of EDB on humans as being of an acute nature and resulting from inhalation, skin contact, or ingestion. The most commonly observed effects were changes in the liver and kidneys. In an epidemiologic study of 161 workers exposed to EDB in chemical plants, Ott et al. (1980), reported equivocal findings, attributed to small population size and multiple chemical exposures of some workers.

Acute toxic signs observed in certain laboratory animals after single or repeated exposures to EDB at various concentrations appear to be similar to those reported in humans. Rats exposed to a concentration of EDB in excess of 200 ppm usually died within 24 hours from respiratory and/or cardiovascular collapse (Rowe et al. 1952). These investigators also reported that animals exposed to EDB at levels of 50 ppm and higher for 70 to 90 days exhibited tissue lesions. The lungs were congested and inflammatory; the liver showed swelling, fatty degeneration, and necrosis; and the kidneys showed interstitial swelling and degeneration of the tubular epithelium. No evidence of an adverse effect was noted in rats, rabbits, guinea pigs, or monkeys exposed to 25 ppm of EDB for 6 months.

In a number of test systems, EDB has been reported to be mutagenic (Vogel and Chandler 1974; Sparrow et al. 1974). The chemical has also been reported to affect spermatogenesis in bulls (Amir and Volcani 1967) and to result in decreased egg weights in laying hens (Bondi et al. 1955). A study of the carcinogenicity of EDB in rats and mice, which involved oral administration of the compound over a 59- to 90-week period, resulted in a high incidence of squamous cell carcinoma of the forestomach in both species (Olson et al. 1973). This effect was noted in both rats and mice as early as 10 weeks after initiation of EDB treatment.

The animal studies appearing in the literature strongly suggest that exposure to EDB may pose carcinogenic, mutagenic, and reproductive hazards to man. The present study was performed under simulated occupational exposure conditions to evaluate the chronic toxicity of EDB at 20 ppm, the exposure limit currently permitted by the Occupational Safety and Health Administration (OSHA). At the time this study was designed, it was believed that a portion of the inhaled dose of EDB was converted to bromoacetaldehyde. (That belief has since been substantiated by investigators at the Southern Research Institute (Hill et al. 1978).) Accordingly, some of the animals were placed on a diet containing 0.05% disulfiram (an inhibitor of aldehyde dehydrogenase that is used in the management of alcoholism in man) on the presumption that the enzyme inhibitor would

block the further oxidation of the bromoacetaldehyde formed and thus increase the toxicity of inhaled EDB.

MATERIALS AND METHODS

ANIMALS AND HOUSING

Weanling Sprague-Dawley CD rats were purchased from Charles-River Breeding Laboratories, Wilmington, Massachusetts. Rats were allowed to become acclimated to the laboratory environment for 10 to 14 days before the inhalation exposure began. Each of the four groups in the study contained 48 male and 48 female rats. The group designations were:

A. Control/Control--exposed to filtered air, fed standard rat diet

B. Control/Disulfiram--exposed to filtered air, fed diet containing 0.05% disulfiram by weight

C. EDB/Control--exposed to 20 ppm 1,2-dibromoethane, fed standard rat diet

D. EDB/Disulfiram--exposed to 20 ppm 1,2-dibromoethane, fed diet containing 0.05% disulfiram by weight.

Table 1 shows the initial body weights in grams (mean ± S.E.) for males and females in each group.

Table 1--Initial body weights of rats by group (mean ± S.E.) (grams)

Group	Males	Females
Control/Control	133.8 ± 2.2	124.1 ± 2.1
Control/Disulfiram	134.4 ± 2.4	120.7 ± 1.5
EDB/Control	133.6 ± 2.0	118.4 ± 1.5
EDB/Disulfiram	131.3 ± 2.2	120.4 ± 1.4

All animals were housed individually in stainless steel cages. Except during the period of inhalation exposure (7 hours/day, 5 days/week), animals were permitted free access to water and diet. The light cycle in the animal holding area of the inhalation building was 12 hours on and 12 hours off. The temperature in the holding area ranged from 70° to 76°F, and relative humidity ranged from 35 to 50%.

A number of early animal deaths due to pneumonia necessitated replacement of some animals. To provide balance in replacement, five male and five female rats of comparable age and weight with those already in the study were randomly replaced in all four groups at the end of the first month of the study. These replacement animals were obtained from the same supplier in a second shipment.

FEED/DISULFIRAM

Disulfiram (tetraethylthiuram disulfide) with a stated purity of 96% was purchased from Aldrich Chemical Company, Milwaukee, Wisconsin, and was mixed with Wayne Lab-Blox mash to yield an 0.05% disulfiram concentration. Mixing was effected by rotating the powdered feed with the disulfiram in a drum for 30 to 40 minutes. At the beginning of the study, moisture was added to both the control and disulfiram-containing diets so the feed could be pelletized; however, this was discontinued because the poor quality of the pellets resulted in feed wastage and made it impossible to estimate food consumption and the dosage of disulfiram. In the eighth week of the study, a decision was made to switch to powdered feed. The disulfiram-containing diet was prepared weekly and was analyzed for disulfiram content twice during the study. The stability of disulfiram in the feed mix was also studied. The methods employed in these analyses and the results obtained appear in Appendix A.

TEST METHODS

Vapor Generation and Exposure Procedures

The inhalation exposures were conducted in 4.5-m^3, stainless steel, Rochester-type inhalation chambers (one EDB chamber and one filtered-air chamber). The EDB, with a stated purity of 99%, was purchased from Aldrich Chemical Company, Milwaukee, Wisconsin. Some contained small quantities of dark particles, which were removed by filtration before the EDB was used. One lot was analyzed for purity by gas-liquid chromatography after completion of the study. This analysis included a determination of the stability of the EDB at the vapor-generation temperature. The dark particles that were filtered out were also analyzed at the conclusion of the study. The results of these purity analyses appear in Appendix B.

Vapor was generated by bubbling nitrogen into a heated glass vessel containing the EDB. The temperature of the vessel was maintained at a nominal 60°C (range was 53° to 65°C) during the vapor generation. The EDB vapor was mixed with a stream of filtered air in the plenum at the top of the chamber. The air from this chamber exited at the bottom and passed through a burner at 1600°C before being released into the environment. Air flow through the chamber was maintained at 26 cubic feet/minute (736.3 liters/minute), which is equivalent to 10 air changes per hour. The air flow was monitored by a critical orifice in the exhaust duct, which was connected to a Magnahelix gauge. The orifice plate was calibrated with a Model 100-SXX air flow transducer (Autotronic Controls Corporation, El Paso, Texas). Uniform exposure to EDB vapors was ensured by rotating the cages daily according to an established schedule.

Control animals were exposed to filtered air in a similar inhalation chamber in which air flow was maintained in the same manner as described for the EDB exposure chamber.

Both chambers were operated with a slight negative pressure of 0.1 to 0.2 inches of water, which was monitored by Magnahelix gauges. The temperature in the inhalation chambers ranged from 70° to 80°F and the relative humidity, from 35 to 50%. After the 7-hour exposures, the chambers were flushed with air for 1 to 2 hours to remove residual EDB, and the interior of each was thoroughly cleaned

daily with a disinfectant soap solution followed by a copious water rinse. The entire inhalation room was also wet-cleaned daily.

When removed from the chambers, the animals were placed in compartmentalized holding racks. Measurements of the animal holding area and the inhalation room indicated that there was no detectable EDB in either area. All personnel involved in the inhalation operation wore masks, gloves, and laboratory coats and underwent annual physical examinations.

Determination of Chamber Concentrations of EDB

The concentration of EDB in the inhalation chamber was monitored with a Varian Model 2700 gas chromatograph equipped with a flame ionization detector. The 10 ft x 1/8 in. stainless steel column was packed with 5% didecyl phthalate on 80/100 mesh Chromosorb W and maintained at a temperature of 120°C. The injector temperature was maintained at 155°C and the detector temperature, at 183°C. The carrier gas was nitrogen, and the flow rate was 46 ml/minute. Under these chromatographic conditions, EDB exhibited a retention time of 2.65 minutes. At least three times a day air samples were collected from the chamber through a 1/4-inch Teflon line connected to an automatic sampler. The Teflon line was continuously purged with carrier gas, and a sample was withdrawn from the chamber periodically and directed into the gas chromatograph via a sampling valve with twin 1-ml sampling loops. Also, a distribution study was performed with the chamber loaded to determine the concentrations of EDB in the eight corners of the chamber relative to a central reference point. Standards of 1,2-dibromoethane were prepared volumetrically in carbon disulfide. A 20-ppm standard was analyzed periodically during the day to verify the analysis and to update the calculation program, if necessary. The gas chromatographic signal was processed by a Varian Model CDS-111 electronic integrator, which was programmed to measure peak area and to calculate parts per million of EDB by an external standard program. A strip chart recorder was used occasionally to visualize the chromatographic signal. To verify the chamber concentrations, the amount of EDB used during each daily exposure was determined by weight. The concentration of EDB in the chamber was based on the rate of air flow, the duration of vapor generation, and the amount of EDB used.

Clinical Observations

During the course of the study, individual body weights of all animals were recorded weekly for the first 14 weeks and monthly thereafter. Food consumption and disulfiram intake were not determined until the beginning of the fourth month of the study because problems associated with pelletizing the feed resulted in wastage. Animals were observed twice daily, once in the morning and once in the afternoon, for clinical signs of toxicity. A weekly palpation was performed on all animals when a tissue mass was first detected, and a data record was established for each animal bearing a detectable mass.

Gross and Microscopic Examinations

Complete gross and histopathological examinations were performed on all animals that survived the 18-month experimental period and on those animals that either died during the course of the study or appeared moribund (based on criteria

established by the principal investigator to reduce the chance of loss of tissues due to autolysis). At necropsy, the weights of the liver, spleen, heart, and kidneys were recorded. Histopathologic evaluations were performed on sections of the following tissues: liver, heart, stomach, kidneys, pancreas, spleen, adrenals, thyroid, pituitary, urinary bladder, brain, skin, sternal bone marrow, each lobe of the lung, mesenteric and tracheobronchial lymph nodes, salivary glands, zymbal glands, testes, prostate, mammary glands, ovaries, uterus, and any abnormally appearing tissues. The zymbal glands were prepared by fixing in formalin, decalcification, paraffin embedding, sectioning, and staining. All other tissues were fixed in formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Microscopic Examination of Nasal Cavities

Following the completion of the study by MRI, the formalin preserved heads of 315 of the rats from this study were sent to Experimental Pathology Laboratories, Incorporated, Herndon, Virginia, for the preparation of histologic sections of the nasal cavity and subsequent histopathologic evaluation. Hematoxylin and eosin stained tissue sections of cross sections through the nasal cavity of each rat were prepared at the following levels:

A. level of the incisor teeth;

B. midway between incisors and first molar; and

C. middle of the second molar (olfactory region).

At the time that the tissue sections were being prepared, it was noted that many of the specimens appeared to be in a rather poor state of preservation, precluding critical examination and classification of many lesions and tumors.

The number of animal heads from each group which were available for nasal cavity examination appears below.

Group	Number of Animals	
	Male	Female
Control/Control	47	47
Control/Disulfiram	45	47
EDB/Control	42	42
EDB/Disulfiram	27	18

Statistical Evaluation of Data

The significance of differences between control and test values for body weight, food consumption, and organ weights was statistically determined by Tukey's Omega procedure. A significance level of 5% was used. Data on tumor incidence were analyzed by Fisher's exact probability test at a significance level of 5%.

RESULTS

CHAMBER CONCENTRATIONS OF 1,2-DIBROMOETHANE

Chamber concentrations of EDB, determined by the gas chromatographic method, ranged from 18.6 ± 3.1 to 20.9 ± 0.4 ppm (mean ± S.E.) during the 18-month exposure period. Figure 1 depicts these data graphically, and Table 2 summarizes them on a monthly basis.

The study of the distribution of EDB in the eight corners of the loaded chamber relative to a central reference point yielded the following results: U-SE (upper southeast), -7%; U-SW, -1%, U-NE, +3%; U-NW, +2%; L-SE (lower southeast), -10.5%; L-SW, -8%; L-NE, +2.4%; and L-NW, -4.5%.

MORTALITY

Rats exposed to 20 ppm EDB alone (EDB/Control) or a combination of 20 ppm EDB and 0.05% disulfiram in the diet (EDB/Disulfiram) exhibited high mortality rates in comparison with those animals in the other two experimental groups. A majority of the animals in the EDB/Disulfiram group died or were terminated within the first 12 months of exposure (58% of the males and 83% of the females). All animals in this group had either died or been terminated by the end of the fifteenth month of the study. Table 3 summarizes the cumulative number of deaths or terminations occurring in each group at the end of 3, 6, 9, 12, 15, and 18 months of exposure.

BODY WEIGHTS

Although feeding of disulfiram alone and exposure to EDB alone resulted in a depression of weight gain in both male and female rats compared with animals in the Control/Control group, the combination of EDB and disulfiram resulted in a more pronounced depression in body weights than that resulting from exposure to either agent alone. Figure 2 shows the average body weights of male and female rats in each group throughout the 18-month experimental period.

FOOD CONSUMPTION AND DISULFIRAM INTAKE

As mentioned, food consumption was not determined until the fourth month of the study because pelleting problems caused feed wastage. A correlation between low food consumption and decreased body weight gain seemed evident in animals in the Control/Disulfiram and EDB/Disulfiram groups; however, this relationship was not evident in the EDB/Control group. Although males and females in this group ate essentially the same quantity of food as those in the Control/Control group, the body weights of the EDB/Control group were consistently lower. Table 4 presents food consumption data for each group from the fourth through the eighteenth month of the study, and Table 5 presents data on mean daily disulfiram intake during the corresponding period.

HISTOPATHOLOGY

Table 6 summarizes the principal histopathologic findings in male and female rats in each group. Exposure to EDB alone resulted in a significant increase in the incidence of hemangiosarcoma of the spleen in males, focal proliferation of the bronchiolar epithelium of the lung in both sexes, and adenocarcinoma of the mammary gland in females compared with those in the Control/Control group. Combined exposure to EDB and disulfiram resulted in a significant increase in the incidence of hepatocellular carcinoma, hemangiosarcoma of the spleen, hemangiosarcoma of the mesentery or omentum, and atrophy of the testes in males compared with the Control/Control and EDB/Control groups. Histopathologic findings in the Control/Control and Control/Disulfiram groups were unremarkable.

Histopathologic changes observed in the evaluation of the nasal cavities, both tumor and nontumor, were either solely observed in the EDB/Control and EDB/Disulfiram groups or were of increased severity in these groups. The proliferative lesions observed ranged from epithelial hyperplasias and dysplasias to malignant tumors. The most commonly diagnosed malignant tumor was classified as an adenocarcinoma and like other malignant tumors they were, on occasion, found to be locally invasive. In all instances in which malignant tumors were seen invading the brain, they were classified as adenocarcinomas. Malignant tumors tended to be located in the more posterior portions of the nasal cavity, while benign tumors tended to be multiple and located in the more anterior regions of the nasal cavity.

It is difficult to assess whether dietary disulfiram enhanced the 1,2-dibromoethane effects on the nasal cavity because of the limited number of animals available for evaluation from the EDB/Disulfiram group. However, based on the animals provided for examination, it does not appear that the addition of disulfiram to the diet altered the EDB-related effects on the nasal cavity. A summary of the neoplastic findings appears in Table 7. Summary incidence tables of neoplastic and hyperplastic changes appear in Appendix C.

DISCUSSION

The results of this study clearly indicate that inhaled 1,2-dibromoethane exerts a carcinogenic effect in both male and female rats exposed to 20 ppm of the chemical under simulated occupational exposure conditions for up to 18 months. Addition of disulfiram to the diet of animals exposed to EDB enhances both the general toxicity and carcinogenicity of the halogenated hydrocarbon and results in effects not observed with either agent alone. For example, the combined exposure resulted in a tenfold increase in the incidence of hepatocellular carcinoma over exposure to EDB alone. Additionally, testicular atrophy was noted in 88% of the males in the EDB/Disulfiram group as opposed to 6%, 2%, and 2% incidence of this change in the Control/Control, Control/Disulfiram, and EDB/Control groups, respectively. Dietary disulfiram alone, although associated with a decrease in body weight gain in both male and female rats, produced no significant histopathologic changes. The finding that chronic disulfiram administration is not associated with any carcinogenic response is consistent with the findings of a National Cancer Institute bioassay of the compound in male and female F344 rats and B6C3F1 mice (National Cancer Institute, 1979).

The mechanism of this toxic interaction between the industrial chemical and the pharmacologic agent is not clear. Hill *et al.* (1978) have identified bromoacetaldehyde as an intermediate of EDB biotransformation in the rat. Based upon this information, one could speculate that disulfiram, by virtue of its ability to block aldehyde dehydrogenase, prevents the oxidation of bromoacetaldehyde to bromoacetate by this enzyme system and causes elevated tissue levels of the aldehyde. The literature contains virtually no information on the toxicity and biological reactivity of bromoacetaldehyde. Nevertheless, chloroacetaldehyde, a closely-related α-haloaldehyde, is known to be a potent mutagen in the Ames assay employing tester strain TA100 without activation (McCann *et al.* 1975), and it reacts nonenzymatically with the nucleic acid bases adenine and cytosine to form so-called "etheno" derivatives (Secrist *et al.* 1972). Such a reaction between bromoacetaldehyde and these bases, if it occurs *in vivo*, could produce significant alterations in nucleic acids. Support for this hypothesis comes from the work of Plotnick *et al.* (1979), who found that the addition of disulfiram (0.05%) to the diet of male rats resulted in significant increases in the ^{14}C content of washed liver nuclei at both 24 and 48 hours after administration of a single oral dose of ^{14}C-1,2-dibromoethane when compared with those of animals receiving a standard diet with no disulfiram added. In the same study, Plotnick and his coworkers found that levels of ^{14}C in the testes of rats receiving the disulfiram-containing diet did not differ significantly at 24 and 48 hours, which suggests that the testicular atrophy noted in animals in the EDB/Disulfiram group is associated with a slower clearance of EDB and/or its metabolites from this organ. It is unknown at this time whether the toxic interaction noted between inhaled EDB and ingested disulfiram is limited to this particular combination or is characteristic of other 1,2-dihaloethanes such as 1,2-dichloroethane. A study designed to determine whether dietary disulfiram enhances the toxicity of inhaled 1,2-dichloroethane is currently in the planning stages, as is a study of the biochemical mechanism of the interaction between disulfiram and EDB.

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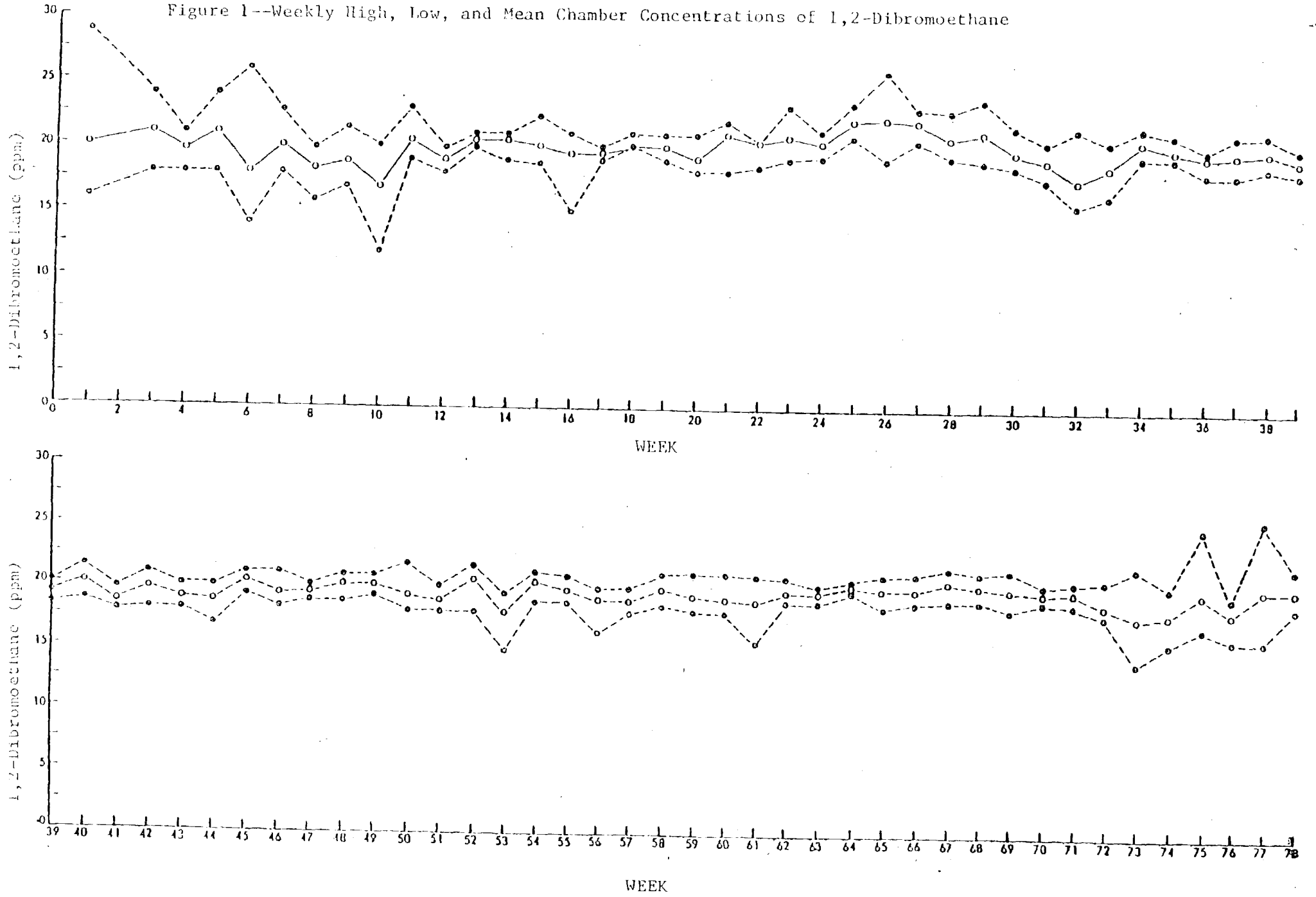
Figure 1--Weekly High, Low, and Mean Chamber Concentrations of 1,2-Dibromoethane



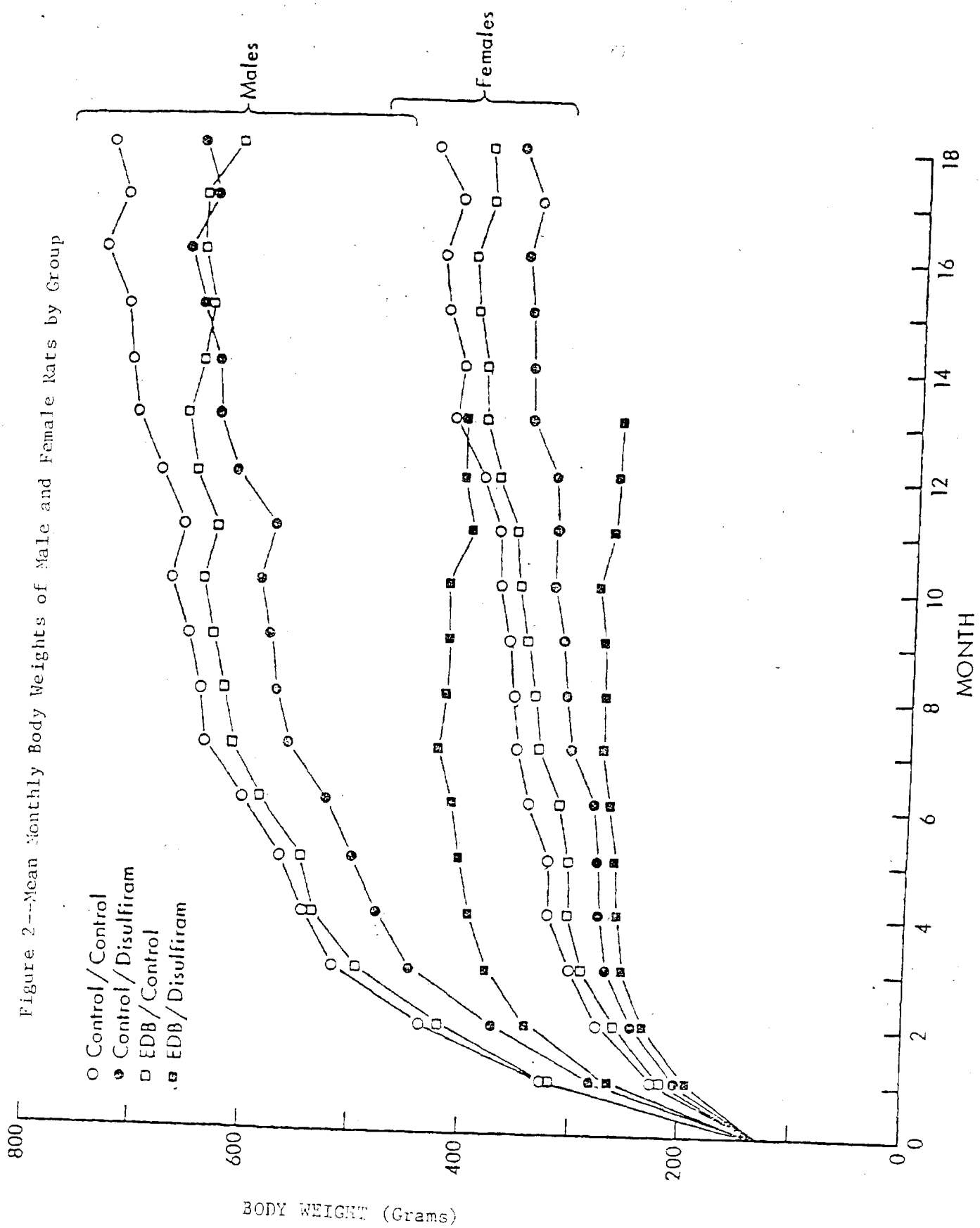


Figure 2--Mean Monthly Body Weights of Male and Female Rats by Group

Table 2--Mean daily chamber concentrations of 1,2-Dibromoethane During the 18-month inhalation exposure period. (ppm ± S.E.)

Month	Mean Daily Concentration
1	19.7 ± 1.5
2	19.7 ± 0.7
3	18.6 ± 3.1
4	20.0 ± 0.3
5	19.6 ± 0.3
6	20.0 ± 0.4
7	20.0 ± 0.8
8	20.9 ± 0.4
9	18.7 ± 0.4
10	19.8 ± 0.2
11	19.4 ± 0.2
12	19.4 ± 0.2
13	19.8 ± 0.2
14	19.2 ± 0.4
15	19.3 ± 0.2
16	19.6 ± 0.3
17	19.8 ± 0.2
18	19.0 ± 0.4

Table 3--Cumulative mortality by group and sex at three-month intervals throughout the study.*

	Group			
	Control/Control	Control/Disulfiram	EDB/Control	EDB/Disulfiram
Male rats				
3 months	0	0	0	0
6 months	0	0	0	1
9 months	0	1	1	8
12 months	0	2	5	28
15 months	1	4	30	48
18 months	5	6	43	48
Female rats				
3 months	0	0	0	0
6 months	0	0	0	0
9 months	0	0	1	12
12 months	2	2	4	40
15 months	3	2	19	48
18 months	6	3	37	48

* All groups consisted of 48 male and 48 female rats at study initiation.

Table 4--Food consumption by group during months 4 through 18.
(g/day per rat)

Month	Control/Control		Control/Disulfiram		EDB/Control		EDB/Disulfiram	
	Male	Female	Male	Female	Male	Female	Male	Female
4	26.0 ± 0.4	18.9 ± 0.3	23.9 ± 0.3	17.9 ± 0.4	26.9 ± 0.3	21.1 ± 0.5	23.9 ± 0.5	21.5 ± 0.7
5	26.7 ± 0.5	19.1 ± 0.5	23.9 ± 0.3	17.3 ± 0.3	27.5 ± 0.4	22.2 ± 0.5	23.4 ± 0.4	21.0 ± 0.5
6	26.8 ± 0.5	18.9 ± 0.5	23.9 ± 0.3	17.2 ± 0.4	26.8 ± 0.4	20.9 ± 0.5	21.5 ± 0.3	19.2 ± 0.6
7	26.9 ± 0.4	19.5 ± 0.4	23.8 ± 0.2	17.4 ± 0.4	27.2 ± 0.5	20.8 ± 0.4	21.3 ± 0.3	19.4 ± 0.5
8	26.7 ± 0.5	18.8 ± 0.4	24.7 ± 0.3	16.5 ± 0.4	25.5 ± 0.4	20.2 ± 0.4	20.5 ± 0.4	18.7 ± 0.6
9	27.2 ± 0.4	16.7 ± 1.3	23.6 ± 0.2	17.0 ± 0.3	26.6 ± 0.5	21.0 ± 0.4	19.7 ± 0.3	18.0 ± 0.7
10	21.4 ± 0.4	18.6 ± 0.4	24.1 ± 0.3	16.4 ± 0.3	25.8 ± 0.4	20.3 ± 0.4	18.6 ± 0.4	17.3 ± 0.6
11	25.5 ± 0.6	19.7 ± 0.5	22.9 ± 0.4	18.9 ± 0.7	23.6 ± 0.5	19.6 ± 0.4	16.4 ± 0.7	16.0 ± 1.1
12	26.3 ± 0.4	20.0 ± 0.5	24.6 ± 0.3	17.5 ± 0.5	26.4 ± 0.5	21.9 ± 0.5	19.4 ± 0.6	18.2 ± 1.1
13	26.4 ± 0.5	20.7 ± 0.4	25.0 ± 0.3	18.3 ± 0.4	26.6 ± 0.5	21.1 ± 0.6	19.6 ± 0.9	19.0 ± 0.0
14	26.1 ± 0.4	20.1 ± 0.5	23.5 ± 0.4	16.7 ± 0.5	24.9 ± 0.5	20.2 ± 0.6		
15	26.6 ± 0.4	19.9 ± 0.5	25.1 ± 0.3	18.1 ± 0.5	26.5 ± 0.8	21.5 ± 0.6		
16	27.8 ± 0.4	21.9 ± 0.4	26.3 ± 0.4	19.4 ± 0.5	28.0 ± 0.9	23.3 ± 0.8		
17	24.5 ± 0.6	27.3 ± 0.7	21.5 ± 0.4	15.6 ± 0.4	28.9 ± 1.0	20.4 ± 1.2		
18	27.3 ± 0.7	21.8 ± 0.3	24.5 ± 0.4	17.9 ± 0.4	28.0 ± 1.0	23.7 ± 1.1		

Table 5--Disulfiram intake during months 4 through 18.
(mg/kg per day)

Month	Control/Control		Control/Disulfiram		EDB/Control		EDB/Disulfiram	
	Male	Female	Male	Female	Male	Female	Male	Female
4	0	0	25.9 ± 0.3	34.6 ± 2.0	0	0	31.0 ± 0.6	41.9 ± 1.4
5	0	0	24.7 ± 0.3	31.0 ± 0.6	0	0	29.2 ± 0.5	40.1 ± 0.9
6	0	0	23.1 ± 0.2	29.6 ± 0.6	0	0	26.2 ± 0.4	35.6 ± 1.1
7	0	0	23.0 ± 0.3	29.3 ± 0.5	0	0	25.7 ± 0.4	35.5 ± 0.9
8	0	0	21.0 ± 0.2	26.8 ± 0.5	0	0	24.8 ± 0.5	34.3 ± 1.3
9	0	0	21.6 ± 0.3	27.3 ± 0.5	0	0	23.9 ± 0.4	32.4 ± 1.2
10	0	0	20.5 ± 0.3	25.8 ± 0.4	0	0	22.5 ± 0.4	30.7 ± 0.9
11	0	0	19.5 ± 0.5	29.1 ± 1.0	0	0	19.9 ± 0.8	28.9 ± 2.0
12	0	0	20.5 ± 0.3	26.9 ± 0.6	0	0	23.3 ± 0.8	34.4 ± 2.8
13	0	0	20.4 ± 0.4	27.3 ± 0.7	0	0	23.2 ± 0.9	53.1 ± 0.0
14	0	0	18.7 ± 0.4	24.3 ± 0.6	0	0		
15	0	0	19.5 ± 0.4	26.1 ± 0.8	0	0		
16	0	0	20.3 ± 0.4	27.5 ± 0.8	0	0		
17	0	0	16.9 ± 0.4	22.2 ± 0.8	0	0		
18	0	0	19.2 ± 0.4	25.3 ± 0.7	0	0		

Table 6--Major histopathologic findings.

Organ/Tissue	Test							
	Control/Control		Control/Disulfiram		EDB/Control		EDB/Disulfiram	
	Male	Female	Male	Female	Male	Female	Male	Female
Liver								
Hepatocellular carcinoma	0	0	0	0	2	4	37*	34*
Hemangiosarcoma	0	0	0	0	0	0	2	4
Hepatocellular neoplastic nodules	0	0	0	0	2	8+	0	0
Spleen								
Atrophy	0	0	0	0	0	0	17*	13*
Hemangiosarcoma	0	0	0	0	12+	2	15	10
Testes								
Atrophy of seminiferous tubules	3	-	1	-	1	-	42*	-
Lung								
Focal proliferation of bronchiolar epithelium	0	0	0	0	13+	19+	25	33*
Bronchogenic carcinoma	0	0	0	0	1	0	4	0
Hemagiosarcoma	0	0	0	0	0	0	1	1
Epidermoid carcinoma	0	0	0	0	2	0	0	0
Metastatic tumor	0	0	0	0	0	0	5	1
Kidney								
Adenoma	0	0	0	0	2	1	10	3
Adenocarcinoma/carcinoma	0	0	0	0	1	0	8	2
Hemangiosarcoma	0	0	0	0	0	0	0	2
Mesentery/Omentum								
Hemangiosarcoma	0	0	0	0	0	0	11*	8*

Mammary gland								
Carcinoma	–	0	–	4	–	11+	–	10
Brain								
Meningioma	0	0	0	0	3	1	3	2
Prostate gland								
Atrophy	0	–	0	–	0	–	12*	–
Heart								
Hemangiosarcoma	0	0	0	0	0	0	1	0
Skeletal muscle								
Hemangiosarcoma	0	0	0	0	1	0	0	0

+ – Significantly different from control/control group ($p<0.05$)
* – Significantly different from EDB/control group ($p<0.05$)

Table 7--Histopathologic Examination of the Nasal Cavity -
Summary of Neoplastic Changes Noted

	Control/Control		Control/Disulfiram		EDB/Control		EDB/Disulfiram	
	Male	Female	Male	Female	Male	Female	Male	Female
Number of Rats with Benign Tumors	0	0	0	0	32	27	22	17
Number of Rats with Multiple Benign Tumors	0	0	0	0	21	19	17	9
Number of Rats with Malignant Tumors	0	0	0	0	27	19	17	3
Number of Rats with Tumors	0	0	0	0	40	38	24	17

APPENDIX A. Analysis of Disulfiram in Rat Diet

INTEROFFICE COMMUNICATION

MIDWEST RESEARCH INSTITUTE

February 3, 1978

To: Dr. Mark Winston

From: David Steele

Subject: Analysis of Disulfiram in Rat Feed

Equipment

1. Princeton Applied Research Corporation (Princeton, New Jersey) Model 174 polarographic analyzer equipped with a P.A.R.C. droptimer and with a Hewlett Packard Model 7030A x-y recorder.

2. Saturated calomel electrode, dropping mercury electrode, platinum coil electrode, Brinkmann EA 825-20 titration vessel with cover.

Reagents

1. Ethanol (95%)

2. Water--deionized, then distilled from dichromater.

3. Disulfiram.

4. Acetate buffer (0.5 N sodium acetate and 0.5 N acetic acid; pH = 4.5).

Basic Procedure

The basic procedure was that outlined in D. G. Prue, C. R. Warner, and B. T. Kho, "Pulse Polarographic Determination of Disulfiram," Journal of Pharmaceutical Sciences, Vol. 61, No. 2, 249-251, February 1972.

Samples of disulfiram on feed (2.5 g) were accurately weighed and extracted with 100 ml ethanol. The extracts were filtered and 15 ml aliquots, measured volumetrically, were added to the polarographic cell with 15 ml aliquots of acetate buffer, also measured volumetrically.

For differential pulse polarography, a dropping mercury electrode was used as the working electrode. A platinum coil electrode was used as the counter electrode. The reference electrode, a saturated calomel electrode, made electrical contact with the solution via a salt bridge containing saturated potassium chloride solution.

The cell was deaerated with prepurified nitrogen which had been passed through a U-tube filled with acetate buffer. While the pulse polarogram was being obtained, the solution in the cell was blanketed with prepurified nitrogen.

The instrument parameters were as follows:

Scan: Negative
Rate: 1 mv/sec
Range: 1.5 v
Initial Potential: -0.2 v
Modulation Amplitude: 100
Current Range: 10 ua/full scale
Droptime: 2 sec
Lowpass Filter: off

Sample Analysis

1. Standard Curve - Pulsed polarograms of standard solutions of disulfiram (0, 4, 8, 12, 16, and 20 µg disulfiram per milliliter ethanol) were obtained. A plot of concentration versus peak height was linear with a slope of 1.6 and an intercept of zero.

2. Sample Analysis

a. Method - Three aliquots of each of the samples of disulfiram mixed with Feed (Top, Middle and Bottom) were weighed, extracted with ethanol, and analyzed by pulsed polarography. The concentration of each aliquot was obtained from the standard curve using sample peak height data.

Extraction efficiency data was obtained during an earlier analysis and found to be 100 ± 2%. (Report to Dr. Paul Peters on the Analysis of Disulfiram in rat feed, dated April 20, 1977.)

b. Results

Sample	Concentration of Disulfiram in Feed/Chemical Sample
Top	0.067 ± 0.003 (δ) %
Middle	0.064 ± 0.006 (δ) %
Bottom	0.063 ± 0.002 (δ) %

DS/sam

April 20, 1977

To: Dr. Paul Peters

From: David Steele

Subject: Analysis of Disulfiram in Rat Feed

Equipment

1. Princeton Applied Research Corporation (Princeton, New Jersey) Model 174 polarographic analyzer equipped with a P.A.R.C. droptimer and with a Hewlett Packard Model 7030A x-y recorder.

2. Saturated calomel electrode, dropping mercury electrode, platinum coil electrode, Brinkmann EA 825-20 titration vessel with cover.

Reagents

1. Ethanol (95%)

2. Water--deionized, then distilled from dichromater.

3. Disulfiram.

4. Acetate buffer (0.5 N sodium acetate and 0.5 N acetic acid; pH = 4.5).

Basic Procedure

The basic procedure was that outlined in D. G. Prue, C. R. Warner, and B. T. Kho, "Pulse Polarographic Determination of Disulfiram," Journal of Pharmaceutical Sciences, Vol. 61, No. 2, 249-251, February 1972.

Samples of disulfiram on feed (2.5 g) were accurately weighed and extracted with 100 ml ethanol. The extracts were filtered and 15 ml aliquots,

measured volumetrically, were added to the polarographic cell with 15 ml aliquots of acetate buffer, also measured volumetrically.

For differential pulse polarography, a dropping mercury electrode was used as the working electrode. A platinum coil electrode was used as the counter electrode. The reference electrode, a saturated calomel electrode, made electrical contact with the solution via a salt bridge containing saturated potassium chloride solution.

The cell was deaerated with prepurified nitrogen which had been passed through a U-tube filled with acetate buffer. While the pulse polarogram was being obtained, the solution in the cell was blanketed with prepurified nitrogen.

The instrument parameters were as follows:

Scan: Negative

Rate: 1 mv/sec

Range: 1.5 v

Initial Potential: -0.2 v

Modulation Amplitude: 100

Current Range: 10 μa/full scale

Droptime: 2 sec

Lowpass Filter: off

Sample Analysis

1. Standard Curve

Pulsed polarograms of standard solutions of disulfiram (0, 4, 8, 12, 16, and 20 µg disulfiram per milliliter ethanol) were obtained. A plot of concentration versus peak height was linear with a slope of 1.2 and an intercept of zero.

2. Sample Analysis

a. Method: Three aliquots of the sample of disulfiram mixed with feed were weighed, extracted with ethanol, and analyzed by pulsed polarography. The concentration of each aliquot was obtained from the standard curve using sample peak height data.

In order to obtain extraction efficiency data, standard mixes of dry disulfiram/feed were prepared, extracted and analyzed using the same method as that employed for the sample aliquots.

b. Results:

Concentration of disulfiram in feed/chemical sample:

0.052 ± 0.002 (6) %

Extraction efficiency:

$100 \pm 2\%$

April 21, 1978

TO: Dr. Mark Winston

FROM: David Steele

SUBJECT: Stability of Disulfiram on Rat Feed

I. Purpose

To determine the stability of disulfiram on rat feed (1% w/w) using a constant time (2 weeks) and variable temperature (-20, 5, 25, 45°C) sample comparison study.

II. Sample Preparation and Storage

Individual samples of disulfiram on rat feed were prepared by accurately weighing 25 mg of disulfiram into small French square bottles, adding 2.5 g rat feed, and shaking thoroughly. The samples were then stored at temperatures of -20°C, 5°C, 25°C, and 45°C for 2 weeks. Samples (2.5 g) of blank feed were also stored along with the samples of disulfiram on feed at each temperature.

III. Sample Analysis

At the end of the 2-week storage period, each sample was extracted with 95% ethanol (100 ml), the extracts filtered, and the filtrates analyzed using the liquid chromatographic system described below:

Instrument: Waters programmable component system
Detector: Schoeffel Model 770 Variable Absorbance detector, analytical wavelength -290 nm
Column: C_{18} μBondapack, 300 mm x 4 mm ID
Solvent system: 47% acetonitrile
53% water
Flow rate: 1.0 ml/min

The detector response (peak height in millimeters per milligram disulfiram injected) for the 5°C, 25°C, and 45°C samples was compared to the average detector response for the -20°C samples. These values were then normalized to a value of 100% for the -20°C samples.

IV. Results and Discussion

Storage Temperature	Detector Response (Peak height mm/µg injected)	% of -20°C Sample
- 20°C	45.36 ± 1.06(σ)	100.0 ± 2.3(σ)
5°C	45.38 ± 0.91(σ)	100.0 ± 2.3(σ)
25°C	42.27 ± 0.46(σ)	93.2 ± 2.3(σ)
45°C	39.10 ± 0.62(σ)	86.2 ± 2.3(σ)

Disulfiram on rat feed at a level of 1% w/w was found to be stable at 5°C for a period of 2 weeks. At 25°C a slight decomposition was found and at 45°C, there was a definite decomposition

MIDWEST RESEARCH INSTITUTE

CHEMICAL SCIENCES DIVISION

David Steele
Assistant Chemist

Approved:

Evelyn Murrill
Senior Advisor for Chemistry

APPENDIX B. Purity Analysis of 1,2-Dibromoethane

PARTICULATE ANALYSIS

Several drops of suspension of particulates contained in 1,2-dibromoethane Lot No. MA102567, supplied by the Aldrich Chemical Company, were placed on a carbon planchet. After evaporation of the solvent, the planchet was examined in a JEOL JXA-50A electron probe microanalyzer equipped with an Ortec energy dispersive spectrometer and an Ortec Model EEDS-II multichannel analyzer. One hundred of the particles found on the planchet were analyzed by energy dispersive X-ray analyses. The results of the analyses are summarized in Table B-1. In 87% of the particles analyzed, the only major peaks found were those for bromine. Since no other major inorganic component was found in these particles, it can be concluded that they were some type of nonvolatile organic bromide. Since the particulates are nonvolatile, their contribution to possible effects on animals exposed to 1,2-dibromoethane by inhalation should not be significant.

GAS CHROMOTOGRAPHIC ANALYSIS FOR PURITY AND STABILITY

The material tested was 1,2-dibromoethane, Lot No. MA102467, supplied by Aldrich Chemical Company, Milwaukee, Wisconsin. The chemical was tested as received, after 24 hours at 60°C, and after 96 hours at 60°C. The 60° stability study was performed by placing a small volume of the material in a glass serum bottle, which was subsequently sealed with a rubber septum and placed in an incubator. Duplicate 3-microliter aliquots of the respective samples were individually injected into a Perkin-Elmer Model 3920B gas chromatograph equipped with a flame ionization detector under the following conditions:

Column: 10 ft x 1/8 in., stainless steel, packed with 20% SP-2100, 0.1% Carbowas 1500 on 100/120-mesh Supelcorport

Carrier gas: Nitrogen at 15.0 ml/min

Column temperature: 60°-140°C at 2°/min, 8-minute delay, 16-minute hold at upper limit

Injector temperature: 200°C

Detector temperature: 200°C

Under these chromatographic conditions, 1,2-dibromoethane exhibited a retention time of approximately 26 minutes. Area normalization analysis with a Perkin-Elmer Sigma-10 chromatography system indicated a purity of greater than 99%; it also indicated that the material is stable for up to 96 hours when heated to 60°C.

Table B-1. Particulate Analyses*

Particle Class	Number Found	Major Components(s)	Trace Component(s)
1	87	Br	Na, S, Cl, Fe, Si Ca, Zn, K, Cu, Mn
2	5	Si	K, Br, Fe, Ca, Cl Cu
3	3	Ca, Br	S, Cl
4	2	Na, Cl	Cu
5	1	Si, Br	Ca, Cu, Zn
6	1	K	Br
7	1	K, Fe, Si, Br	Cu

Note: Not all trace elements appear in each particle in a particular class.

* Performed by Lloyd E. Stettler, Ph.D., Biological Support Branch, Division of Biomedical and Behavioral Science, NIOSH.

APPENDIX C. - Neoplastic and Hyperplastic Changes in the Nasal Cavity

Summary Incidence Tables

Key to Group Designations:

Group I - Control/Control

Group II - Control/Disulfiram

Group III - EDB/Control

Group IV - EDB/Disulfiram

NEOPLASM AND HYPERPLASIA
SUMMARY INCIDENCE TABLE

Toxicity of Inhaled 1,2-Dibromoethane
With and Without Disulfiram in the Diet

	Group I		Group II		Group III		Group IV			
M = Male; F = Female	M	F	M	F	M	F	M	F		
NASAL CAVITY (NO. EXAMINED)	(47)	(47)	(45)	(45)	(42)	(42)	(27)	(18)		
Squamous Cell Carcinoma					2			1		
Adenocarcinoma					2	8	9	1		
Anaplastic Carcinoma					1	1	1			
Carcinoma, NOS					1	2	1	1		
NASAL BONES (NO. EXAMINED)	(47)	(47)	(45)	(45)	(42)	(42)	(27)	(18)		
Adenocarcinoma, Invasive					7	10	9	1		
Carcinoma, NOS, Invasive					1	2		1		
Anaplastic Carcinoma, Invasive						1	1			
Squamous Cell Carcinoma, Invasive					1	1				
NASAL SEPTUM (NO. EXAMINED)	(47)	(47)	(45)	(45)	(42)	(42)	(27)	(18)		
Adenocarcinoma					2					
Adenocarcinoma, Invasive					3	7	3			
Carcinoma, NOS						1				
Carcinoma, NOS, Invasive					1	1				
Squamous Cell Carcinoma, Invasive					2	1				
Anaplastic Carcinoma, Invasive						1				
Papillary Adenoma						1				
Squamous Cell Carcinoma					1					
Papillary Hyperplasia, Focal					1					

NEOPLASM AND HYPERPLASIA
SUMMARY INCIDENCE TABLE

Toxicity of Inhaled 1,2-Dibromoethane
With and Without Disulfiram in the Diet

	Group I		Group II		Group III		Group IV			
M = Male; F = Female	M	F	M	F	M	F	M	F		
NASAL TURBINATE (NO. EXAMINED)	(47)	(47)	(45)	(45)	(42)	(42)	(27)	(18)		
Adenocarcinoma					1					
Squamous Cell Carcinoma					1					
Adenocarcinoma, Invasive					1					
Papillary Adenoma, Multiple					7	6	7	3		
Papillary Adenoma					4	13	7	9		
Papillary Adenoma, Focal					12					
MAXILLOTURBINATE (NO. EXAMINED)	(47)	(47)	(45)	(45)	(42)	(42)	(27)	(18)		
Papillary Adenoma, Multifocal					3		3			
Papillary Adenoma					6	8	7	5		
Adenocarcinoma, Invasive					1					
ETHMOTURBINATE (NO. EXAMINED)	(47)	(47)	(45)	(45)	(42)	(42)	(27)	(18)		
Adenocarcinoma					12	5	3			
Squamous Cell Carcinoma						1				
Solid Carcinoma					1					
Carcinoma, NOS					4	1				
Sarcoma, NOS					1					
Papillary Adenoma					4	3	1			
Papillary Adenoma, Multiple					1					

NEOPLASM AND HYPERPLASIA
SUMMARY INCIDENCE TABLE

Toxicity of Inhaled 1,2-Dibromoethane
With and Without Disulfiram in the Diet

	Group I		Group II		Group III		Group IV			
M = Male; F = Female	M	F	M	F	M	F	M	F		
RESPIRATORY EPITHELIUM (NO. EXAMINED)	(47)	(47)	(45)	(45)	(42)	(42)	(27)	(18)		
Adenocarcinoma							3			
Papillary Adenoma					11	7	3	8		
Seromucous Glands/Adenoma, NOS					1					
Papillary Adenoma, Multiple					7	6	11	2		
Epithelial Hyperplasia/Dysplasia, Multifocal					20	17	7	11		
Papillary Hyperplasia, Multifocal					1					
Epithelial Hyperplasia/Dysplasia, Focal					4	3	3	3		
MAXILLARY RECESS (NO. EXAMINED)	(47)	(47)	(45)	(45)	(42)	(42)	(27)	(18)		
Papillary Adenoma						1		1		
BRAIN (NO. EXAMINED)					(1)		(4)			
Adenocarcinoma, Invasive					1		4			

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