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REPORT ON THE CARCINOGENESIS BIOASSAY OF CHLOROFORM

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REPORT ON CARCINOGENESIS BIOASSAY OF

CHLOROFORM

Carcinogenesis Program, Division of Cancer Cause and Prevention

National Cancer Institute

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CONTRIBUTORS: This report presents a synopsis of results of a carcinogenesis bioassay conducted by the Carcinogen Bioassay and Program Resources Branch, Carcinogenesis Program, Division of Cancer Cause and Prevention, National Cancer Institute (NCI), Bethesda, Maryland. This research was conducted at the Hazleton Laboratories America, Incorporated, Vienna, Virginia, initially under direct contract to the NCI and currently under a subcontract to Tracor Jitco, Incorporated, Prime Contractor for the NCI Carcinogenesis Bioassay Program.

The results of this study were reviewed and this report was prepared by Drs. N. P. Page[1] and U. Saffiotti[1]. Ms. J. W. Chase[1] functioned as Executive Secretary for the report review, while Ms. P. A. Steinour[1] was responsible for the consolidation and technical preparation of the report. The experimental design, including dose levels were determined by the NCI project officers, Drs. J. H. Weisburger[1,2] and E. K. Weisburger[1]; principal investigators for the contract were Drs. M. B. Powers[3], R. W. Voelker[3], W. A. Olson[3,4] and W. M. Weatherholtz[3,5]; chemical analysis was performed by Dr. C. L. Guyton[3,6]; technical supervisor of animal treatments and experiments was Ms. K. J. Petrovics[3]; the pathology was supervised by Dr. R. W. Voelker; microscopic diagnosis was conducted by Drs. R. A. Renne[7], J. F. Ferrell[7], and R. T. Habermann[3], and reviewed by Drs. C. N. Barron[8] and R. A. Squire[1]; data collection and data preparation was performed by EG&G/Mason Research Institute[9]; statistical analysis was performed by Drs. K. C. Chu[1] and K. M. Patel[9], and reviewed by Dr. J. J. Gart[10]. This report was reviewed by a panel of consultants as well as members of the contributing organizations[1,3,7,8,9].

A technical report is in preparation which will provide additional details of the design, materials and methods used, conduct and results of the study.

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Summary: A carcinogenesis bioassay of USP grade chloroform was conducted using Osborne-Mendel rats and B6C3F$_1$ mice. Chloroform was administered orally (by gavage) in corn oil to 50 animals of each sex and at two dose levels five times per week for 78 weeks. Rats were started on test at 52 days of age and sacrificed after 111 weeks. The dose levels for males were 90 and 180 mg/kg body weight. Female rats were started at 125 and 250 mg/kg, reduced to 90 and 180 mg/kg after 22 weeks, with an average level of 100 and 200 mg/kg for the study. A decrease in survival rate and weight gain was evident for all treated groups. The most significant observation ($P = .0016$) was kidney epithelial tumors in male rats with incidences of: 0% in controls, 8% in the low dose and 24% in the high dose groups. Although an increase in thyroid tumors was also observed in treated female rats, this finding was not considered biologically significant. Mice were started on test at 35 days and sacrificed after 92-93 weeks. Initial dose levels were 100 and 200 mg/kg for males and 200 and 400 mg/kg for female mice. These levels were increased after 18 weeks to 150/300 and 250/500 mg/kg respectively so that the average levels were 138 and 277 mg/kg for males and 238 and 477 mg/kg for female mice. Survival rates and weight gains were comparable for all groups except high dose females which had a decreased survival. Highly significant increases ($P < .001$) in hepatocellular carcinoma were observed in both sexes of mice with incidences of: 98% and 95% for males and females at the high dose; 36% and 80% for males and females at the low dose as compared with 6% in both matched and colony control males, 0% in matched control females and 1% in colony control females. Nodular hyperplasia of the liver was observed in many low dose male mice that had not developed hepatocellular carcinoma.

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I. INTRODUCTION:

Chloroform ($CHCl_3$), also known as trichloromethane, is primarily used (93%) in the manufacture of fluorocarbons for refrigerants, propellants, and plastics. The remainder is used for many purposes including extracting and purifying antibiotics, as an industrial solvent, in preparation of dyes, drugs and pesticides, as a component of some toothpastes, cough medicines, liniments, salves, in photographic processing and in industrial drycleaning (1). Chloroform was selected for carcinogenesis bioassay as one agent in a study of halogenated alkanes that occur in the general and occupational environment of humans. Chloroform was included in this study because of its chemical structure, use, and prior suspicion of carcinogenicity (2).

II. MATERIALS AND METHODS:

Design of Chronic Studies - The experiment's basic design consisted of administering chloroform at two dose levels to groups of 50 animals of each sex and species. Thus, 400 treated animals divided into 8 groups were used. Treatment was by oral gavage 5 times per week for 78 weeks with sacrifice of surviving rats at 111 weeks from start of study and mice at 92-93 weeks. Rats were started on treatment at 52 days and mice at 35 days of age. The initial highest dose level was the estimated maximum tolerated dose (MTD) based upon a preliminary

toxicity study in which chloroform was administered for 6 weeks at various dose levels followed by an additional 2 weeks of observation. The parameters evaluated in the toxicity study were mainly survival, weight differences and clinical/necropsy observations.

The dose levels for male rats were 180 and 90 mg/kg throughout the chronic study. For female rats, it was necessary to lower the doses from starting levels of 250 and 125 mg/kg to 180 and 90 mg/kg after 22 weeks. The initial dose levels for mice were 200 and 100 mg/kg for males and 400 and 200 mg/kg for females. These were increased slightly after 18 weeks to 300/150 mg/kg for males and 500/250 mg/kg for females since it was considered that the animals could tolerate a higher dose. Actual doses, days on treatment at each dose, "time weighted average dose levels", and estimated average daily doses for each group are presented in Table I. The average doses ranged from 36-90 milligrams for rats and 4-14 milligrams for mice.

Three types of controls were used in this study, "matched" controls, "colony" controls and "positive" controls. The "matched" controls were animals as nearly identical to the chloroform-treated animals as possible. They were from the same source, with identical animal care, housed in same room and received a like quantity of the vehicle, corn oil, as the treated animals. Rats were assigned to treated and matched control groups in a randomized manner, such that the average

Table I. Dosage Schedule - Chloroform

SPECIES	SEX	DOSAGE GROUP	DOSE	DOSE LEVEL[1] (MG/KG)	TREATMENT PERIOD (DAYS)	TIME WEIGHTED AVE. DOSE LEVEL (MG/KG)[2]	ESTIMATED AVE. DOSE/ANIMAL/DAY (MG)[3]
Rat (OM)	M	Low	Initial Final	90	546	90	50
	M	High	Initial Final	180	546	180	90
	F	Low	Initial Final	125 90	154 392	-- 100	36
	F	High	Initial Final	250 180	154 392	-- 200	70
Mice (B6C3F1)	M	Low	Initial Final	100 150	126 420	-- 138	4
	M	High	Initial Final	200 300	126 420	-- 277	8
	F	Low	Initial Final	200 250	126 420	-- 238	7
	F	High	Initial Final	400 500	126 420	-- 477	14

NOTES: 1 - Dose administered in corn oil 5 x/week
2 - Time-weighted average dose = Σ(dose x treatment period in days)/ Σ(no. days receiving each dose).
3 - Based upon average weight as presented in Figures 2 and 9.

weight in each group was approximately the same. The matched control groups of mice were started on the vehicle treatment 1 week earlier than the chloroform-treated mice but were otherwise comparable.

"Colony" control animals were of same strain and source, and were started on test within 3 months of the chloroform-treated animals. They were maintained in the same manner and received corn oil as described for the chloroform "matched" controls. The colony control included the chloroform-matched controls plus matched controls to other chemicals that were tested simultaneously. The "matched" controls consisted of 20 for each sex of each species, whereas the colony controls consisted of 99 male and 98 female rats and 77 male and 80 female mice. All colony control mice were housed in the same room whereas colony control rats were housed in two different rooms.

"Positive" control animals were of same strain and source, also housed in the same way. These, however, received a known carcinogen, carbon tetrachloride, and were included as a control for the entire series of halogenated chemicals on test. The purpose of the positive control was to verify the sensitivity of the test animals to carcinogenicity by halogenated chemicals and to serve as a check on procedures and techniques. The experimental design for the carbon tetrachloride test was essentially the same as the chloroform study

except that the dose levels were: 47 and 94 mg/kg for male rats; 80 and 160 mg/kg for female rats; and 1250 and 2500 mg/kg for both male and female mice. A comparison of chloroform and CCl_4 dose levels is presented in Table II.

Table II. Comparison of Dose Levels for Chloroform and Carbon Tetrachloride-Treated Groups

EXPERIMENTAL GROUP		CHLOROFORM	CCl_4
Rats		(mg/kg)*	(mg/kg)*
Males	Low Dose	90	47
	High Dose	180	94
Females	Low Dose	100	80
	High Dose	200	160
Mice			
Males	Low Dose	138	1250
	High Dose	277	2500
Females	Low Dose	238	1250
	High Dose	477	2500

* Mg/kg body weight. Single dose administered by gavage 5 x/week for 78 weeks.

In evaluating suspected treatment-related effects, the matched controls were initially compared to the test groups. Since the matched control groups were small, 20 per sex and species, in comparison with the treated groups, comparisons were also made with the larger groups of colony controls. Complete data on all tumors are presented for the matched controls and for the chloroform-treated groups. For colony and CCl_4 controls, only the data relating to total tumors, and/or specific lesions of concern are presented in the analysis tables and comparison figures.

Chemicals - The material tested was USP grade chloroform purchased from Aldrich Chemical Company, Inc., 940 West Saint Paul Avenue, Milwaukee, Wisconsin. USP grade chloroform should be at least 99.0% chloroform and 0.5-1.0% ethyl alcohol. Ethyl alcohol is added by the manufacturer as a stabilizer. The purity was checked by Hazleton Laboratories America, Inc., using gas-liquid chromatography (glc) with flame ionization detector and infrared spectrometry. Approximately 98% of the glc peak area was chloroform with ethyl alcohol accounting for the remainder. Infrared spectrometric and glc analysis at intervals during the bioassay indicated no significant change in chemical composition.

Chloroform was administered by oral gavage using corn oil as a vehicle. Fresh solutions of chloroform in corn oil were prepared weekly in amounts sufficient to treat all animals, sealed, and refrigerated until use. The concentration of chloroform in corn oil was 10% for rats and 2-5% for mice. The corn oil was purchased from a distributor, C. F. Sauer Company, Richmond, Virginia. For safety purposes, the test solutions were maintained cold to minimize volatilization, and dosing was conducted under a hood.

Animals - Rats and mice of both sexes, obtained through contracts of the Division of Cancer Treatment, NCI, were used in these tests. The rats were Osborne-Mendel strain, procured from Battelle Memorial Institute, Columbus, Ohio, and the mice were $B6C3F_1$ hybrids obtained

from Charles River Breeding Laboratories, Inc., Wilmington, Massachusetts. Upon receipt, animals were quarantined for 7-10 days, determined to be free from observable disease or parasites and randomly assigned to the experimental groups.

Animal Maintenance - All animals were housed in temperature and humidity-controlled rooms. Incoming air was filtered through 2-inch thick disposable fiberglass filters at a rate providing 12 changes of room air per hour. Lighting was provided on a 12-hour per day cycle. Rats were individually housed in suspended steel, wire-mesh cages and mice in polypropylene cages. Ten mice were housed in each cage. Clean cages with bedding (Sani-chips, manufactured by Shurfire) were provided twice each week for mice, while the rat cages were changed weekly.

Food containers were changed and sterilized once a week for the first 10 weeks and once a month thereafter. Sterile glass water bottles were provided three times a week for mice and twice a week for rats. Food (Wayne Laboratory Blox Meal) and water were consumed ad libitum. Racks were rotated in the room and positioned at random. The rats were housed in a room in which 1,1,2,2-tetrachloroethane, 3-chloropropene, ethylene dibromide and carbon tetrachloride were also on test. Chloroform-treated mice were housed in the same room as mice receiving 1,1,2,2-tetrachloroethane, 3-chloropropene, chloropicrin, 1,1-dichloroethane, trichloroethylene, sulfolene, iodoform, ethylene dichloride,

methylchloroform, 1,1,2-trichloroethane, tetrachloroethylene, hexachloroethane, carbon disulfide, trichlorofluoromethane, carbon tetrachloride, ethylene dibromide and dibromochloropropane. Vehicle matched control groups were housed in the same room as their respective treated groups.

Clinical/Pathology Examinations - All animals were inspected twice daily. Body weights and food consumption were recorded weekly for the first 10 weeks and monthly thereafter. Animals appearing moribund when examined were sacrificed and immediately necropsied. In the chronic study a necropsy was performed on each animal regardless of whether it died, was sacrificed early or survived to termination. Animals were anesthetized, exsanguinated and immediately necropsied. The following tissues were taken from sacrificed animals and where possible from those found dead: brain, pituitary, adrenal, thyroid, parathyroid, trachea, esophagus, thymus, salivary gland, lymph nodes (mesenteric and cervical), heart, nasal passages, lung, spleen, liver, kidney, stomach, small intestine, large intestine, pancreas, urinary bladder, prostate or uterus, testis with epididymus, seminal vesicles, ovary, skin with mammary gland, muscle, nerve, bone, bone marrow, and tissue masses.

Tissues were preserved in 10% buffered formalin, embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined

microscopically. Because some tissues (especially small organs) were lost during the gross autopsy, and the histologic preparation process, the denominator used for a particular organ, tissue or lesion in Appendixes A and B, does not necessarily equal the number of animals placed on experiment in each group.

The pathologic findings of the Experimental Pathology Laboratories and Hazleton Laboratories America, Inc., were reviewed by pathologists at Tracor Jitco, Inc., and the National Cancer Institute, with special attention given to hepatic and renal lesions.

Data Recording and Statistical Analysis - Pertinent data on this experiment have been recorded in an automatic data processing system, the Carcinogenesis Bioassay Data System (3). The data elements include descriptive information on the chemical, animals, experimental design, clinical observations, survival, animal weights, and individual pathologic results, as recommended by the International Union Against Cancer (UICC) (4). Data tables were generated for statistical review and verification of data transcription.

Survival probabilities were estimated by the product-limit procedure of Kaplan and Meier (5). The statistical analysis of tumor incidence reported in Tables III and VII was performed using the Armitage Test

for linear trend in proportions (6a). This analysis determines if the slope of a dose-response plot is statistically different from zero ($P < .05$), assuming a linear trend. If the associated statistic which detects departure from linear trend was significant, then the Fisher Exact Test (6b) was used to compare controls to each dose level. A correction for simultaneous comparison of controls was made using the Bonferroni inequality (7). Thus, a corrected P value < .05 was also deemed significant.

III. RESULTS:

A. Rats

1. Survival - As illustrated in Figure 1, the survival rate for both male and female rats treated with chloroform was considerably less than for controls. While decreased survival appeared dose-related, the difference between high and low dose females became substantial only after 70 weeks. At approximately 90 weeks, the death rate for male controls increased, probably due to respiratory and renal conditions.

2. Body Weights, Food Consumption and Clinical Signs - As evident in Figure 2, treated animals of both sexes gained less weight than did the controls. The weight gain appears directly related to the dose level. Food consumption was also slightly depressed in treated groups.

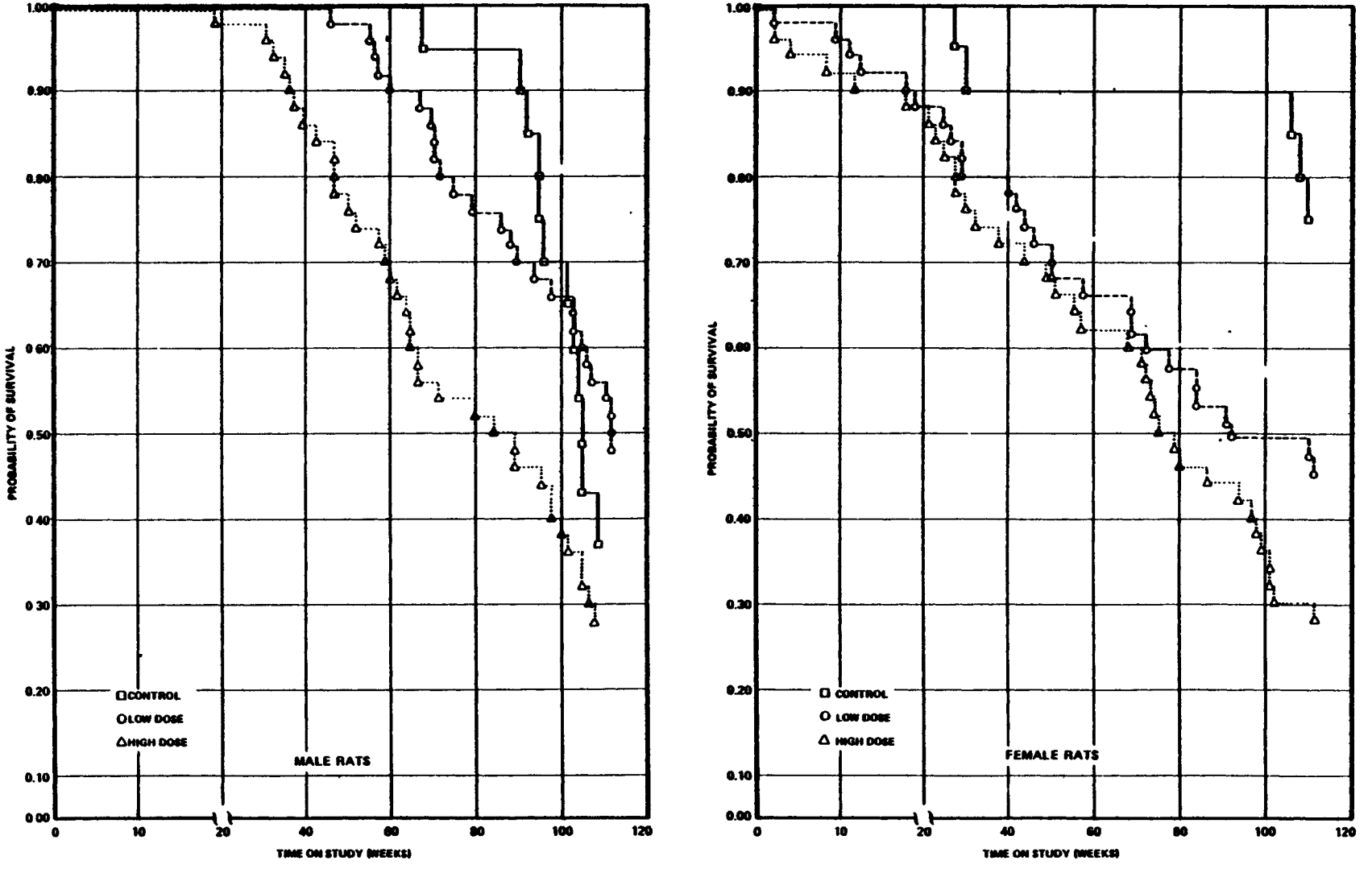


Figure 1. Survival Curves for Rats (Chloroform)

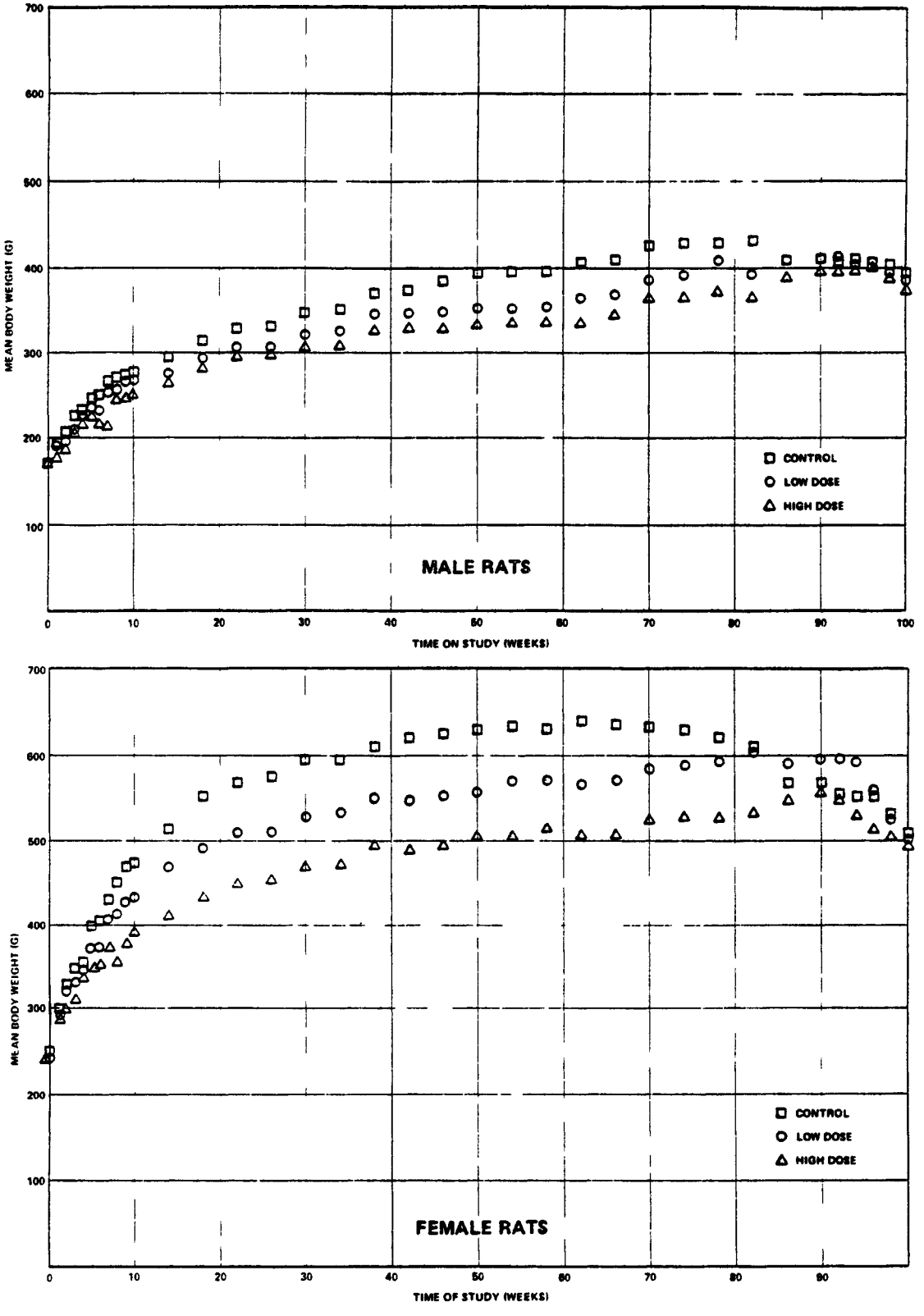


Figure 2. Growth Curves for Rats - Chloroform

During the first 10 weeks of the study in all treatment groups, low to moderate numbers of animals developed a hunched appearance, urine stains on the lower abdomen, redness of the eyelids and apparent weight loss. During the remainder of the first year of the study, a large percentage of the test animals were hunched and wheezing, and the urine staining continued. The first palpable nodule was noted at week 50 in the low dose male group.

During the second year of the study, the incidence of the above described clinical signs gradually increased in all test groups. In addition, rough haircoat, stains on haircoat, localized alopecia on extremities or trunk, sores on the body, head, and particularly on the tail were also noted. Both matched and colony control groups began to exhibit similar signs in the 70th week and by 110 weeks clinical observations were essentially the same in the test and control groups. Several palpable nodules and tissue masses were noted in all groups during the latter part of the second year. Occasionally, the small nodules palpated at one observation period were not palpable at a later time or were replaced by small sores. These nodules were apparently small subcutaneous abscesses which drained and healed.

3. Pathology - Of the 200 treated and 40 matched control rats entered into the study, four were lost (two missing and two

autolyzed). Histopathologic findings of tumors are tabulated in Appendix A. From an examination of Appendix A, differences in tumor incidences between chloroform-treated and controls were apparent only for kidney tumors in males and females and thyroid tumors in female rats. These data were statistically analyzed and the results presented in Table III. In addition to those results and that of total tumor incidence, the incidence of hepatocellular carcinomas are also presented for comparison purposes as highly statistical differences of this tumor type were observed in mice.

The total incidence of animals with tumors of any kind did not vary greatly between treated and control groups. A slightly higher percentage of matched controls had tumors than in the high dose males and the females of both doses. As the survival of all treated groups was considerably less, this slight (and not significant) negative trend is likely attributable to fewer treated animals at risk for the development of spontaneous tumors that appear late in life. As the pathology diagnosis for colony controls are undergoing review to standardize nomenclature, the data on total tumors are not presented. However, there was no indication of an unusual response in the matched controls from other control groups included in the colony control.

A statistically significant increase ($P < .05$) in epithelial tumors of renal tubular-cell origin was observed in treated males,

Table III. Analysis of Total Tumors and Specific Liver, Kidney and Thyroid Tumors - Rats (Chloroform)

	MALE				FEMALE			
	CONTROLS				CONTROLS			
TREATMENT[1] :	COLONY	MATCHED	LOW	HIGH	COLONY	MATCHED	LOW	HIGH
Total Tumor-Bearing Animals/Animals[2]	--	9/19 47%	24/50 48%	20/50 40%	--	12/20 60%	24/49 49%	24/48 50%
P Value[3]	--	.2347#	--	--	--	.2733#	--	--
Time to Tumor (weeks)[4]	--	95	70	42	--	108	73	49
Hepatocellular Carcinoma/Animals[2]	1/99 1%	0/19 0%	0/50 0%	1/50 2%	0/98 0%	0/20 0%	0/49 0%	0/48 0%
P Value[3]	.3366	.1497	--	--	1.000	1.000	--	--
Time to Tumor (weeks)[4]	97	--	--	111	--	--	--	--
Kidney Epithelial Tumors/ Animals[2]	0/99 0%	0/19 0%	4/50 8%	12/50 24%	0/98 0%	0/20 0%	0/49 0%	2/48 4%
P Value[3]	.0000*	.0016*	--	--	.0592[6]	.1662[6]	--	--
Time to Tumor (weeks)[4]	--	--	102	80	--	--	--	102
Thyroid Tumors/ Animals[2]	8/99 8%	4/19 21%	3/49 6%	4/48 8%	1/98 1%	1/19 5%	8/49 16%	10/46 22%
P Value[3]	.4874#	.1123#	--	--	.0000*	.0574	--	--
Time to Tumor (weeks)[4]	103	103	111	111	110	110	73	49
Survival at Terminal Sacrifice (111 weeks)	26%	37%	48%	28%	51%	75%	45%	29%

1 - Oral dose of chloroform in corn oil administered by gavage five times per week.
2 - Based on animals whose tissues were examined from a specific organ.
3 - One-tail P value from Armitage test for linear trend in proportions, unless otherwise stated.
4 - Time to detection of first tumor (at death).
5 - Data departure from linear trend (for departure statistic; $P < .05$). Fisher Exact Test is used comparing controls to a dose level. Bonferroni (7) correction for simultaneous comparison of controls is included.
6 - P value computed using exact test (Cox, Analysis of Binary Data) as the number of tumors is too small for Armitage method.
* - Statistically significant ($P < .05$).
# - P value given in direction of negative trend.

as shown by the data presented in Table III. Primary epithelial tumors were observed in the kidneys and renal pelvis of 18 rats, all chloroform-treated. Of these, 16 were in males: 12 in the high dose group and 4 at the low dose. The other two tumors were in the high dose female group. The observation of two kidney tumors in the high dose female group was not significant when compared with colony controls (P = .0592). No primary epithelial tumor of the kidney was found in any of the 49 low dose females or 197 controls. Figure 3 illustrates the percentages of animals with these tumors according to experimental group. In addition to the purely epithelial tumors, four malignant mixed tumors, and three hamartomas were also observed. However, these were found in both the colony control and treated groups, and not considered treatment related.

Two male rats had more than one primary renal tumor: a low dose male with both a malignant mixed tumor and a tubular cell adenoma in the left kidney, and a high dose male with both a tubular cell carcinoma and a tubular cell adenoma in the right kidney.

Of the 13 tumors of renal tubular-cell epithelium observed in 12 of the 50 high dose male rats, ten were carcinomas and three adenomas; two of the carcinomas were found to have metastasized. Two carcinomas and two adenomas of renal tubular epithelium were observed among the 50 low dose male rats. One carcinoma of renal

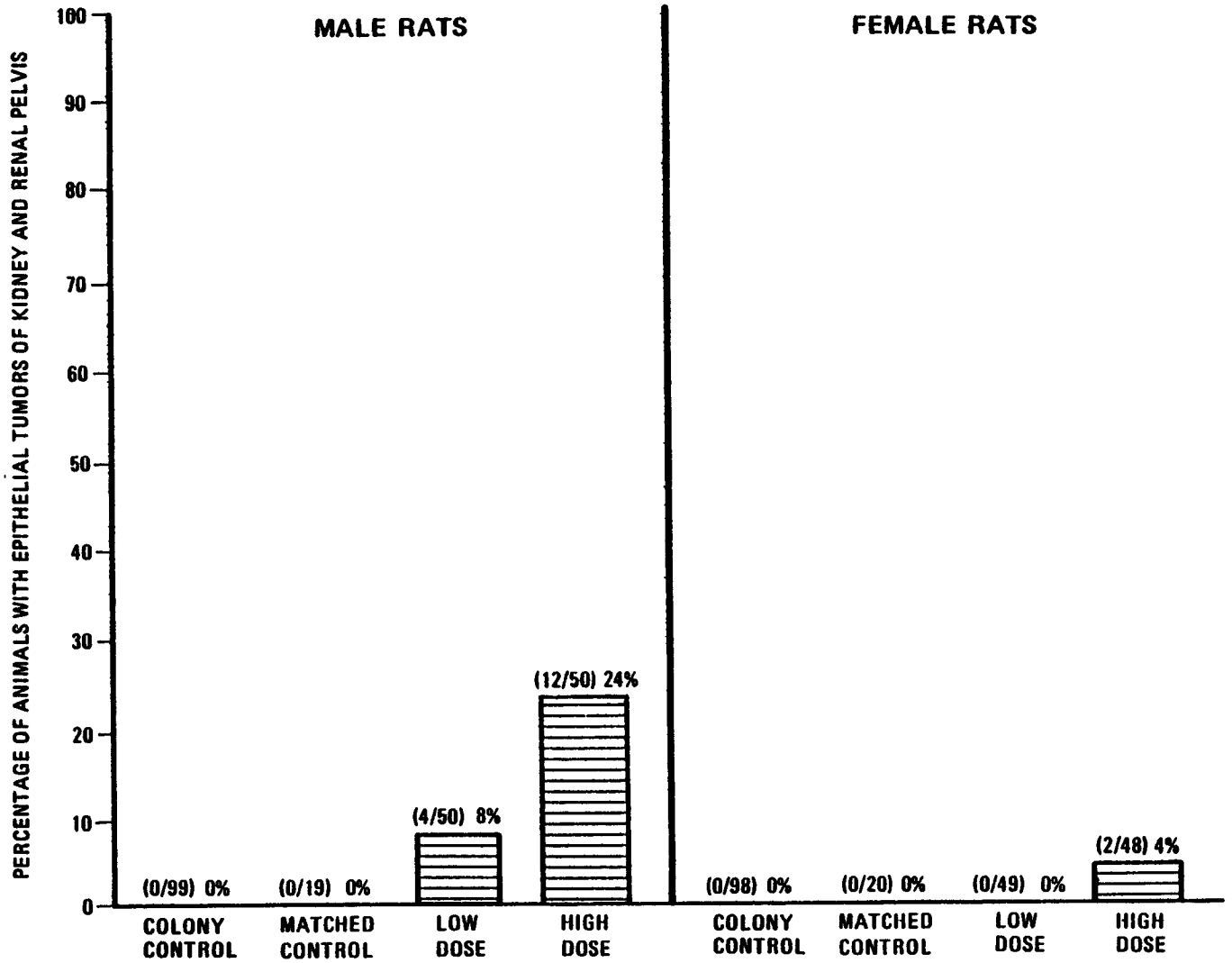


Figure 3. Comparison of Incidences of Epithelial Tumors of Kidney and Renal Pelvis (Chloroform)

tubular epithelium and one squamous cell carcinoma arising from renal pelvic transitional epithelium were observed among the 48 high dose female rats. The tubular-cell adenocarcinoma widely metastasized.

Microscopically, the appearance of these epithelial tumors varied from circumscribed, well-differentiated tubular-cell adenomas to highly pleomorphic, poorly differentiated carcinomas which had invaded and metastasized. The cells in adenomas were relatively uniform and polygonal, with abundant eosinophilic cytoplasm. Nuclei were central or basal in location, with minimal atypia and little increase in mitotic index (Figure 4). Most carcinomas were vary large and replaced a considerable portion of the renal parenchyma. They were poorly circumscribed and infiltrated surrounding normal tissues. These were of irregular sheets, nests, and tubular arrangements of cells with varying degrees of anaplasia and increased nuclear/cytoplasmic ratio (Figures 5 and 6). The nests of cells were often surrounded by a delicate fibrovascular stroma, and central necrosis was sometimes present in the more anaplastic neoplasms. Rarely, a papillary glandular pattern was observed.

The seven renal tumors that were <u>not purely epithelial</u> contained renal epithelial, stromal, and fatty tissue components. Four of these (two in low dose male rats and two in male colony controls) were

histologically malignant and were classified as malignant mixed tumors (Figure 7). The other three tumors (one each from low dose male, high dose male and male colony controls) appeared benign and were classified as hamartomas. In addition to these seven tumors, one hemangioma also occurred in the kidney of a high dose female rat.

Criteria for differentiating malignant from benign primary tumors of the kidney, both purely epithelial and mixed types, included: loss of normal cellular architecture; evidence of invasion of renal parenchyma, vessels, or adjacent tissues; cellular atypia including nuclear/cytoplasmic ratio; prominent nucleoli; numerous and/or abnormal mitotic figures; and abnormal size and shape of neoplastic cells. Evidence of metastasis, although observed in several tumors, was not a requirement for classification of tumors as malignant.

Malignant mixed tumors and hamartomas have been seen in a low spontaneous incidence at several laboratories in aged Osborne-Mendel rats used on the Bioassay Program, occurring with equal frequency in control and test rats. In contrast, purely epithelial tumors of the renal tubules or renal pelvic transitional epithelium rarely occur spontaneously in these Osborne-Mendel rats.

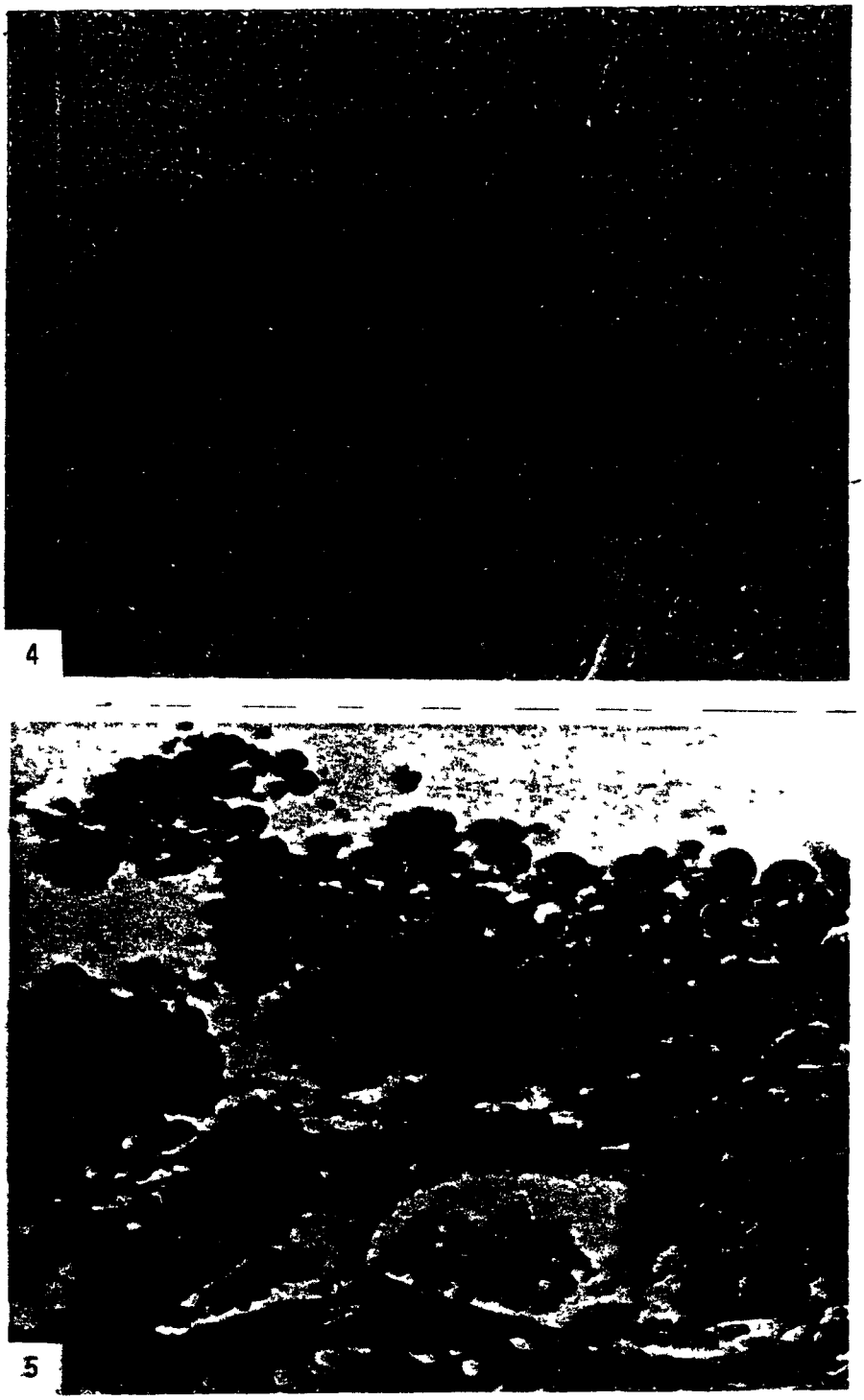


Figure 4. Well differentiated tubular cell adenoma with distinct margin, kidney. Rat, high dose male. Hematoxylin and eosin, X250.

Figure 5. Tubular cell carcinoma, kidney. Rat, high dose male. Hematoxylin and eosin, X250.

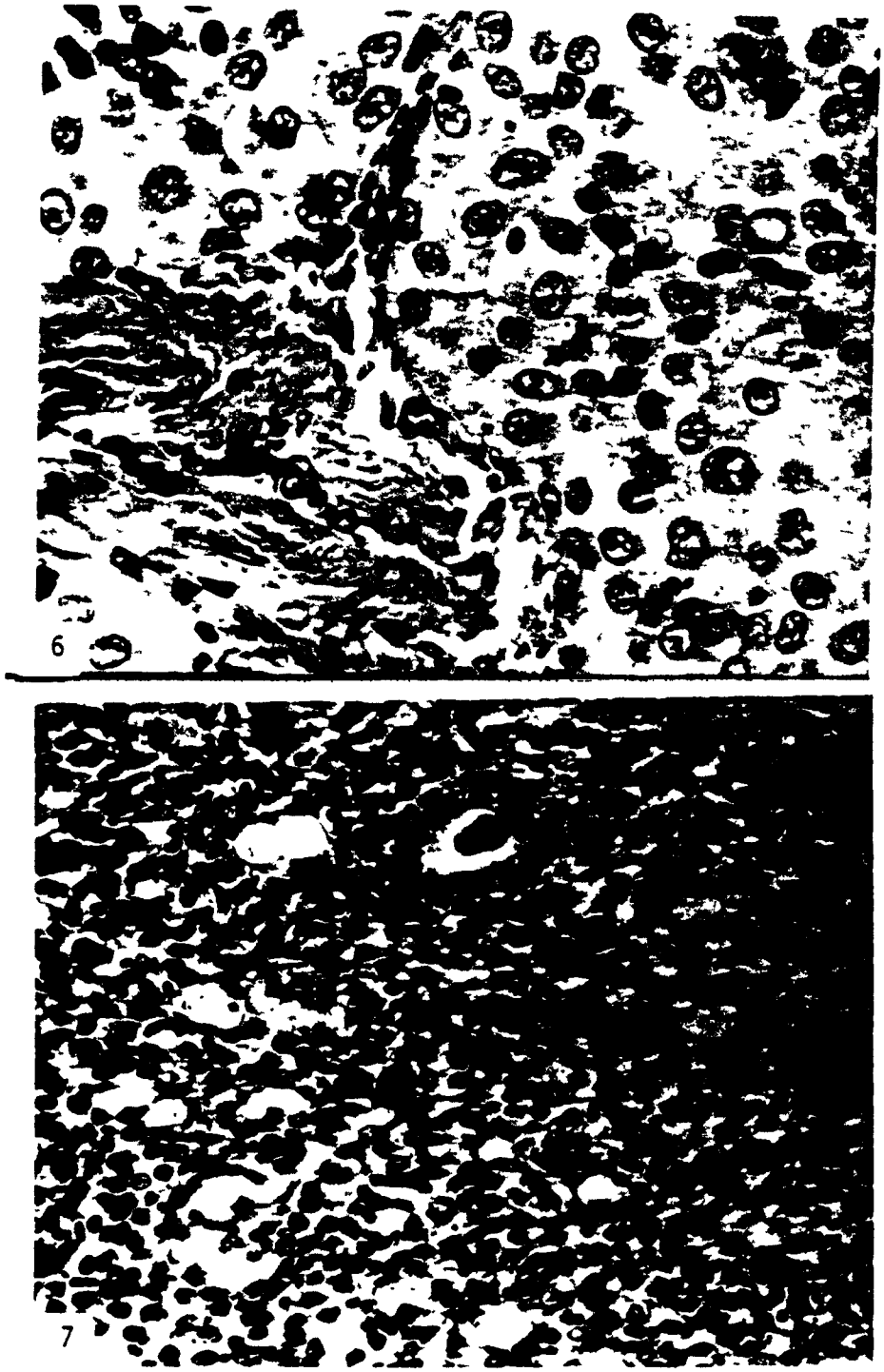


Figure 6. Tubular cell carcinoma, kidney. Rat, high dose male. Hematoxylin and eosin, X400.
Figure 7. Malignant mixed tumor, kidney. Rat, low dose male. Hematoxylin and eosin, X250.

Follicular cell and C-cell tumors of the thyroid gland were observed in both control and test groups. Follicular cell adenomas appeared microscopically as well circumscribed, usually single masses composed of enlarged follicles lined by hyperbasophilic follicular cells. The cells were increased in number, either by papillary infolding of simple cuboidal or columnar epithelium into the follicular lumen, or stratification of follicular cells surrounding the lumen. Distinct compression of surrounding normal thyroid parenchyma, usually with some evidence of fibrous encapsulation, was present. Follicular architecture and cytology within the mass differed markedly from that of the adjacent normal thyroid parenchyma. Follicular cell lesions were classifed as carcinoma based upon the presence of anaplasia and histologic arrangement in disorderly nests and/or sheets. Areas with papillary patterns were also often present. Fibrous stroma often intermingled with, but did not encapsulate the tumors. Some of the carcinomas encompassed the entire thyroid lobe, and the fibrous stroma present made it impossible to recognize the normal thyroid capsule.

C-cell lesions were classified as adenomas when the proliferating C-cells were present in nodular masses which widely separated thyroid follicles and distorted normal follicular architecture. In the larger, more discrete, nodular lesions, the proliferating

C-cells were present as interlacing bundles of elongated, spindling cells, rather than the polyhedral to spherical shape characteristic of normal C-cells. In the one rat in which the C-cell lesion was classified as a carcinoma, microscopic evidence of capsular invasion and multiple pulmonary metastases was present.

The incidences of female rats with thyroid tumors was statistically higher than controls at both dose levels (P = .05) as was the departure from linear trend when comparing treated with the colony controls. In contrast, the incidence in males was reversed (not significant at P = .05) with a higher percentage of controls with thyroid tumors than chloroform-treated animals. The evaluation of "total" thyroid tumors was not considered valid since two epithelial cell types of the thyroid (follicular cell and C-cell) were observed, having distinctly different embryonic origins and physiologic functions. Based upon this and the variabil'ty of observed spontaneous incidence of these tumors in this rat strain and laboratory with opposite and inconsistent effects in males and females, the thyroid differences were not considered of biological significance. The incidences of these different cell types is presented in Table IV.

Table IV. Incidence of Thyroid Tumors - Rats

	MALES				FEMALES			
	CONTROLS		LOW	HIGH	CONTROLS		LOW	HIGH
TUMOR	COLONY	MATCHED	DOSE	DOSE	COLONY	MATCHED	DOSE	DOSE
Follicular-cell	4/99	3/19	1/49	2/48	1/98	1/19	2/49	6/49
C-Cell	4/99	1/19	2/49	2/48	0/98	0/19	6/49	4/49
Total Thyroid	8/99	4/19	3/49	4/48	1/98	1/19	8/49	10/49

Only two hepatocellular carcinomas were observed among all rats in the study, one in a male colony control dying at 97 weeks, and the other a high dose male rat that died at 111 weeks. Neoplastic nodules occurred in the liver of 10/197 test rats (5.0%) and 2/197 colony control rats (1%). Such nodules have recently been defined morphologically and designated as neoplastic nodules (8). As such, they have been categorized and coded as neoplasms when observed in this study.

Table V shows a comparison of the survival of rats receiving chloroform and the known carcinogen, carbon tetrachloride with pooled colony controls at 90 and 110 weeks.

Table V. Comparison of Survival of Colony Controls, Chloroform and Carbon Tetrachloride-Treated Rats

		CHLOROFORM			CARBON TETRACHLORIDE		
ANIMAL GROUP		INITIAL NO.	78 WEEKS	111 WEEKS	INITIAL NO.	78 WEEKS	110 WEEKS
Males	Controls	100	67	26	100	67	26
	Low Dose	50	39	27	50	34	14
	High Dose	50	27	14	50	34	7
Females	Controls	100	75	51	100	75	51
	Low Dose	50	28	23	50	38	20
	High Dose	50	25	15	50	21	14

The incidences of both hepatocellular carcinomas and neoplastic nodules in colony controls and in rats receiving chloroform or carbon tetrachloride are given in Table VI.

Table VI. Incidences of Liver Tumors - Colony Controls, Chloroform and Carbon Tetrachloride - Treated Rats

ANIMAL GROUP		HEPATOCELLULAR CARCINOMA		NEOPLASTIC NODULE	
		CHLOROFORM	CCl_4	CHLOROFORM	CCl_4
MALES	Controls	1/99	1/99	0/99	0/99
	Low Dose	0/50	2/50	1/50	2/50
	High Dose	1/50	2/50	2/50	1/50
FEMALES	Controls	0/98	0/98	2/98	2/98
	Low Dose	0/49	4/49	4/49	2/49
	High Dose	0/48	1/49	3/48	3/49

Numerous other neoplasms, that often occur spontaneously in aged laboratory rats, were observed in test and control groups without significant differences in frequency. These included fibrous histiocytomas of subcutis, hemangiomas and hemangiosarcomas of spleen and other organs, pituitary adenomas, adrenal tumors, and islet cell tumors of pancreas; hematopoietic tumors, mesenchymal and epithelial mammary tumors, endometrial stromal polyps and astrocytomas of the brain.

In addition to tumors, numerous inflammatory, degenerative, and proliferative lesions commonly seen in aged rats occurred with approximately equal frequency in treated and control animals.

These included pericholangitis and biliary hyperplasia, chronic nephritis with tubular dilatation and epithelial hyperplasia of the renal pelvis, subacute to chronic prostatitis, and atrophy of seminiferous epithelium of the testes.

Non-neoplastic, possibly treatment-related lesions were observed in the lungs, liver, urinary bladder, and spleen as described in the following paragraphs.

Although inflammatory pulmonary lesions occurred in all groups of control and test rats, there was a distinct difference in the nature and severity of the lesions between treated and control groups. Control rats of both sexes had pulmonary lesions characteristic of the Mycoplasma-associated chronic pneumonia observed very commonly in aged laboratory rats; i.e , peribronchial and perivascular lymphoid aggregates and accumulation of alveolar macrophages in interstitium and alveoli. While the compound-treated rats of both sexes and at both dose levels had lesions similar to the controls, the lesions were more severe and occurred in a higher incidence. In addition, lungs of many animals (approximately 30%) contained foreign-body giant cells and large marcophages filled with a fine granular material which in some sections stained brown with hematoxylin and eosin.

Necrosis of hepatic parenchyma occurred in chloroform-treated rats as follows: 3/50 low dose males, 4/50 high dose males, 3/49 low dose females, and 11/48 high dose females.

Hyperplasia of the epithelium of the urinary bladder occurred in 1/18 matched control males, 7/45 low dose males, 1/45 high dose males, 6/43 low dose females, and 2/40 high dose females.

A possible increase in splenic hematopoiesis was observed in male rats: 1/18 matched control males, 3/45 low dose males and 6/45 at the high dose.

B. Mice

1. Survival - As illustrated in Figure 8, survival was comparable in both treated and control groups with the exception of the high dose females. The earlier deaths in female high dose mice cannot be explained with certainty, but the incidence of hepatocellular carcinomas was very high in this group. In addition, pulmonary inflammation was observed in 8, and cardiac thrombosis in 9 of the 41 high dose females. This latter lesion was not seen in either the control or low dose females.

2. Body Weights, Food Consumption and Clinical Signs - As illustrated in Figure 9, there was very little difference in the growth curves for control or treated mice of both sexes. Food consumption was also comparable with no treatment-related effect evident.

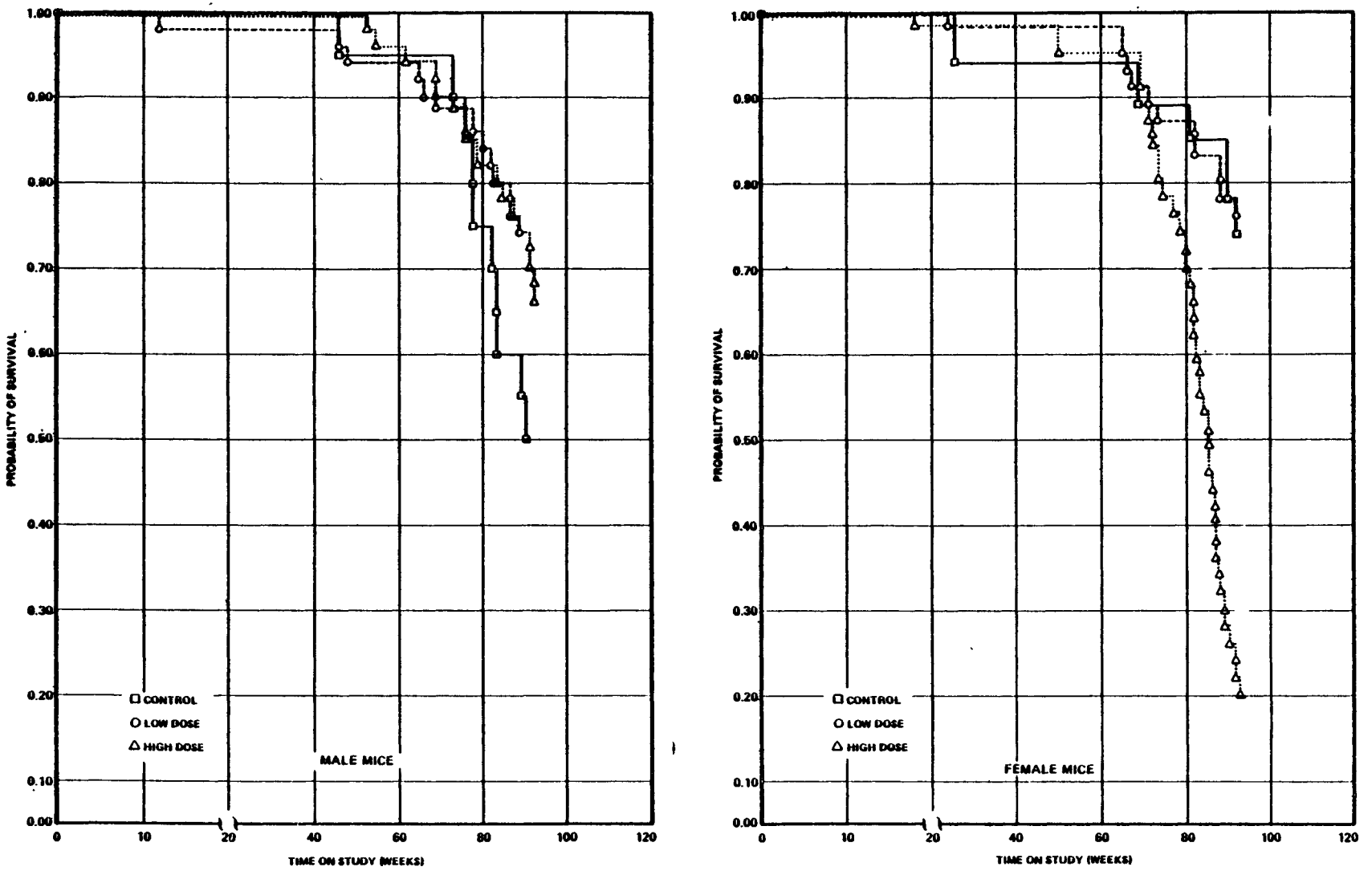
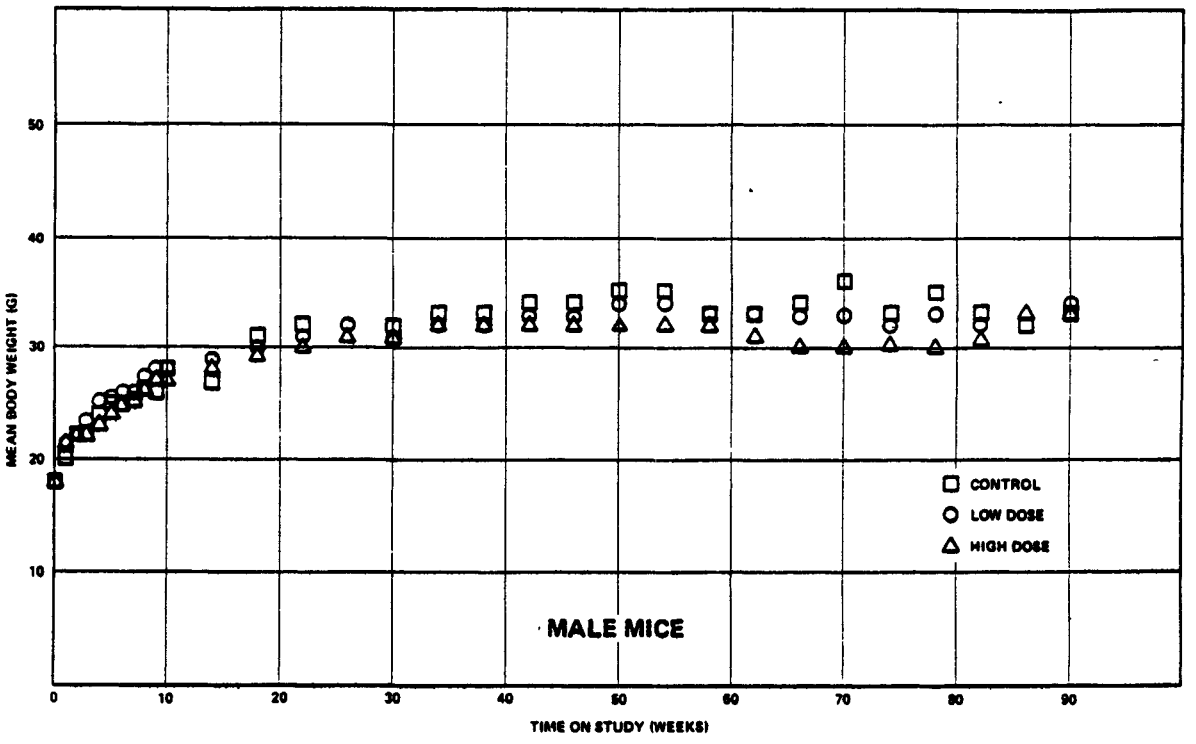


Figure 8. Survival Curves for Mice (Chloroform)



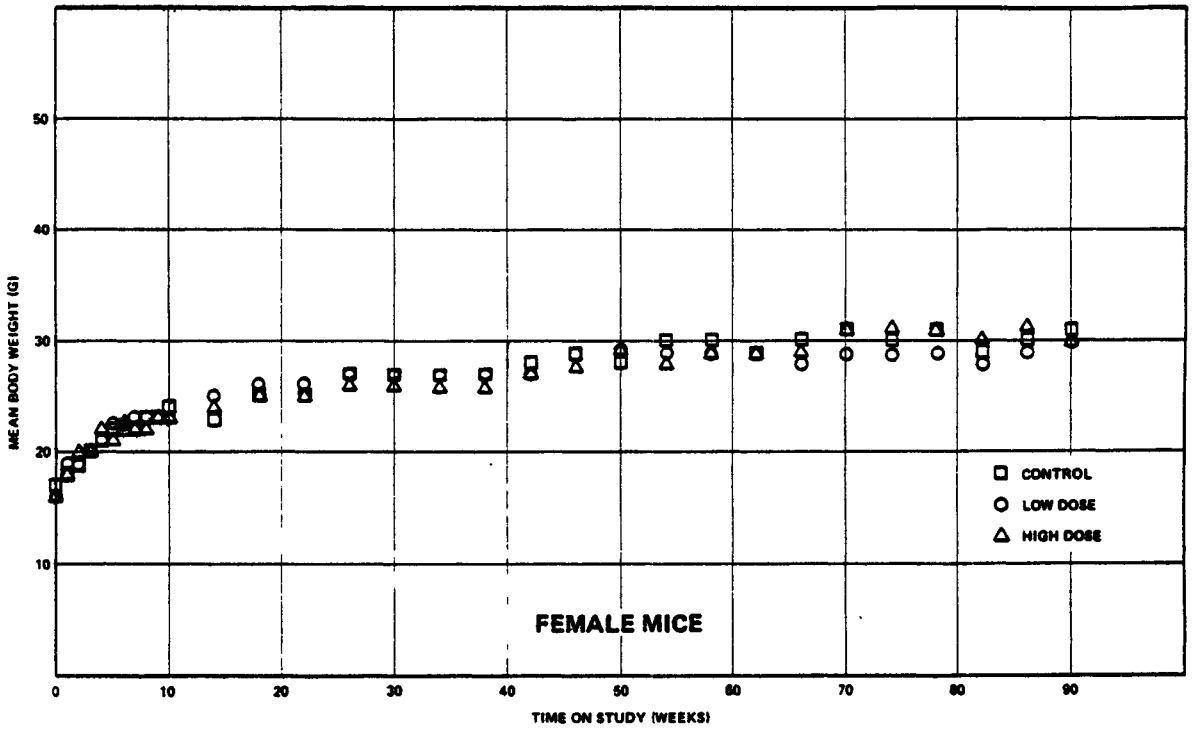


Figure 9. Growth Curves for Mice - Chloroform

During the first 10 months of the study, the appearance and behavior of the treated and control mice were generally comparable. Alopecia (generalized and/or localized), sores on the back and other parts of the body, small palpable nodules on lower midline and/or inguinal areas were noted in increasing numbers of male mice, beginning at week 9 and persisting during the study. After 42 weeks of treatment, bloating or abdominal distension was noted in the high dose females and beginning in week 78 in the high dose males. By week 86, nearly all high dose females and more than 50% of the high dose males had abdominal distention. This was also apparent in eight low dose females. Necropsy of these animals confirmed the presence of liver lesions, the majority of which were subsequently diagnosed as hepatocellular carcinomas.

3. Pathology - Twenty of the 240 treated and control animals were lost to the study. Of these, 15 (6%) were autolyzed, 4 were missing, and 1 was accidentally killed. Although most losses were in the high dose groups, the influence on the results was negligible. Histopathologic findings of all tumors observed are tabulated in Appendix B. From an examination of Appendix B, differences in tumor incidences between chloroform-treated and controls were apparent only for total tumors and hepatocellular carcinoma in both males and females. These data were statistically analyzed and the results presented in Table VII. In addition to those results

Table VII. Analysis of Total Tumors and Specific Liver, Kidney and Thyroid Tumors - Mice (Chloroform)

	MALE				FEMALE			
	CONTROLS				CONTROLS			
TREATMENT[1] :	COLONY	MATCHED	LOW	HIGH	COLONY	MATCHED	LOW	HIGH
Total Tumor-Bearing Animals/Animals[2]	--	4/18 22%	26/50 52%	44/45 98%	--	2/20 10%	37/45 82%	39/41 95%
P Value[3]	--	.0000*	--	--	--	.0000*	--	--
Time to Tumor (weeks)[4]	--	72	66	54	--	27	66	67
Hepatocellular Carcinoma/Animals[2]	5/77 6%	1/18 6%	18/50 36%	44/45 98%	1/80 1%	0/20 0%	36/45 80%	39/41 95%
P Value[3]	.0000*	.0000*	--	--	--	--	.0000*[5]	.0000*[5]
Time to Tumor (weeks)[4]	72	72	80	54	90	--	66	67
Kidney Epithelial Tumors/Animals[2]	1/77 1%	1/18 6%	1/50 2%	2/45 4%	0/80 0%	0/20 0%	0/45 0%	0/40 0%
P Value[3]	.1414	.4873	--	--	1.000	1.000	--	--
Time to Tumor (weeks)[4]	92	92	92	92	--	--	--	--
Thyroid Tumors/ Animals[2]	0/77 0%	0/17 0%	0/48 0%	0/43 0%	0/80 0%	0/20 0%	0/41 0%	0/36 0%
P Value[3]	1.000	1.000	--	--	1.000	1.000	--	--
Time to Tumor (weeks)[4]	--	--	--	--	--	--	--	--
Survival At Terminal Sacrifice (92 weeks)	48%	50%	65%	65%	81%	75%	75%	20%

1 - Oral dose of chloroform in corn oil administered by gavage five times per week.
2 - Based on animals whose tissues were examined from a specific organ.
3 - One-tail P value from Armitage test for linear trend in proportions, unless otherwise stated.
4 - Time to detection of first tumor(at death).
5 - Data departure from linear trend (for departure statistic; $P < .05$). Fisher Exact Test is used comparing controls to a dose level. Bonferroni (7) correction for simultaneous comparison of controls is included.
* - Statistically significant ($P < .05$).

the incidence of kidney epithelial and thyroid tumors are presented for comparison purposes as statistical differences of these tumor types were observed in rats.

The incidence of total tumors was greatly elevated in both male and female mice at both dose levels. The increase is due to the occurrence of a specific type of tumor, hepatocellular carcinoma. A significantly increased incidence of hepatocellular carcinomas was found in all treated groups of mice ($P < .001$). These lesions were observed in treated animals dying as early as 54-60 weeks. Figure 10 illustrates the incidences of hepatocellular carcinomas.

The hepatocellular carcinomas observed in the various test and control groups comprised the full spectrum of morphology of this entity. The tumors varied from those composed of well-differentiated hepatocytes with a relatively uniform arrangement to those which were very anaplastic and poorly differentiated with numerous mitotic figures. Various types of hepatocellular carcinomas described in the literature were seen, including those with an orderly cord-like arrangement of neoplastic cells (Figure 11), those with a pseudo-glandular pattern resembling adenocarcinoma, and those composed of sheets of highly anaplastic cells with little tendency to form a cord or gland-like arrangement. The diagnosis of hepatocellular carcinoma was primarily based on histologic characteristics of the

neoplasm. Hepatocellular carcinomas were found to have metastasized to the lung in two low dose males (Figure 12), and two high dose females, and to the kidney in a high dose male.

Few mice receiving carbon tetrachloride survived until the planned termination of the test, compared with a considerable number in each of the chloroform-treated groups as shown in Table VIII.

Table VIII. Comparison of Survival of Colony Control - Vehicle and Chloroform- and Carbon Tetrachloride-Treated Mice

		CHLOROFORM			CARBON TETRACHLORIDE		
ANIMAL GROUP		INITIAL NO.	78 WEEKS	90 WEEKS	INITIAL NO.	78 WEEKS	91-92 WEEKS
Males	Controls	77	53	38	77	53	38
	Low Dose	50	43	37	50	11	0
	High Dose	50	41	35	50	2	0
Females	Controls	80	71	65	80	71	65
	Low Dose	50	43	36	50	10	0
	High Dose	50	36	11	50	4	1

Hepatocellular carcinomas were found in practically all mice receiving carbon tetrachloride, including those dying before termination of the

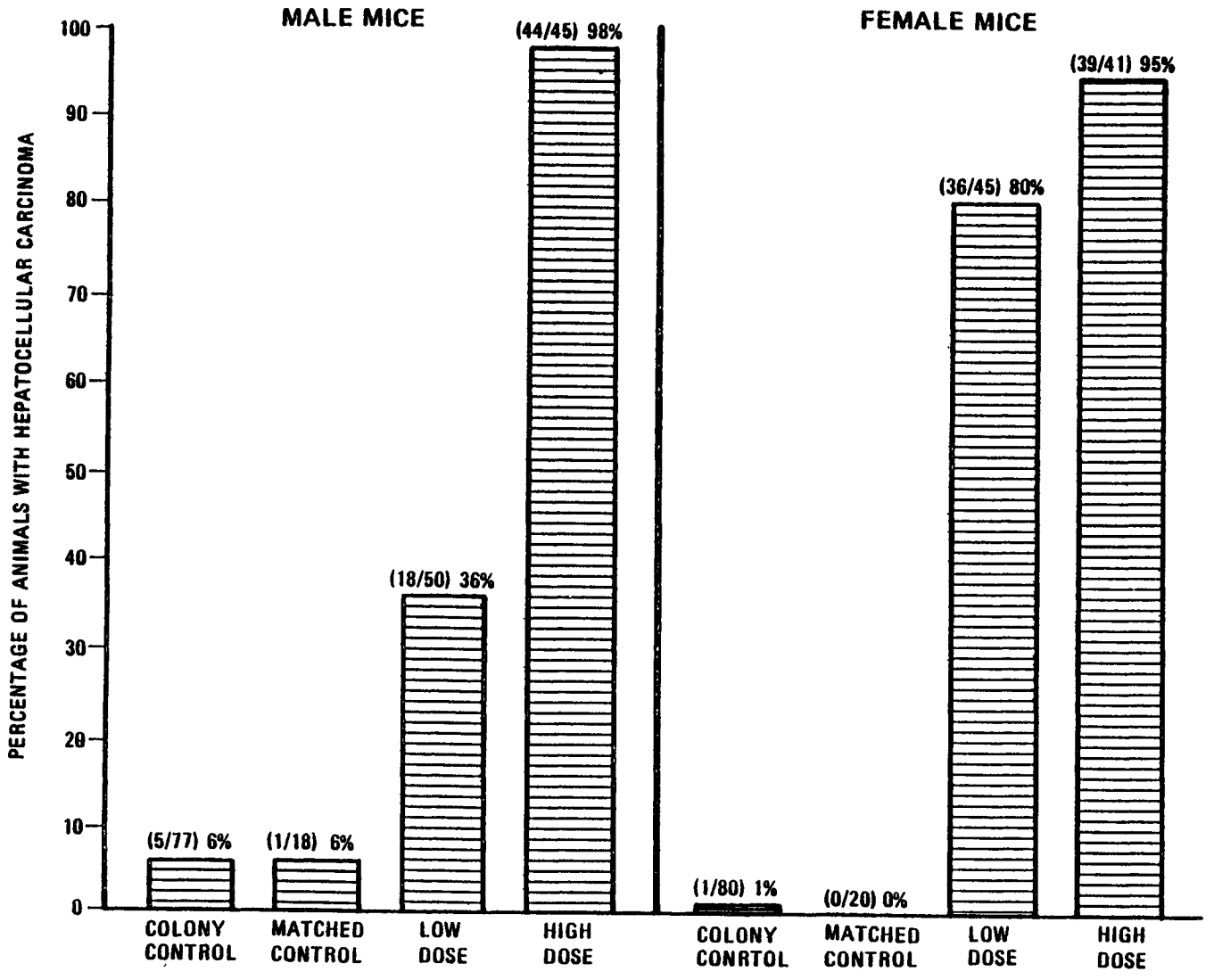


Figure 10. Comparison of Incidences of Hepatocellular Carcinoma (Chloroform)

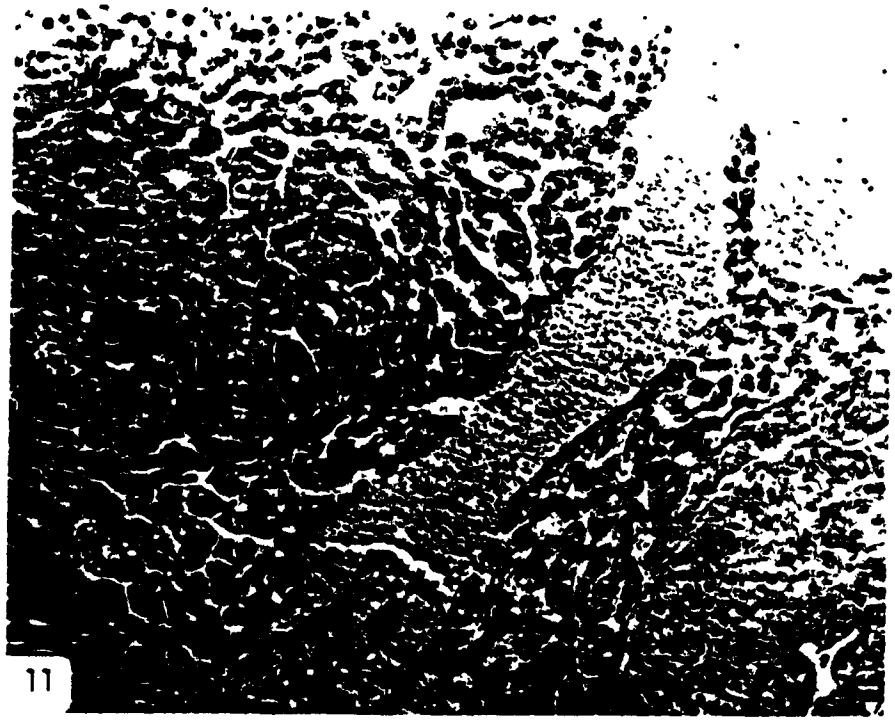


Figure 11. Well differentiated trabecular hepatocellular carcinoma, liver. Mouse, high dose male. Hematoxylin and eosin.
Figure 12. Metastatic hepatocellular carcinoma, lung. Mouse, low dose male. Hematoxylin and eosin.

test. The incidence of liver tumors was somewhat greater in carbon tetrachloride-treated mice (especially at the lower dose levels) than in chloroform-treated mice as shown in Table IX.

Table IX. Comparison of Hepatocellular Carcinoma Incidence in Colony Control - Vehicle Treated and Chloroform- and Carbon Tetrachloride-Treated Mice

ANIMAL GROUP		CHLOROFORM	CARBON TETRACHLORIDE
Males	Controls	5/77	5/77
	Low Dose	18/50	49/49
	High Dose	44/45	47/48
Females	Controls	1/80	1/80
	Low Dose	36/45	40/40
	High Dose	39/41	43/45

These liver tumors in carbon tetrachloride-treated mice varied greatly in appearance from lesions which contained well differentiated hepatic cells that had a relatively uniform arrangement of the cords to very anaplastic liver cells having large hyperchromatic nuclei, often with inclusion bodies, and with vacuolated, pale cytoplasm. Arrangement of the neoplastic liver cells varied from short stubby cords to nests of hepatic cells and occasionally acinar arrangements. Mitotic figures were often present. Some of the tumors were characterized by discrete areas of highly anaplastic cells surrounded by relatively well differentiated tumor cells. The neoplasms occurring in the CCl_4-treated mice were similar in appearance to those noted in the chloroform-treated mice.

The test week at which the first animal died in which a hepatocellular carcinoma was observed in each group is given in Table X.

Table X. Comparison of Time to Liver Tumor Detection in Colony Control - and Chloroform- and Carbon Tetrachloride-Treated Mice

ANIMAL GROUP		CHLOROFORM	CARBON TETRACHLORIDE
Males	Controls	72	72
	Low Dose	80	48
	High Dose	54	26
Females	Controls	90	90
	Low Dose	66	16
	High Dose	67	19

In addition to the higher incidence, hepatocellular carcinomas were observed much earlier in carbon tetrachloride-treated mice than in the chloroform-treated mice. Tumors in control mice were observed much later than with either other compound.

A very small number of non-hepatic spontaneous tumors were observed in the various control and test groups, but no significant differences were observed.

Non-neoplastic hepatic proliferative changes were found in both the high and low dose mice of both sexes. Of these, lesions of the liver classified as nodular hyperplasia occurred in 10 of 50 low dose males, 6 of 45 low dose females, and 1 of 41 high dose females. Hepatic necrosis was observed in six mice (all treated), 1 low dose male, 4 low dose females and 1 high dose female.

A variety of inflammatory, degenerative, and proliferative lesions occurred in both control and treated groups of mice. There was a generally low incidence of such lesions, and most did not occur more commonly in test than in control animals. Examples of such spontaneously occurring lesions included testicular atrophy or mineralization, and mild inflammatory alterations of the seminal vesicle, lung, lymph node, skin, urinary bladder, epididymus, testis, ovary, and uterus. Cystic endometrial hyperplasia occurred very commonly in both control and treated female mice. Cardiac atrial thrombosis occurred in 9 of 41 high dose females, all of which died on study and had concurrent hepatocellular carcinoma.

Inflammatory alterations of the kidney, primarily chronic, occurred in 10 of 18 controls, 2 of 50 low dose males, and 1 of 50 high dose males. Significant renal inflammation did not occur in any control or treated female mice. No explanation can be given for this effect.

IV. DISCUSSION:

This study clearly indicates that chloroform has induced hepatocellular carcinomas in both male and female mice ($P < .001$) and renal epithelial tumors ($P = .0016$) in male rats. While there was also a statistically significant ($P < .05$) incidence of total thyroid tumors in treated female

rats, the pathologists did not attach any biological significance to those findings (see page 23). The observation of liver cancer was not totally unexpected, on the basis of earlier studies with chloroform (9, 10), however, the increased incidence of kidney tumors had not been predicted.

The previous chloroform studies were conducted 30 years ago (1945-1946) by Eschenbrenner and Miller (9, 10) and suggested the potential hepatocarcinogenicity of chloroform. In those studies chloroform was administered by stomach tube to Strain A mice. Thirty doses (at five different concentrations) were given at 4-day intervals for a 120-day treatment period with sacrifice 1 month following the last treatment. Hepatomas were found in 7/15 female mice at the highest dose levels; no hepatic tumor was observed in any male nor female at the lower dose levels.

The results of the present study clearly support and extend the findings of Eschenbrenner and Miller, that chloroform administered by gastric gavage can induce hepatocellular proliferative lesions, including hepatocellular carcinomas, in mice. In this study a high incidence of hepatocellular carcinomas was observed in both males and females, while a high incidence was found only in females in the Eschenbrenner and Miller study. This might be attributed not only to a sex difference in susceptibility of the Strain A mouse, but also to the shorter duration of treatment (120 days) and earlier sacrifice (at 150 days after start of treatment) in the Eschenbrenner and Miller study.

The term "hepatocellular carcinoma" was used for proliferative lesions of the livers in mice which, in the judgment of the pathologists, had the potential or the capacity for progressive growth, invasion, and metastasis and for causing death of the host. This judgment was based upon the cytologic and histologic features of the neoplasms and the knowledge that lesions with the same morphologic characteristics have exhibited malignant biologic behavior. The observation of nodular hyperplasia in many male mice at the low dose without hepatocellular carcinomas, while virtually all high dose males had hepatocellular carcinomas, would tend to support the hypothesis that nodular hyperplasia is a stage in the development of carcinoma.

The terms "neoplastic nodule" and "hepatocellular carcinoma" used to diagnose proliferative hepatic lesions in rats were based on the morphologic criteria and nomenclature recently reported from a workshop on the classification of specific hepatocellular lesions in rats (8).

The observation of kidney tumors in rats and liver tumors in mice illustrates species differences in organ specificity and sensitivity.

In regard to the choice of animal models, the Osborne-Mendel rat was selected because of the experience gained by the Food and Drug Administration, where this strain has been used for many years as a general purpose test animal. In addition, it was known to be

sensitive to the carcinogenic effects of CCl_4 administered by subcutaneous injection (11). The $B6C3F_1$ strain of mouse has been extensively used by NCI for carcinogenesis bioassays. Current experience with this strain in our Program indicates an incidence of hepatocellular carcinomas in control mice of approximately 5-10% in males and 1% in females. The matched and colony control animals in this study conformed well to this expected incidence.

From the relatively low response of the rats to CCl_4 (< 5% with hepatocellular carcinomas), it would appear that the Osborne-Mendel rats used in these studies were less sensitive to hepatocarcinogenicity than those used by Reuber (11). In contrast, nearly 100% of the CCl_4-treated mice developed hepatocellular carcinomas with many occurring in animals dying in the first year. While it would appear that the mouse was more sensitive to CCl_4 than chloroform, the greater dose levels of CCl_4 (5-9 x that of chloroform), should be considered.

A concern in any testing program is the possible influence of extraneous factors. Because several other compounds were on test in the same rooms with the present test animals, the possibility of a low level exposure to these compounds in the air must be considered. The absence of an increased incidence of tumors in controls is evidence against any direct pronounced effect of such respiratory exposure, but the possibility cannot be eliminated that the effects observed were accentuated by

concurrent exposures to these contaminants. No experimental studies of cross contamination or simultaneous administration are available. We would not expect a protective effect from simultaneous exposure to other halogenated solvents, and it is highly unlikely that an interaction of possible airborne contaminant amounts of solvents with the high doses of chloroform used would bring about false positives.

With mice, stringent precautions against cross contamination were employed. The mice were kept in cages with filter tops which limited the amount of expired chemical in the air available for inhalation by other animals, the total air in each room was changed 10 to 15 times per hour, and the mouse racks were transported to another room with a large hood for the daily intubations. Furthermore, the hepatocarcinomas in mice were present at a greater than $P = 0.01$ level of significance and were produced by doses of chloroform of 90-477 mg/kg, which are several thousand-fold greater than any possible contamination could have been. A dose related effect was observed and, any possible chemical in general room air did not affect controls. Thus, although this room arrangement is not desirable as is stated in the NCI Guidelines for Carcinogen Bioassay in Small Rodents (12), there is no evidence the results would have been different with a single compound in a room.

The methodology used in these studies differs from that currently adopted by NCI (12) in that: (a) the testing for subchronic toxicity was for 42 rather than 90 days; (b) the dosage was changed during the test; (c) the period of treatment was for 18 rather than 24 months; (d) the number of matched controls was 20 rather than 50; and (e) several volatile compounds were tested in the same room. In spite of these limitations, this bioassay is considered a valid test for carcinogenic effect. While the induction of hepatocellular carcinoma in mice, and epithelial tumors of the kidney in rats were highly significant, even using the small matched control groups, the use of pooled colony controls further increased the validity of these differences.

Due to changes in dosage of chloroform during the study and the use of only two dose levels, a quantitative assessment of a dose-response relationship is not considered feasible. However, a linear dose trend was seen for both hepatocellular carcinomas in mice and renal epithelial tumors in the male rat.

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APPENDIX A

SUMMARY OF TUMORS OBSERVED IN RATS

(CHLOROFORM)

TABLE A1. *MALE RATS* WITH PRIMARY TUMORS BY ANATOMIC SITE (CHLOROFORM)

(PERCENTAGES BY SYSTEM AND ORGAN ARE BASED ON THE EFFECTIVE NUMBER OF ANIMALS)

	CONTROL	LOW DOSE	HIGH DOSE
EFFECTIVE NUMBER OF ANIMALS*	19 (100%)	50 (100%)	50 (100%)
ANIMALS WITH PRIMARY TUMORS	9 (47%)	24 (48%)	20 (40%)
INTEGUMENTARY SYSTEM	2 (11%)*	6 (12%)	1 (2%)
SUBCUT TISSUE	2 (11%)	4 (8%)	1 (2%)
FIBROUS HISTIOCYTOMA	1	1	1
MALIGNANT FIBROUS HISTIOCYTOMA	1	2	
FIBROMA		1	
SKIN	0 (0%)	2 (4%)	0 (0%)
KERATOACANTHOMA		1	
SQUAMOUS CELL CARCINOMA		1	
RESPIRATORY SYSTEM	0 (0%)	1 (2%)	0 (0%)
LUNG	0/19 (0%)	1/50 (2%)	0/48 (0%)
ALVEOLAR-CELL ADENOMA		1	
CIRCULATORY SYSTEM			
NONE			
DIGESTIVE SYSTEM	0 (0%)	1 (2%)	4 (8%)
LIVER	0/19 (0%)	1/50 (2%)	3/50 (6%)
NEOPLASTIC NODULE		1	2
HEPATOCELLULAR CARCINOMA			1
SMALL INTESTINE	0/19 (0%)	0/50 (0%)	1/50 (2%)
FIBROSARCOMA			1
URINARY SYSTEM	0 (0%)	6 (12%)	13 (26%)
KIDNEY	0/19 (0%)	6/50 (12%)	13/50 (26%)
TUBULAR-CELL ADENOCARCINOMA		2	10**(2)
TUBULAR-CELL ADENOMA		2	3
HAMARTOMA		1	1
MIXED TUMOR MALIGNANT		2**(1)	
ENDOCRINE SYSTEM	7 (37%)	9 (18%)	6 (12%)
THYROID	4/19 (21%)	3/49 (6%)	4/48 (8%)
FOLLICULAR-CELL CARCINOMA	2		2
FOLLICULAR-CELL ADENOMA	1	1	
C-CELL ADENOMA	1	2	2

* COLUMNS ARE OFFSET ACCORDING TO ORGAN SYSTEM, SPECIFIC ORGAN AND TUMOR TYPE.
**(X) NUMBER IN PARENTHESIS INDICATES THE NUMBER OF METASTASIZED TUMORS

TABLE A1. MALE RATS WITH PRIMARY TUMORS BY ANATOMIC SITE (CHLOROFORM) (CONTINUED)

(PERCENTAGES BY SYSTEM AND ORGAN ARE BASED ON THE EFFECTIVE NUMBER OF ANIMALS)

	CONTROL	LOW DOSE	HIGH DOSE
ENDOCRINE SYSTEM (CONT)			
PITUITARY	0/16 (0%)	4/44 (9%)	1/47 (2%)
CHROMOPHOBE ADENOMA		4	1
ADRENAL	2/19 (11%)	0/49 (0%)	0/49 (0%)
HEMANGIOSARCOMA	1		
PHEOCHROMOCYTOMA	1		
PANCREATIC ISLETS	1/16 (6%)	2/50 (4%)	1/49 (2%)
ISLET-CELL CARCINOMA		1	
ISLET-CELL ADENOMA	1	1	1
HEMATOPOIETIC SYSTEM	1 (5%)	4 (8%)	2 (4%)
SPLEEN	1/17 (6%)	4/49 (8%)	2/48 (4%)
HEMANGIOSARCOMA	1	1	
HEMANGIOMA		3	2
REPRODUCTIVE SYSTEM	1 (5%)	0 (0%)	0 (0%)
MAMMARY GLAND	1/19 (5%)	0/50 (0%)	0/49 (0%)
ADENOCARCINOMA	1		
NERVOUS SYSTEM	0 (0%)	1 (2%)	1 (2%)
BRAIN	0/18 (0%)	1/50 (2%)	1/50 (2%)
ASTROCYTOMA		1	1
MUSCULOSKELETAL SYSTEM			
NONE			
SPECIAL SENSE ORGANS			
NONE			
ALL OTHER SYSTEMS	0 (0%)	0 (0%)	1 (2%)
MULTIPLE ORGANS	0 (0%)	0 (0%)	1 (2%)
RETICULUM-CELL SARCOMA			1

COLUMNS ARE OFFSET ACCORDING TO ORGAN SYSTEM, SPECIFIC ORGAN AND TUMOR TYPE.

TABLE A1. *MALE RATS* WITH PRIMARY TUMORS BY ANATOMIC SITE (CHLOROFORM) (CONTINUED)

(PERCENTAGES BY SYSTEM AND ORGAN ARE BASED ON THE EFFECTIVE NUMBER OF ANIMALS)

	CONTROL	LOW DOSE	HIGH DOSE
TUMOR SUMMARY			
TOTAL ANIMALS WITH BENIGN TUMORS	5 (26%)	16 (32%)	10 (20%)
TOTAL BENIGN TUMORS	5	18	11
TOTAL ANIMALS WITH MALIGNANT TUMORS	4 (21%)	10 (20%)	14 (28%)
TOTAL MALIGNANT TUMORS	6	11	18

* COLUMNS ARE OFFSET ACCORDING TO ORGAN SYSTEM, SPECIFIC ORGAN AND TUMOR TYPE.

TABLE A2. FEMALE RATS WITH PRIMARY TUMORS BY ANATOMIC SITE (CHLOROFORM)

(PERCENTAGES BY SYSTEM AND ORGAN ARE BASED ON THE EFFECTIVE NUMBER OF ANIMALS)

	CONTROL	LOW DOSE	HIGH DOSE
EFFECTIVE NUMBER OF ANIMALS*	20 (100%)	49 (100%)	48 (100%)
ANIMALS WITH PRIMARY TUMORS	12 (60%)	24 (49%)	24 (50%)
INTEGUMENTARY SYSTEM	0 (0%)*	2 (4%)	0 (0%)
SUBCUT TISSUE	0 (0%)	1 (2%)	0 (0%)
LIPOMA		1	
SKIN	0 (0%)	1 (2%)	0 (0%)
PAPILLOMA		1	
RESPIRATORY SYSTEM	0 (0%)	1 (2%)	0 (0%)
LUNG	0/20 (0%)	1/49 (2%)	0/47 (0%)
MALIGNANT FIBROUS HISTIOCYTOMA		1	
CIRCULATORY SYSTEM			
NONE			
DIGESTIVE SYSTEM	2 (10%)	5 (10%)	4 (8%)
LIVER	2/20 (10%)	5/49 (10%)	3/48 (6%)
NEOPLASTIC NODULE	2	4	3
MALIGNANT FIBROUS HISTIOCYTOMA		1	
PANCREAS	0/20 (0%)	1/49 (2%)	0/48 (0%)
MALIGNANT FIBROUS HISTIOCYTOMA		1	
BILE DUCT	0/20 (0%)	0/49 (0%)	1/48 (2%)
HAMARTOMA			1
URINARY SYSTEM	0 (0%)	1 (2%)	3 (6%)
KIDNEY	0/20 (0%)	1/49 (2%)	2/48 (4%)
MALIGNANT FIBROUS HISTIOCYTOMA		1	
HEMANGIOMA			1
TUBULAR-CELL ADENOCARCINOMA			1**(1)
RENAL PELVIS	0/20 (0%)	0/49 (0%)	1/48 (2%)
SQUAMOUS CELL CARCINOMA			1

* COLUMNS ARE OFFSET ACCORDING TO ORGAN SYSTEM, SPECIFIC ORGAN AND TUMOR TYPE.
**(X) NUMBER IN PARENTHESIS INDICATES THE NUMBER OF METASTASIZED TUMORS

TABLE A2. FEMALE RATS WITH PRIMARY TUMORS BY ANATOMIC SITE (CHLOROFORM) (CONTINUED)

(PERCENTAGES BY SYSTEM AND ORGAN ARE BASED ON THE EFFECTIVE NUMBER OF ANIMALS)

	CONTROL	LOW DOSE	HIGH DOSE
ENDOCRINE SYSTEM	9 (45%)	17 (35%)	12 (25%)
PITUITARY	6/20 (30%)	10/45 (22%)	3/45 (7%)
CHROMOPHOBE ADENOMA	6	10	3
THYROID	1/19 (5%)	8/49 (16%)	10/46 (22%)
FOLLICULAR-CELL ADENOMA	1	1	4
FOLLICULAR-CELL CARCINOMA		1	2
C-CELL ADENOMA		6	3
C-CELL CARCINOMA			1**(1)
ADRENAL	2/20 (10%)	1/48 (2%)	0/48 (0%)
CORTICAL ADENOMA	1	1	
PHEOCHROMOCYTOMA	1		
PANCREATIC ISLETS	2/20 (10%)	0/49 (0%)	0/48 (0%)
ISLET-CELL CARCINOMA	1		
ISLET-CELL ADENOMA	1		
HEMATOPOIETIC SYSTEM	0 (0%)	1 (2%)	0 (0%)
SPLEEN	0/20 (0%)	1/48 (2%)	0/48 (0%)
HEMANGIOMA		1	
REPRODUCTIVE SYSTEM	8 (40%)	15 (31%)	14 (29%)
MAMMARY GLAND	7/20 (35%)	13/48 (27%)	11/46 (24%)
FIBROSARCOMA	1		
FIBROADENOMA	7	9	7
FIBROMA		3	1
ADENOMA		2	2
ADENOCARCINOMA			1
UTERUS	1/20 (5%)	1/48 (2%)	3/46 (7%)
SQUAMOUS CELL CARCINOMA	1		
ENDOMETRIAL STROMAL POLYP		1	2
HEMANGIOSARCOMA			1
OVARY	0/20 (0%)	1/48 (2%)	1/48 (2%)
MALIGNANT FIBROUS HISTIOCYTOMA		1	
GRANULOSA-CELL TUMOR			1
NERVOUS SYSTEM			
NONE			

* COLUMNS ARE OFFSET ACCORDING TO ORGAN SYSTEM, SPECIFIC ORGAN AND TUMOR TYPE.
**(X) NUMBER IN PARENTHESIS INDICATES THE NUMBER OF METASTASIZED TUMORS

TABLE A2. *FEMALE RATS* WITH PRIMARY TUMORS BY ANATOMIC SITE (CHLOROFORM) (CONTINUED)

(PERCENTAGES BY SYSTEM AND ORGAN ARE BASED ON THE EFFECTIVE NUMBER OF ANIMALS)

	CONTROL	LOW DOSE	HIGH DOSE
MUSCULOSKELETAL SYSTEM	0 (0%)	0 (0%)	1 (2%)
MAXILLA	0/20 (0%)	0/49 (0%)	1/47 (2%)
OSTEOMA			1
SPECIAL SENSE ORGANS			
NONE			
ALL OTHER SYSTEMS	0 (0%)	1 (2%)	0 (0%)
MESENTERY	0/20 (0%)	1/49 (2%)	0/48 (2%)
MALIGNANT FIBROUS HISTIOCYTOMA		1	
PLEURA	0/20 (0%)	1/49 (2%)	0/47 (0%)
MALIGNANT FIBROUS HISTIOCYTOMA		1	
TUMOR SUMMARY			
TOTAL ANIMALS WITH BENIGN TUMORS	12 (60%)	23 (47%)	23 (46%)
TOTAL BENIGN TUMORS	17	36	27
TOTAL ANIMALS WITH MALIGNANT TUMORS	3 (15%)	6 (12%)	9 (19%)
TOTAL MALIGNANT TUMORS	5	6	10

* COLUMNS ARE OFFSET ACCORDING TO ORGAN SYSTEM, SPECIFIC ORGAN AND TUMOR TYPE.

APPENDIX B

SUMMARY OF TUMORS OBSERVED IN MICE

(CHLOROFORM)

TABLE B1. *MALE MICE* WITH PRIMARY TUMORS BY ANATOMIC SITE (CHLOROFORM)

(PERCENTAGES BY SYSTEM AND BY ORGAN ARE BASED ON THE EFFECTIVE NUMBER OF ANIMALS)

	CONTROL	LOW DOSE	HIGH DOSE
EFFECTIVE NUMBER OF ANIMALS *	18 (100%)	50 (100%)	45 (100%)
ANIMALS WITH PRIMARY TUMORS	4 (22%)	26 (52%)	44 (98%)
INTEGUMENTARY SYSTEM	0 (0%) *	1 (2%)	1 (2%)
SKIN	0/18 (0%)	1/49 (2%)	0/44 (0%)
FIBROSARCOMA		1	
SUBCUT TISSUE	0/18 (0%)	0/49 (0%)	1/44 (2%)
FIBROSARCOMA			1**(1)
RESPIRATORY SYSTEM	1 (6%)	3 (6%)	3 (7%)
LUNG	1/18 (6%)	3/50 (6%)	3/44 (7%)
ALVEOLAR-CELL ADENOMA	1	3	2
RETICULUM-CELL SARCOMA			1
CIRCULATORY SYSTEM			
NONE			
DIGESTIVE SYSTEM	2 (11%)	20 (40%)	44 (98%)
LIVER	2/18 (11%)	19/50 (38%)	44/45 (98%)
HEPATOCELLULAR CARCINOMA	1	18**(2)	44**(1)
RETICULUM-CELL SARCOMA			1
HEMANGIOMA	1		
LYMPHOSARCOMA		1	2
LARGE INTESTINE	0/18 (0%)	1/50 (2%)	0/45 (0%)
RETICULUM-CELL SARCOMA		1	
URINARY SYSTEM	1 (6%)	2 (4%)	3 (7%)
KIDNEY	1/18 (6%)	2/50 (4%)	3/45 (7%)
LYMPHOSARCOMA		1	1
TUBULAR-CELL ADENOMA	1	1	1
TUBULAR-CELL ADENOCARCINOMA			1
ENDOCRINE SYSTEM	0 (0%)	0 (0%)	2 (5%)
ADRENAL	0/18 (0%)	0/50 (0%)	2/44 (5%)
PHEOCHROMOCYTOMA			1
LYMPHOSARCOMA			1

* COLUMNS ARE OFFSET ACCORDING TO ORGAN SYSTEM, SPECIFIC ORGAN AND TUMOR TYPE.
**(X) NUMBER IN PARENTHESIS INDICATES THE NUMBER OF METASTASIZED TUMORS

TABLE B1. *MALE MICE WITH PRIMARY TUMORS BY ANATOMIC SITE (CHLOROFORM) (CONTINUED)*

(PERCENTAGES BY SYSTEM AND BY ORGAN ARE BASED ON THE EFFECTIVE NUMBER OF ANIMALS)

	CONTROL	LOW DOSE	HIGH DOSE
HEMATOPOIETIC SYSTEM	0 (0%)	1 (2%)	3 (7%)
LYMPH NODE	0/18 (0%)	1/50 (2%)	3/45 (7%)
RETICULUM-CELL SARCOMA		1	2
LYMPHOSARCOMA			1
SPLEEN	0/18 (0%)	1/49 (2%)	2/45 (4%)
RETICULUM-CELL SARCOMA		1	1
LYMPHOSARCOMA			1
BONE MARROW	0/18 (0%)	0/50 (0%)	1/45 (2%)
LYMPHOSARCOMA			1
REPRODUCTIVE SYSTEM	0 (0%)	1 (2%)	0 (0%)
TESTIS	0/18 (0%)	1/50 (2%)	0/45 (0%)
SERTOLI-CELL TUMOR		1	
NERVOUS SYSTEM	0 (0%)	2 (4%)	0 (0%)
BRAIN	0/18 (0%)	2/50 (4%)	0/44 (0%)
LYMPHOSARCOMA		2	
MUSCULOSKELETAL SYSTEM			
NONE			
SPECIAL SENSE ORGANS			
NONE			
ALL OTHER SYSTEMS	0 (0%)	1 (2%)	0 (0%)
MULTIPLE ORGANS	0 (0%)	1 (2%)	0 (0%)
LYMPHOSARCOMA		1	
TUMOR SUMMARY			
TOTAL ANIMALS WITH BENIGN TUMORS	3 (17%)	5 (10%)	4 (8%)
TOTAL BENIGN TUMORS	3	5	4
TOTAL ANIMALS WITH MALIGNANT TUMORS	1 (6%)	22 (44%)	44 (98%)
TOTAL MALIGNANT TUMORS	1	24	50

* COLUMNS ARE OFFSET ACCORDING TO ORGAN SYSTEM, SPECIFIC ORGAN AND TUMOR TYPE.

TABLE B2. FEMALE MICE WITH PRIMARY TUMORS BY ANATOMIC SITE (CHLOROFORM)

(PERCENTAGES BY SYSTEM AND BY ORGAN ARE BASED ON THE EFFECTIVE NUMBER OF ANIMALS)

	CONTROL	LOW DOSE	HIGH DOSE
EFFECTIVE NUMBER OF ANIMALS*	20 (100%)	46 (100%)	41 (100%)
ANIMALS WITH PRIMARY TUMORS	2 (10%)	37 (80%)	39 (95%)
INTEGUMENTARY SYTEM			
NONE			
RESPIRATORY SYSTEM	0 (0%)*	3 (7%)	0 (0%)
LUNG	0/20 (0%)	3/46 (7%)	0/41 (0%)
ALVEOLAR-CELL ADENOMA		1	
MYELOSARCOMA		2	
CIRCULATORY SYSTEM			
NONE			
DIGESTIVE SYSTEM	0 (0%)	37 (80%)	39 (95%)
LIVER	0/20 (0%)	37/45 (82%)	39/41 (95%)
HEPATOCELLULAR CARCINOMA		36	39**(2)
MYELOSARCOMA		2	
URINARY SYSTEM			
NONE			
ENDOCRINE SYSTEM	0 (0%)	0 (0%)	1 (2%)
ADRENAL	0/20 (0%)	0/43 (0%)	1/41 (2%)
PHEOCHROMOCYTOMA			1
HEMATOPOIETIC SYSTEM	0 (0%)	1 (2%)	0 (0%)
SPLEEN	0/19 (0%)	1/46 (2%)	0/41 (0%)
MYELOSARCOMA		1	
REPRODUCTIVE SYSTEM	2 (10%)	0 (0%)	0 (0%)
OVARY	1/20 (5%)	0/40 (0%)	0/36 (0%)
TERATOMA	1		

* COLUMNS ARE OFFSET ACCORDING TO ORGAN SYSTEM, SPECIFIC ORGAN AND TUMOR TYPE.
**(X) NUMBER IN PARENTHESIS INDICATES THE NUMBER OF METASTASIZED TUMORS

TABLE B2. FEMALE MICE WITH PRIMARY TUMORS BY ANATOMIC SITE (CHLOROFORM) (CONTINUED)

(PERCENTAGES BY SYSTEM AND BY ORGAN ARE BASED ON THE EFFECTIVE NUMBER OF ANIMALS)

	CONTROL	LOW DOSE	HIGH DOSE
UTERUS	1/20 (5%)	0/42 (0%)	0/31 (0%)
ADENOCARCINOMA	1		
ENDOMETRIAL STROMAL SARCOMA	1**(1)		
NERVOUS SYSTEM			
NONE			
MUSCULOSKELETAL SYSTEM			
NONE			
SPECIAL SENSE ORGANS			
NONE			
ALL OTHER SYSTEMS	0 (0%)	4 (9%)	0 (0%)
MULTIPLE ORGANS	0 (0%)	4 (9%)	0 (0%)
RETICULUM-CELL SARCOMA		2	
LYMPHOCYTIC LYMPHOSARCOMA		1	
LYMPHOBLASTIC LYMPHOSARCOMA		1	
TUMOR SUMMARY			
TOTAL ANIMALS WITH BENIGN TUMORS	1 (5%)	1 (2%)	1 (2%)
TOTAL BENIGN TUMORS	1	1	1
TOTAL ANIMALS WITH MALIGNANT TUMORS	1 (5%)	37 (80%)	39 (95%)
TOTAL MALIGNANT TUMORS	2	42	39

* COLUMNS ARE OFFSET ACCORDING TO ORGAN SYSTEM, SPECIFIC ORGAN AND TUMOR TYPE.
**(X) NUMBER IN PARENTHESIS INDICATES THE NUMBER OF METASTASIZED TUMORS

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