The acute toxicity and chemical analysis of bromodichloromethane

by

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PART I. ACUTE TOXICITY OF BROMODICHLOROMETHANE

INTRODUCTION

There has been an increase in governmental and public awareness of the possible deleterious effects of low level contaminants in food, water, and the atmosphere.

Products and process by-products are being increasingly investigated for their chronic effects because of the long-term, low-level exposure of the general population.

Chlorination of drinking water, a popular water purification process used throughout the United States, has been suspected of producing by-products for many years. Because of advancements in chemical analytical techniques and sensitivity of the detecting devices, very low levels of a group of chlorinated and brominated compounds have been detected in chlorine-treated water or finished water.

Bromodichloromethane is one member of this family of haloforms found in chlorinated drinking water. Toxicologic data for many of the haloforms are scanty or nonexistent.

The present work provides data for the acute toxicity of bromodichloromethane in the rat, thus providing some data needed for a chronic study. Clinical signs of acute exposure in the rat to bromodichloromethane are presented as well as clinical chemical effects.

REVIEW OF PREVIOUS WORK

Chlorination of sewage as a bactericide and viricide has been used in the United States for many years, Laubusch, 1971. Free chlorine was recognized as an effective and economical germicide as compared to other disinfectants; e.g., bromine, iodine, ozone, and silver on charcoal. Since the beginning of wastewater chlorination, chemical products of the reactions between chlorine and organic compounds in the water were noted. As early as 1931, Adam reported a malodorous water condition due to chlorination of phenols in water. Ettinger and Ruchoft, 1951; Burttschell et al., 1959; Lee and Morris, 1962; Shervchenko, 1963; and Trakhtman, 1966; all reported chlorination reaction products of a variety of organic compounds.

Kleopfer and Fairless, 1963, reported the presence of trihalogenated compounds, including bromoform, in chlorinated municipal water.

Rook, 1974, showed that chlorinated drinking water consistently contained low levels of trihalogenated methane derivatives. He concluded that these trihalogenated methane derivatives, or haloforms, were formed as a result of chlorination processes acting upon naturally occurring humic substances in natural waters, rather than being added as a contaminant of the chlorine source itself. The reactions

of ammonia with aqueous chlorine, as a result of water treatment, were reported by Barnhart and Campbell, 1972.

Bellar, et al., 1974, reported finding chloroform and other haloforms in chlorinated waters at levels as high as 150 micrograms per liter (µg/l) in waters. They also suggested a mechanism for the formation of the haloforms, involving the oxidation of ethanol and the intermediate formation of chloral, (Cl_3-C-$\overset{O}{\overset{\|}{C}}$-H), leading to the production of chloroform. No evidence was given to support this hypothesis.

Bunn, et al., 1975, tested Bellar's proposed mechanism using chloride, iodide, fluoride, and bromide added to natural waters. Haloforms were produced for all halogens except fluoride, which did not oxidize.

After initial discovery of haloforms in finished water, research turned to area surveys and water source analysis. A joint Federal/State survey in 1975 of 83 city water supplies found haloforms in concentrations from less than 1 to 366 µg/l.

Other surveys, Bush, et al., 1976, and Nicholson and Meresz, 1975, indicated similar levels of chloroform, carbon tetrachloride, bromodichloromethane, and other unidentified compounds present in treated drinking water.

The discovery of the haloforms in drinking water was a result of increasingly sensitive detection methods available

to the analyst. Methods were sensitive to parts-per-million (ppm) and parts-per-billion (ppb) concentration levels in water, yet little was known of their toxicologic significance to human health. Some of the compounds of interest or related compounds had been investigated for their acute toxic properties, Table I-1.

Much of the early work reported involved industrial solvents, airborne vapors, industrial pollutants and volatile anesthetics, particularly chloroform ($CHCl_3$), and carbon tetrachloride (CCl_4).

Early work by Kimura et al., 1971, reported lethal dose values resulting in 50% population mortality, (LD_{50}), in various age rats for 16 industrial solvents. Included were chloroform, 1314 milligrams per kilogram body weight (mg/kg), and methylene chloride, 2136 mg/kg body weight, both in young rats by oral gavage. Kutob and Plaa, 1962, using mice and a subcutaneous injection route of exposure, calculated LD_{50} values of 6455 mg/kg body weight for methylene chloride, 3738 mg/kg body weight for methylene bromide, 3283 mg/kg body weight for chloroform, 1819 mg/kg body weight for bromoform, 30.76 grams/kg body weight for carbon tetrachloride, and 298 mg/kg body weight for carbon tetrabromide.

Thompson et al., 1974, found oral LD_{50} values for chloroform, 1060 mg/kg body weight undiluted and 1280 mg/kg

Table I-1. Toxicity data of haloforms and related compounds

U.S. Department of Health, Education and Welfare, 1974

Compound	Parameter	Species	Route	LD Value
$CHCl_3$	LD_{50}	Rat	Oral	300 mg/kg
	LD_{Lo}	Mouse	Oral	2400 mg/kg
	LD_{Lo}	Dog	Oral	1000 mg/kg
$CHBr_2Cl$	No data currently available			
$CHBrCl_2$	No data currently available			
$CHBr_3$	LD_{50}	Mouse	Subcu	1820 mg/kg
CCl_4	LD_{Lo}	Dog	Oral	1000 mg/kg
CBr_4	LD_{Lo}	Rat	Oral	1000 mg/kg
CH_2Cl_2	LD_{Lo}	Dog	Oral	3000 mg/kg
CH_2Br_2	LD_{50}	Mouse	Subcu	3738 mg/kg
$ClCH_2CH_2Cl$	LD_{Lo}	Hamster	Oral	845 mg/kg
	LD_{50}	Rat	Oral	680 mg/kg
	LD_{Lo}	Mouse	Oral	600 mg/kg
	LD_{Lo}	Dog	Oral	2000 mg/kg

body weight when diluted in corn oil, using young female rats.

Torkelson et al., 1976, determined an acute oral LD_{50} of 2000 mg/kg body weight for chloroform in young male rats.

Bowman et al., 1978, reported oral LD_{50} values in male mice of 1120 mg/kg body weight for trichloromethane, 450 mg/kg body weight for bromodichloromethane, 800 mg/kg body weight for dibromochloromethane, and 1400 mg/kg body weight for tribromomethane. They found higher values in groups of females for every compound.

Butler, 1961, and Slater and Sawyer, 1971, both concluded that metabolism of the haloform by the enzyme systems was a prerequisite to their toxicity. They suggested that the haloform's toxicity depended directly on the bond-dissociation energy of the halogen involved. This was given as the reason for increased toxicity of bromo-haloforms compared to chloro-haloforms.

Kutob and Plaa, 1962, reported chloroform, bromoform, carbon tetrachloride and carbon tetrabromide to be potent hepatotoxins, and that acute ethanol intoxication increased mice susceptibility to chloroform-induced liver damage by increasing liver lipid and thus exposing liver cells to an increased amount of the haloform.

Thompson et al., 1974, concluded that no teratogenic effects were produced by chloroform at any dose tested in

rabbits or rats. However, reduced birth weights were noted only in the highest dose groups receiving 20 or 50 mg/kg body weight per day. They also found marked hepatotoxic and nephrotoxic action with gastric erosions at levels of oral exposure or 316 mg/kg body weight/day. Renal changes were characterized by tubular cell swelling, fatty degeneration, necrosis, and marked cast formation. Mild centrilobular hydropic and fatty degeneration were observed in the liver.

Schwetz et al., 1974, reported that 100 or 300 ppm of inhaled chloroform for 7 hours/day on days 6 through 15 of gestation produced a high incidence of fetal resorption and retarded fetus development in rats.

Koch et al., 1974, supported earlier workers, Butler, 1961, and Slater and Sawyer, 1971, in their conclusion that the halogen bond energy was directly related to the haloform's acute toxicity but noted that trichloromethane did not fit the pattern as expected and might have extrahepatic sites of action.

Timms and Moser, 1975, presented their work and others, Stahl et al., 1966, and Hall and Hine, 1966, with accidental and deliberate haloform poisoning in humans resulting in diffuse pulmonary damage.

Torkelson et al., 1976, reported skin hyperemia and exfoliation upon dermal application of chloroform to rabbits. Rats exposed to 85 ppm chloroform by inhalation for 144 days,

(7 hours of exposure per 24 hours), had normal serum pyruvate transaminase (SGPT), blood urea nitrogen (BUN), and serum alkaline phosphatase (ALK. PHOS.) levels, despite marked central lobular degeneration of the liver.

Ahmed et al., 1977, studied the evidence to support the hypothesis that the haloforms are metabolized to carbon monoxide via a cytochrome P-450-dependent mixed function oxidase system, and also found the degree of metabolism was related to the bond dissociation energy of the halide involved.

Bowman et al., 1978, found fatty infiltration, pale kidneys and hemorrhages in the brain, lungs, and adrenal glands following oral exposure in mice to trichloromethane, bromodichloromethane, dibromochloromethane and tribromomethane.

Brown et al., 1974, concluded that chloroform hepatotoxicity occurred when haloform exposure significantly reduced the glutathion levels in the liver to the point that continued exposure produced free radicals, which covalently bound the microsomal protein. Also, it was suggested that an autocatalytic lipoperoxidative reaction led to cellular necrosis (destruction of phospholipid-rich intracellular membranes), and triglyceride accumulation.

Docks and Krishna, 1976, found no glutathion reduction after exposure to carbon tetrachloride and

bromotrichloromethane. They concluded that glutathion depletion must be due to formation of metabolites other than the trichloromethyl free radical.

Finally, Roe, 1976, and researchers at the National Cancer Institute, 1976, identified chloroform and trichloroethylene as carcinogenic in at least one species, and several other haloforms were considered prime suspects.

Morris, 1975, presented an excellent review of aqueous chlorination chemistry. Chlorine chemistry in water involved reactions of hypochlorite rather than the chlorine itself because of an almost instantaneous and complete hydrolysis according to eq. I-1.

$$Cl_2 + HCO_3^- \rightarrow HOCl + Cl^- + CO_2 \qquad \text{(I-1)}$$

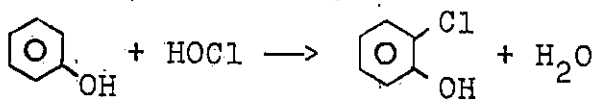
$$HOCl \rightleftarrows H^+ + OCl^-$$

The hypochlorite will participate in four principal types of reactions:

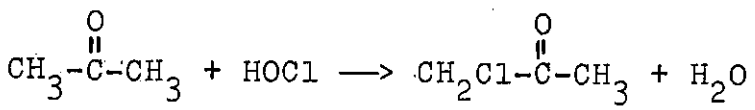
(1) addition to olefinic bonds

$$R_1\text{-}CH{=}CH\text{-}R_2 + HOCl \longrightarrow R_1\underset{Cl}{CH}\text{-}\underset{OH}{CH}\text{-}R_2$$

(2) activated ion substitution



or



(3) oxidation with reduction of the hypochlorite to chloride

$$R\text{-}\overset{O}{\overset{\|}{C}}\text{-}H + HOCl \longrightarrow R\text{-}\overset{O}{\overset{\|}{C}}\text{-}OH + H^+ + Cl^-$$

(4) and substitution of chlorine for hydrogen in a nitrogen atom, as reported by Barnhart and Campbell, 1972.

$$NH_3 + 3\ HOCl \longrightarrow NCl_3 + 3\ H_2O + 3\ Cl^-$$

Of particular interest to haloform research is the haloform reaction, which generally occurs in alkaline solutions of acetyl-bearing compounds, $CH_3\text{-}C{=}O$, or alcohols which can be oxidized to acetyls.

By successive replacement of hydrogen with halide, mono, di, and tri halogenated compounds are formed, eq. 2-8.

$$R\text{-}\overset{O}{\overset{\|}{C}}\text{-}CH_3 \xrightarrow{k_1} R\text{-}\overset{O^-}{\overset{|}{C}}{=}CH_2 + H^+ \qquad \text{(I-2)}$$

$$R\text{-}\overset{O^-}{\overset{|}{C}}{=}CH_2 + CH_2 + HOCl \xrightarrow{k_2} R\text{-}\overset{O}{\overset{\|}{C}}\text{-}CH_2Cl \qquad \text{(I-3)}$$

$$R\text{-}\overset{O}{\overset{\|}{C}}\text{-}CH_2Cl \xrightarrow{k_3} R\text{-}\overset{O^-}{\overset{|}{C}}{=}CHCl + H^+ \qquad \text{(I-4)}$$

$$R\text{-}\overset{O^-}{\overset{|}{C}}{=}CHCl + HOCl \xrightarrow{k_4} R\text{-}\overset{O}{\overset{\|}{C}}\text{-}CHCl_2 \qquad \text{(I-5)}$$

$$R\text{-}\overset{O^-}{\overset{|}{C}}{=}CCl_2 + HOCl \xrightarrow{k_6} R\text{-}\overset{O}{\overset{\|}{C}}\text{-}CCl_3 \qquad \text{(I-6)}$$

$$R\text{-}\overset{O}{\overset{\|}{C}}\text{-}CCl_3 + OH^- \xrightarrow{k_8} R\text{-}\overset{O}{\overset{\|}{C}}\text{-}OH + CCl_3^- \qquad \text{(I-7)}$$

$$CCl_3^- + H^+ \xrightarrow{k_9} CHCl_3 \qquad \text{(I-8)}$$

K_1 is much slower than all other reaction rates and is the rate determining step for the whole haloform reaction.

Brominated products were produced depending on the ratio of chlorine to bromine in solution. Ethanol, acetaldehyde, methyl ketones, and secondary alcohols are examples of compounds subject to haloform reactions.

MATERIALS AND METHODS

Materials

Test animals

Male and female Sprague-Dawley specific pathogen free rats were purchased from the Blue Spruce Farms, Inc.[1] Ten week old animals with mean body weights of approximately 220 grams (g) for females and 320 g for males were used.

Dosing reagents

Bromodichloromethane, purity greater than 97%, was obtained from Aldrich Chemical Co.[2] Purity was verified by low temperature gas-liquid chromatography, using 0.2% Carbowax 1500[3] liquid phase on carbopack 100/120 support at 100°C.

Reagent grade propylene glycol, Baker Chemical Co.,[4] was used as a dosing vehicle. Electron-capture gas chromatographic analysis of the propylene glycol showed no halogenated compounds in detectable concentrations, i.e., less than 1 part-per-million, ppm.

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[1]Blue Spruce Farms, Inc., Altamont, New York.

[2]Aldrich Chemical Co., Milwaukee, Wisconsin.

[3]Supelco Co., Bellefonte, PA.

[4]Baker Chemical Co., Chicago, Illinois.

Feed, housing, and supplemental supplies

Wayne Lab-Blox®, a commercially available lab animal feed, was obtained from Allied Mills, Inc.[1] Feed proximate analysis values were 24% protein, 4% fat, 4.5% fiber, and all required vitamins and minerals, as specified by the National Academy of Science, 1972.

Wood shavings used for bedding were obtained from the Laboratory Animal Resources group within the Iowa State University College of Veterinary Medicine.

Stainless steel shoebox style rodent cages were used throughout the studies. Inside dimensions of the cages were 12 inches long, 12 inches wide and 7 inches deep, with overhead food and water holders.

Methods

General

The rats were acclimated for 2 weeks after receiving them. Animals were housed 2 per cage in the initial screen test and 3 per cage in the later LD_{50} test. All animals received food and water *ad lib*. Temperature, humidity, noise, and other environmental conditions were maintained at constant levels throughout the study. The animal rooms were lighted 9 hours out of each 24 hours.

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[1] Wayne Lab-Blox®, Allied Mills, Inc., Chicago, Illinois.

Animal weights were obtained using a single pan displacement balance with dampened movement. Accuracy of body weights was ±0.5 grams due to animal movement during weighing operations. Weights were obtained before dosing to determine the dose volume, and at death of necropsy.

All animals were tattooed on the tails, using indelible ink, with the study designations, their dose levels and their individual identification numbers. An example of rat number 1 from the 1600 mg/kg dose group in the initial or prescreen test would be P-16-01. The LD study was designated by L in place of P.

Solutions for dosages were prepared on the day of administration and mixed on a magnetic stirrer for at least 1 hour prior to administration.

Doses were administered by oral gavage using a curved dosing needle and disposable 3 milliliter polyethylene syringes.

The dose volume administered was based on the actual rat weight to insure equal vehicle administration as well as bromodichloromethane per gram of body weight. Every rat received 1 ml dose/110 g body weight.

Rats were lightly anesthetized by exposure to diethyl ether prior to dosing or bleeding to reduce the possibility of death from aspiration or cardiac tamponade. Both control and dose groups were treated identically.

Initial screen

Six dose groups and a control group of 3 males and 3 females each were used to initially define the range of toxicity. Doses in mg/kg body weight were 100, 300, 900, 2700 and 8100, plus a control group which received propylene glycol only. Each animal was weighed, dosed according to its weight at a rate of 1 ml dose/110 g body weight, and observed for 14 days. Animals were caged 2 per cage and the sexes were segregated.

Clinical signs were observed and noted on a special form, Form 1, throughout the study. Animals which died were necropsied immediately. Surviving rats were killed by exposure to water saturated diethyl ether and necropsied after 14 days. Samples of blood were collected by heart puncture in heparinized tubes,[1] when possible, and immediately stored at -10°C. Liver, kidney, brain and lung were collected from all rats for histologic examination. In addition, eye, stomach, adrenal gland and sciatic nerve were collected in representative animals of each group of the prescreen study. Tissues were fixed in 10% neutral, buffered formalin, processed by routine paraffin technique and embedded in Altman's paraffin mixture consisting of

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[1]Vacutainer blood collection tubes, Becton-Dickinson Co., Rutherford, New Jersey.

beeswax, and 100 parts steric acid. Seven micron tissue sections were cut. Mayers routine hematoxylin and eosin staining procedures were used.

Blood, liver, kidney, and brain were collected and stored at -10°C for later chemical analysis for bromodichloromethane. Record of collected tissues was made on Form 2.

LD_{50} study

Six groups of 9 male and 9 female rats per group were used to define the LD_{50} in the rat. Doses in mg/kg body weight were 0, 400, 800, 1600, 3200 and 6400 bromodichloromethane administered in propylene glycol. Animals were caged 3 per cage. Dosing was again done by weight, 1 ml/100 g body weight, and observation was maintained for 14 days. Necropsy and sampling procedures were as previously defined. Specimens were collected and stored at -10 C for later chemical analysis or fixed in 10% neutral, buffered formalin and processed as previously described. Record of collected tissues was entered on Form 2.

Data collection

Individual animal data and observations were collected and recorded on Forms 1 and 2. The forms for

prescreen rat number 1, dose level 900 mg/kg, are set out herein identified as Forms 1 and 2.

Blood chemistry methods

Two groups of 9 female Sprague-Dawley rats were acclimated, housed, fed and cared for according to procedures already described. One group was dosed with 1200 mg/kg bromodichloromethane in propylene glycol. The other group served as a control group and received propylene glycol only. BUN was chosen to monitor renal function. SGPT, ALK. PHOS. and serum albumin were used to monitor hepatic functions.

Initial blood profiles for the 4 tests were conducted in all animals prior to dosing. A second survey was conducted at 48 hours post dose administration when signs were evident in the majority of the dose group. A final survey was conducted on the surviving animals on the 14th day post dose. Each survey included both the control and dose group.

Blood was collected by heart puncture using a 22 ga. disposable needle and a 3 ml disposable syrings. The blood was immediately transferred to a capped test tube and allowed to clot. The serum was separated from the clot with centrifugation. Clinical chemistry analyses

Experiment Pre LD_{50} Screen

Animal No. P 9 1 Ear Tag No. Tattoo No.

Body Tag No. Other Identification

Species Rat Breed Sprague Dawley Sex ♀ Age ≤ 11 wks

Initial Weight 225 Other Observations

after = 234

Date	TREATMENT, OBSERVATION, DISPOSITION (All Entries Dated & Signed)
10/17	12:00 noon dosed with 900 mg/kg $BrCl_2CH$ in propylene glycol
10/17	2:00 pm no signs
10/17	4:00 pm no signs
10/17	10:00 pm "
10/18	8:00 am "
10/18	12:00 noon "
10/19	5:00 pm "
10/19	8:00 am no signs at all
10/20	8:00 am "
10/20	12:00 noon a bit twitchy, nervous, touch [redacted], hind feet and tail rapid breathing, looks kind of staining
10/21	8 am same
10/22	8 am better, little scruffy.
10/24	8 am no signs "
10/25	12 noon " "
10/26	" "
10/27	" "
10/28	" "
10/29	" "

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Form 1

Form 1 was used to record daily observations for each individual rat.

ANIMAL NECROPSY FORM

Toxicology Section; Veterinary Diagnostic Laboratory; IOWA STATE UNIVERSITY, Ames, Iowa

Proj. Code	Test Type	Repl.	Species	Grp.	Animal Id.			Date				Date 10/29
P			9		1							
1	2	3	4	5	6	7	8	14	15	16	17	

Veterinarian(s) _____ Technician(s) Walt Hyde

Tissue Code 9 10 11 12 13	Histo.	Chem.	SYSTEM: tissue	Wt.	Tissue Code 9 10 11 12 13	Histo.	Chem.	SYSTEM: tissue	Wt.
			INTEGUMENT					UROGENITAL	
			hair			✓	✓	kidney	
			skin					ureter	
			fat (subcutan.)					bladder	
			lymph nodes					urine	
			MUSCULOSKELATAL					testicle	
			bone					epididymis	
			joint					ductus deferens	
			muscle					prostate	
			RESPIRATORY					bulbourethra	
			nasal cavity					seminal vesicles	
			larynx					penis	
			trachea					prepuce	
			bronchi					ovary	
			pleura					oviduct	
	✓		lungs					uterus	
			thoracic cav.					cervix	
			lymph nodes					vagina	
			DIGESTIVE					vulva	
			oral cavity					mammary gland	
			tongue					CARDIOVASCULAR	
			teeth					blood, EDTA	
			salivary gland				✓	blood, Heparin	
			pharynx					blood, clot	
			esophagus					heart	
			abdominal cav.					spleen	
			peritoneum					artery	
	✓		stomach			✓	✓	vein	
			duodenum					thoracic duct	
			jejunum			✓	✓	NERVOUS	
			ileum			✓	✓	cerebrum	
			cecum					basal gang.	
			colon					cerebellum	
			rectum			✓		medulla	
			anus					spinal cord	
		✓	pancreas					cranial n.	
	✓		liver			✓		nerve sciatic	
			fat (abdominal)					special sense eye	
			lymph node			✓		ENDOCRINE	
			feces					adrenal	
								thyroid	
								thymus	
								pituitary	

Record GROSS and HISTOPATHOLOGICAL observations on reverse side. (2 of 2)

Form 2

Form 2 was used to record tissues collected from each rat.

were conducted immediately using an American Instrument Co. Rotochem® [1] analyzer.

A Student "t" test was used to determine if there was a significant difference between the 2 groups of blood data, or if they actually belonged to the same population. A certainty condition minimum was set at the 95% confidence level, p=.05.

Statistical methods

Several methods of analysis were available for calculating the LD_{50} of bromodichloromethane.

The dose level, the number of animals per group and the number dying during the 14 day observation period, were used in the method of Litchfield and Wilcoxon, 1949, and also in the statistical analysis system[2] computer program available at the Iowa State University Computation Center through the Analysis Of Variance (ANOVA) procedures and the probit procedures (PROC PROBIT).

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[1] American Instrument Co., Rotochem Analyzer,® Silver Springs, Maryland.

[2] PROC PROBIT and ANOVA are operating at Iowa State University within the Statistical Analysis System under license from the SAS Institute Inc., P.O. Box 1006, Raleigh, NC.

RESULTS

Clinical Results

Clinical signs observed in the 8100 mg/kg initial screen animals commenced within an hour of dosing (0 hr). At 1 hr post dose, animals had loss of coordination with lessened response to external stimuli. Dosed rats lost eye color compared with the control rats. The dosed rats rapidly became moribund, but retained some muscle tone. There was no response to external stimuli. The next phase was signaled by the presence of a completely limp body. Respiration went from normal at 0 hr to very rapid and shallow at 1-2 hrs post dose. Breathing further slowed after 2 hrs post dose to a very slow dyspnic breathing just prior to death.

The 2700 mg/kg dose group in general were sluggish and incoordinated at approximately 2 hrs post dose. They were totally debilitated with no response to external stimuli at 4 hrs post dose. Very rapid shallow breathing was noted at 10 hrs post dose. Survivors had lessened clinical signs. Sluggishness and breathing both improved from 1-2 days post dose until the end of this study.

The 900 mg/kg group had first signs 3 days post dose. They appeared nervous with rapid breathing. They seemed somewhat depressed and developed very rough hair coats.

Petechial hemmorhages were present in the foot pads of some of the rats. The rat's general appearance improved after 4 days. Some rats had red, irritated, swollen snouts and oral mucous membranes.

The 300 mg/kg group had very mild clinical signs 3-4 days post dose. Some had slightly pink oral mucous membranes and feet and others developed a rough, uncared-for appearance.

Both the 100 mg/kg group and the control group had no clinical signs throughout the 2 week trial.

Gross lesions were observed during necropsy in some of the rats receiving higher dose levels. Livers were dark brown and the subcapsular surface of the kidneys was mottled dark brown. There was a general loss of color in the lungs of the dosed rats to a pale flesh color at death compared to lungs of rats in the control group. Subdural hemorrhage was noted in many rats of the 3 highest dose groups. Stomach contents varied from full of feed to empty, with the animals dying early generally having the fullest stomachs. Many of the 2 or 3 day fatalities had empty, contracted stomachs. Blood was noted in the urine of a few 1600 mg/kg dosed rats.

Signs observed in the LD_{50} study were nearly identical to the initial study at the various dose levels. One discrepancy appeared at 4 days in several of the 800 and 1600 mg/kg male rats. During the night, 7 of 18 rats were

found dead wedged between the cage and lid, or were found dead on the floor. No similar instances occurred before or after in any of the studies.

LD_{50} statistical results

The LD_{50} values for bromodichloromethane in male rats by oral gavage were 1633 mg/kg body weight with confidence intervals (CI) at p=.05 of 814 to 3089 mg/kg using the SAS PROC. PROBIT procedure, and 1740 mg/kg body weight with CI of 800 to 3900 mg/kg using the method of Litchfield and Wilcoxon, 1949.

The LD_{50} value in female rats was 1504 mg/kg body weight with CI of 1116 to 2255 mg/kg at p=.05 using the SAS PROC. PROBIT procedures.

The LD_{50} of the total population, disregarding sex, was 1580 mg/kg body weight with CI at p=.05 of 1256 to 2011 mg/kg, using the SAS PROC. PROBIT procedures.

The LD_{50} of the total screen and LD_{50} study combined population, disregarding sex, was 1706 mg/kg body weight, using the SAS PROC. PROBIT system.

The ANOVA study resulted in an insignificant F value for animal weight at the time of dose and sex. Dose level received and time (hours-to-death) were both found to have significant F values at p=.05.

Clinical Chemistry Results

Results for the clinical chemistry values in the control group and dose group were compared statistically. Table I-2 summarizes the results of blood chemistry analyses at 3 sampling times: before dose, after dose at 48 hours, and at the conclusion of the 2 week observation period. Table I-3 represents the pertinent statistical results of the study.

None of the control group chemistries differed from the dose group results prior to dosing, at the p=.05 level.

BUN was affected statistically, p=.05 or lower, by the administered dose at the 48 hour testing. Neither SGPT, serum albumin, nor ALK. PHOS. showed differences between the control and dose groups at this time.

All 4 blood chemistry analyses did show a significant statistical difference between the 2 groups after 2 weeks at the p=.05 significance level.

Histopathology Results

Tissue sections were referred to a certified veterinary toxicologist[1] and were not completed. The experience and judgement necessary to evaluate histologic changes in tissues was not deemed fundamental to the present study.

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[1]Dr. W. E. Lloyd, Veterinary Diagnostic Laboratory, Iowa State University, Ames, Iowa.

Table I-2. Blood chemistry results - 1200 mg/kg dose

Time	SGPT[a]		ALK. PHOS.[b]		Albumin[c]		BUN[d]	
	Control	Dose	Control	Dose	Control	Dose	Control	Dose
	14.6	19.9	75.6	108.4	4.5	4.7 4.7	24.0	17.0
0 hr	23.2	26.5	79.8	168.0 193.0	4.5 4.8	4.4 4.2	18.8 14.0	19.0 17.4
	18.1	15.1	186.7	124.2	4.5	4.9	15.0	19.0 21.9
	15.4	14.9	142.5	93.6	4.9	5.0	15.0	17.1
	16.0	13.7	81.4	78.5	4.4	4.3	12.0	19.8
	16.3	33.9	176.8	163.0	4.2 4.3	4.7 4.5	19.0	23.7
	17.4	13.8	101.1	91.6	4.4	4.4	25.0	16.0
	13.2	21.1	176.8	142.6			15.0	16.5
	9.7	27.6	109.7	134.2				
	21.2	21.2	131.9	79.3	4.8	5.1	25.0	17.1
48 hr	47.8	47.8	130.1	104.7 116.1	4.8 4.4	4.7	19.7 25.6	18.4 16.0
	25.3 15.8	25.3 15.8	162.9 170.0	180.5 105.6	5.1 4.4	4.5	23.0	19.9
	18.0		207.5		4.6	3.8	22.3	20.1
	18.1		142.5		4.7		25.3	
	20.5							
	15.9							
	19.9							
	16.9							

[a] SGPT expressed in IU of enzyme activity.

[b] ALK. PHOS. expressed in IU of enzyme activity.

[c] Albumin expressed in g/dl.

[d] BUN expressed in mg/dl.

Table I-2. (Continued)

Time	SGPT[a]		ALK. PHOS.[b]		Albumin[c]		BUN[d]	
	Control	Dose	Control	Dose	Control	Dose	Control	Dose
	14.9	13.2	56.4	103.8	5.0	4.8	20.2	15.1
	16.4		37.3	86.3	5.3	4.7	21.8	18.4
14	17.4	12.5	78.1	73.1	5.6	4.7	22.1	20.6
days	16.9	12.2	72.6					
	14.3		50.5		5.4		18.3	
	18.9		83.5		5.1		24.4	
					5.1		20.9	
	15.6		63.5		5.5		22.9	
	12.1		48.6		5.5		22.8	

Table I-3. Blood chemistry statistical results

Dose	SGPT	ALK. PHOS.	Albumin	BUN
Predose	Not significant at p=.05 t = 1.85 with 16 df	Not significant at p=.05 t = 0.63 with 17 df	Not significant at p=.05 t = 0.75 with 17 df	Not significant at p=.05 t = 1.51 with 17 df
48 hr post dose	Not significant at p=.05 t = 0.77 with 9 df	Not significant at p=.05 t = 2.0 with 9 df	Not significant p=.05 t = 0.77 with 9 df	Not significant p=.05 t = 4.67 with 9 df dose level depressed[a]
14 days	Significant at p=.05 with 9 df dose level depressed[a]	Significant at p=.05 t = 2.7 with 9 df dose level elevated[a]	Significant at p=.05 t = 4.5 with 9 df dose level depressed[a]	Significant at p=.05 t = 2.86 with 9 df dose level depressed[a]

[a]Dose blood level was compared with control blood level.

The histologic examination was not complete but will be presented in other publications.

DISCUSSION

The rats were divided into groups, dosed, and observed. A model describing the relationship between effect and dose was formulated. The model also included other variables, e.g., sex and dosed body weight.

Equation 9 outlined the suspected relationships between effect (dead or alive), and the variables, sex, weight, and dose.

$$\text{Effect} = \text{dose factor (dose level)} + \text{sex factor (sex)} + \text{weight factor (wt)} \quad \text{(I-9)}$$

For statistical analysis, equation 9 became equation 10

$$y = u + d_i + b\,(wt_i - wt) + c\,(sex) + e \quad \text{(I-10)}$$

where y_i = observed or predicted effect

u = overall mean

d_i = effect due to dose level

$b\,(wt_1 - wt)$ = effect due to different animal weights

$c\,(sex)$ = effect due to different sexes

e = error in model

If each sex was considered separately in a group then eliminating c (sex) was justified and equation 11 was justified:

$$y_{ij} = u_j + d_{ij} + b\ (wt_{ij} - wt_j) + e_j \qquad \text{(I-11)}$$

In this manner, it was possible to test the significance of dose-to-effect, and weight-to-effect directly; and sex-to-effect by comparison.

Using an "F" test or analysis of variance, it was possible to examine data and conclude whether a variable was significant and necessary to the model or merely occurred randomly due to chance. Since an arbitrary boundary had to be chosen for what was significant and what was not, a value of P = .05 was chosen as the break-off point of significance.

Of initial interest was analysis of how randomly the animals had been assigned to the different dose groups. If the animals had been randomly assigned, each group would be expected to behave or respond similarly to an external stimuli. If, however, the groups were dissimilar by weight, breeding, or some other factor, they might have been expected to respond dissimilarly, thus affecting the significance of variable testing results. In this study, due to the nature of the breeding, weight appeared to be the most probable source of initial bias. The results of

the Analysis Of Variance (ANOVA), see Appendix, for the dependent variable, weight, between dose groups supported the hypothesis that both sexes had been randomly assigned. The males showed 9% prob > F while the females showed 80% prob > F. This disparity was attributed to the small number of observations at each dose level. Examination of the individual weights among the males showed that one group, 1600 mg/kg, had an extremely low group mean weight due primarily to 2 light weight members. The ANOVA results proved weight to be random between animals and allowed the simplified equation 12 to be used, eliminating the consideration of weight effects. The General Linear Models procedure

$$y_{ij} = u_j + d_{ij} + e_j \qquad \text{(I-12)}$$

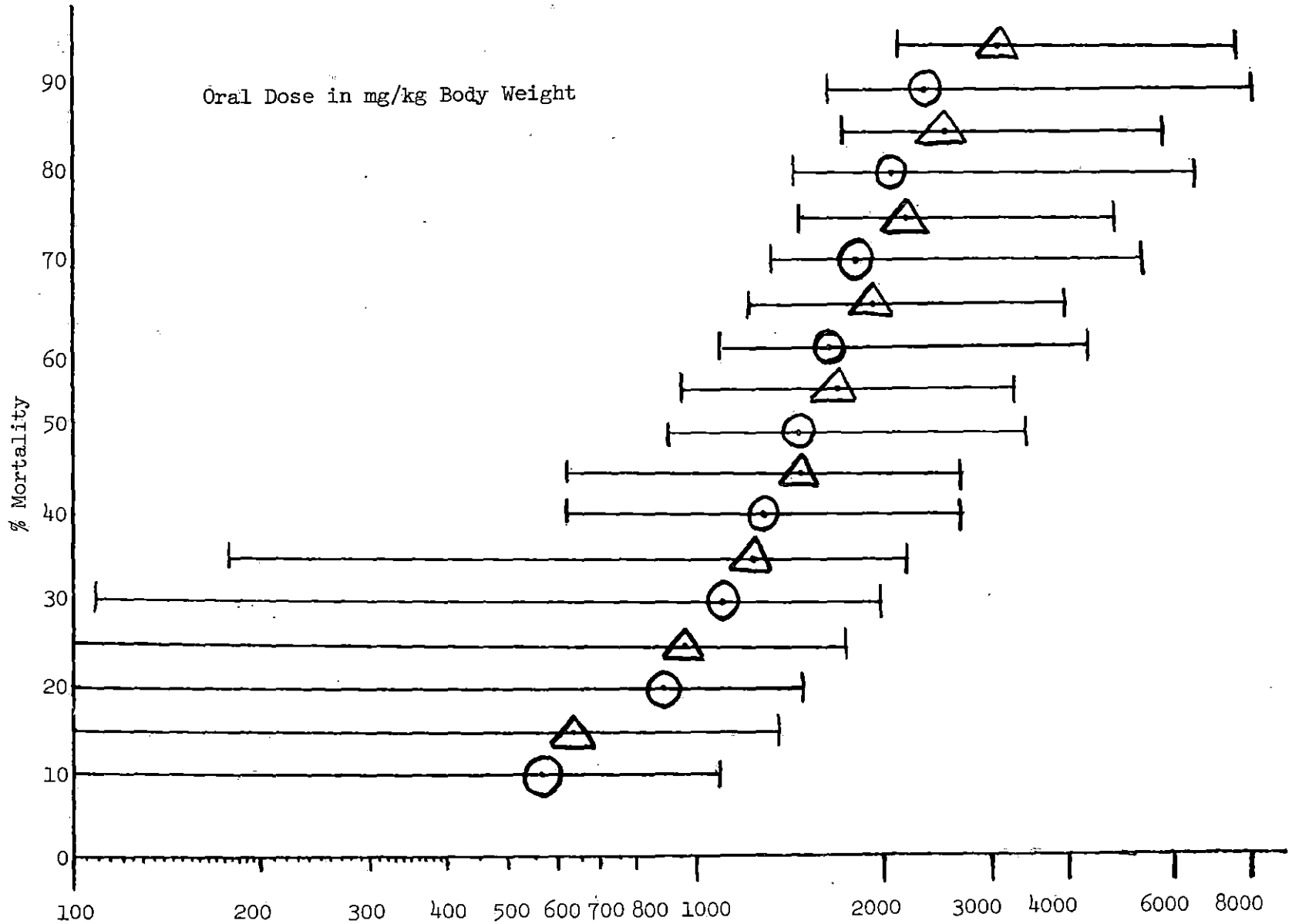
(GLM) was used to test the hypothesis that the dose was a significant variable in equation 12. The relationship between effect and dose was analyzed in 2 ways. First, the quantal, dead or alive, response versus dose level was checked. Second, the hours-to-death, (HTD), and reciprocal HTD, (RHTD), were analyzed as a function of dose level. The "F" test results, see Appendix, proved a very strong effect dependence on dose when the analysis was considered both ways. Both the F test for quantal comparison and the HTD or RHTD confirmed the hypothesis that dose was a significant variable to be considered.

The results supported equation 12. To actually define equation 12, the PROBIT procedure was used, as well as the older classical procedure of Litchfield and Wilcoxon, 1949. The PROBIT procedure used the doses, the total number of rats per dose group and the percent mortality in each dose group to produce a relationship between the mortality and dose. Since all other variables had been eliminated, this program was justified.

As discussed previously, the sexes were separated and analyzed in 2 groups. Both groups showed high dose-to-effect correlation. Figure I-1 shows mortality as a function of dose for both sexes, as calculated by the PROBIT procedure, SAS. The graph illustrates the overlap of confidence intervals of LD values for the two sexes. It would be very difficult indeed to conclude that there were sex differences in dose-effect from this data due to the extremely small numbers of observations taken, and the correspondingly large confidence interval values. Larger groups of animals would produce finer definitions of the confidence intervals of the functions allowing a valid conclusion of whether there were sex differences. The PROBIT procedure calculated the LD_{50} values to be 1633 mg/kg body weight for males and 1504 mg/kg body weight for females. The confidence intervals were ±69% and ±88% of the LD value. The manual method for Litchfield and Wilcoxon, Appendix,

Figure I-1. Mortality versus dose

Note the almost complete overlap of male -⊙- and female -△- confidence intervals, making differentiation between sexes difficult


Oral Dose in mg/kg Body Weight
% Mortality
90
80
70
60
50
40
30
20
10
0
100
200
300
400
500
600
700
800
1000
2000
3000
4000
6000
8000

produced an LD_{50} for males of 1740 mg/kg body weight with CI of ±43%. The manual method for females was seriously hampered due to limited data between 0 and 100 per cent mortality. The PROBIT procedure was used on the data as a whole, disregarding sex, since it was shown to be an insignificant factor. The LD_{50} in 10 week old Sprague-Dawley rats was 1581 mg/kg with CI of +21%,-28% of the LD_{50}.

Lastly, the time effect of dosing bromodichloromethane was of interest. What effect, if any, did the dose level have on how fast death occurred? Figures I-2 and I-3 illustrated per cent mortality in each dose group as a function of time. Clearly, there was a relationship and using the GLM procedures, its significance was tested. Both hours-to-death and reciprocal hours-to-death resulted in an F value supporting the hypothesis that there was a high positive correlation between dose and death. This relationship was predicted in Figure I-4 for acute oral exposures using average hours-to-death. Again, due to the small nature of the present study, confidence intervals were large on these values. However, the least square means agreed excellently with the actual observations, Table I-4.

The results of the clinical chemistry analyses did not support a conclusion that bromodichloromethane was acting as either a hepatotoxic or nephrotoxic agent. The statistical

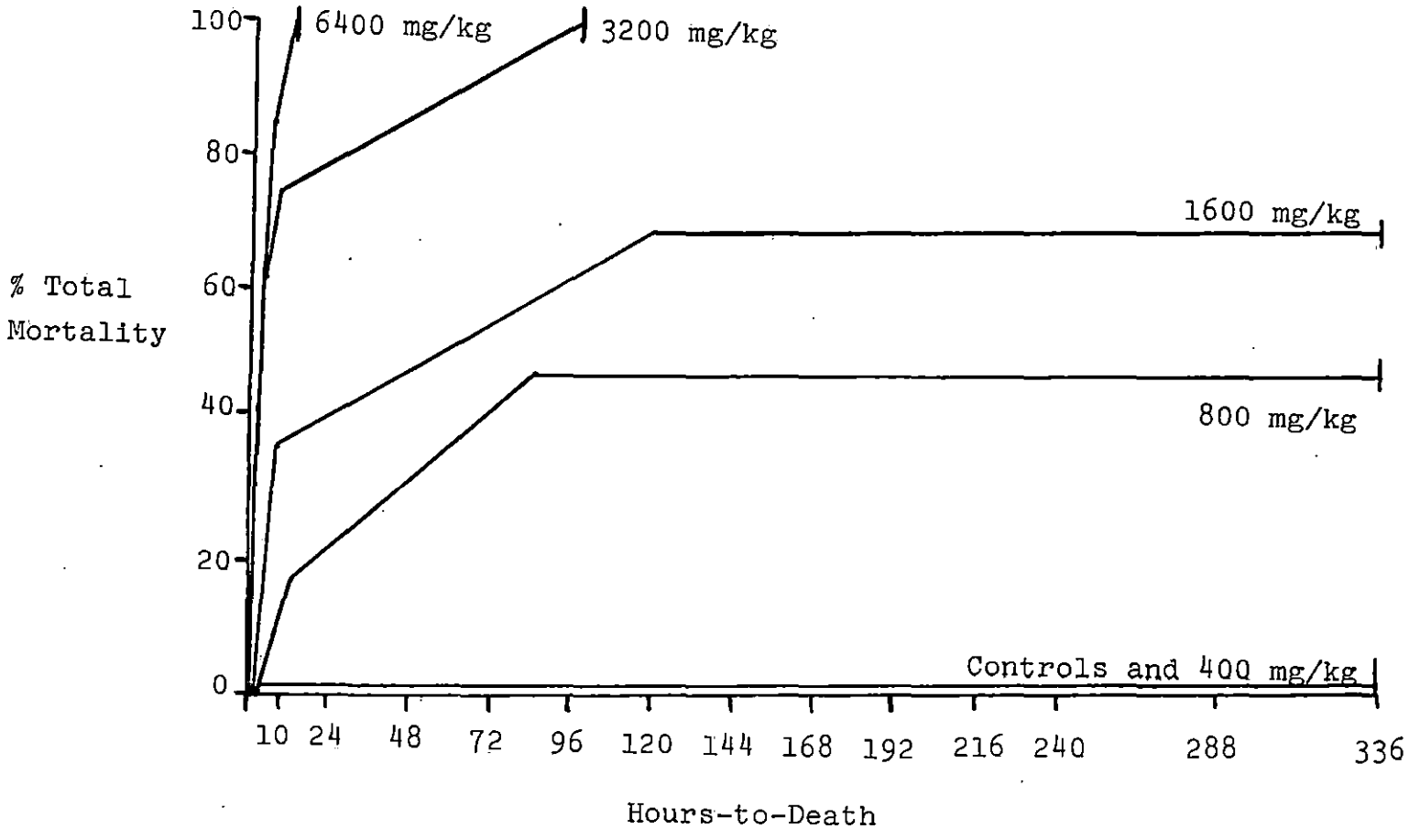


Figure I-2. Observed mortality rate as a function of dose for females. Each line represents a dose's actual mortality rate as a function of time. Note that all effects of a single oral dose seem to display themselves by 120 hours. The study was terminated at 336 hours

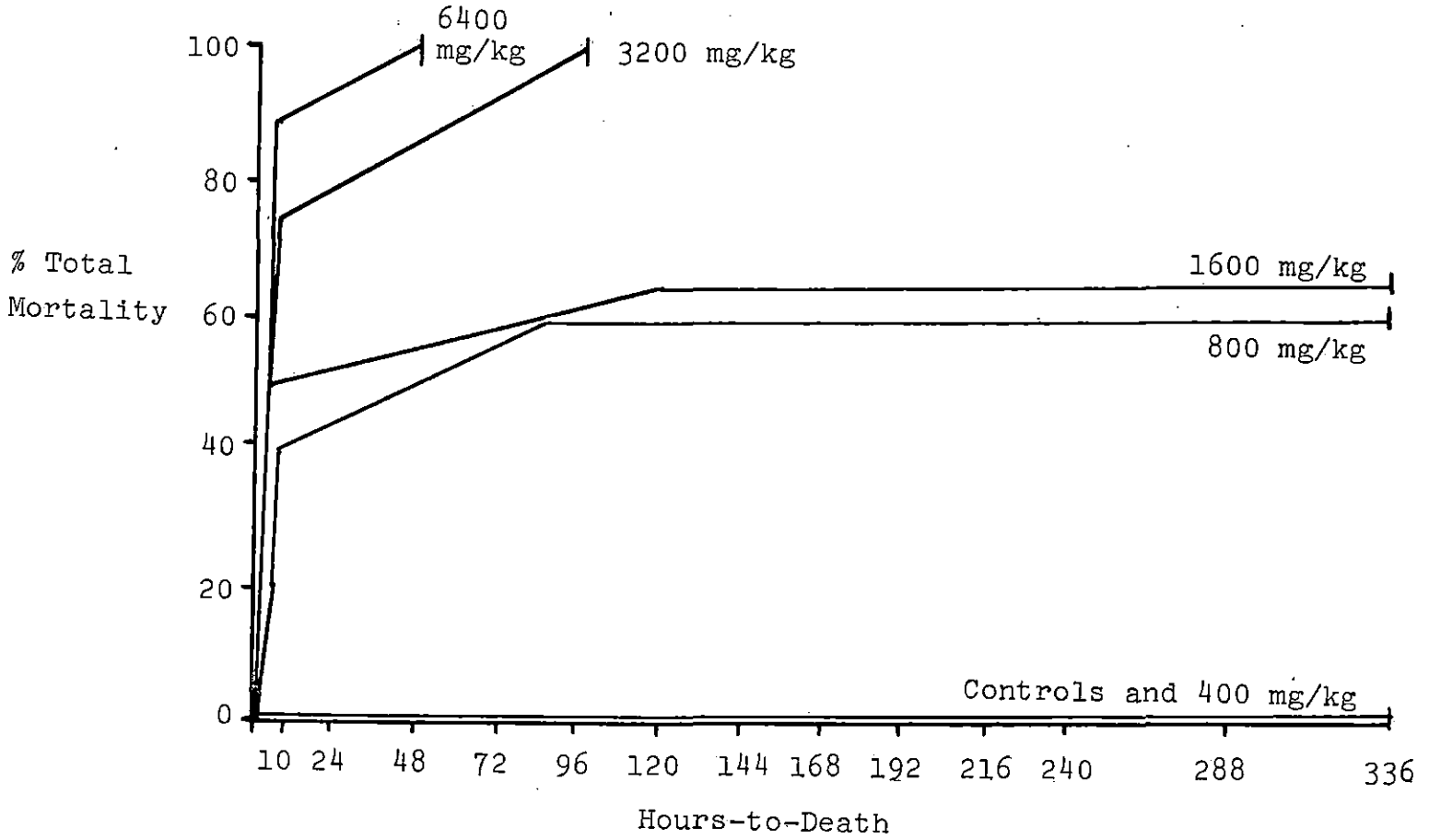


Figure I-3. Observed mortality rate as a function of dose for males. Each line represents a dose's actual mortality rate as a function of time. Note that all effects of a single oral dose again seem to display themselves by 120 hours. The study was terminated at 336 hours

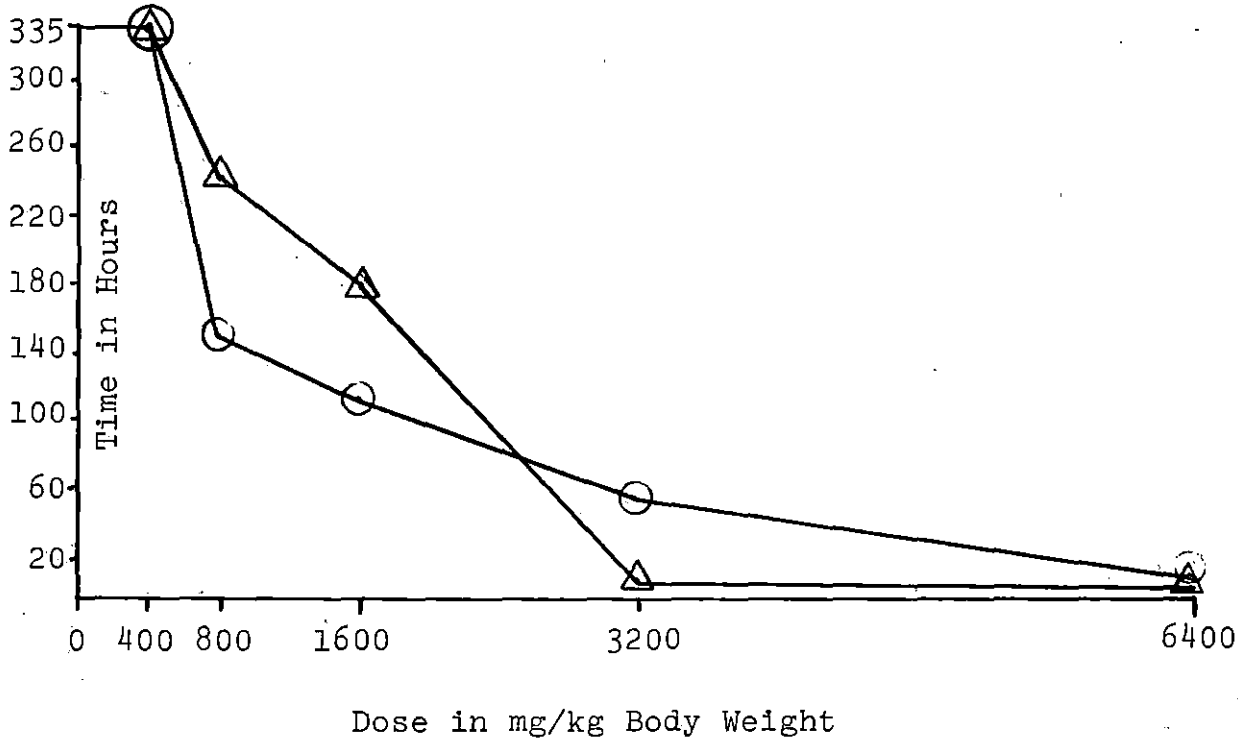


Figure I-4. Predicted hours-to-death

Circled data ⊙ reflects HTD in males

Triangled data △ reflects HTD in females

Table I-4. Comparison of calculated least square and observed hours-to-death (HTD)

Dose	Sex	HTD Observed	Least Square HTD Calculated
0	F	336.00	324.19
	M	336.00	338.42
400	F	336.00	329.75
	M	336.00	335.26
800	F	155.00	157.88
	M	245.33	243.75
1600	F	118.33	135.31
	M	183.44	182.87
3200	F	58.56	64.77
	M	11.33	10.21
6400	F	12.67	4.62
	M	8.67	10.72

Student "t" tests between the dosed group results and the control group results showed statistically significant differences in all 4 blood chemistry analyses, p=.05, at 2 weeks post dose, Table I-3. However, the control group blood levels did not differ from the dose group levels enough to be of diagnostic significance.

Compiled data by Melby and Altman, 1974, were used to determine that blood chemistry results in the control groups were within normal ranges as reported by other workers. All 4 blood chemistry analyses were in good agreement with workers cited by Melby and Altman.

ALK. PHOS. was used as a monitor for liver injury. The results of the clinical chemistry analyses for ALK. PHOS. showed a statistical difference between the dosed group and control group at 48 hrs and 2 weeks post dose. However, the average blood ALK. PHOS. levels differed by only 20% between the 2 groups. Since ALK. PHOS. levels generally fluctuate more than this in normal healthy populations, no clinical significance was attributed to the findings.

SGPT was also used as a liver injury monitor. SGPT differed statistically at 2 weeks from the control group but not at 48 hrs post dose. However, again the differences between the 2 groups were within the normal ranges expected in healthy individuals. Control and dosed group chemistry levels were in agreement with data collected by Melby and

Altman, 1974, for normal animals, and thus showed no evidence of a hepatotoxic mode of action.

Serum albumin was also used as a monitor of liver injury. Serum albumin levels were statistically different between the two groups but the difference again was not considered to be of diagnostic significance. Therefore, serum albumin results did not indicate hepatotoxic injury.

BUN was used as a monitor of renal function. BUN in blood differed statistically at both 48 hrs and 2 weeks post dose from the control group levels. However, the values obtained all fell well within normal ranges and were not considered diagnostic of a nephrotoxic mode of action.

The clinical chemistry data were limited. Because of the size of the groups and the health of the dose group during the last 2 blood samplings, the number of samples available for chemistry and statistical analysis was smaller than originally hoped. Also, some difficulty was experienced in bleeding the animals without killing them. A minimum of 2 ml of nonhemolyzed blood was required to provide the necessary 1 ml of serum for the 4 blood analyses. Even after repeated attempts, to the point of endangering the animals' lives, adequate blood was not obtained in a few cases, resulting in only 3 or 4 samples for statistical analysis. As the number of data got smaller and smaller, the certainty or precision of the statistical analysis decreased

accordingly. As has been pointed out, statistical significance does not necessarily indicate diagnostic significance.

One factor of the LD_{50} study was especially perplexing. At 4 days post dose, 7 of 18 male rats in the 800 and 1600 mg/kg dose level groups were found dead. The rats had climbed halfway out of their cages and died with their bodies bent over the edge of the cage. half in, half out, or were found on the floor of the animal room. Dr. Ronald Flatt and Mr. Ronald Moses, both experienced in rodent testing, have not encountered this problem with the cage used or any breed of rat. These rat deaths were discounted in the statistical workup. The deaths may reflect behavior effects that should be considered in future studies.

SUMMARY

Acute death due to bromodichloromethane was clearly dose-related. Weights of animals in groups were found to be randomly assigned. The data were insufficient to conclude that sex contributed significantly to the model. A study involving large numbers of animals could answer this question more precisely.

There was a definite relationship between hours-to-death and dose level. Although not tested specifically, it was obvious during observation that order of appearance and severity of clinical signs were dose-related also. Clinical signs included incoordination, weakness, depressed respiration, and depressed appetite.

Post mortem examinations of dosed rats revealed pale lungs, mottled kidneys, subdural cranial hemorrhage, hemorrhage of foot pads, and dark brown livers as compared to the control group. Clinical chemistry analyses did not show a diagnostic difference between the dose and control groups and did not give evidence for a simple nepho- and hepatotoxic mode of action for bromodichloromethane. However, numbers of samples in the clinical chemistry study dwindled from 10 rats per test group to 3 or 4 per group in the 48 hr and 2 week testing due to sampling difficulties and death of the subjects. The blood chemistry study should be done with larger groups of rats over a longer period of time, possibly

a chronic exposure study. The LD_{50} for the total population was 1581 mg/kg body weight with CI of +21%, -28%. Due to the small number of animals observed, the 95% confidence intervals on the LD values are large. A study involving a larger number of subjects than in the present study is needed to seriously use the LD_{01} value from the statistical procedures. Also, the doses used should be geometrically spaced between 500 and 2500 mg/kg for a mortality spread of 5% to 95% mortality. Also, the unexplained behavior of the 800 mg/kg and 1600 mg/kg groups should be reinvestigated for possible CNS or behavior effects.

PART II: CHEMICAL ANALYSIS OF BROMODICHLOROMETHANE

INTRODUCTION

Chemical analysis of trace contaminants has been an increasing concern in recent years because of an awareness of their detrimental effects on people or animals exposed over long periods of time. Halogenated methane derivatives (haloforms) and homologs have been investigated since their discovery in drinking water by Kleopfer and Fairless, 1963. However, the majority of the chemical analysis methods have dealt primarily or solely with the analysis of haloforms in water.

Little research has been directed to the problems surrounding the analysis of the haloforms in biological tissues and fluids. The present study provides methodology for the routine extraction of bromodichloromethane from blood, liver, kidney and brain and its quantitation using gas-liquid chromatography. Interferences and recovery data for the methods are included.

REVIEW OF PREVIOUS WORK

Rook, 1972, developed a static gas head-space analysis method in which 10 ml of water were heated for 12 hrs after which the head-space gas was forced through a small trap of activated silica gel. The organic compounds were eluted from the trap and separated by gas chromatography. Rook's work was with lower alkanes, freons, chlorinated solvents and substituted benzenes.

Mieure and Dietrich, 1973, developed procedures for sampling air or water for trace organics. They relied on a salting-out effect using sodium chloride to decrease the solubility of haloforms and other organic compounds in water. They used methylene chloride to extract the less volatile components of finished drinking water.

For more volatile compounds, Mieure and Dietrich used gas stripping techniques with collection of the organics on Tenax®.[1] gas chromatographic (GC) packing in tubes. This trapping tube was then directly connected to the gas chromatograph and thermally transferred onto the GC column to be separated and identified. They also investigated the possibility of direct resin column collection using Chromosorb 102®.[1] The water sample was fed through the

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[1]Tenax® column packing and Chromosorb 102® column packing available from Supelco, Inc., Bellefonte, PA.

column packing, the packing was dried, and then the column was connected directly to the gas chromatograph and the temperature programmed.

Glaze et al., 1973, used XAD-2® [1] resin, a macroreticular resin, to extract and trap organics from large volumes of chlorine-treated sewage water. Diethyl ether or acetone was then used to elute the trapped organics from the resin column. These eluents were further concentrated for gas chromatographic separation and mass spectroscopy structural identification. Glaze's work was limited to organic phenols and other relatively nonvolatile compounds, since his concentration step lost many of the volatile components.

Grob, 1973, reported a continuous loop, recycling gas-stripping process followed by collection on charcoal. The charcoal trap was then eluted with carbon disulfide. Bellar and Sigsby, 1970, and Bellar and Lichtenberg, 1974, used gas stripping and resin column trapping to collect volatile water contaminants. Various trapping materials, silica gel, Porpak Q®, [2] Chromosorbs®, and Tenax® were compared for trapping effectiveness. Using these

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[1] XAD-2 resin, Rohm and Haas, Philadelphia, PA.

[2] Porpak^{R} column packing, Supelco, Inc., Bellefonte, PA.

techniques, Bellar, Lichtenberg, and Kroner, 1974, found unequivocal qualitative identification of chloroform, bromodichloromethane, and dibromochloromethane in laboratory tap water. Various untreated waters were tested with levels of these 3 contaminants ranging from 1 to 152 ppb.

Junk et al., 1974, proposed a method for large sample testings utilizing up to 4000 liters of water. XAD-2® resin was used as a trapping material.

Dowty, Carlisle and Laseter, 1975, used sample heating and helium gas stripping with resin collection to determine 13 halogenated hydrocarbons in drinking water and blood plasma from New Orleans, La. U.S.A. Blood plasma was collected in EDTA Vacutainer® [1] tubes to prevent clotting. Both tetrachloroethylene and carbon tetrachloride were confirmed in the plasma samples.

Kopfler et al., 1975, investigated the effects of heating water samples to achieve higher haloform recoveries. They found both time and temperature increased the concentration of haloforms in the water artificially. They suggested storing and shipping samples at 4 degrees centigrade to prevent haloform concentration changes.

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[1] EDTA Vacutainer® tubes, Becton-Dickinson Co., Rutherford, NJ.

Nicholson and Meresz, 1975, used direct aqueous injection gas chromatography to analyze the haloforms in water at levels of 10 ppm and below. Surprisingly, they reported very little degradation of the scandium tritide electron capture detector from the water injections.

Bunn et al., 1975, using the Bellar and Lichtenberg method, 1974, reported the formation of all 10 possible trihalomethanes of chlorine, bromine and iodine in laboratory tests.

Kissinger and Fritz, 1976, reported a novel approach to haloform analysis with resin collection on acetylated XAD-2 resin followed by stripping with pyridine. Chriswell, Kissinger and Fritz, 1976, showed that the pyridine solvent could be completely eliminated when injected on a copper chloride/chromosorb column. The obvious advantage is a lack of a solvent peak to interfere with the very early eluting haloform peaks. Also, Kissinger suggested ascorbic acid as a water sample preservative to half haloform concentration changes during sample shipment and storage.

Richard and Junk, 1977, used pentane for liquid extraction of water. The resulting pentane, after being dried over a suitable desiccant, could be directly injected for gas chromatographic analysis.

Kaiser and Oliver, 1976, modified the head space procedure by slightly evacuating the space before thermal

equilibration. They claimed an improved recovery, but did not address the reported problems of artificially elevated haloform concentrations.

Nicholson, Meresz and Lemyk, 1977, suggested that the direct injection technique be used to determine total potential haloform content of water. However, as the authors point out, the final concentration depends on temperature, pH, and other environmental factors. Also, total potential haloform concentrations are achieved under conditions unlikely to be encountered in nature so that potential haloform content is of doubtful significance.

Davies, 1978, used an n-heptane liquid-liquid extraction of blood to determine levels of chloroform and other organic compounds in water.

MATERIALS AND METHODS

Materials

Chemicals

Pentane, nanograde or distilled-in-glass quality, Mallinckrodt.[1]

Bromodichloromethane, purity 98%, Aldrich Chemical Company.[2]

Bromoform, purity 97%, Aldrich Chemical Company.[2]

Carbon tetrachloride, Baker Chemical Company.[3]

Carbon tetrabromide, purity 98%, Aldrich Chemical Company.[2]

1,2-Dibromomethane, purity 95%, Aldrich Chemical Company.[2]

1,2-Dichloroethane, Baker Chemical Company.[3]

Chloroform, Baker Chemical Company.[3]

Methylene Chloride, Baker Chemical Company.[3]

Sodium sulfate, Baker Chemical Company.[3]

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[1]Mallinckrodt Chemical Company, St. Louis, MO.

[2]Aldrich Chemical Company, Milwaukee, WI.

[3]Baker Chemical Company, Chicago, IL.

Equipment and instrumentation

Packard model 427 gas liquid chromatograph with a nickel-63 electron capture detector.[1]

Column packing: 0.2% carbowax 1500 on 80/100 mesh carbopack^{R}, Supelco Co., lot #F-13182.[2]

GC columns: 2 meters in length, glass.

Graduated, glass-stoppered 15 ml Pyrex® extraction tubes.[3]

Sorval® high-speed blender/homogenizer, DuPont Company.[4]

Glass wool, prewashed or preextracted.

Disposable pipets, Fisher Chemical Company.[3]

Glass tissue grinder with gas-stripping modifications, Figure II-1.

Stainless steel resin traps, Figure II-2.

Thermal desorption unit, Figure II-2.[5]

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[1]Packard Instrument Co., Inc., Downers Grove, IL.

[2]Supelco Co., Bellefonte, PA.

[3]Fisher Chemical Company, Chicago, IL.

[4]DuPont Instruments, Newtown, CT.

[5]J. Wilkes, Union Carbide, Chicago, IL.

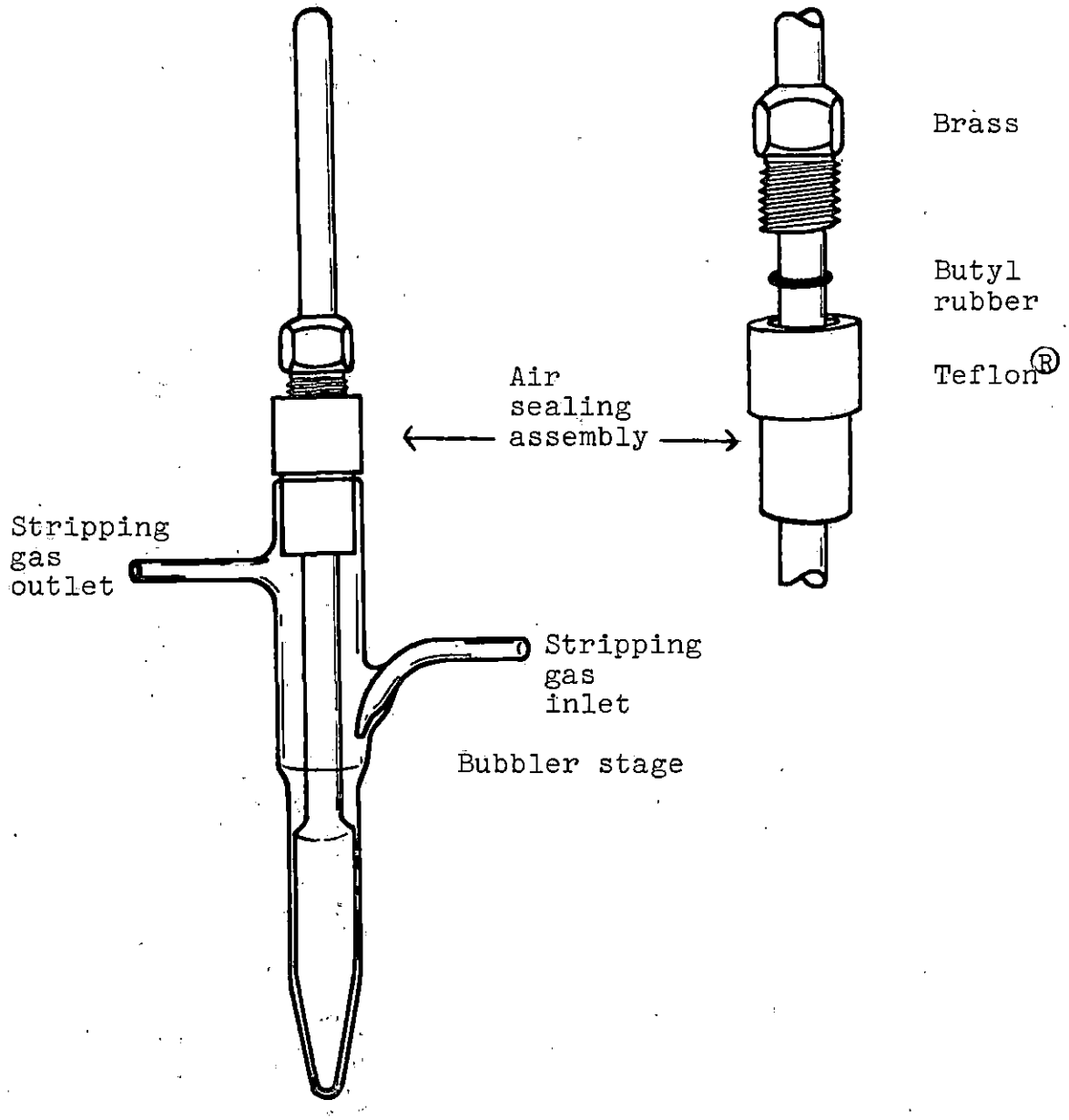

Figure II-1. Gas stripping tissue grinder[1]

Tissue samples were ground to a suspended aqueous slurry in the modified grinder. Nitrogen gas bubbling through the slurry removed volatile organic compounds and swept them out of solution to be trapped and analyzed

[1]Kontes, Vineland, NJ.

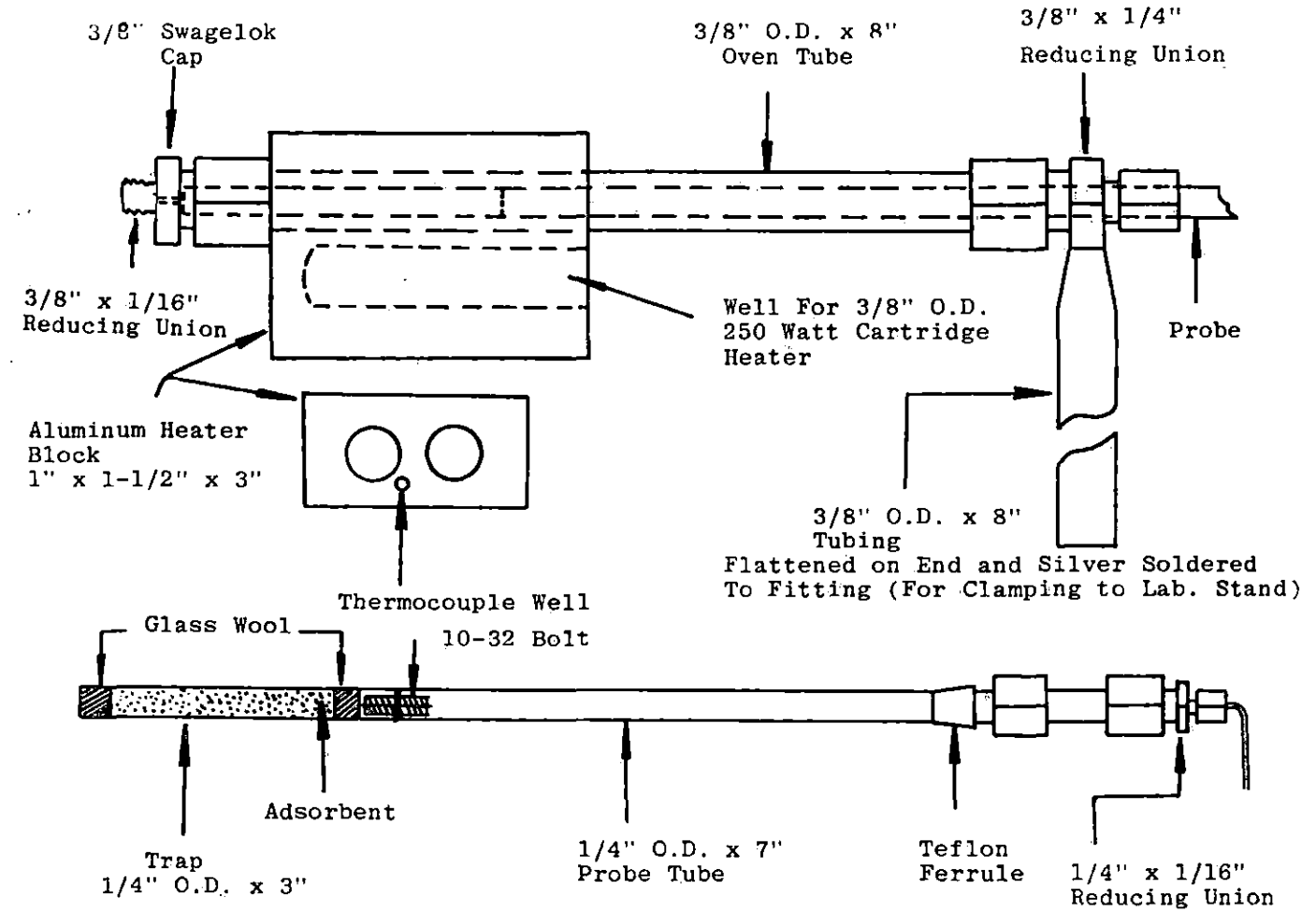

Figure II-2. Resin trap and thermal desorption apparatus

The volatile organic compounds were swept from solution into the trap where they adhered to adsorbant resin. The trap was then connected to the probe tube, lower figure. The whole assembly was placed into the oven tube as illustrated in the upper figure, for thermal stripping of the organic compounds into the GLC inlet

Methods

Analysis of biological materials for bromodichloromethane was approached in 2 ways. Each method had advantages making it appropriate in certain circumstances. Each also had disadvantages.

Liquid-liquid extraction - primary method

Collection Tissues collected during necropsy were wrapped in aluminum foil and stored at -10°C until analysis. Blood samples were heparinized in stoppered collection tubes and stored at -10°C until analysis. Because of the extremely volatile nature of bromodichloromethane, all samples were kept frozen until immediately prior to analysis.

Blood analysis at levels of 20 ppb or greater

Frozen blood samples were thawed, mixed with the aid of a vortex mixer, and sampled for analysis. One milliliter (ml) was volumetrically pipetted into a 15 ml glass-stoppered tube. Eight ml of pentane were added volumetrically and the tube tightly stoppered. The tubes were mixed for 30 minutes on a Roto-rak® [1] at approximately

[1] Roto-rak®, Fisher Instruments, Chicago, Illinois.

30 revolutions per minute (rpm). After mixing, the pentane layer was pipetted through a mini-drying column made by plugging a disposable Pasteur pipet with glass wool and filling with 5 cm of sodium sulfate, Figure II-3. This pentane extract was immediately ready for separation and analysis by GLC using electron capture detection, see GLC section.

Liver, kidney and brain at levels of 20 ppb or greater Two grams of tissue or, in the case of small animals, the whole tissue, was weighed by difference into a Sorval^{R} stainless steel tissue cup, 25 ml size. Ten ml of pentane were pipetted volumetrically into the cup and the homogenizer unit assembled. The tissue and pentane were mixed at high speed for approximately 2 minutes. The pentane layer was decanted into glass centrifuge tubes and centrifuged at 680 x g for 10 minutes. The pentane extract was then dried through a mini-drying column of sodium sulfate stoppered and stored for gas chromatographic analysis.

Gas stripping with collection - secondary method

An apparatus described by Wilkes, 1978, was built at the Iowa State University Physics Instrument Shop, Figure II-4. A Kontes® tissue homogenizer was modified

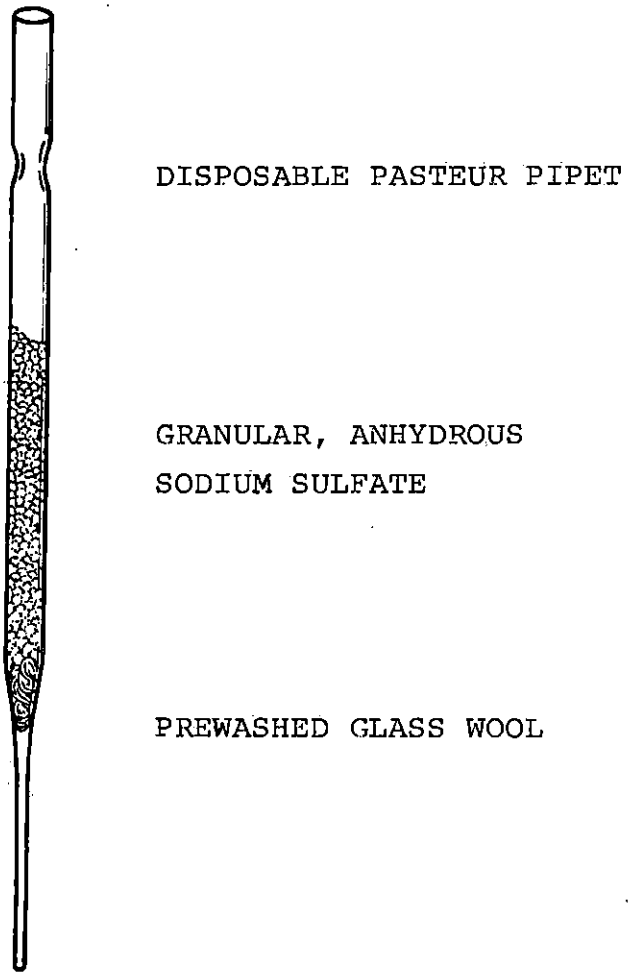

Figure II-3. Mini-drying column

The sample extract was passed through the mini-drying column to remove traces of water prior to analysis using gas chromatography and electron-capture detection

by the Iowa State University Glass Shop for gas stripping, Figure II-3.

The resin traps were filled with a mixture of 50% each Tenax®, and silica gel. These were thermally stripped before use to insure minimum background.

The sample to be analyzed was weighed into the tissue grinder. An aliquot of 2% sodium chloride solution was added and the grinder assembled to give an air-tight seal in the sample chamber. A clean resin trap was placed in-line for collection, and the nitrogen gas flow was adjusted to 20 ml per minute. The sample and the 2% chloride solution were ground together and the haloforms were bubbled out of solution and onto the trap. After collection, the trap was assembled on the thermal desorption probe, Figure II-4. The desorption unit was connected to the gas chromatograph and carrier gas was used to flush the probe free of air. Finally, the probe was inserted fully into the heater chamber to thermally strip the haloforms from the resin onto the GC column.

Gas chromatography

All the present work was done using a 2 m x 5 mm glass column and Supelco, Inc., 0.2% Carbowax 1500 on 80/100 mesh Carbopak® column packing.[1] Ultrapure

[1]All column supports were obtained from Supelco, Inc., Bellefonte, PA.

nitrogen from Matheson[1] was used at a flow rate of 40 ml/min for the GC carrier gas. The GC injector temperature was 250°C and the electron capture detector was maintained at 260°C. The column oven was operated at 110°C in most cases. Overnight conditioning of the column was accomplished at 145°C.

Hamilton® glass syringes[2] were used throughout this study for GC injection.

A 1 millivolt strip chart recorder was used to record chromatograms.

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[1]Matheson Scientific, Chicago, IL.

[2]Hamilton Company, Reno, NV.

RESULTS

Liquid-Liquid Extraction - Primary Method

General

Figure II-4 illustrates a typical gas chromatographic separation of bromodichloromethane and various related compounds, with almost complete baseline resolution of the components from each other. Carbon tetrabromide, which was not illustrated in the figure, eluted at about 90 minutes. The peaks represented 0.1 to 0.6 nanograms of haloform injected, depending on the compound.

Figure II-5 illustrates the usable sensitivity and excellent linearity over a wide concentration range obtained with the described techniques.

Blood

Blood was collected in heparinized Vacutainers® and stored at -10°C for later analysis. Studies were initiated to determine the effects, if any, of long-term storage on blood levels of haloforms under refrigeration and freezing storage conditions.

A large pooled collection of blood was sampled, sealed, refrigerated overnight, then sampled again. Table II-1 presents the data, which illustrated the rapid loss of the haloform from the stored blood.

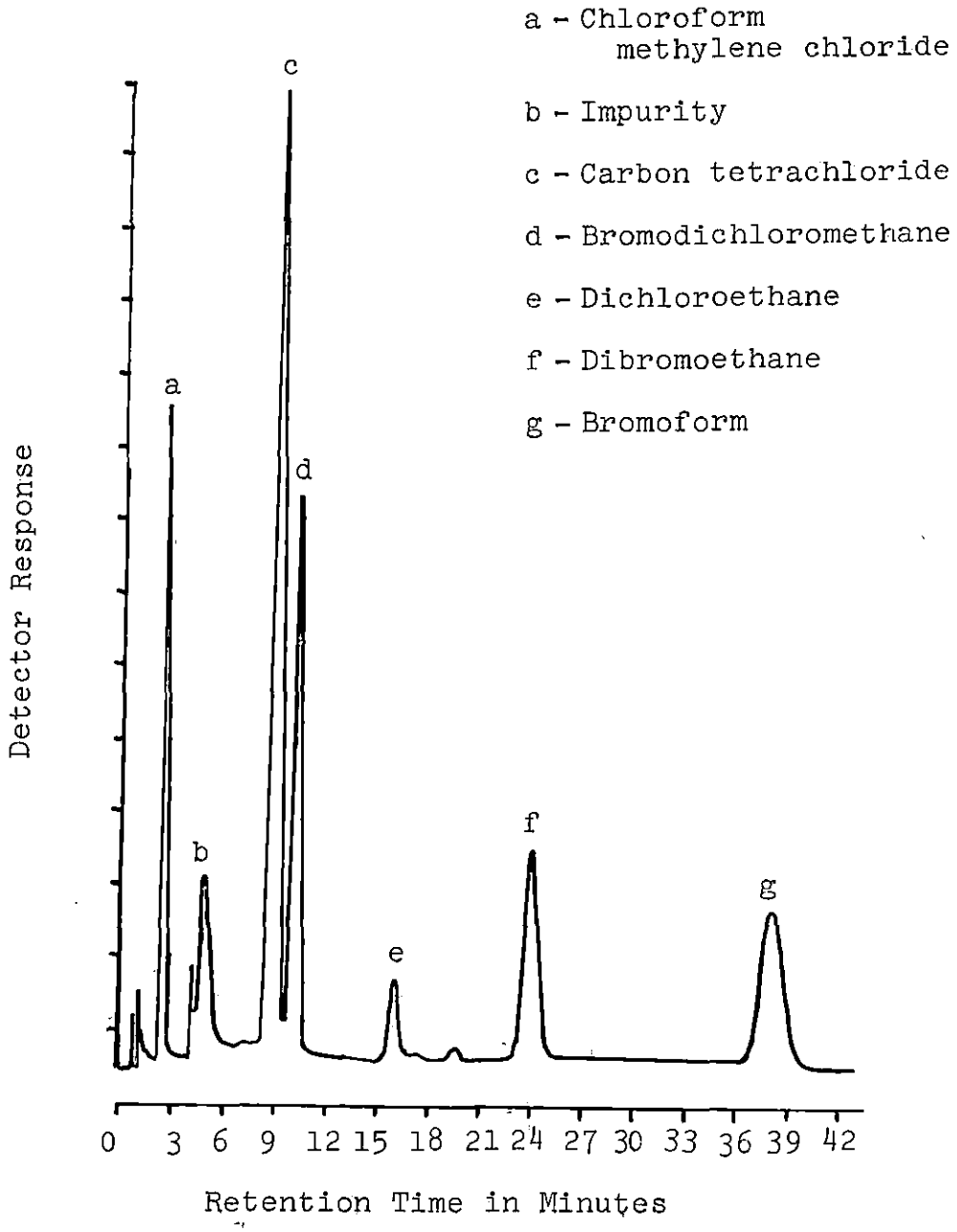

Figure II-4. Gas chromatographic separation of haloforms

2 m glass column, 0.2% Carbowax 1500 on 100/120 Carbopak, Supelco, Inc.; column temperature: 100°C, injector port and E.C. detector 285°C; carrier gas = 40 ml/min Matheson nitrogen; amounts injected ranged from 0.1 to 0.6 ng of compound

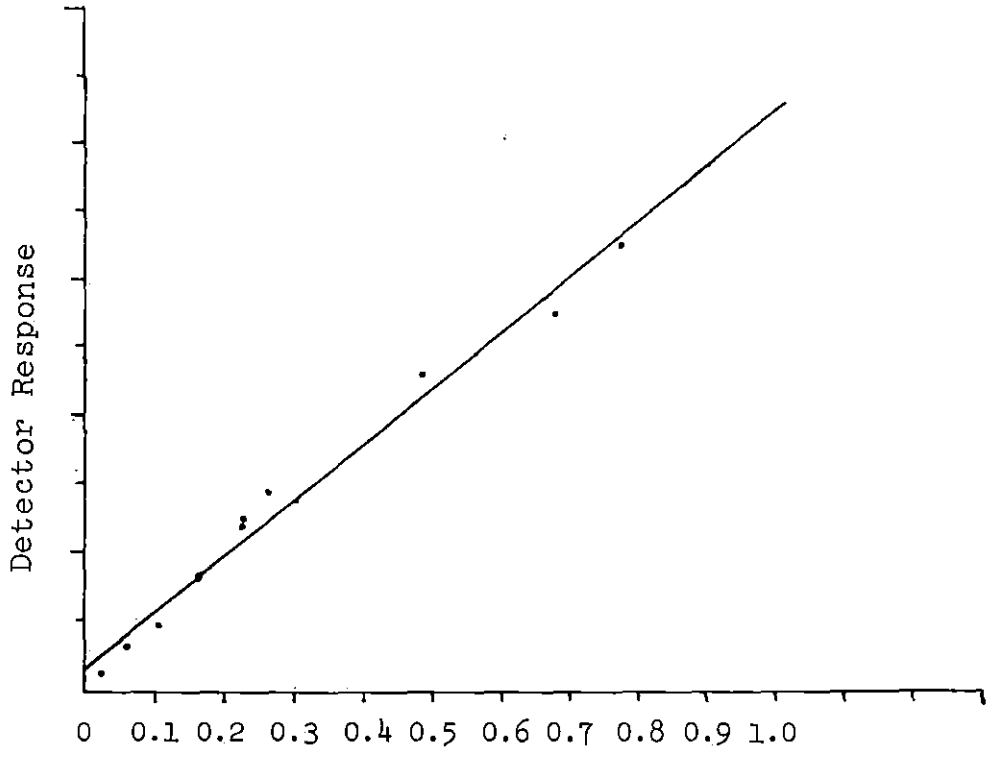

Figure II-5. Standard curve of bromodichloromethane

2 m glass column, 0.2% Carbowax 1500 100/120 Carbopak, Supelco, Inc.; column temperature 100°C, injector port and E.C. detector 285°C; carrier gas 40 ml/min Matheson nitrogen

Table II-1. Effects of refrigeration storage on $BrCl_2CH$ levels in blood

	Trial #	First day conc. (ppm)	Average (ppm)	Second day conc. (ppm)	Average (ppm)	% Change
Conc.	1	0.087		0.077		
Level	2	0.083	0.084	0.082	0.079	-6%
1	3	0.081				
Conc.	1	0.370		0.169		
Level	2	0.360	0.36	0.160	0.165	-54%
2	3	0.350				
Conc.	1	2.44		1.21		
Level	2	2.12	2.56	1.22	1.21	-53%
3	3	2.13				

Table II-2 presents data of pooled blood sampled at various times indicating that -10°C is a suitable storage method for blood up to at least 228 hrs.

The extraction method was tested using hexane, pentane, and heptane, using different ratios of blood to solvent, and using a surfactant, TX-100®,[1] to completely hemolyze the red blood cells. Pentane was chosen as the extracting solvent at a ratio of 1 ml blood to 8 ml pentane. Bromodichloromethane was added to blood at a level of 0.1 ppm with recoveries averaging 94% and a range of values from 85% to 107%, Table II-3. Blood levels of bromodichloromethane ranged from no detectable amount (NDA) in the lowest dose groups and control group to 482 ppm in some of the highest dose rats, Table II-4. No interferences or other peaks were observed in blood extract analysis from these feeding studies.

Liver, kidney, and brain Tissues were stored in aluminum foil at -10°C until analysis.

Studies were initiated to determine the effects, if any, of long-term storage at freezing temperatures on tissue levels. Levels did not change after 4 months of storage at -10°C. However, levels in samples tested after 11 months had decreased by 60 to 80% of original levels.

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[1]Triton X-100, Rohm and Haas Co., Philadelphia, PA.

Table II-2. Effects of freezer storage on $BrCl_2CH$ levels in blood

Hours storage at -10°C	12	36	60	228
Concentration in ppm	4.48 4.00 3.90	4.10 4.40 4.40	4.20 4.70	4.11 4.70 4.50
Average, $\bar{x}$	4.13	4.30	4.40	4.40
% Change in concentration	0	4.1	6.5	6.5

Table II-3. Recoveries of bromodichloromethane from biological matrices

Trials %	Matrix			
	Blood	Liver	Kidney	Brain
	93	102	86	78
	103	85	101	71
	92	107	87	102
	85	105	101	104
	92	95	78	107
	107	94	101	95
	89	110	72	75
	97	112	78	86
	99	83	88	
	91			
	89			
Average, $\bar{x}$	94	99	88	90
Standard deviation	7	11	11	14
Relative standard deviation	7	11	12	16
Range	85-107	83-112	72-101	71-107

Table II-4. Blood levels of bromodichloromethane (blood concentration ppm)[a]

Dose Level mg/kg					
0	100	300	800	900	1600
P-0-2 NDA	P-1-2 NDA	P-3-1 NDA	L-8-6 .03	P-9-1 .02	L-16-1 NDA
		P-3-2 NDA	L-8-9 NDA	P-9-3 .07	L-16-3 .04
P-0-3 NDA	P-1-3 NDA	P-3-3 .03	L-8-10 .03	P-9-4 .06	L-16-4 NDA
	P-1-4 .04	P-3-4 .05	L-8-17 NDA	P-9-6 .04	L-16-6 NDA
P-0-4 .04		P-3-6 .04			L-16-8 .07
	P-1-5 .02				L-16-14 .02
	P-1-6 .06				L-16-16 314.2
					L-16-17 .11

[a]Animal tatto coding is illustrated by an example from both the prescreen and LD_{50} groups.

P-0-2 represents number 2 rat in the 0 dose control group in the prescreen study.

L-8-6 represents number 6 rat in the 800 mg/kg dose group in the LD_{50} study.

2700		3200		6400		8100	
P-27-1	.07	L-32-2	176.7	L-64-1	391.7	P-81-5	272.0
P-27-3	108.8	L-32-5	482.7	L-64-5	280.0	P-81-15	338.0
P-27-5	215.1	L-32.6	216.1	L-64-10	406.8		
P-27-6	.09	L-32-12	241.7	L-64-14	403.0		
		L-32-16	349.3	L-64-18	313.6		
		L-32-19	414.4				

The recovery method was evaluated by adding bromodichloromethane to pooled samples of brain, liver and kidney. Table II-3 summarizes the recovery data for all three tissues and blood in detail. Liver recoveries averaged 99.2% with a range of 83% to 110%. Kidney recoveries averaged 88.0% with a range of 72% to 101%. Brain recoveries averaged 90.0% with a range of 71% to 107%.

No interferences were observed in the chromatograms of liver or kidney extracts and column integrity, as well as electron capture detector sensitivity did not seem affected by numerous injections, over an 8 hour period.

Extractions of brain samples did, however, coextract some interfering compounds which affected both column life and detector sensitivity after only a few injections. Extracts were redried over additional sodium sulfate to eliminate water as the problem with little improvement. The brain extracts were observed to be slightly cloudy compared to liver, kidney and blood extracts. Filtering with glass-fiber filter paper did not remove the cloudiness problem. Centrifugation was tried and worked very well. Centrifuging the brain extracts at 680 x g for 10 minutes cleared the extract and eliminated most of the problems experienced in the GC analysis. Detector sensitivity was still slightly affected by numerous injections over a 5 to 6 hour period. Standards were injected regularly

to monitor the detector sensitivity over long periods of time.

Tissue levels of bromodichloromethane ranged from NDA in many of the control group rats and lowest dose level groups to over 1000 ppm in tissues of a few rats in the highest 2 dose level groups, Table II-5.

Gas stripping with collection

Figure II-6 illustrates the results of the work with resin collection and subsequent thermal desorption into the GC injection port.

Limited modifications to both the desorption unit and the manner in which it connects to the Packard GC failed to improve the chromatographic separation of a mixture of the haloforms.

Figure II-6 also illustrates the lack of sensitivity due to band broadening. The amounts of the individual haloforms injected ranged from 0.1 to 0.6 µg of compound.

Table II-5. Representative tissue bromodichloromethane levels

	Dose, mg/kg					
	0	100	800	1600	3200	6400
Brain, ppm	0.27	1.1	233.0	112.8	772.7	238.6
	0.16	0.1	8.3	0.8	146.7	57.0
	0.07	0.4	7.1	43.7	21.7	74.3
	0.26		1.4	5.3		
Liver, ppm	0.18	0.81	246.7	632.8	1201.7	1279.0
	0.53	0.05	1.1	734.4	762.0	1947.0
	0.03	0.15	2.8	0.8	737.5	838.0
	0.14		0.3	85.7	1512.5	
				0.8		
Kidney, ppm	2.07	38.1	44.3	294.5	153.8	316.0
	4.3	0.87	126.4	2.3	762.0	109.6
	2.9	1.24	9.1	59.3	160.3	126.3
	0.75		2.2	2.9	102.7	

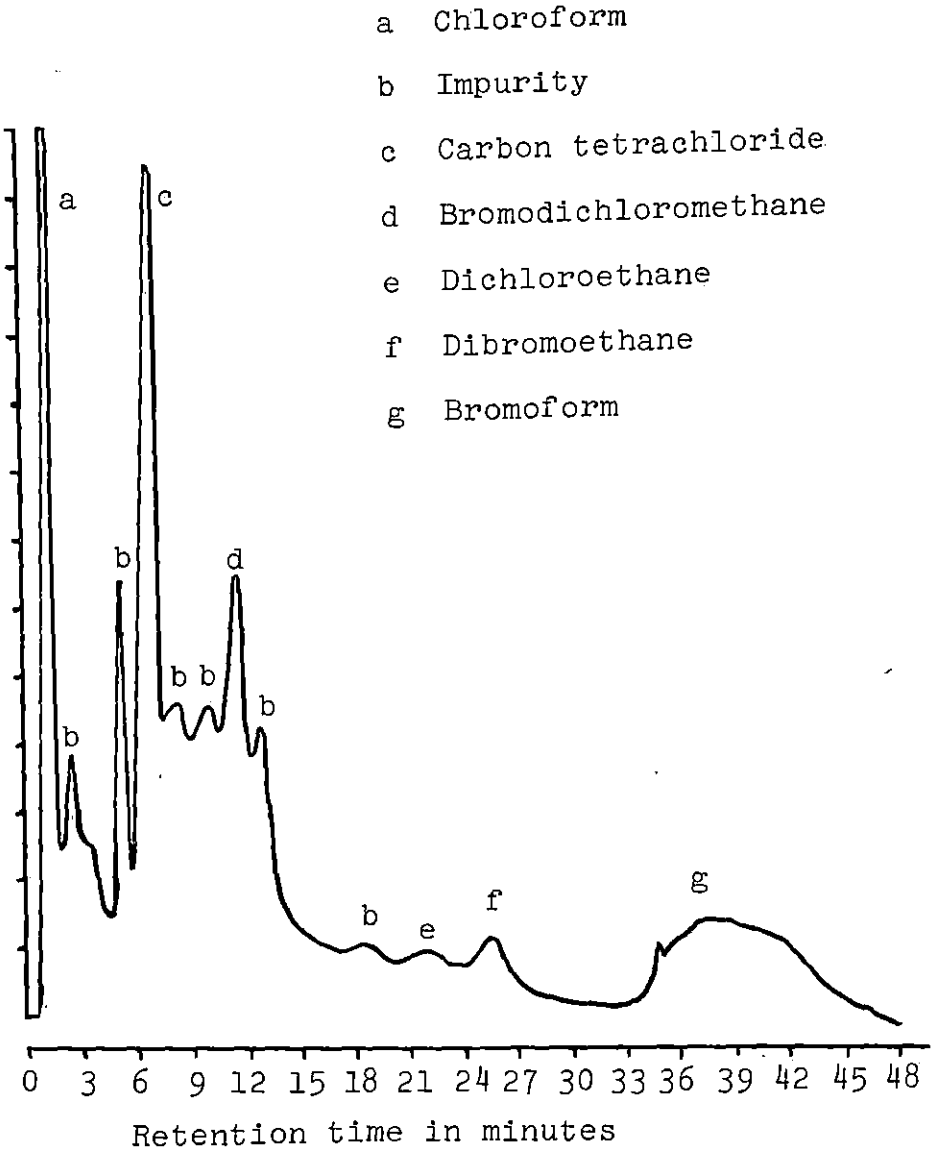

Figure II-6. Gas chromatographic separation of haloforms using thermal desorption introduction to the gas chromatograph

GC Conditions: 2 m glass column, 0.2% Carbowax 1500 on 100/200 Carbopack, Supelco, Inc.
Column Temp = 100°C, injector port and E.C. detector = 285 detector = 285°C
Carrier Gas = 40 ml/min nitrogen, Matheson
Haloform amounts injected range from 0.1 to 0.6 µg

DISCUSSION

The present work was approached in two ways. For large numbers of samples at levels greater than 20 parts-per billion (ppb), the advantages of a single liquid-liquid extraction were numerous. No elaborate instrumentation, aside from the normal gas chromatographic equipment, was necessary. Sample preparation was speedy and was kept to a minimum, and normal use of the GC was possible.

If levels were very low, then the use of gas-stripping with collection and concentration was used to advantage. This approach offered the advantage of lower detection limits and freedom from many possible interferences experienced with the simpler liquid extraction. However, the assembled equipment was subject to contamination, interfered with the normal use of the gas chromatograph, and was much less efficient or speedy to work with for large numbers of samples. Also, the initial results indicated that the design of the thermal desorption unit and its connection to existing chromatographic equipment allowed too much dead space resulting in excessive chromatographic band broadening. Figure II-6 illustrates the chromatogram of the haloform mixture introduced via the desorption unit. Although the chromatogram resembled the one obtained with normal injection techniques in order of

compound elution, the sensitivity and compound resolutions were entirely unacceptable.

Modification of the hardware and perfection of the extracting methodology are goals of future subacute work with the haloform compounds.

The pentane liquid-liquid extraction of bromodichloromethane from blood, liver, kidney and brain was very appropriate for levels encountered in this study. The method presented was relatively free of interferences, very rapid, and complete as supported by recoveries of added bromodichloromethane.

The GC determination of bromodichloromethane in pentane extracts of blood, liver, kidney and brain was both selective in its ability to differentiate the various haloforms from each other and sensitive in its ability to analyze subnanogram amounts of the haloforms.

Impurities noted in a few chromatograms led to investigation of solvent purity. Solvent purity was checked by gas chromatography prior to use. Solvents absorbed fumes and vapors from laboratory environments even when tightly capped. Many of the impurities were removed by adsorption column chromatography using activated basic alumina or silica. Figure II-7 shows solvent contamination and the effects of a single elution through a basic alumina chromatography column.

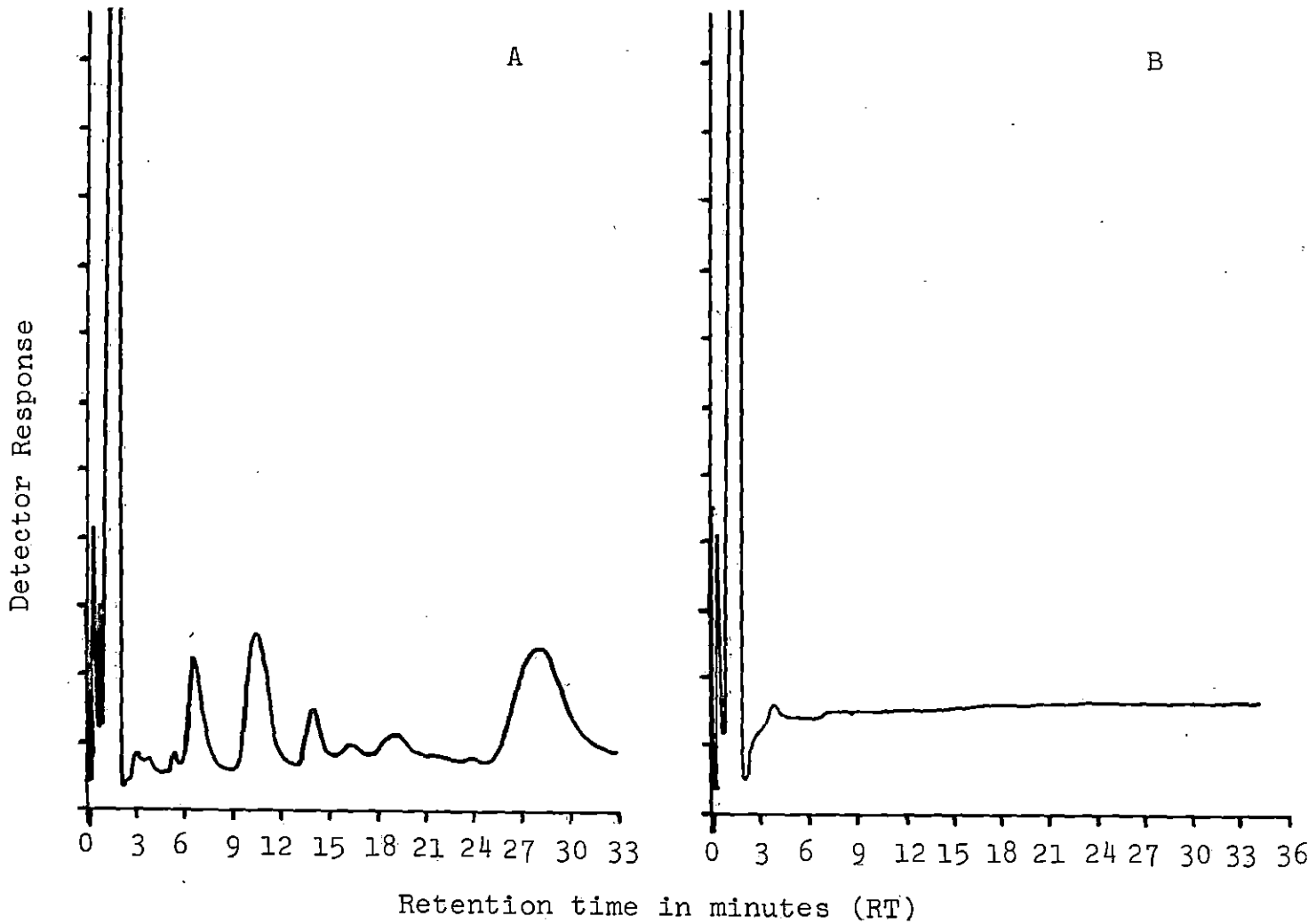

Figure II-7. Alumina chromatography of pentane

A shows impurity peaks in pentane solvent. B illustrates the effectiveness of single pass through an alumina chromatograph column in removing them

All glassware was thoroughly washed in hot, soapy water, rinsed three times with double distilled water or equivalent (Millipore Milli-Q® [1] deionized water) and fired at 500°C overnight in a muffle oven.

Bromodichloromethane is very volatile with a boiling point of 87°C. Obvious problems of haloform loss with evaporation of solvent to gas headspace or into the laboratory environment when containers are unstoppered must be recognized and avoided when possible.

No long-term storage conditions were found. Freezing the tissues and blood at -10°C was adequate for short periods of time, blood at least 9 days, and tissues at least 4 months.

For blood extractions, pentane was chosen because it produced a better chromatographic separation from the early eluting haloforms than did hexane or heptane. TX-100® was also investigated because of its hemolyzing properties on red blood cells. In most cases, a ratio of 1 ml blood to 4 ml pentane was less clearly divided even after centrifugation. In every case, the addition of any TX-100® to the blood followed by pentane extraction produced a solid proteineous plug which could not be separated by filtration or centrifugation into the biphase layers.

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[1] Millipore, Bedford, MA.

However, reextractions and spike addition studies indicated that a pentane extraction was successfully removing the blood haloforms produced by dosing animals with bromodichloromethane, Table II-3. Pentane also proved to be superior to hexane and heptane for the extraction of the tissues because of better GC separation.

Blood levels fell into two 2 groups, Table II-4. Animals surviving through the 2 week study generally had blood levels of 100 ppb or less. Animals which died during the trials generally had much higher blood concentrations, often in the hundreds of ppm. The level in these latter animals reflected both the dose level received and the length of time they survived. The larger doses resulted in higher blood concentrations. The longer-living animals generally had lower blood levels due to elimination, metabolism, or compartmentation into other tissues.

Tissue levels of the dosed animals reflected both the initial dose received and the length of time the animal survived, Table II-5. As with the blood levels, the tissue levels were very low or at the control levels if the animal survived the 2 week observation period. Animals dying sooner had much higher levels, Table II-5. Measurable levels of bromodichloromethane in the control group were thought to be due to inhalation by the control group of exhaled bromodichloromethane from the other dose groups. Further studies

involving the chronic toxicity of the haloforms should consider this source of cross-contamination and separate cages in well-ventilated areas.

SUMMARY

The proposed liquid extraction methodology provided quantitative extraction of bromodichloromethane from blood, liver, kidney and brain at ppb concentration levels. Interferences were noted in some brain tissues but centrifugation largely solved the problem. The chromatographic conditions used produced well-resolved chromatograms of bromodichloromethane and other haloforms.

Storage at -10°C was found to be adequate for storage periods of 4 months or less. Tissue levels in the various matrices reflected both the dose level received and the length of time the animal survived.

The proposed gas-stripping with thermal desorption methodology resulted in unacceptable GC separation due to basic equipment design and methods available for connecting the equipment to the gas chromatograph.

SUGGESTIONS FOR FUTURE WORK

Future work could better define the effects of exposure to bromodichloromethane on blood chemistry values.

Also, using data from the present work, a subacute toxicity study could investigate low level longer term effects of haloform exposure.

Modification and refinement of the trap-and-purge concept should be areas of future work as well.

REFERENCES

Adam, B. A. 1931. Substances producing taste in chlorinated water. Water and Water Eng. 33: 387.

Ahmed, A. E., V. L. Kubic and M. W. Anders. 1977. Metabolism of haloforms to carbon monoxide. Drug Metabolism and Disposition 5(2): 198-204.

Barnhart, B. L. and C. R. Campbell. 1972. Effect of chlorination on selected organic chemicals. NTIS Publication #PB211-160.

Bellar, T. A. and J. E. Sigsby. 1970. Non-cryogenic trapping techniques for gas chromatography. Internal Report. U.S. Environmental Protection Agency, Research Triangle Park, North Carolina.

Bellar, T. A. and J. J. Lichtenberg. 1974. The determination of volatile organic compounds at the µg/liter level in water by gas chromatography. EPA-670/4-74-009. (U.S. Environmental Protection Agency, Cincinnati, Ohio.)

Bellar, T. A., J. J. Lichtenberg and R. C. Kroner. 1974. The occurrence of organohalides in chlorinated drinking waters. EPA-670/4-74-008. (U.S. Environmental Protection Agency, Cincinnati, Ohio.)

Bowman, F. J., J. Borzelleca and A. Munson. 1978. The toxicity of some halomethanes in mice. Toxicology and Applied Pharmacology 44: 213-215.

Brown, B. R., I. G. Sipes and A. M. Segalyn. 1974. Mechanisms of acute hepatic toxicity. Anesthesiology 41: 554-561.

Bunn, W. W., B. B. Haas, E. R. Deane and R. D. Kleopfer. 1975. Formation of trihalomethanes by chlorination of surface water. Environmental Letters 10(3): 205-213.

Burttschell, R. H., A. A. Rosen, F. M. Middleton and M. B. Ettinger. 1959. Chlorine derivatives of phenol causing taste and odor. J. Am. Water Works Assoc. 51: 205-213.

Bush, B., R. S. Narang and S. Syrotynski. 1976. Screening for halo-organics in New York State drinking water. Unpublished paper. Division of Laboratory Research, New York Department of Health, Albany, New York.

Butler, T. C. 1961. Reduction of carbon tetrachloride in vivo and reduction of carbon tetrachloride and chloroform in vitro by tissues and tissue constituents. J. Pharmacol. Exp. Ther. 134: 311.

Chriswell, C. D., L. D. Kissinger and J. S. Fritz. 1976. Use of copper II salts as amine abstractors in chromatography. Analytical Chemistry 48: 1123.

Davies, D. D. 1978. A method of gas chromatography using E.C.D. for the determination of blood concentrations of halothane, chloroform and trichloroethane. Br. J. Anaesth. 50: 147-155.

Docks, E. L. and G. Krishna. 1976. The role of glutathione in chloroform-induced hepatotoxicity. Experimental and Molecular Pathology 24: 13-22.

Dowty, B., D. Carlisle and J. L. Laseter. 1975. Halogenated hydrocarbons in New Orleans drinking water and blood plasma. Science 137: 75-77.

Ettinger, M. B. and C. C. Ruchoft. 1951. Effect of stepwise chlorination on taste- and odor-producing intensity of some phenolic compounds. J. Am. Water Works Assoc. 43: 561.

Glaze, W. H., J. E. Henderson, IV, J. E. Bell and V. A. Wheeler. 1973. Analysis of organic materials in wastewater effluents after chlorination. J. Chromatogr. Sci. 11: 580-584.

Grob, K. 1973. Organic substances in potable water and in its precursor. J. Chromatogr. 84: 255.

Hall, F. B. and C. H. Hine. 1966. Trichloroethane intoxication: a report of two cases. J. Forensic Sci. 11: 404-413.

Joint federal/state survey of organics and inorganics in selected drinking water supplies. 1975. U.S. Environmental Protection Agency, Cincinnati, Ohio.

Junk, G. A., J. J. Richard, M. D. Grieser, D. Witiak, J. L. Witiak, M. D. Arguello, R. Vick, H. J. Svec, J. S. Fritz and G. V. Calder. 1974. The use of macroreticular resins in the analysis of water for trace organic contaminants. J. Chromatogr. 99: 745.

Kaiser, K. L. and B. G. Oliver. 1976. Determination of volatile halogenated hydrocarbons in water by gas chromatography. Analytical Chemistry 48: 2207-2209.

Kimura, E., D. M. Ebert and P. W. Dodge. 1971. Acute toxicity and limits of solvent residue for sixteen organic solvents. Toxicology and Applied Pharmacology, 19: 699-704.

Kissinger, L. D. and J. S. Fritz. 1976. Analysis of drinking water for haloforms. J. Am. Water Works Assoc. 68: 435-437.

Kleopfer, R. D. and B. J. Fairless. 1963. Characterization of organic compounds in a municipal water supply. Environ. Sci. Technol. 6: 1036-1037.

Koch, R. R., E. A. Glende, Jr. and R. O. Recknagel. 1974. Hepatotoxicity of bromotrichloromethane - bond dissociation energy and lipoperoxidation. Biochemical Pharmacology 23: 2907-2915.

Kopfler, F. C., R. G. Melton, R. D. Lingg and W. E. Coleman. 1975. GC/MS determination of volatiles for the national organics reconnaissance survey (NORS) drinking water. U.S. Environmental Protection Agency, Cincinnati, Ohio.

Kutob, S. D. and G. L. Plaa. 1962. A procedure for estimating the hepatotoxic potential of certain industrial solvents. Toxicology and Applied Pharmacology 4: 354-361.

Laubusch, E. J. 1971. Chlorination and other disinfectants, water quality and treatment. McGraw-Hill, New York. 160-226.

Lee, G. F. and J. C. Morris. 1962. Kinetics of chlorination of phenol chlorophenolic tastes and odors. Int. J. Air and Water Poll. 6: 419-431.

Litchfield, J. T. and F. Wilcoxon. 1949. A simplified method of evaluating dose effect experiments. J. Pharmacol. Exp. Ther. 96: 99.

Luna, L. G., ed. 1968. Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology. 3rd ed. McGraw-Hill Book Co., New York.

Melby, E. C. and N. H. Altman, ed. 1974. Handbook of Laboratory Animal Science Vol. II. CRC Press, Inc., Cleveland, Ohio.

Mieure, J. P. and M. W. Dietrich. 1973. Determination of trace organics in air and water. J. Chromatogr. Sci. 11: 559.

Morris, J. C. 1975. Formation of halogenated organics by chlorination of water supplies. EPA #P5-01-1805-J. (Environmental Protection Agency (RD-683), Washington, D.C.)

National Academy of Science. 1972. Nutrient Requirements of lab animals. Printing and Publishing Office, National Academy of Science, 2101 Constitution Avenue, Washington, D.C.

National Cancer Institute. 1976. Carcinogenisis bioassay of trichloroethylene. DHEW #(NIH)76-802. (U.S. Dept. of HEW, Washington, D.C.)

Nicholson, A. A. and O. Meresz. 1975. Analysis of volatile halogenated organics in water by direct aqueous injection-gas chromatography. Bulletin of Environmental Contamination and Toxicology 14: 453-456.

Nicholson, A. A., O. Meresz and B. Lemyk. 1977. Determination of free and total potential haloforms in drinking water. Analytical Chemistry 49: 814-819.

Richard, J. J. and G. A. Junk. 1977. Liquid extraction for the rapid determination of haloforms in water. J. Am. Water Works Assoc. 69: 62-64.

Roe, F. J. C. Preliminary report of long-term tests of chloroform in rats, mice and dogs. (Unpublished) Hazelton Labs., Vienna, Va., 1976.

Rook, J. J. 1972. Production of potable water from a highly polluted river. Water Treat. Exam. 21: 259.

Rook, J. J. 1974. Formation of haloforms during chlorination of natural waters. Water Treat. Exam. 23: 234-243.

Schwetz, B. A., B. K. Leong and P. J. Gohring. 1974. Embryo- and fetotoxicity of inhaled chloroform in rats. Toxicology and Applied Pharmacology 28: 442-451.

Shervchenko, M. V. 1963. Chlorination of natural waters containing phenols, humous materials, and petroleum products (in Russian). Ukr. Khim Zh. 29: 1105. Chemical Abstracts 60: 10390b (1964).

Slater, T. F. and B. C. Sawyer. 1971. The stimulatory effects of carbon tetrachloride on peroxidative reactions in rat liver fractions in vitro. Biochem. J. 123: 815-821.

Stahl, C. J., V. F. Abdullah and A. M. Dominguez. 1966. Trichloroethane poisoning: observations on the pathology and toxicology in six fatal cases. J. Forensic Sci. 14: 393-397.

Timms, R. M. and K. M. Moser. 1975. Toxicity secondary to intravenously administered chloroform in humans. Arch. Intern. Med. 135: 1601-1603.

Thompson, D. J., S. D. Warner and V. B. Robinson. 1974. Teratology studies on orally administered chloroform in the rat and rabbit. Toxicology and Applied Pharmacology 29: 348.

Torkelson, T. R., F. Oyen and V. K. Rowe. 1976. The toxicity of chloroform as determined by single and repeated exposures of laboratory animals. American Industrial Hygiene Association Journal 37: 697-705.

Trakhtman, N. N. 1966. Effect of chlorine on 3,4-benzopyrene in water chlorination (in Russian, English summary). Gig. Sanit. 31: 21.

U.S. Department of Health, Education and Welfare. 1974. Toxic Substances List. U.S. Department of HEW (National Institute of Occupational Safety and Health, Rockville, Maryland).

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None of the work would have been possible without the dedicated support and financial assistance given me by Drs. Seaton and Stahr of the Veterinary Diagnostic Lab. I very much appreciated it.

Finally, I thank my lucky stars I am a citizen of a country where pursuit of educational excellence and freedom of thought is possible.

APPENDIX

Classical LD_{50} Determination

The method of Litchfield, 1949, is considered a standard in the field of toxicity determination. The method is relatively fast, straightforward and easily used. Its accuracy increases as the number of groups which have some mortality but not all, increases.

Dose Level (mg/kg)	# Dead/total tested	% Mortality	% Mortality Corrected	% Mortality Expected
0	0/7	0		
400	0/9	0	1	3.2
800	2/9	22.22		16.5
1600	3/9	33.33		46.0
3200	7/9	77.78		81.0
6400	9/9	100.0	99	93.0

$(X)^2$ test for line homogeneity

Expected - Observed (or corrected) = contribution to $(X)^2$

3.2 - 1 = 2.2 = 0.16

16.5 = 22.22 = - 5.72 = .025

46 - 33.33 = 12.67 - .065

81 - 77.78 = 3.22 = .0061

93 - 99 = 6 = .034

.146

Average # of animals/group = 8.67

$(X)^2$ 2.146 x 8.67 = 1.283 df = 3

$(X)^2$ = 1.283 less than p (.05) of 7.82

Therefore, graphed line is a good fit of data. The LD_{50} can be read directly from the graph

LD_{16} = 800 mg/kg

LD_{50} = 1740 mg/kg

LD_{84} = 3900 mg/kg

Calculation of CI for LD_{50}

$$S = \frac{\frac{3900}{1740} + \frac{1740}{800}}{2} = 2.208$$

N = 27

So, $f_{LD_{50}} = S^{2.208\sqrt{N}} = 1.52$

upper CI = 1.52 x LD_{50} = 2644.5 at p (.05)

upper CI = LD_{50} x 1.52 = 1740

SAS, ANOVA, GLM, and PROBIT Procedure[1]

[1]Barr, Goodnight, Sall and Helwig, SAS Institute, Inc., P. O. Box 10066, Raleigh, NC 27605)

PROBIT ANALYSIS ON DOSE

ITERATION	INTERCEPT	SLOPE	MU	SIGMA
0	4.60504348	0.00050647	779.82071502	1974.44697676
1	3.65605281	0.00085544	1571.06287129	1168.99152540
2	3.34006617	0.00101934	1628.44596346	981.03064894
3	3.29984961	0.00104097	1633.23498908	960.64148381
4	3.29922477	0.00104131	1633.29574201	960.32427839
5	3.29922462	0.00104132	1633.29575381	960.32420039

COVARIANCE MATRIX

	INTERCEPT	SLOPE
INTERCEPT	0.19666568	-0.00009113
SLOPE	-0.00009113	0.00000007

COVARIANCE MATRIX

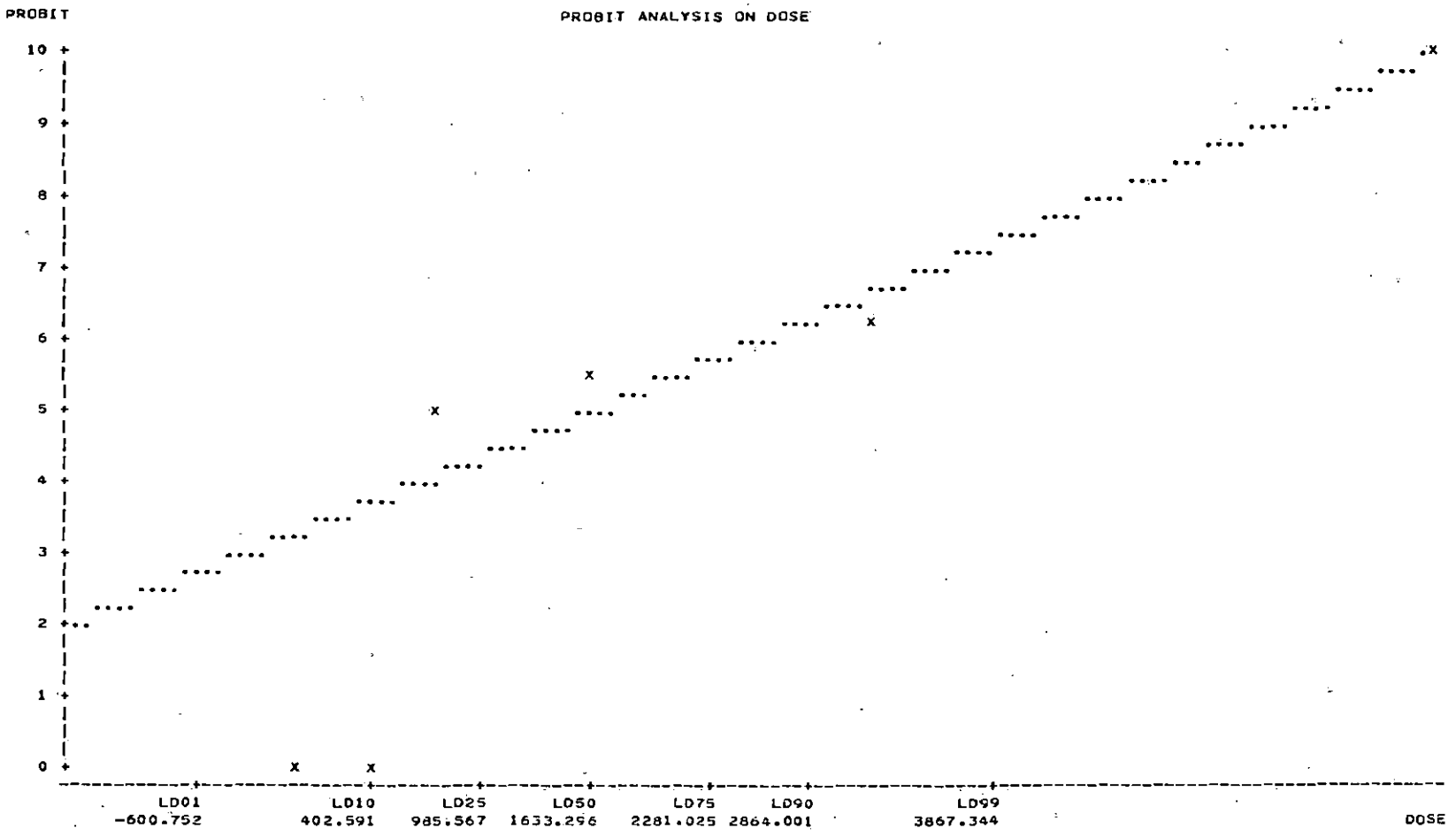
	MU	SIGMA
MU	78149.12856839	20016.23005504
SIGMA	20016.23005504	59221.61390951

CHI-SQ = 5.3447 WITH 4 DF PROB > CHI-SQ = 0.2537

NOTE: SINCE THE CHI-SQUARE IS SMALL (P > 0.10), FIDUCIAL LIMITS WILL BE COMPUTED USING A T VALUE OF 1.96 .

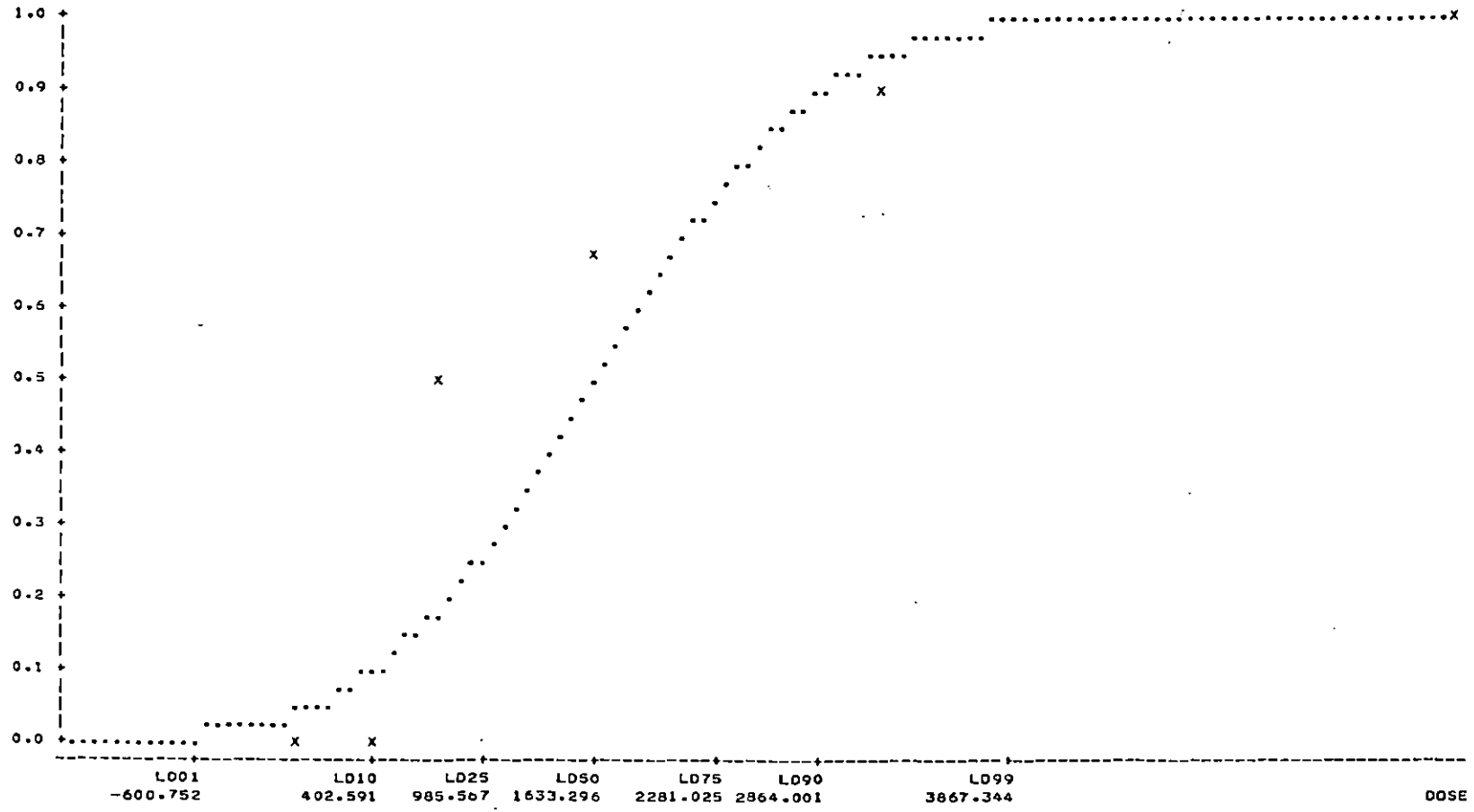
LD50 MALES FINAL

OBS	DOSE	N	MORT
1	0	8	0
2	400	10	0
3	800	4	2
4	1600	6	4
5	3200	9	8
6	6400	9	9


PROBIT ANALYSIS ON DOSE
PROBIT
10
9
8
7
6
5
4
3
2
1
0
LD01 -600.752
LD10 402.591
LD25 985.567
LD50 1633.296
LD75 2281.025
LD90 2864.001
LD99 3867.344
DOSE

PROBABILITY

PROBIT ANALYSIS ON DOSE



PROBIT ANALYSIS ON DOSE

PROBABILITY	DOSE	95 PERCENT FIDUCIAL LIMITS LOWER	UPPER
0.01	-600.75240444	-2622.15409993	-170.02373978
0.02	-338.96902332	-2121.23096552	364.12492687
0.03	-172.87586104	-1806.24035035	490.10496051
0.04	-47.93044491	-1571.15687751	586.74652995
0.05	53.70301237	-1381.37245975	665.79484291
0.06	140.20904727	-1221.02825347	736.12067126
0.07	216.05791708	-1081.47332973	797.94143857
0.08	283.97153340	-957.44671385	854.22273568
0.09	345.73625030	-845.50014632	906.25900109
0.10	402.59077341	-743.24582827	954.95114947
0.15	637.98368944	-329.73690114	1166.40092311
0.20	825.06651699	-15.75756940	1349.11895515
0.25	985.56692487	239.87542881	1519.60806830
0.30	1129.70125161	456.28944296	1685.86498000
0.35	1263.26318564	644.28301323	1852.47285923
0.40	1390.00039998	810.89243368	2022.34563270
0.45	1512.62012158	961.24641276	2197.54207029
0.50	1633.29575381	1099.40816822	2379.76925263
0.55	1753.97138603	1228.80112804	2570.76523060
0.60	1876.59110763	1352.47225139	2772.64452393
0.65	2003.32832197	1473.31703727	2988.28193196
0.70	2136.89025600	1594.34586452	3221.85465421
0.75	2281.02458275	1719.08823497	3479.78310962
0.80	2441.52499062	1852.34453000	3772.64892594
0.85	2628.60781917	2001.85865887	4119.79216085
0.90	2864.00073420	2183.54041673	4563.10910376
0.91	2920.85525731	2226.55715781	4671.03882911
0.92	2982.61997421	2272.97200602	4788.60581384
0.93	3050.53359053	2323.65813828	4918.22859457
0.94	3126.38246035	2379.87243746	5063.38998644
0.95	3212.88849524	2443.52668661	5229.40577193
0.96	3314.52195252	2517.75517986	5425.01000980
0.97	3439.46736865	2608.28586197	5666.20436958
0.98	3605.56053093	2727.57444176	5987.88643859
0.99	3867.34391205	2913.60180170	6496.88340015

PROBIT ANALYSIS ON DOSE

ITERATION	INTERCEPT	SLOPE	MU	SIGMA
0	4.27825570	0.00036377	1984.06085878	2748.98030082
1	3.72826446	0.00067317	1889.18196320	1485.51480198
2	3.26822218	0.00105504	1641.33481338	947.82520750
3	3.01365475	0.00129204	1537.37575197	773.97209251
4	2.93081566	0.00137331	1506.71819152	728.17011283
5	2.92248020	0.00138141	1503.90865385	723.89618173
6	2.92240679	0.00138148	1503.88507447	723.85925452
7	2.92240679	0.00138148	1503.88507274	723.85925178

COVARIANCE MATRIX

	INTERCEPT	SLOPE
INTERCEPT	0.29470096	-0.00019205
SLOPE	-0.00019205	0.00000017

COVARIANCE MATRIX

	MU	SIGMA
MU	50201.10711631	22678.74907795
SIGMA	22678.74907795	45975.52013406

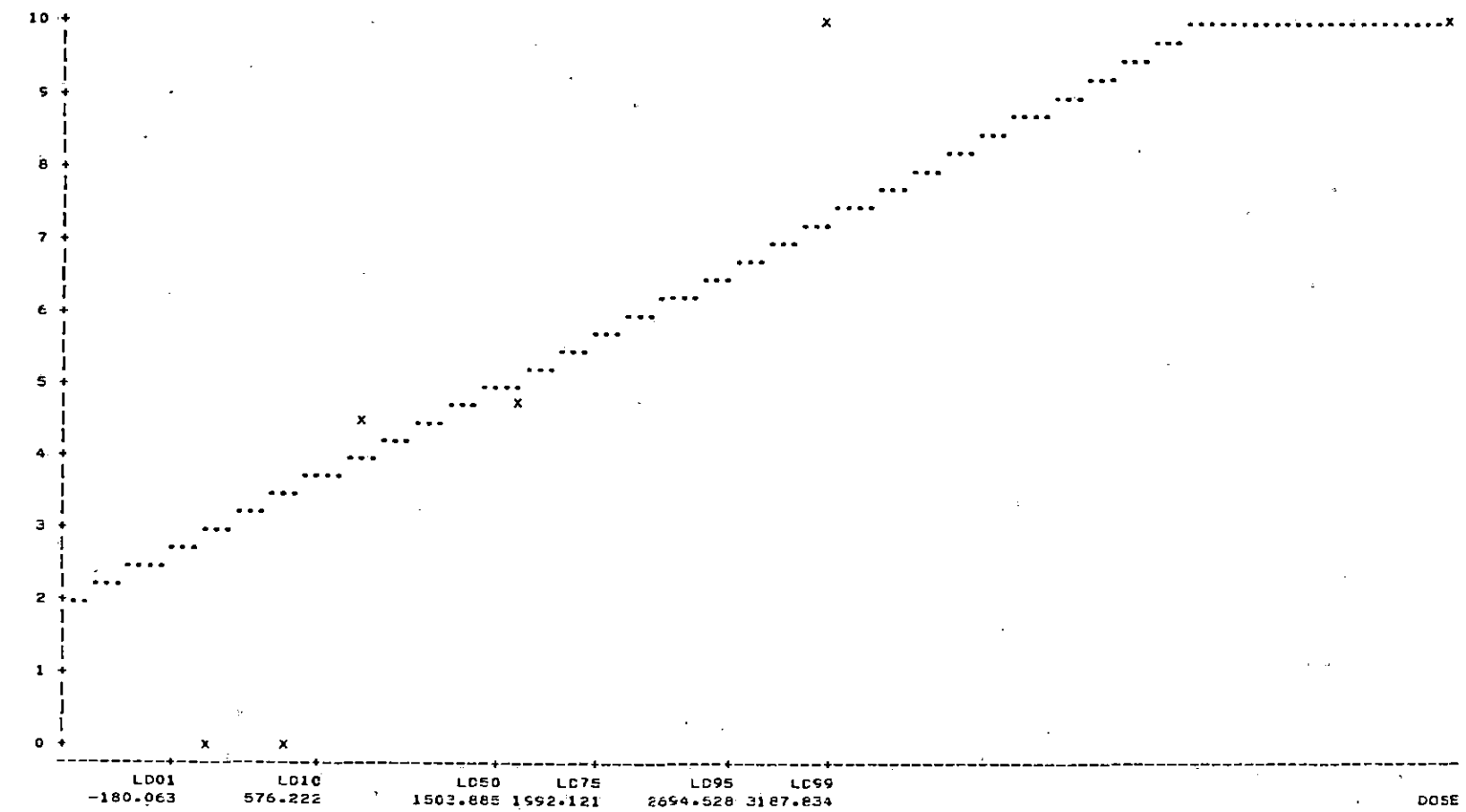
CHI-SQ = 3.0307 WITH 4 DF PROB > CHI-SQ = 0.5527

NOTE: SINCE THE CHI-SQUARE IS SMALL (P > 0.10), FIDUCIAL LIMITS WILL BE COMPUTED USING A T VALUE OF 1.96 .

LD50 FEMALES FINAL

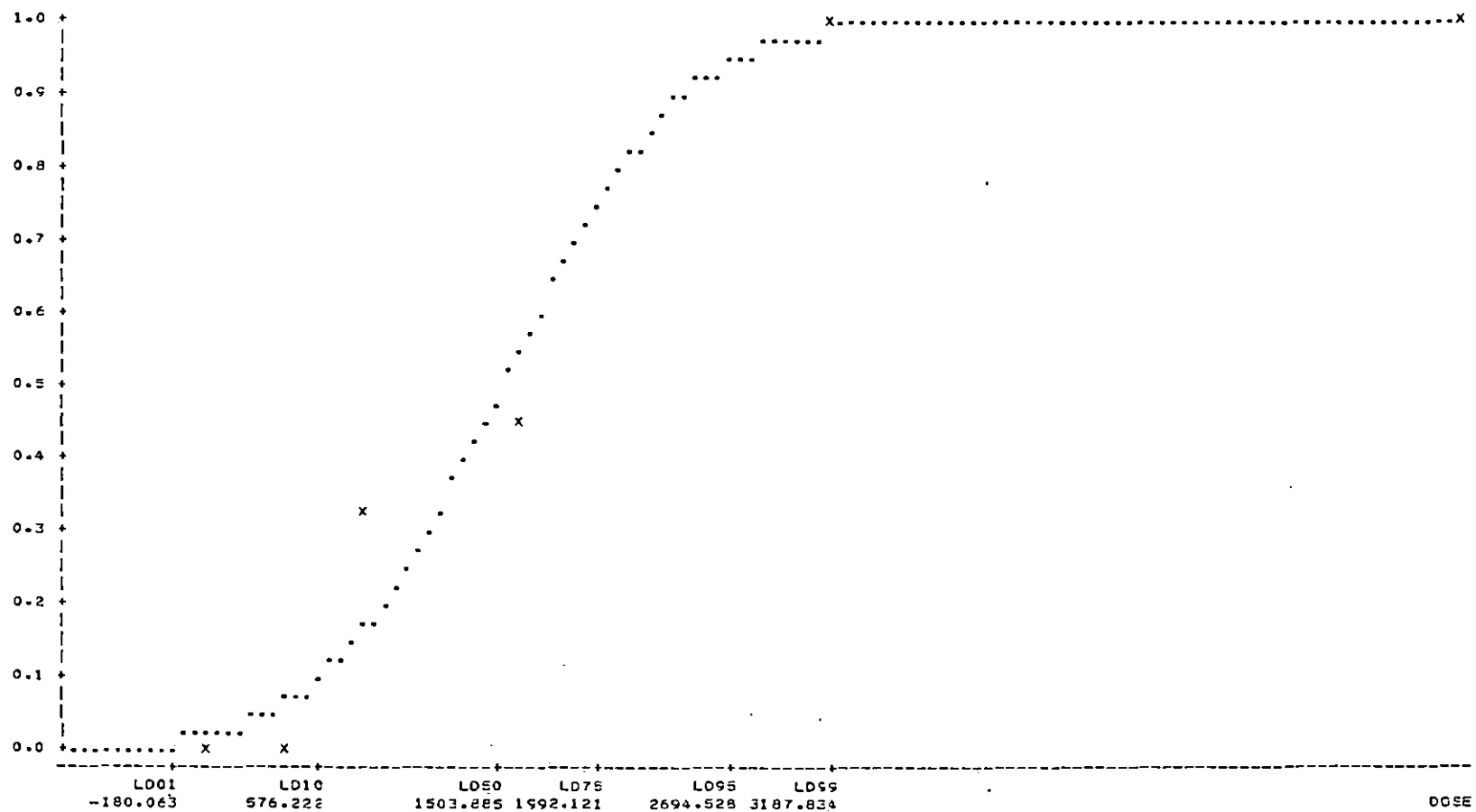
OBS	DOSE	N	MORT
1	0	7	0
2	400	8	0
3	800	9	3
4	1600	9	4
5	3200	9	9
6	6400	9	9

PROBIT ANALYSIS ON DOSE



PROBABILITY

PROBIT ANALYSIS ON DOSE



PROBIT ANALYSIS ON DOSE

PROBABILITY	DOSE	95 PERCENT FIDUCIAL LIMITS LOWER	UPPER
0.01	-180.06335595	-2110.34157548	400.84463642
0.02	17.25952541	-1654.65334965	540.51022012
0.03	142.45522105	-1368.14526737	631.66966328
0.04	236.63476520	-1154.34752543	702.00657999
0.05	313.24255902	-981.84429236	760.62497254
0.06	378.44782373	-836.22158045	811.72306447
0.07	435.62008507	-709.61849451	857.60563987
0.08	486.81102608	-597.25678769	899.68427019
0.09	533.36713884	-496.00692879	938.89171893
0.10	576.22211692	-403.70437006	975.88004203
0.15	753.65317615	-33.33215816	1140.80816686
0.20	894.66975734	242.15422786	1290.72128068
0.25	1015.64942765	459.78736501	1438.11735254
0.30	1124.29291062	636.94487060	1588.73143710
0.35	1224.96728871	784.31827725	1745.08725646
0.40	1320.49742854	909.58683161	1908.02803583
0.45	1412.92394439	1018.70372166	2077.75701158
0.50	1503.88507274	1116.34160720	2254.54395596
0.55	1594.84620110	1206.17621828	2439.13425482
0.60	1687.27271695	1291.16657617	2632.98976272
0.65	1782.80285678	1373.84160546	2838.52406716
0.70	1883.47723487	1456.60024924	3059.49464939
0.75	1992.12071784	1542.07841571	3301.78807308
0.80	2113.10038815	1633.73345969	3575.12223810
0.85	2254.11696934	1737.08723637	3897.20796125
0.90	2431.54302856	1863.29554161	4306.29585275
0.91	2474.40300665	1893.28402726	4405.60238894
0.92	2520.95911941	1925.68096212	4513.66286171
0.93	2572.15006042	1961.10307994	4632.68098103
0.94	2629.32232176	2000.43936084	4765.83036148
0.95	2694.52758647	2045.04178039	4917.94874576
0.96	2771.13538029	2097.12764453	5096.98450724
0.97	2865.31492444	2160.75041892	5317.49639150
0.98	2990.51022006	2244.72808841	5611.22624766
0.99	3187.83350144	2375.96397246	6075.30417293

PROBIT ANALYSIS ON DOSE

ITERATION	INTERCEPT	SLOPE	MU	SIGMA
0	3.95183523	0.00080857	1296.32154155	1236.75358651
1	3.32071056	0.00107882	1556.59424915	926.93624502
2	3.15934172	0.00115803	1580.83906136	863.53585214
3	3.16010332	0.00116357	1581.24914142	859.42279329
4	3.16006724	0.00116360	1581.24693071	859.40473935

COVARIANCE MATRIX

	INTERCEPT	SLOPE
INTERCEPT	0.10823809	-0.00005732
SLOPE	-0.00005732	0.00000005

COVARIANCE MATRIX

	MU	SIGMA
MU	31676.53317743	10150.76241527
SIGMA	10150.76241527	25291.01917326

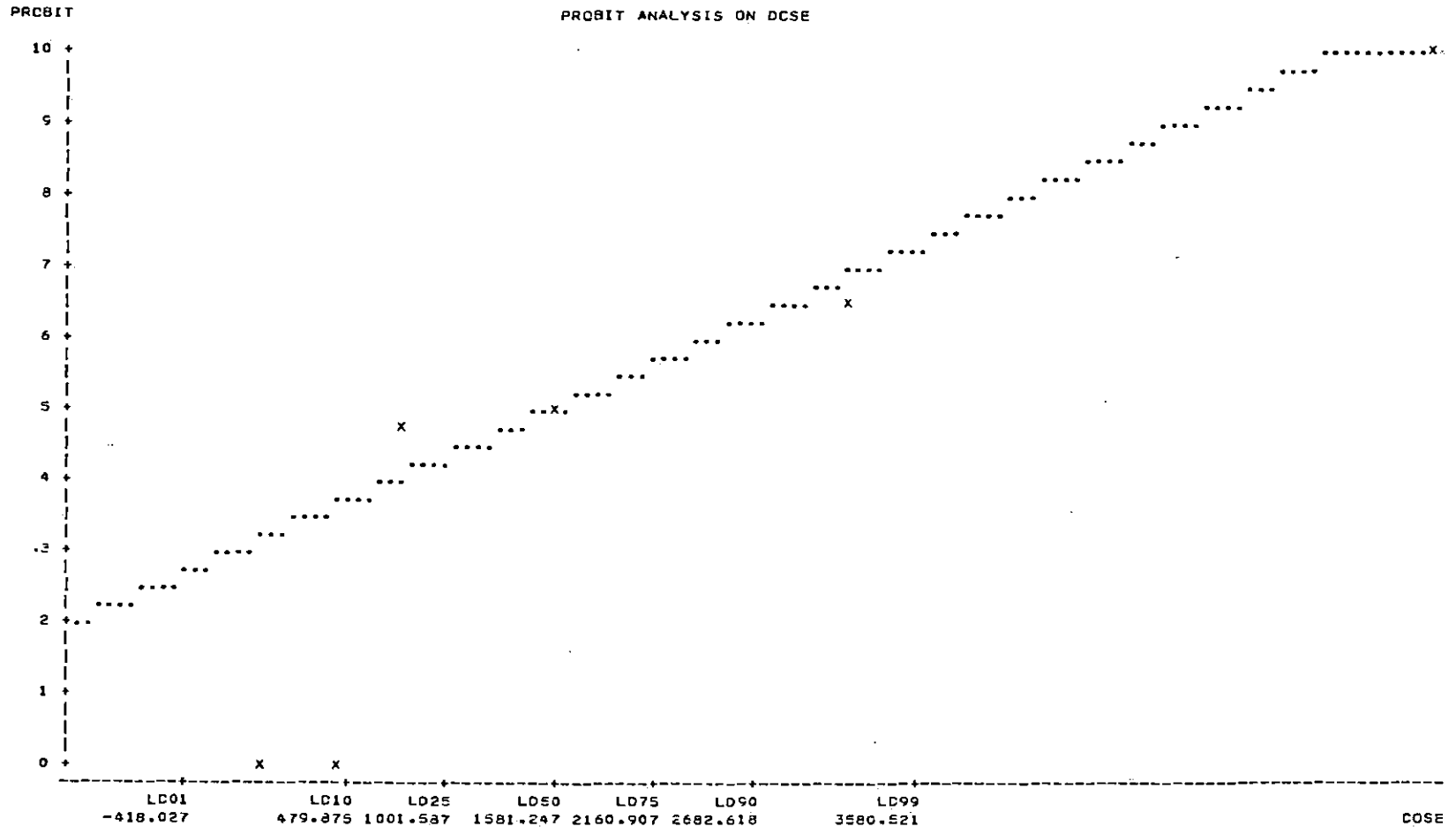
CHI-SQ = 6.2255 WITH 4 DF PROB > CHI-SQ = 0.1829

NOTE: SINCE THE CHI-SQUARE IS SMALL (P > 0.10), FIDUCIAL LIMITS WILL BE COMPUTED USING A T VALUE OF 1.96 .

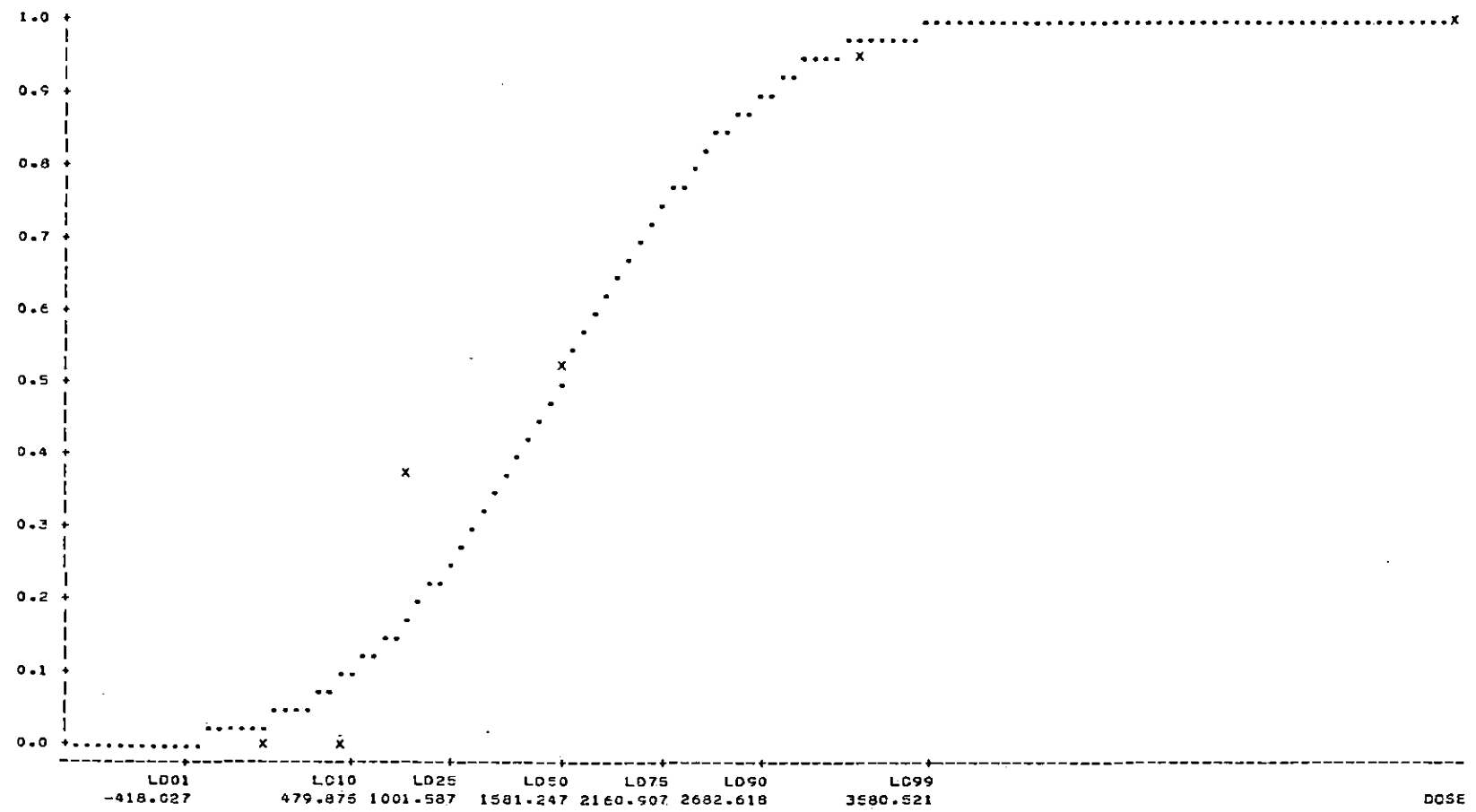
LD50 TOTAL POPULATION FINAL

OBS	DOSE	N	MORT
1	0	15	0
2	400	18	0
3	800	13	5
4	1600	15	8
5	3200	18	17
6	6400	18	18

PROBIT ANALYSIS ON DOSE



PROBIT ANALYSIS ON DOSE



PROBIT ANALYSIS ON DOSE

PROBABILITY	DOSE	95 PERCENT FIDUCIAL LIMITS LOWER	UPPER
0.01	-418.02745430	-1442.86270315	105.62855024
0.02	-183.75461359	-1087.21220034	289.49603428
0.03	-35.11600720	-863.35666713	407.94736621
0.04	76.69902645	-696.14752824	498.24234804
0.05	167.65193051	-561.04960181	572.60415902
0.06	245.06713711	-446.81771195	636.65540386
0.07	312.94512890	-347.31649853	693.47362283
0.08	373.72177465	-258.81402053	744.93655211
0.09	428.99570236	-178.86360459	792.27907422
0.10	479.87544339	-105.77050497	836.35933267
0.15	690.53116524	190.65930831	1025.05852807
0.20	857.95365505	417.14578663	1184.13720715
0.25	1001.58724372	603.11366906	1328.94989803
0.30	1130.57464553	762.35049131	1466.76425399
0.35	1250.10069627	902.71378209	1601.66314929
0.40	1363.51922595	1029.34449499	1736.22933303
0.45	1473.25297386	1145.97448585	1872.31036247
0.50	1581.24693071	1255.53827233	2011.45095853
0.55	1689.24088756	1360.50638873	2155.18722466
0.60	1798.97463147	1463.11190930	2305.29272439
0.65	1912.39316515	1565.55472072	2464.04680961
0.70	2031.91921589	1670.24645293	2634.61726349
0.75	2160.90661770	1780.18580040	2821.72909423
0.80	2304.54020637	1899.66656400	3033.02890413
0.85	2471.96269618	2035.90974443	3282.25088090
0.90	2682.61841803	2203.88090562	3599.50872839
0.91	2733.49815906	2243.99545251	3676.56753958
0.92	2788.77208577	2287.40471620	3760.45121394
0.93	2849.54873252	2334.94792310	3852.87341432
0.94	2917.42672431	2387.83407801	3956.30669180
0.95	2994.84193091	2447.90354748	4074.52035702
0.96	3085.79483496	2518.17580625	4213.70783566
0.97	3197.60986861	2604.17330543	4385.21445720
0.98	3346.24847601	2717.91573253	4613.77889525
0.99	3580.52131572	2896.05858059	4975.11403404

PROBIT ANALYSIS ON DOSE

ITERATION	INTERCEPT	SLOPE	MU	SIGMA
0	3.68059498	0.00080911	1630.69504270	1235.92454221
1	3.26900149	0.00101702	1702.02632955	983.26267978
2	3.21528674	0.00104640	1705.57441877	955.55739242
3	3.21439148	0.00104692	1705.58129660	955.18209994
4	3.21439122	0.00104692	1705.58128181	955.18195461

COVARIANCE MATRIX

	INTERCEPT	SLOPE
INTERCEPT	0.09278360	-0.00004495
SLOPE	-0.00004495	0.00000003

COVARIANCE MATRIX

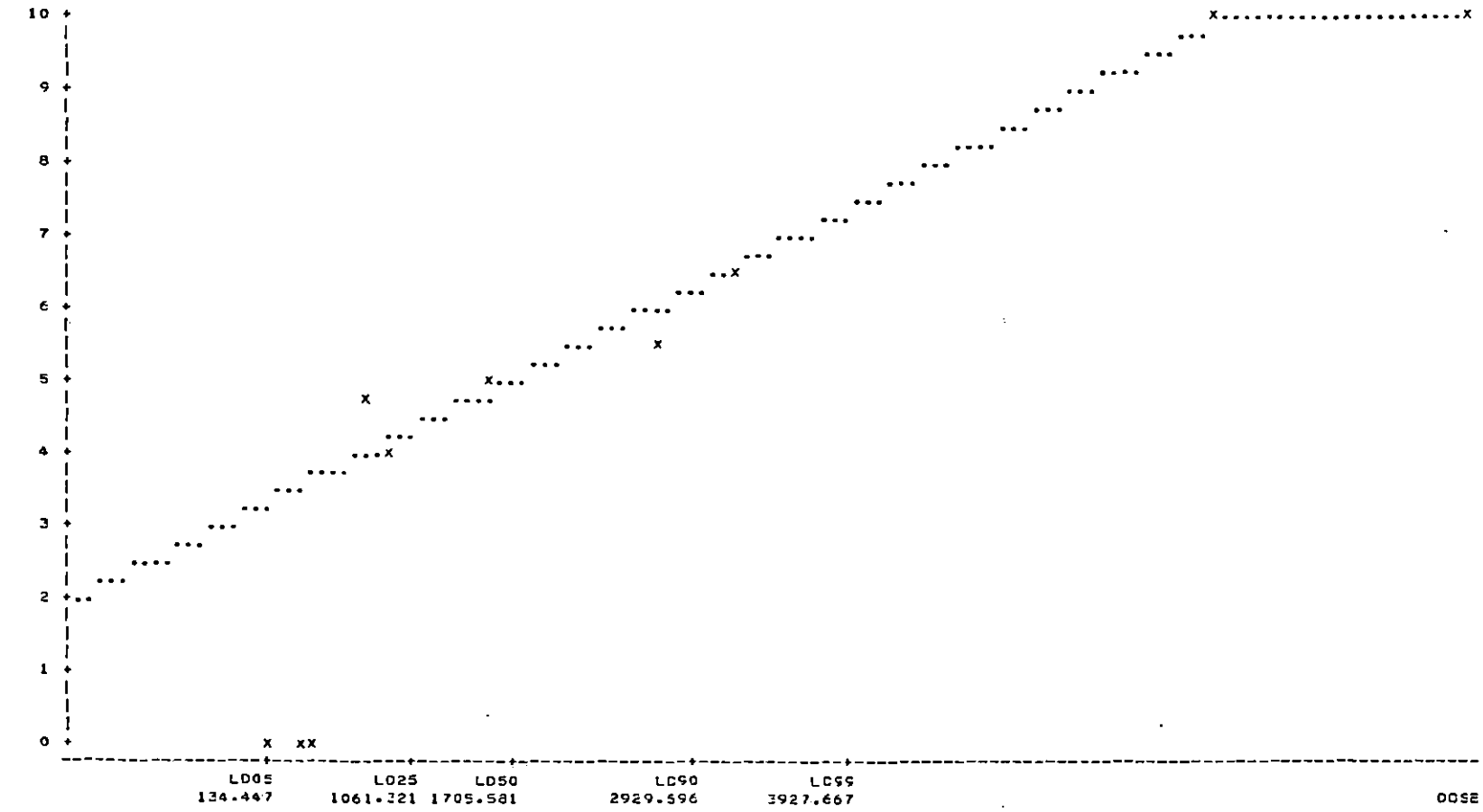
	MU	SIGMA
MU	29137.47246896	8086.15945325
SIGMA	8086.15945325	26468.22567793

CHI-SQ = 9.6061 WITH 8 DF PROB > CHI-SQ = 0.2966

NOTE: SINCE THE CHI-SQUARE IS SMALL (P > 0.10), FIDUCIAL LIMITS WILL BE COMPUTED USING A T VALUE OF 1.96 .

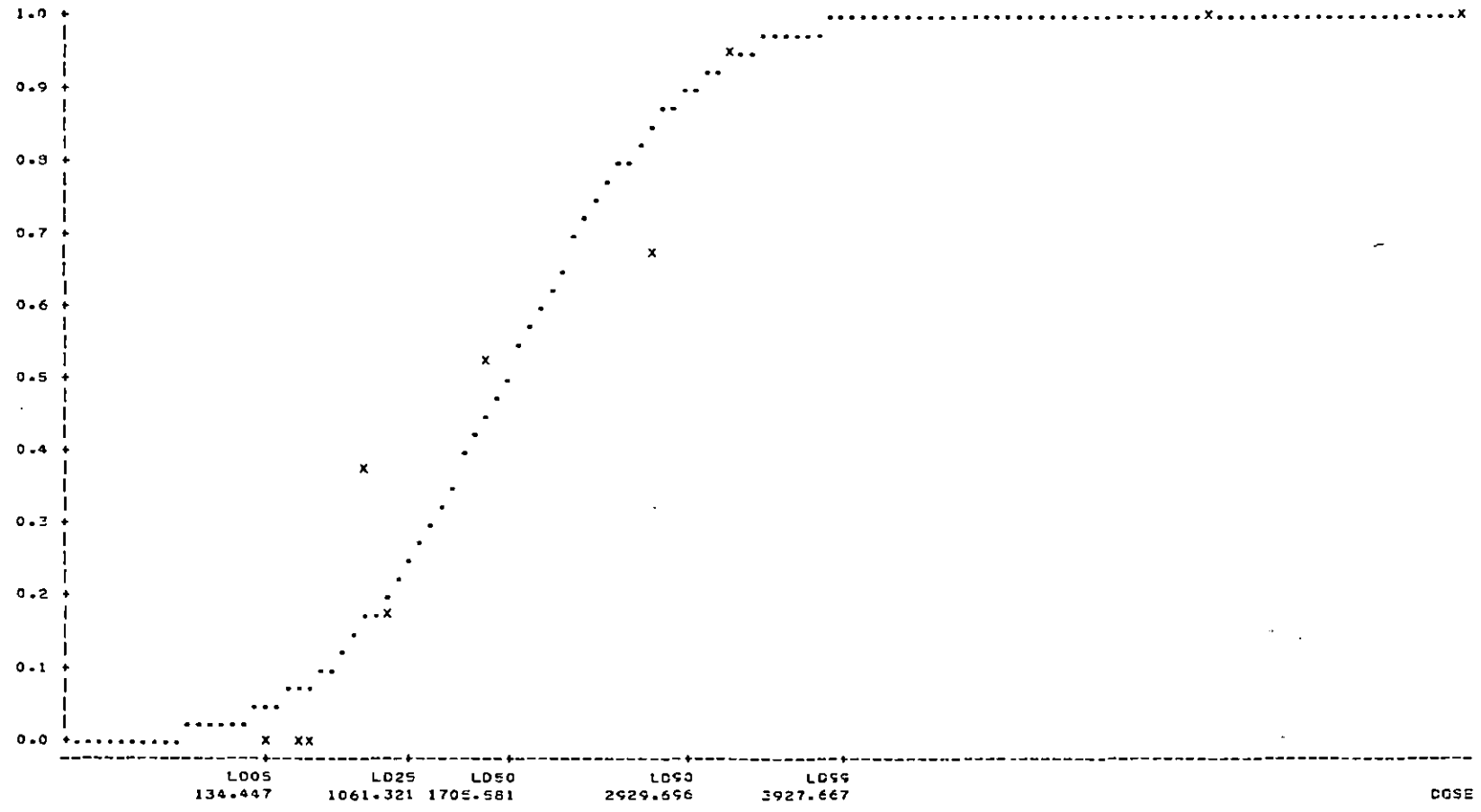
OBS	DOSE	N	MORT
1	100	6	0
2	200	6	0
3	400	18	0
4	800	13	5
5	900	5	1
6	1600	15	8
7	2700	6	4
8	3200	18	17
9	6400	18	18
10	8100	6	6

PROBIT ANALYSIS ON DOSE



PROBABILITY

PROBIT ANALYSIS ON DOSE



PROBIT ANALYSIS ON DOSE

PROBABILITY	DOSE	95 PERCENT FIDUCIAL LIMITS LOWER	UPPER
0.01	-516.50422394	-1559.39529657	42.16731143
0.02	-256.12261368	-1178.96330732	247.81646237
0.03	-90.91863007	-939.11310816	379.81595631
0.04	33.35754112	-759.68827349	480.11916103
0.05	134.44678196	-614.51135120	562.47945956
0.06	220.49960320	-491.58223482	633.28018149
0.07	295.93232524	-384.35246634	695.80130952
0.08	363.48228468	-286.83844637	752.33212518
0.09	424.91627019	-202.42824296	804.20088947
0.10	481.46635452	-123.31253815	852.37120101
0.15	715.59891255	198.95710129	1057.09967746
0.20	901.67986822	447.17757161	1227.72060347
0.25	1061.32084493	652.65575165	1381.67104693
0.30	1204.58337592	829.93867209	1526.97633629
0.35	1337.93012618	987.21058835	1668.72273634
0.40	1463.58870105	1129.77160122	1809.90164422
0.45	1585.55183111	1261.46862538	1952.72589003
0.50	1705.58128181	1385.37115871	2098.99219563
0.55	1825.61073250	1504.12306224	2250.40913104
0.60	1947.57386256	1620.17160547	2408.88185779
0.65	2073.63243744	1735.97257544	2576.82040851
0.70	2206.47918769	1854.24725479	2757.56444351
0.75	2349.84171865	1978.39361676	2956.10629314
0.80	2509.48269329	2113.27943092	3180.54910268
0.85	2695.56375096	2267.08461615	3445.58831378
0.90	2929.69620909	2456.75018821	3782.91795760
0.91	2986.24629342	2502.05684374	3864.85721843
0.92	3047.68027893	2551.09025833	3954.14277154
0.93	3115.23023837	2604.80018082	4052.47798468
0.94	3190.67296041	2664.55488711	4162.53387490
0.95	3276.71578165	2732.43729343	4288.33134639
0.96	3377.80502249	2811.86504635	4436.43077478
0.97	3502.08129369	2905.08935145	4618.93450908
0.98	3667.28517729	3037.71752364	4862.15602998
0.99	3927.66678755	3239.29854681	5245.65614902

OBS	DOSE	AN	SEX	WT	HTD	RHTD	S
1	0	1	2	249	336	0.002976	1
2	0	4	2	234	336	0.002976	1
3	0	5	2	238	336	0.002976	1
4	0	6	2	244	336	0.002976	1
5	0	7	2	270	336	0.002976	1
6	0	8	2	247	336	0.002976	1
7	0	9	2	284	336	0.002976	1
8	0	10	1	433	336	0.002976	1
9	0	11	1	421	336	0.002976	1
10	0	12	1	410	336	0.002976	1
11	0	13	1	407	336	0.002976	1
12	0	14	1	425	336	0.002976	1
13	0	15	1	463	336	0.002976	1
14	0	16	1	444	336	0.002976	1
15	0	17	1	405	336	0.002976	1
16	4	1	2	266	336	0.002976	1
17	4	3	2	236	336	0.002976	1
18	4	4	2	257	336	0.002976	1
19	4	5	2	260	336	0.002976	1
20	4	6	2	289	336	0.002976	1
21	4	7	2	236	336	0.002976	1
22	4	8	2	250	336	0.002976	1
23	4	9	2	280	336	0.002976	1
24	4	10	1	422	336	0.002976	1
25	4	11	1	402	336	0.002976	1
26	4	12	1	404	336	0.002976	1
27	4	13	1	418	336	0.002976	1
28	4	14	1	452	336	0.002976	1
29	4	15	1	412	336	0.002976	1
30	4	16	1	406	336	0.002976	1
31	4	17	1	470	336	0.002976	1
32	4	18	1	386	336	0.002976	1
33	8	1	2	243	336	0.002976	1
34	8	2	2	259	336	0.002976	1
35	8	3	2	255	336	0.002976	1
36	8	4	2	254	336	0.002976	1
37	8	5	2	271	84	0.011905	0
38	8	6	2	293	336	0.002976	1
39	8	7	2	270	84	0.011905	0
40	8	8	2	245	24	0.041667	0
41	8	9	2	260	336	0.002976	1
42	8	10	1	396	336	0.002976	1
43	8	15	1	436	84	0.011905	0
44	8	16	1	386	12	0.083333	0
45	8	17	1	431	336	0.002976	1
46	8	18	1	390	7	0.142857	0
47	16	1	2	258	84	0.011905	0
48	16	2	2	238	120	0.008333	0
49	16	3	2	271	336	0.002976	1
50	16	4	2	258	336	0.002976	1
51	16	5	2	259	84	0.011905	0
52	16	6	2	281	336	0.002976	1
53	16	7	2	255	7	0.142857	0
54	16	8	2	249	336	0.002976	1

OBS	DOSE	AN	SEX	WT	HTD	RHTD	S
55	16	9	2	261	12	0.083333	0
56	16	10	1	379	84	0.011905	0
57	16	11	1	388	84	0.011905	0
58	16	12	1	385	7	0.142857	0
59	16	13	1	388	84	0.011905	0
60	16	14	1	430	336	0.002976	1
61	16	15	1	427	120	0.008333	0
62	16	16	1	411	7	0.142857	0
63	16	17	1	355	336	0.002976	1
64	16	18	1	350	7	0.142857	0
65	32	1	2	255	7	0.142857	0
66	32	2	2	242	12	0.083333	0
67	32	3	2	235	12	0.083333	0
68	32	4	2	260	12	0.083333	0
69	32	5	2	287	7	0.142857	0
70	32	6	2	279	7	0.142857	0
71	32	7	2	247	31	0.032258	0
72	32	8	2	272	7	0.142857	0
73	32	9	2	264	7	0.142857	0
74	32	10	1	363	48	0.020833	0
75	32	11	1	397	7	0.142857	0
76	32	12	1	417	12	0.083333	0
77	32	13	1	392	7	0.142857	0
78	32	14	1	443	336	0.002976	1
79	32	15	1	444	96	0.010417	0
80	32	16	1	422	7	0.142857	0
81	32	17	1	369	7	0.142857	0
82	32	18	1	386	7	0.142857	0
83	64	1	2	241	7	0.142857	0
84	64	2	2	247	12	0.083333	0
85	64	3	2	264	7	0.142857	0
86	64	4	2	273	12	0.083333	0
87	64	5	2	256	7	0.142857	0
88	64	6	2	235	7	0.142857	0
89	64	7	2	245	12	0.083333	0
90	64	8	2	251	7	0.142857	0
91	64	9	2	266	7	0.142857	0
92	64	10	1	384	7	0.142857	0
93	64	11	1	430	7	0.142857	0
94	64	12	1	445	48	0.020833	0
95	64	13	1	426	7	0.142857	0
96	64	14	1	427	12	0.083333	0
97	64	15	1	380	7	0.142857	0
98	64	16	1	439	7	0.142857	0
99	64	17	1	474	7	0.142857	0
100	64	18	1	387	12	0.083333	0

SEX=1

GENERAL LINEAR MODELS PROCEDURE

MEANS

DOSE	N	S	HTD	RHTD	WT
0	8	1.00000000	336.000000	0.00297619	426.000000
4	9	1.00000000	336.000000	0.00297619	419.111111
8	5	0.40000000	155.000000	0.04880952	407.800000
16	9	0.22222222	118.333333	0.05317460	390.333333
32	9	0.11111111	58.555556	0.09242725	403.666667
64	9	0.00000000	12.666667	0.11607143	421.333333

SEX=1

GENERAL LINEAR MODELS PROCEDURE

LEAST SQUARES MEANS

DOSE	S LSMEAN	HTD LSMEAN	RHTD LSMEAN
0	0.97489159	324.187494	0.00994787
4	0.98671235	329.748589	0.00666568
8	0.40612127	157.879813	0.04710987
16	0.25831484	135.313467	0.04315302
32	0.12432484	64.772086	0.08875828
64	-0.01710080	4.621422	0.12081969

SEX=2

GENERAL LINEAR MODELS PROCEDURE

MEANS

DOSE	N	S	HTD	RHTD	WT
0	7	1.00000000	336.000000	0.00297619	252.285714
4	8	1.00000000	336.000000	0.00297619	259.250000
8	9	0.66666667	245.333333	0.00925926	261.111111
16	9	0.44444444	183.444444	0.03002646	258.888889
32	9	0.00000000	11.333333	0.11072709	260.111111
64	9	0.00000000	8.666667	0.12301587	253.111111

SEX=2

GENERAL LINEAR MODELS PROCEDURE

LEAST SQUARES MEANS

DOSE	S LSMEAN	HTD LSMEAN	RHTD LSMEAN
0	1.00988463	338.423902	0.00428125
4	0.99699755	335.263741	0.00257978
8	0.66022032	243.752565	0.00840816
16	0.44211021	182.872046	0.02971827
32	-0.00459590	10.206331	0.11012030
64	0.00835727	10.716031	0.12411927

SEX=1

ANALYSIS OF VARIANCE PROCEDURE

DEPENDENT VARIABLE: WT

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE	C.V.
MODEL	5	7725.69886621	1545.13977324	2.07	0.0878	0.194028	6.6410
ERROR	43	32091.68888889	746.31834625		STD DEV		WT MEAN
CORRECTED TOTAL	48	39817.38775510			27.31882769		411.36734694

SOURCE	DF	ANOVA SS	F VALUE	PR > F
DOSE	5	7725.69886621	2.07	0.0878

SEX=2

ANALYSIS OF VARIANCE PROCEDURE

DEPENDENT VARIABLE: WT

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE	C.V.
MODEL	5	583.43744164	116.68748833	0.47	0.7983	0.049394	6.1314
ERROR	45	11228.48412698	249.52186949		STD DEV		WT MEAN
CORRECTED TOTAL	50	11811.92156863			15.79626125		257.62745098

SOURCE	DF	ANOVA SS	F VALUE	PR > F
DOSE	5	583.43744164	0.47	0.7983

SEX=1

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: HTD

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE	C.V.
MODEL	6	848695.41900858	141449.23650143	18.40	0.0001	0.724410	52.4377
ERROR	42	322872.54017509	7687.44143274		STD DEV		HTD MEAN
CORRECTED TOTAL	48	1171567.95918367			87.67805559		167.20408163

SOURCE	DF	TYPE I SS	F VALUE	PR > F	DF	TYPE IV SS	F VALUE	PR > F
DOSE	5	827781.73696145	21.54	0.0001	5	756561.74611969	19.68	0.0001
WT	1	20913.68204713	2.72	0.1065	1	20913.68204713	2.72	0.1065

SEX=1

ANALYSIS OF VARIANCE PROCEDURE

DEPENDENT VARIABLE: HTD

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE	C.V.
MODEL	5	827781.73696145	165556.34739229	20.71	0.0001	0.706559	53.4765
ERROR	43	343786.22222222	7995.02842377		STD DEV		HTD MEAN
CORRECTED TOTAL	48	1171567.95918367			89.41492282		167.20408163

SOURCE	DF	ANOVA SS	F VALUE	PR > F
DOSE	5	827781.73696145	20.71	0.0001

SEX=2

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: HTD

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE	C.V.
MODEL	6	925906.74943616	154317.79157269	20.83	0.0001	0.739591	48.3528
ERROR	44	326010.23095599	7409.32343082		STD DEV		HTD MEAN
CORRECTED TOTAL	50	1251916.98039216			86.07742695		178.01960784

SOURCE	DF	TYPE I SS	F VALUE	PR > F	DF	TYPE IV SS	F VALUE	PR > F
DOSE	5	923594.75816993	24.93	0.0001	5	922254.20927222	24.89	0.0001
WT	1	2311.99126623	0.31	0.5793	1	2311.99126623	0.31	0.5793

SEX=2

ANALYSIS OF VARIANCE PROCEDURE

DEPENDENT VARIABLE: HTD

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE	C.V.
MODEL	5	923594.75816993	184718.95163399	25.32	0.0001	0.737744	47.9817
ERROR	45	328322.22222222	7296.04938272		STD DEV		HTD MEAN
CORRECTED TOTAL	50	1251916.98039216			85.41691509		178.01960784

SOURCE	DF	ANOVA SS	F VALUE	PR > F
DOSE	5	923594.75816993	25.32	0.0001

SEX=1

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: S

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE	C.V.
MODEL	6	8.57249435	1.42874906	16.90	0.0001	0.707159	64.7530
ERROR	42	3.54995463	0.08452273		STD DEV		S MEAN
CORRECTED TOTAL	48	12.12244898			0.29072793		0.44897959

SOURCE	DF	TYPE I SS	F VALUE	PR > F	DF	TYPE IV SS	F VALUE	PR > F
OCSE	5	8.47800454	20.06	0.0001	5	7.71370364	18.25	0.0001
WT	1	0.09448982	1.12	0.2964	1	0.09448982	1.12	0.2964

SEX=1

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: RHTD

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE	C.V.
MODEL	6	0.09964935	0.01660823	7.52	0.0001	0.517864	86.5138
ERROR	42	0.09277451	0.00220892		STD DEV		RHTD MEAN
CORRECTED TOTAL	48	0.19242386			0.04699911		0.05407556

SOURCE	DF	TYPE I SS	F VALUE	PR > F	DF	TYPE IV SS	F VALUE	PR > F
DOSE	5	0.09236449	8.36	0.0001	5	0.08786023	7.96	0.0001
WT	1	0.00728486	3.30	0.0765	1	0.00728486	3.30	0.0765

SEX=1

ANALYSIS OF VARIANCE PROCEDURE

DEPENDENT VARIABLE: RHTD

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE	C.V.
MODEL	5	0.09236449	0.01847290	7.94	0.0001	0.480005	89.2059
ERROR	43	0.10005937	0.00232696		STD DEV		RHTD MEAN
CORRECTED TOTAL	48	0.19242386			0.04823859		0.05407556

SOURCE	DF	ANOVA SS	F VALUE	PR > F
DOSE	5	0.09236449	7.94	0.0001

SEX=2

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: RHTD

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE	C.V.
MODEL	6	0.13349406	0.02224901	23.93	0.0001	0.765412	62.1598
ERROR	44	0.04091403	0.00092986		STD DEV		RHTD MEAN
CORRECTED TOTAL	50	0.17440809			0.03049368		0.04905688

SOURCE	DF	TYPE I SS	F VALUE	PR > F	DF	TYPE IV SS	F VALUE	PR > F
DOSE	5	0.13282384	28.57	0.0001	5	0.13344299	28.70	0.0001
WT	1	0.00067022	0.72	0.4005	1	0.00067022	0.72	0.4005

SEX=2

ANALYSIS OF VARIANCE PROCEDURE

DEPENDENT VARIABLE: RHTD

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE	C.V.
MODEL	5	0.13282384	0.02656477	28.75	0.0001	0.761569	61.9667
ERROR	45	0.04158425	0.00092409		STD DEV		RHTD MEAN
CORRECTED TOTAL	50	0.17440809			0.03039892		0.04905688

SOURCE	DF	ANOVA SS	F VALUE	PR > F
DOSE	5	0.13282384	28.75	0.0001

SEX=1

ANALYSIS OF VARIANCE PROCEDURE

DEPENDENT VARIABLE: S

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE	C.V.
MODEL	5	8.47800454	1.69560091	20.01	0.0001	0.699364	64.8418
ERROR	43	3.64444444	0.08475452		STD DEV		S MEAN
CORRECTED TOTAL	48	12.12244898			0.29112630		0.44897959

SOURCE	DF	ANOVA SS	F VALUE	PR > F
DOSE	5	8.47800454	20.01	0.0001

SEX=2

ANALYSIS OF VARIANCE PROCEDURE

DEPENDENT VARIABLE: S

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F	R-SQUARE	C.V.
MODEL	5	8.52287582	1.70457516	18.17	0.0001	0.668718	62.4877
ERROR	45	4.22222222	0.09382716		STD DEV		S MEAN
CORRECTED TOTAL	50	12.74509804			0.30631219		0.49019608

SOURCE	DF	ANOVA SS	F VALUE	PR > F
DOSE	5	8.52287582	18.17	0.0001