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Animal Toxicology

Evaluation of 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) toxicity in C57BL/6 mice:

Reproduction and Fertility in Treated Male Mice and Evaluation of Congenital Malformations in Their Offspring

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES (continued)

LIST OF FIGURES

ABSTRACT

This study was undertaken to determine the effects of mixtures (simulated Agent Orange) of 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on reproduction and fertility of treated male mice.

Male C57BL/6 mice were given feed containing varying concentrations of 2,4-D, 2,4,5-T and TCDD such that daily doses of approximately 40 mg/kg 2,4-D, 40 mg/kg 2,4,5-T and 2.4 μg/kg TCDD (Group II) or 40 mg/kg 2,4-D, 40 mg/kg 2,4,5-T and 0.16 μg/kg TCDD (Group IV) or 20 mg/kg 2,4-D, 20 mg/kg 2,4,5-T and 1.2 μg/kg TCDD (Group III) would be achieved. Controls (Group I) were given a diet with only the corn oil vehicle added to the feed. In the treated animals, dose-related liver and thymus toxicity were found and body weight gain was significantly reduced. Liver and thymus toxicity showed significant or complete recovery when the mice were returned to a control diet. Sperm concentration, motility and percent sperm abnormalities were evaluated and no significant effect was noted during or after the dosing period.

At the conclusion of an eight week dosing period treated males were mated to untreated virgin females (three per male per week for eight weeks). Mating frequency, average fertility, percent implantation and resorption sites and percent fetal malformations were all measured in relation to the treatment. No significant decrement in fertility or reproduction was noted in the study. There was no evidence of germ cell toxicity. Survival of offspring and neonatal development were apparently unaffected by paternal exposure to the simulated mixtures of Agent Orange.

INTRODUCTION

Chlorinated phenoxyacetic acid compounds are used extensively as herbicides in forestry and agriculture. The Department of Defense tested and used a number of different herbicides containing chlorinated phenoxy acids in Vietnam as defoliants; these included Herbicide Orange, Herbicide White, Herbicide Purple, Herbicide Pink and Herbicide Green (Young et al., 1978). The herbicide most extensively used was Herbicide Orange, a 1:1 mixture of the n-butyl esters of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). It has been estimated that 107 million pounds were sprayed with the majority used in the years 1967 to 1969 (77% of total herbicide sprayed) (Young et al., 1978).

During the synthesis of 2,4,5-trichlorophenol (TCP) and subsequently 2,4,5-T, but not 2,4-D, a highly toxic contaminant is formed. This contaminant, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), has been found in Herbicide Orange at an average concentration of 2 ppm with individual analysis of up to 47 ppm reported (Young et al., 1978). Occupational or environmental exposure to humans to TCDD has been associated with a number of clinical disorders (Firestone, 1977; IARC, 1978). The heaviest exposures have involved industrial accidents which occurred in plants synthesizing TCP. The most consistently documented clinical manifestation has been chloracne, a severe form of pustular folliculitis which is most frequently observed on the face, neck and upper extremities. Other less common clinical findings following TCDD exposure include porphyria cutanea tarda, central and peripheral nervous system disorders, depression and irritability, hepatic dysfunction and altered serum lipid concentrations (Firestone, 1977; IARC 1978; Young et al., 1978).

A number of Vietnam Veterans have expressed concern as to health effects that may have resulted from exposure to Herbicide Orange either through application of the herbicide or through inhabiting defoliated areas (Holden, 1979; Rawls, 1979). A particular concern is that Herbicide Orange exposure may be related to reported decreases in both libido and fertility (low sperm counts and abnormal sperm forms) and that it may also be responsible for birth defects observed in offspring sired by veterans who were exposed to Agent Orange (Bogen, 1979; Holden, 1979).

The toxicity of TCDD and the phenoxy acids 2,4-D and 2,4,5-T has been studied in some detail. The biological effects of these chemicals are well documented in a number of mammalian test systems (Gehring and Betso, 1978; Moore, 1978). 2,4-D, 2,4,5-T and TCDD have all been investigated for teratogenicity and fetotoxicity when given to pregnant females. 2,4-D acid and 2,4-D esters show signs of fetotoxicity and embryotoxicity in hamsters and rats at high dose levels, but it is unclear whether the compounds are actually teratogenic (Collins and Williams, 1971; Khera and McKinley, 1971; Schwetz et al., 1971). Exposure of mice to 2,4,5-T during pregnancy results in congenital malformations (Courtney and Moore, 1971; Neubert and Dillman, 1972; Hood et al., 1979). Studies in rats (Sparschu et al., 1971) and monkeys (Dougherty et al., 1975), indicate that the teratogenicity of 2,4,5-T may be a species-dependent phenomenon, since gestational exposure to this compound produced fetotoxic but not teratogenic effects (Gehring and Betso, 1978).

Early studies with 2,4,5-T samples which were contaminated with 30 ppm of TCDD indicated that the herbicide was teratogenic in rats (Courtney et al., 1970). Subsequent studies by Courtney and Moore (1971), using

purified 2,4,5-T, showed that both 2,4,5-T and TCDD were teratogenic in three strains of mice but in rats only TCDD was fetotoxic and possibly teratogenic. TCDD has been shown by other laboratories to be teratogenic and/or embryotoxic in mice at levels above 0.1 μg/kg/day (Smith et al., 1976) and rats at 0.125-2.0 μg/kg/day (Sparschu et al., 1971).

Although fetotoxicity and teratogenicity associated with gestational exposures to these compounds have been extensively studied, there is a paucity of data as to the effects of male exposure on fertility and development of their offspring. Investigations in male rats undertaken to determine whether dominant lethal mutations could be caused by TCDD were negative. However, the incidence of fertile matings was decreased but it was not determined whether this was due to the systemic toxicity of TCDD or a direct effect on reproduction (Khera and Ruddick, 1971). Other studies that have considered reproductive competence in males have generally been multigeneration studies of animals treated during their entire lives with one of the compounds of interest. In those studies neither 2,4-D (Hansen et al., 1971) nor 2,4,5-T (Smith et al., 1978) significantly reduced fertility when males and females were given feed containing the herbicides. Three-generation studies with TCDD demonstrated that ingestion of levels greater than 0.1 μg/kg/day decreased fertility and litter survival in the f_0 generation; exposure to 0.01 μg/kg/day decreased fertility in the f_1 and f_2 generations, but not the f_0 generation (Murray et al., 1979). In that case, an increase in the percentage of resorbed implantation sites could be related to female exposure to TCDD, but not to male exposure (Murray et al., 1979).

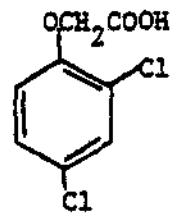
The consequences of chemical toxicity on male reproductive capabilities might include loss or decrease in fertility, abnormal sperm morphology, decreased sperm concentration and/or motility, or lesions in the reproductive tract and accessory sex glands (Gomes, 1970; Manson and Simons, 1980). In addition to effects on reproduction or fertility, chemical exposure might cause genetic mutations in the male germ cells which could be expressed in the offspring as an inherited anomaly, or embryo and fetal death (Joffe, 1979; Manson and Simons, 1980). Another mechanism to explain fetal effects via the male would be that the chemical might actually be transmitted to the female in the seminal plasma which could then result in a direct exposure of the ova.

The dominant lethal (Epstein, 1973; Generoso, 1973) and sperm morphology (Wyrobek, 1979) assays, used routinely in mice to evaluate potential chemical mutagenicity in male germ cells, were employed in this study. Both of these test systems involve chemical exposure followed by fertility testing or sperm evaluation of the animals for the duration of the spermatogenic cycle (approximately 35 days in mice). This approach is necessary since male germ cells are constantly dividing and differentiating during transit from spermatogonia to spermatozoa with each developmental stage varying in its sensitivity and susceptibility to chemical toxicity or mutagenicity. Experimental designs which are directed at determining male germ cell toxicity must consider this sperm maturation process to assure that all stages of development are tested.

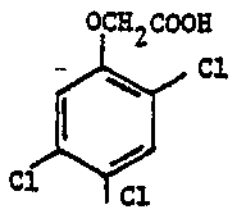
The following investigations were undertaken to determine if composite exposure to 2,4-D, 2,4,5-T plus TCDD (i.e., Herbicide Orange), could affect reproductive function in male mice.

MATERIALS AND METHODS

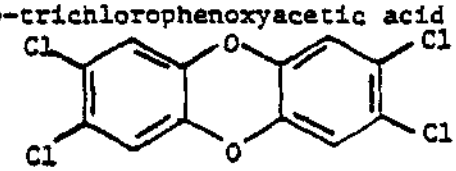
Test Chemicals and Purity



2,4-dichlorophenoxyacetic acid (2,4-D)



2,4,5-trichlorophenoxyacetic acid (2,4,5-T)



2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)

2,4-dichlorophenoxyacetic acid (2,4-D) (AGR 171114, 98.5% pure) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (AGR 133711, 98.7% pure) were supplied by the Dow Chemical U.S.A., Midland, Michigan. Both samples were analyzed by Dow Chemical for TCDD contamination who reported no TCDD or other dioxin detected in the samples (Tables 1 and 2).

The free acid was used to eliminate the volatility problem associated with the butyl ester which would compromise quantification of dose administered and pose an exposure risk to laboratory personnel. The free acid form is readily absorbed from the gastrointestinal tract.

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was synthesized by the Environmental Chemistry Branch, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina. The TCDD was reported to be of greater than 98% purity by gas chromatographic analysis

TABLE 1

Analysis of 2,4-D Sample for Dioxins

	Concentration	Detection Limit
2,3,7,8-Tetrachlorodibenzo-p-dioxin	Not detected	1 ppb
Hexachlorodibenzo-p-dioxin	Not detected	1 ppb
Heptachlorodibenzo-p-dioxin	Not detected	20 ppb
Octachlorodibenzo-p-dioxin	Not detected	5 ppb

TABLE 2

Analysis of 2,4,5-T Sample for Dioxins

	Concentration	Detection Limit
2,3,7,8-Tetrachlorodibenzo-p-dioxin	Not Detected	0.5 ppb
Hexachlorodibenzo-p-dioxins	Not detected	0.03 ppm
Octachlorodibenzo-p-dioxin	Not detected	0.3 ppm

performed by NIEHS. The principal contaminate was 2,3,7-trichlorodibenzo-p-dioxin.

Animals and Husbandry

Four week old male and 10 week old female C57BL/6N inbred mice (Cesarean-Originated, Barrier Sustained) were purchased from the Charles River Breeding Laboratories, Inc., Wilmington, Massachusetts. Upon arrival the mice were eartagged and housed in plastic cages with stainless tops (males, one per cage; females, ten per cage). Absorb-dri hardwood bedding (Barnes Supply, Durham, NC) was used, and cages were cleaned once each week. The animals were kept in constant temperature (20 ± 2°C) and humidity (R.H. 50 ± 10%) on a fixed cycle of 12 hours light-12 hours darkness. The mice were allowed food and water ad libitum. The powdered diet was open formula NIH-31 prepared by Zeigler Bros. Co. of Gardners, Pa.

Preparation of Diets

Stock solution of the test chemicals were prepared in a corn oil vehicle. The calculated and analytical values are given in Table 3. The test diets were prepared each week for 8 weeks by adding the appropriate stock solution into the feed (2% vol/wt). The concentration of chemicals in the feed was not changed during the 8 week exposure period study. Feed consumption (gm/mouse) was measured once a week. Approximate dose levels were projected using consumption of 5 gm feed/day by a mouse weighing 25 gm. The controls (Group I) were given a diet containing 2% corn oil. Group II consisted of mice that received about 40 mg/kg/day of 2,4-D, 40 mg/kg/day of 2,4,5-T and 2.4 µg/kg/day of TCDD for a total dose of 2.24 gm/kg of 2,4-D and 2,4,5-T each and 0.13 mg/kg of TCDD over the entire 8 weeks. Group III mice were treated at a rate of 40 mg/kg/day

TABLE 3

2,4-D, 2,4,5-T and TCDD

Concentrations in Stock Corn Oil Solutions*

and Projected Dosages in Feed

Treatment Group	2,4-D[1] ppm*	2,4-D[1] mg/kg/day**	2,4,5-T[1] ppm*	2,4,5-T[1] mg/kg/day**	TCDD[2] ppb*	TCDD[2] µg/kg/day**
I (Control)	0(0)	0	0(0)	0	0(0)	0
II	10,000(9380)	40	10,000(9590)	40	600(505)	2.4
III	10,000(9310)	40	10,000(9480)	40	40(39)	0.16
IV	5,000(4880)	20	5,000(4830)	20	300(271)	1.2

*Calculated prepared concentrations and in ( ) amounts detected by analysis.

**Dose levels based on ideal concentrations in feed and an average feed consumption of 5 gm feed/day/25 gm mouse.

[1]Samples of oil collected for 2,4-D and 2,4,5-T analyses were extracted with ethyl ether, derivatized with diazomethane, extracted with hexane and analyzed on an electron capture gas chromatograph (ECGC) by the Midwest Research Institute.

[2]Samples of oil collected for TCDD analysis were saponified in ethyl alcohol and potassium hydroxide, then extracted with hexane and analyzed with an ECGC by the Midwest Research Institute.

of 2,4-D and 40 mg/kg/day of 2,4,5-T (same as Group II), but received only 0.16 μg/kg/day of TCDD; the total dosages of 2,4-D and of 2,4,5-T were 2.24 gm/kg and 0.009 mg/kg of TCDD. The mice in Group IV received 20 mg/kg/day of 2,4-D, 20 mg/kg/day 2,4,5-T, and 1.2 μg/kg/day of TCDD, resulting in a total 8 week exposure of 1.12 gm/kg of 2,4-D and of 2,4,5-T and 0.067 mg/kg of TCDD. After the 8 week exposure period all mice were fed standard pelleted NIH 31 diet.

Experimental Design

Two hundred male mice were weighed and sorted (by weight) into eight groups (25 per group). One half of the male mice (4 groups of 25 each) were used for toxicity evaluation, while the other half (4 groups of 25 each) were used for fertility and reproductive studies.

All males were then acclimatized on NIH-31 laboratory chow for three weeks before the chemical exposures were begun. Chemical exposure began when the males were eight weeks old. Body weights and food consumption were recorded on a weekly basis. The males were assigned to experimental and control groups such that weight differences between groups were minimized. The mice were then treated with one of the three treated or control diets for eight consecutive weeks.

Toxicopathology

One hundred of the mice were studied during and after the 8 week feeding period. Four animals from each of the four dose groups were killed by decapitation at 1, 4, 5, 8, 12 and 16 weeks after first receiving treated feed. Each mouse received a gross autopsy examination; body and organ (brain, liver, spleen, kidney, thymus and testis/epididymis) weights were measured. These organs as well as lung, duodenum, ear,

prostate, seminal vesicle, coagulating gland and urinary bladder were fixed in 10% neutral buffered formalin, dehydrated and embedded in paraffin blocks. Six μm histologic sections were prepared and stained with hematoxylin and eosin. The tissues were examined for evidence of histopathologic change.

Also at sacrifice, the vas deferens were removed and spermatozoa milked into a 1.0 ml volume of 0.9% saline. The concentration of sperm per vas deferens was estimated with a hemocytometer immediately after collection. Additionally, the percent motile (any movement vs. no movement) sperm was enumerated. The sperm sample was then stained with 0.25% eosin Y for 30 minutes. The sperm were evenly distributed within the staining solution using a Pasteur pipette and four slide preparations were prepared from each sample. The smears were allowed to air dry, were cover slipped and were examined at 400 X magnification. Three hundred sperm were studied for each sample and sperm were classified as normal or abnormal using the criteria of Wyrobek and Bruce (1975) and Soares et al (1979).

Fertility and Reproduction

Fertility and reproduction assessments were conducted on the remaining 100 mice (four groups of 25).

After the male mice had been treated with the test chemicals for eight weeks they were returned to control feed. Beginning the next day, each male was housed with three virgin female mice (fourteen weeks of age) for up to 5 days each week for eight weeks. Each female was examined each morning for evidence of mating by detection of a vaginal plug (Day 0 of pregnancy). Each mated female was removed from the cage, weighed and placed in a cage with other mated females in that group. Females which did not appear to have mated during the 5-day cohabitation period

were observed for three more weeks to permit detection of possible pregnancies for which vaginal plugs were not observed. Apparent failure of a female to mate or conceive was verified three weeks after cohabitation by killing the animal, removing the uterus, and staining it with ammonium sulfide to better identify the presence of implantation sites (Kopf et al., 1964).

For each weekly mating trial one female bred to each male was put in a group to be sacrificed on day 18 of pregnancy for teratology examination. A second bred female from each male was placed in a group which was allowed to deliver and rear her offspring. All remaining dams found to have plugs were placed in a "teratology backup group" to be subjected to teratological evaluation if the dam selected for day 18 sacrifice was found not to have any live fetuses. The above pregnant mice were systematically distributed to the teratology, postnatal or "backup" groups such that no one group was biased with dams which mated first, second or third. However, if less than three dams had mated, priority was generally given to the teratology group. All mated dams were weighed on days 0, 7, 11, 15 and 18 of gestation.

On day 18 of gestation, the dams designated for teratology examination were coded to permit identification only by number so that laboratory personnel conducting the teratogenic analysis did not know the test group. The mice were killed by cervical dislocation and their reproductive status was determined. Implantation sites in each uterine horn were counted and the general condition of each conceptus was recorded. The detection of implantation sites in the uteri of apparently nonpregnant females was achieved through use of ammonium sulfide (Kopf et al., 1964). Live fetuses were weighed individually, sexed internally (surgical incision below navel), and examined for external malformations. Live

fetuses weighing <0.5 g, or weighing less than two-thirds the mean of their larger littermates, were designated as being "stunted". At least one-half of the fetuses of each litter, all "stunted" fetuses and fetuses having external malformations, were examined for visceral alterations (Staples, 1974). The bodies of all fetuses were then processed for skeletal examination (Staples and Schnell, 1964). The heads of each fetus subjected to visceral examination (with the exception of any fetuses which had external head malformations) were cut off at the base and examined by the free-hand sectioning technique described by Wilson (1965).

The remaining dams (postnatal group) were allowed to deliver their litters. Live and dead offspring as well as birth weight were recorded (day 0). The pups were reweighed on days 4, 7 and 21 and viability also was recorded. The dams and their offspring were killed on day 21.

The above procedures were repeated weekly for eight weeks resulting in a total of eight sets of data. Four weeks after the conclusion of the breeding study, (week 20 of the experiment), the male mice were killed and autopsied in a manner identical to that described for the males sacrificed for toxicopathologic evaluation.

Statistical Evaluation

Statistical evaluations of possible pairwise treatment-control differences in food consumption, body weights, organ weights, fertility, mating efficiency, and sperm number, motility and abnormalities were made by Dunnett's test (Miller, 1966). Analysis of variance procedures were employed to assess the significance of differences among groups, week-to-week variability, and week by group interactions. Analyses of abnormalities among the offspring were carried out employing pairwise

comparison of control versus treated groups with the Mann Whitney U test. The analysis of malformations considered the average percent malformed fetuses per litter.

RESULTS

Feed Consumption and Body Weight

The projected food consumption of 35 gm/week (5 gm/day) proved to be a conservative estimate in all groups throughout the period of chemical exposure (Figure 1). Lower food consumption for all groups is indicated in week-2 because only a fraction of the week (5 days) was measured. The addition of 2,4-D, 2,4,5-T or TCDD did not significantly decrease feed consumption during the full eight week dosing period in any treatment group, as compared to the controls. Statistically significant changes in feed consumption were only found in sporadic cases and no general trend of decreased feed consumption could be attributed to the addition of either phenoxy acids or TCDD.

Body weight and weight gain, however, showed significant reductions in the treated animals when compared to controls (Figures 2 and 3). This reduction in body weight was most pronounced in group II (2.4 μg TCDD/kg/day and 80 mg phenoxy acid/kg/day) from weeks 3 through 8 of the study. The Group II animals recovered most of their weight deficit when returned to control diet.

Generally all of the mice appeared healthy throughout the course of the study. Only two animals died during the twenty weeks, one in group IV at 5 weeks and one in group II at 19 weeks. Their death did not appear to be treatment-related.

Organ Weights and Histopathology

The mean organ weights of animals killed on weeks 1, 4, 5, 8, 12, 16, and 20 are shown in Figures 4-9. Statistically significant increases in liver weight (Figure 4) were observed in all treated groups and was positively correlated with the amount of TCDD exposure (i.e., 2.4 > 1.2

> .16 μg/kg TCDD/day, groups II, IV and III respectively). After conclusion of exposure the liver weight returned toward normal, although Groups II and IV continued to show significantly elevated values even at week 20. The livers of treated mice were enlarged, lighter in color than normal and mottled. The thymus was decreased in weight, which also appeared to be a function of the level of TCDD rather than phenoxy acid exposure (Figure 5). Although the thymus weights were significantly ($p<.01$) reduced in Groups II and IV relative to controls throughout the treatment period (weeks 1-8), thymic recovery appeared complete and weights were not statistically different from the controls by 4 weeks after the last exposure. No significant treatment-related effects were observed in the spleen (Figure 6), testis (Figure 7), kidney (Figure 8), or brain (Figure 9). Histopathological evaluation showed no treatment-related changes in any organs, with the exception of the liver. Even the thymus, which had decreased in size to as much as one-third that of the control thymus, appeared histologically normal. The mild toxic effects observed in the liver included hepatocellular swelling, scattered single cell necrosis, increased numbers of mitotic figures, excess extramedullary hematopoiesis, and leukocytic infiltration. These changes were most apparent in group II (2.4 μg/kg/day TCDD; 80 mg/kg/day phenoxy acids) and least apparent in Group III (0.16 μg/kg/day TCDD; 80 mg/kg/day phenoxy acid). These signs of toxicity diminished substantially by the end of the twelfth week of the study (four weeks after chemical exposure was concluded).

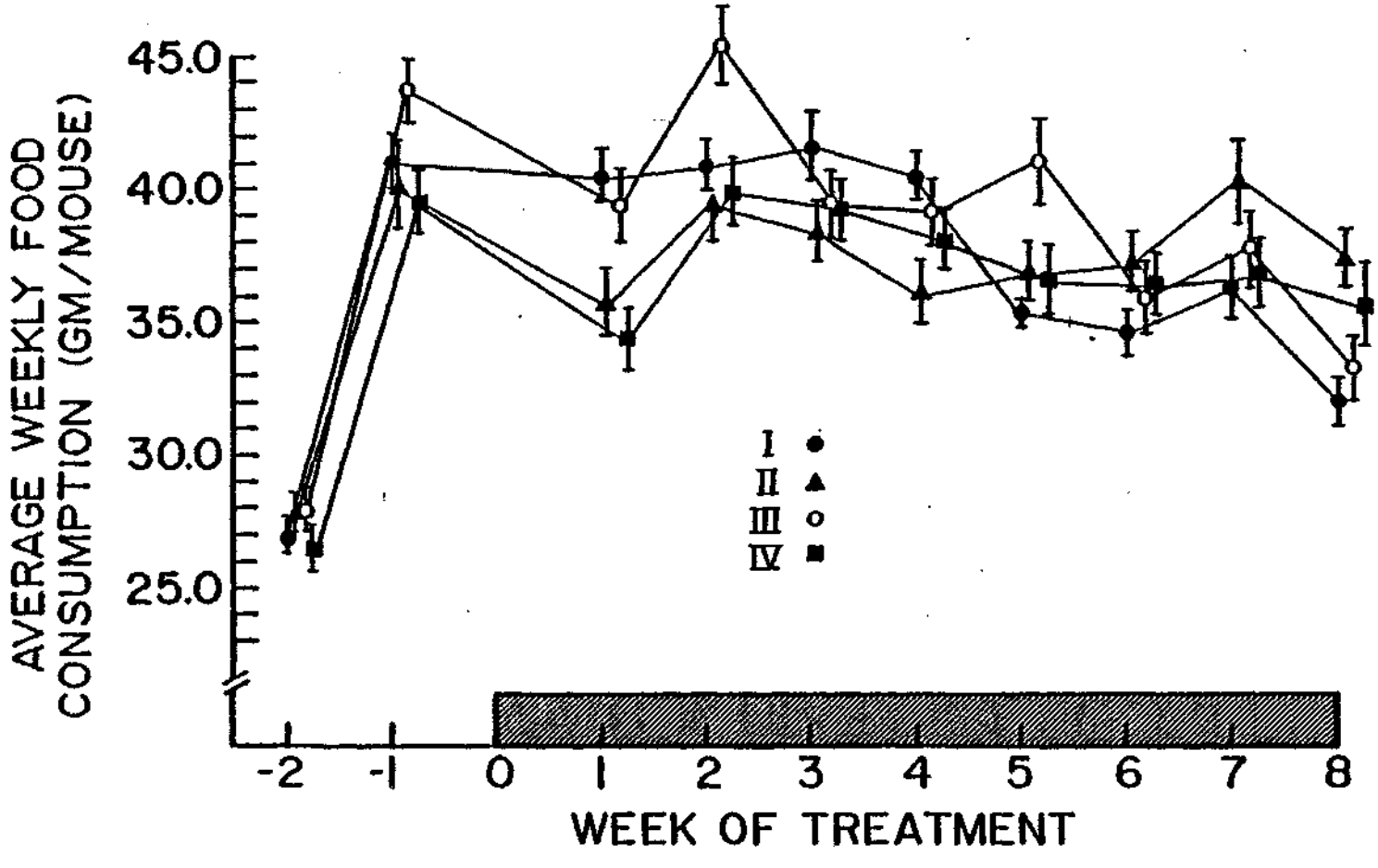


Figure 1. Average weekly food consumption before and during the treatment period of the study for the males. Values are mean ± S.E.M.

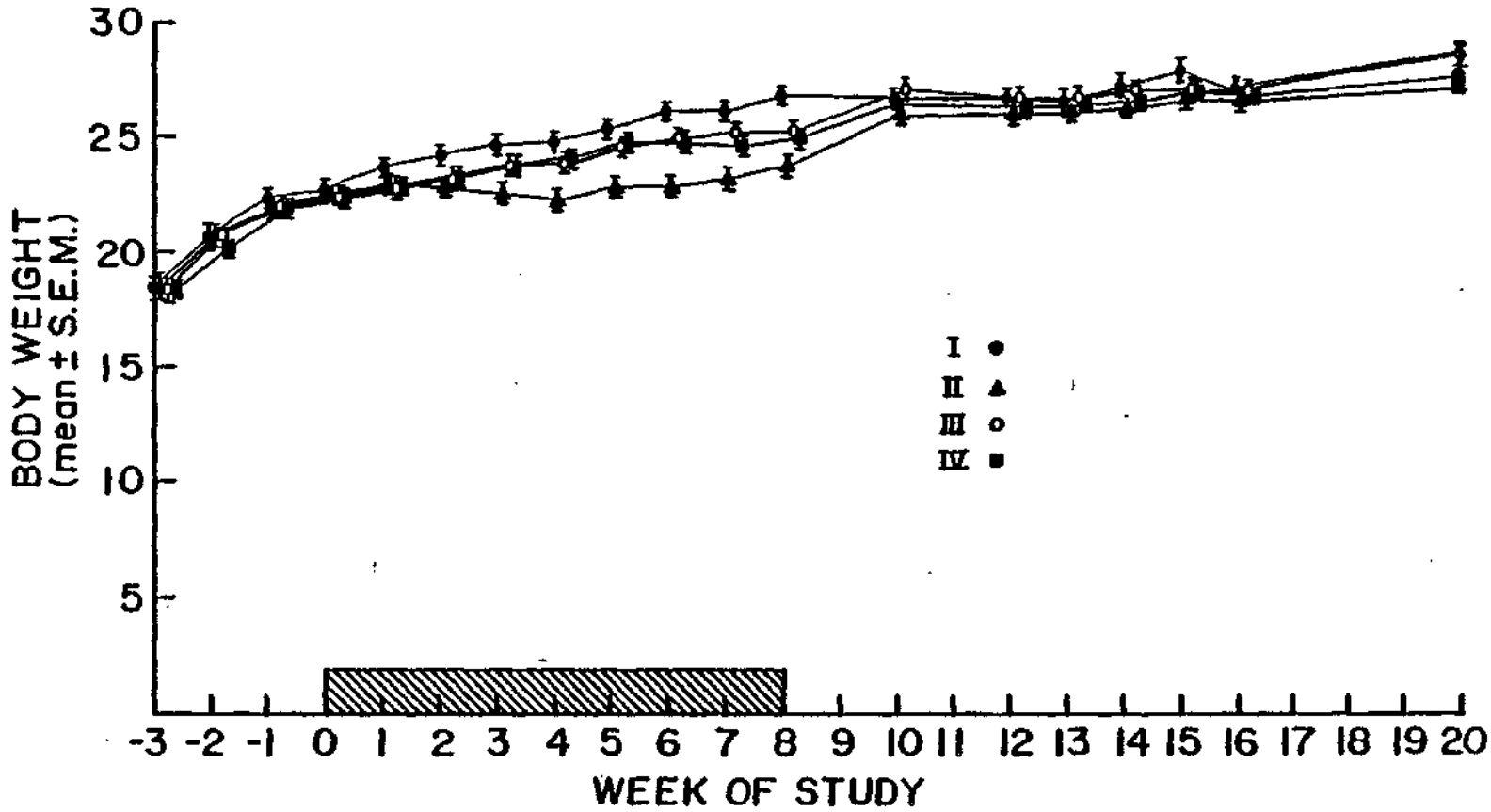


Figure 2. Mean body weights for male mice in all four treatment groups. Significant reductions ($p<0.05$) in weight, as compared to control, for groups II and III were present in weeks 3-8, group IV in weeks 6-8. Values are mean ± S.E.M.

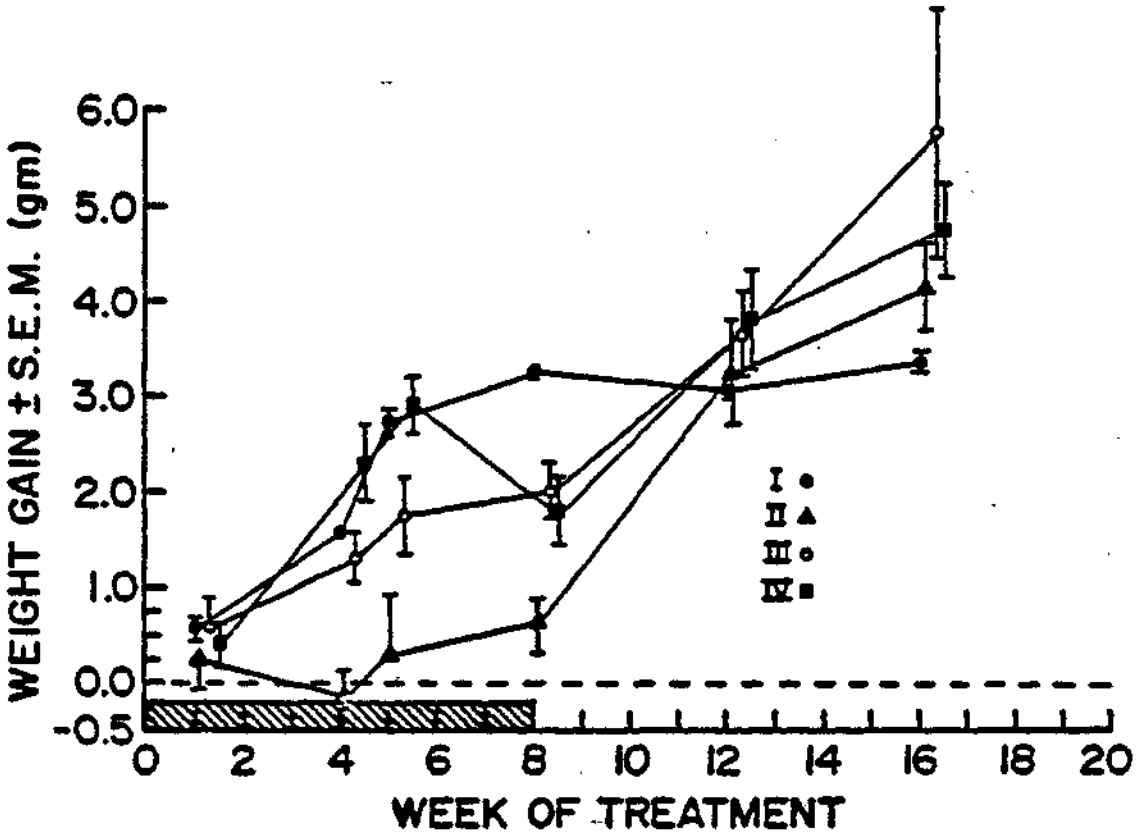


Figure 3. Average weekly weight gain. Weight gain was significantly ($p<0.01$) reduced in Group II relative to control during weeks 4, 5 and 8. Rapid recovery was observed such that no significant difference was present by week 12 in these animals. Values are mean $\pm$ S.E.M., n = 4 per group.

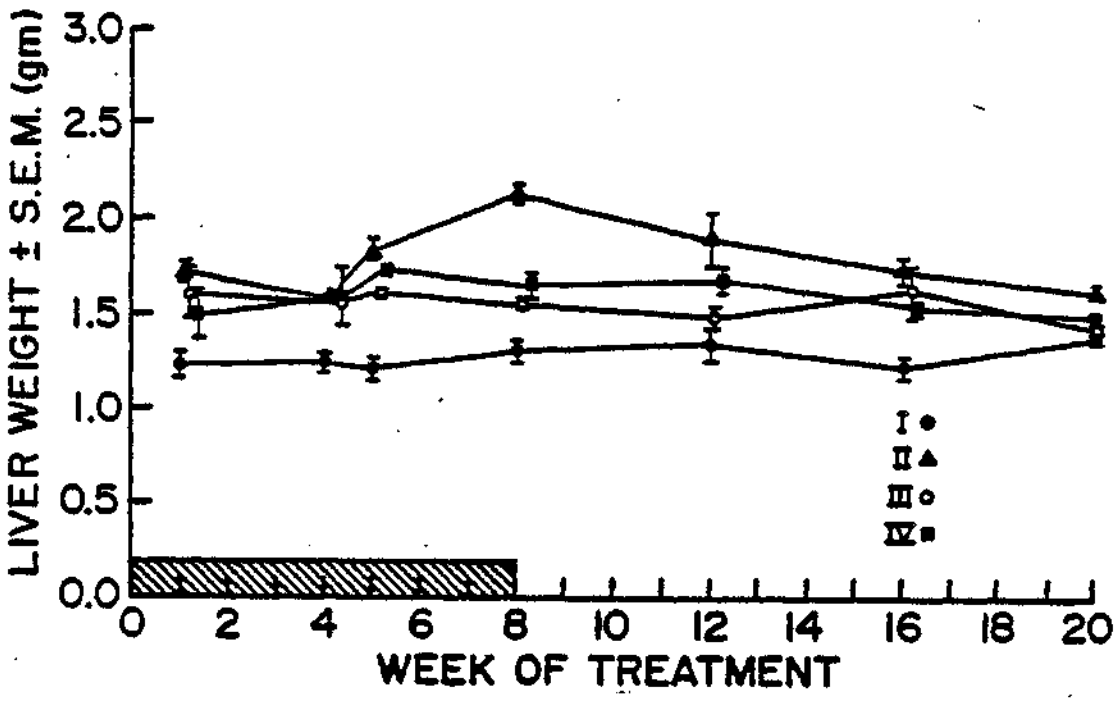


Figure 4. Average liver weight. Liver weight was significantly ($p<.05$) increased in Group II (weeks 1-20), Group III (weeks 1-5, week 16) and Group IV (weeks 4-20) relative to controls. Values are mean ± S.E.M., n = 4 per group, weeks 1-16, n = 25 per group in week 20.

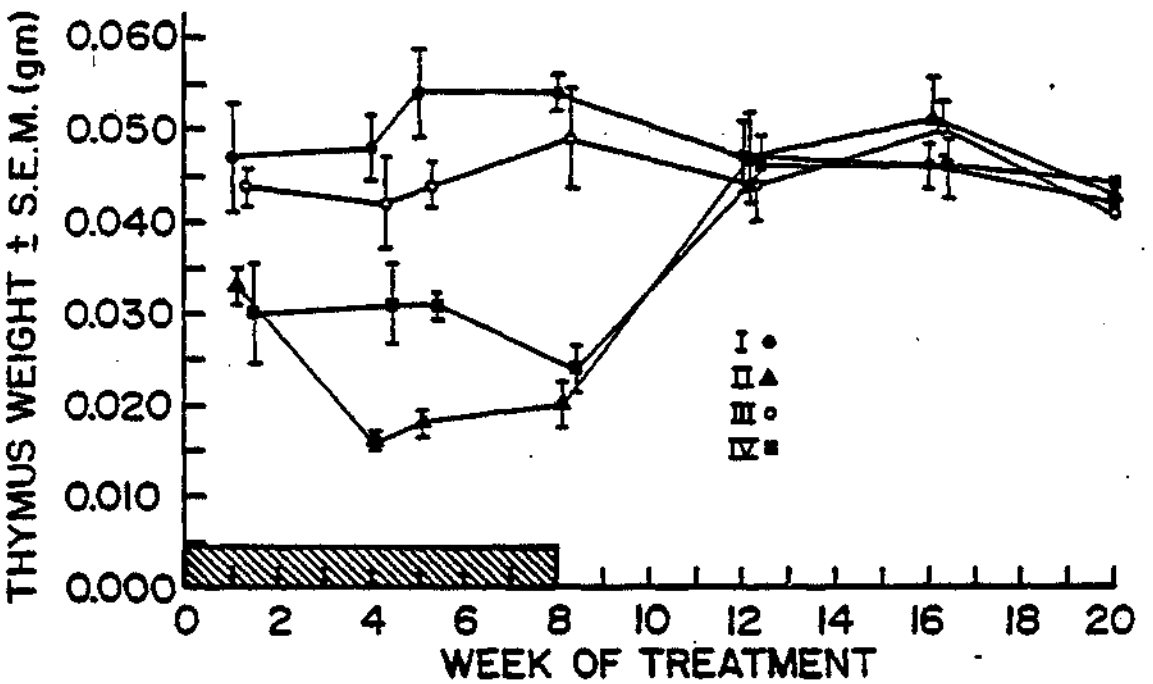


Figure 5. Average thymus weight. Thymus weight was significantly ($p<.01$) reduced in Groups II and IV relative to controls (weeks 1-8). Complete recovery was observed in all groups by week 12. Values are mean ± S.E.M., n = 4 per group on week 1-16, n = 25 per group in week 20.

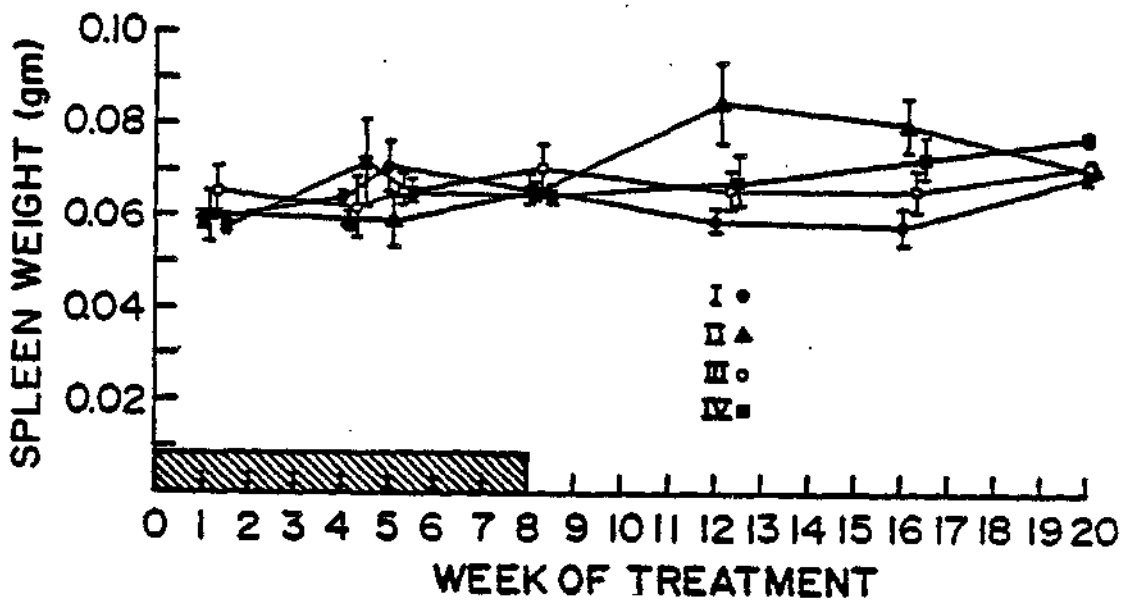


Figure 6. Average spleen weight was not significantly changed by treatment. Values are mean ± S.E.M., n = 4 per group in weeks 1-16, n = 25 per group in week 20.

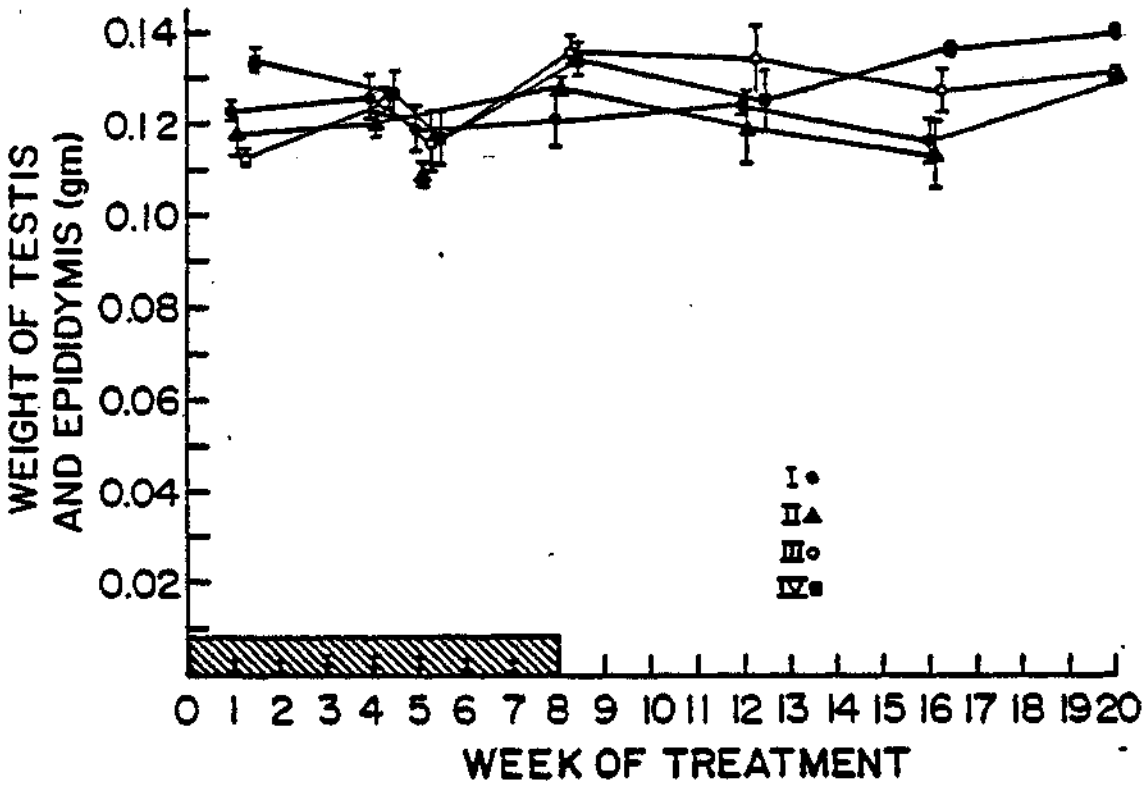


Figure 7. Average testis and epididymis weight was not significantly affected by treatment. Values are mean ± S.E.M., n = 4 per group in weeks 1-16, n = 25 per group in week 20.

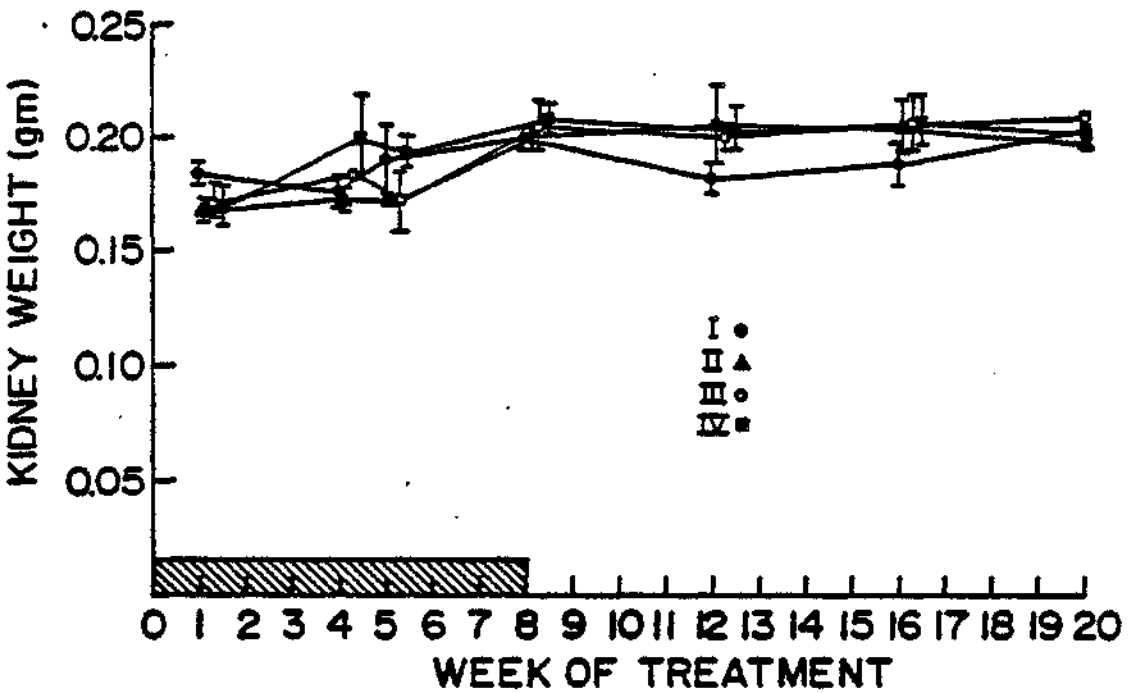


Figure 8. Average kidney weight was not significantly changed by treatment. Values are mean ± S.E.M., n = 4 per group in weeks 1-16, n = 25 per group in week 20.

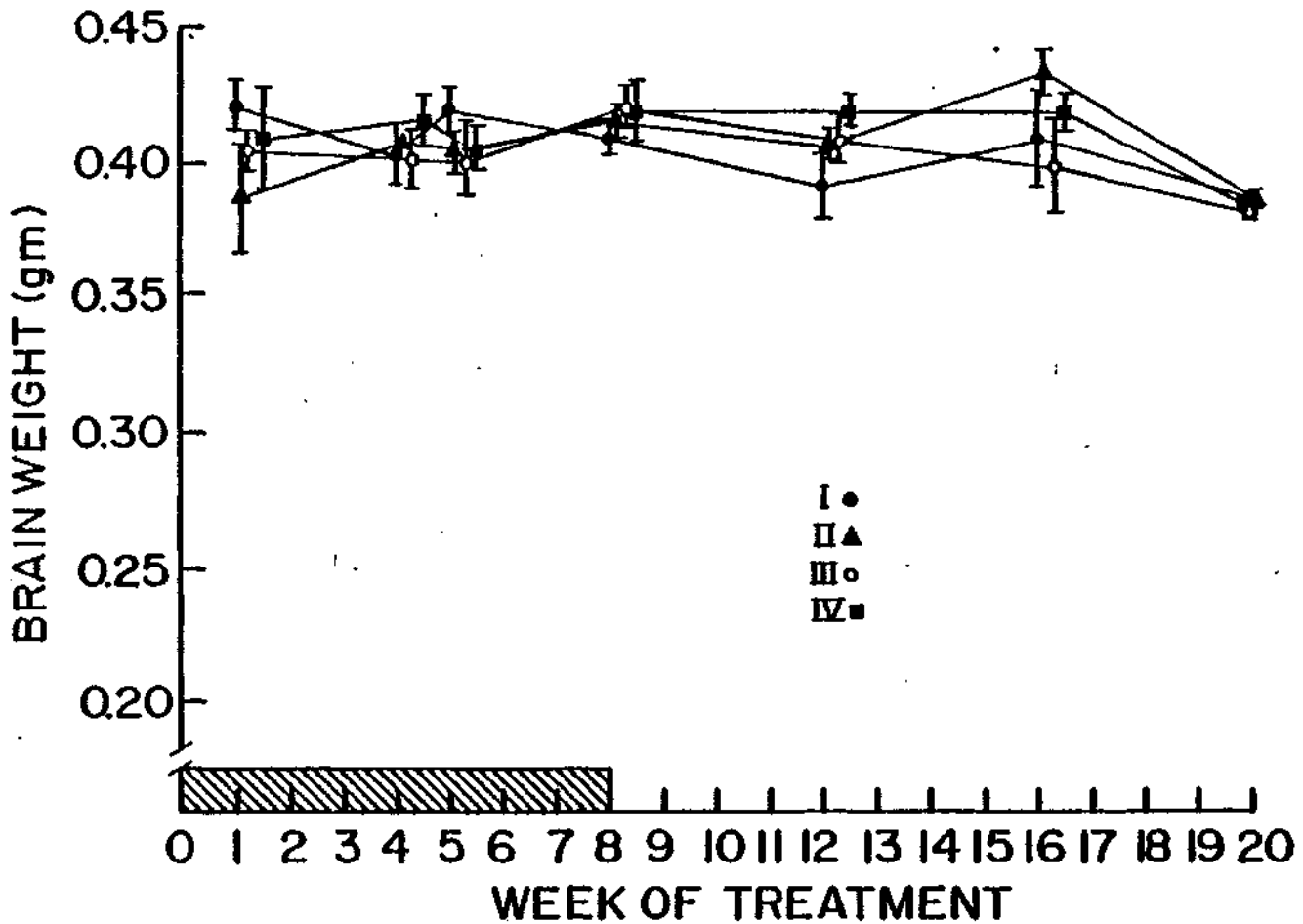


Figure 9. Average brain weight was not significantly altered by chemical treatment. Values are mean ± S.E.M., n = 4 per group in weeks 1-16, n = 25 per group in week 20.

Fertility

The average percent matings and overall fertility of the males through 8 weeks of the study are given in Table 4. There was a significant reduction in the mating frequency in males from group III (80 mg/kg/day phenoxy acid, 0.16 µg/kg/day TCDD). This effect was not significant in Group II whose phenoxy acid exposure was similar and whose TCDD exposure was 15-fold greater. Therefore, no dose-related effect could be attributed to this decrease. Also, the percent fertile matings and total fertility were not significantly reduced in Group III or in any group when compared to the control.

When fertility was evaluated on a week by week basis, no treatment-related changes were observed. Fertility was also studied on an individual per male basis and no significant changes were detected within treated males as compared to controls. At the conclusion of the study sperm concentration and motility and percent abnormal sperm were measured (week 20, Figures 10-12). These values were analyzed, on an individual male basis, to determine whether any correlation existed between low fertility performance (plug frequency, percent fertile matings and total fertility) and low values for sperm quality (concentration, motility, percent abnormal). In all groups there was no correlation between the parameters measured. This would indicate that, even though there was considerable variability within these parameters, variations in fertility could not generally be attributed to specific changes in sperm quality. The values for sperm concentration (Figure 10) and sperm motility (Figure 11) fluctuated considerably from week to week. Percent abnormal sperm were less variable (Figure 12). No treatment-related changes were observed in these parameters. The marked reductions in sperm concentration (Figure 10) and increase in sperm motility (Figure 11) which were

TABLE 4

Fertility and Mating Efficiency in Treated and Control C57Bl/6 Mice
8 Week Total

Treatment Group[1]	Mating[2] Frequency	Fertile Matings (percent)[3]	Total[4] Fertility (percent)
I(Control)	74.6 ± 1.6	56.2 ± 2.2	42.0 ± 1.9
II(80;2.4)	70.3 ± 2.4	58.3 ± 2.9	41.0 ± 2.6
III(80;0.16)	67.8 ± 2.4*	55.3 ± 2.3	37.7 ± 2.2
IV(40;1.2)	73.0 ± 2.0	60.8 ± 2.9	44.2 ± 2.3

[1]Calculated daily exposure is given in parentheses as total mg phenoxy acids/kg/day; [2]μg TCDD/kg/day
[3]Percent plugs observed/total females housed with males.
[4]Percent fertile matings/females with plugs.
*P<.05 relative to controls

Values are mean ± standard error of the mean, n = 25 per group.

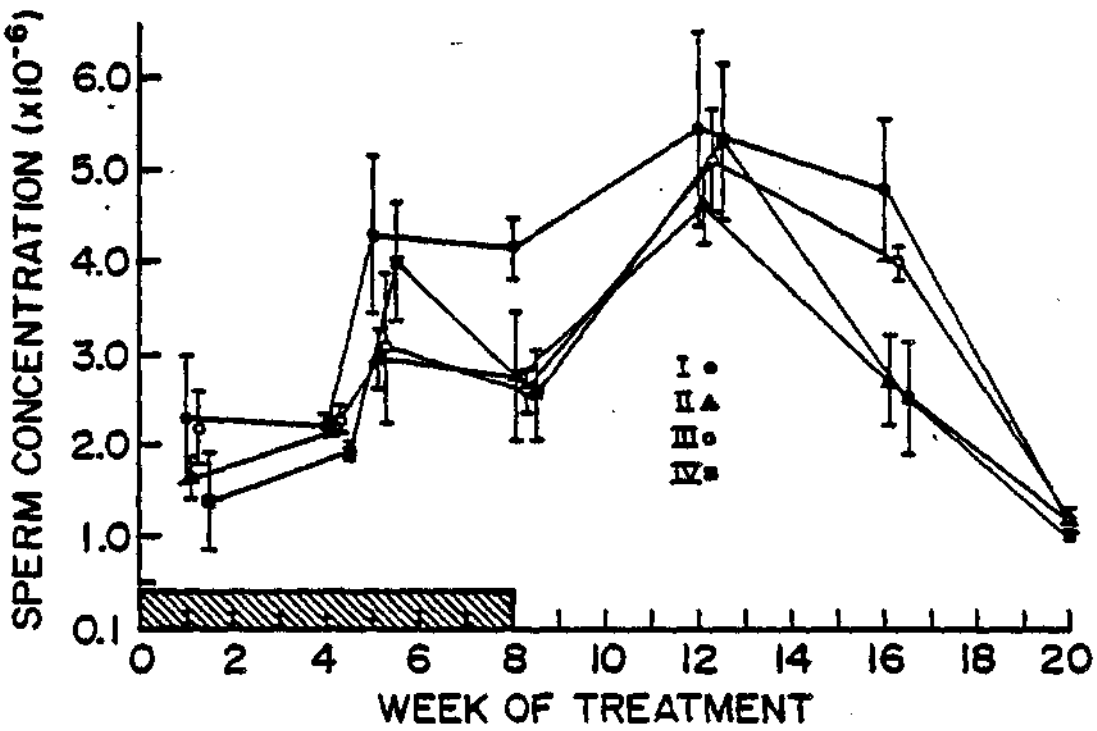


Figure 10. Average sperm concentration. Sperm concentration was quite variable and the only significant reduction relative to controls was in week 16 ($p<.05$, Group II; $p<.01$, Group IV). The marked reduction in all groups at 20 weeks may have been related to the mating of those animals. Values are mean $\pm$ S.E.M., n = 4 per group in weeks 1-16, n = 25 per group in week 20.

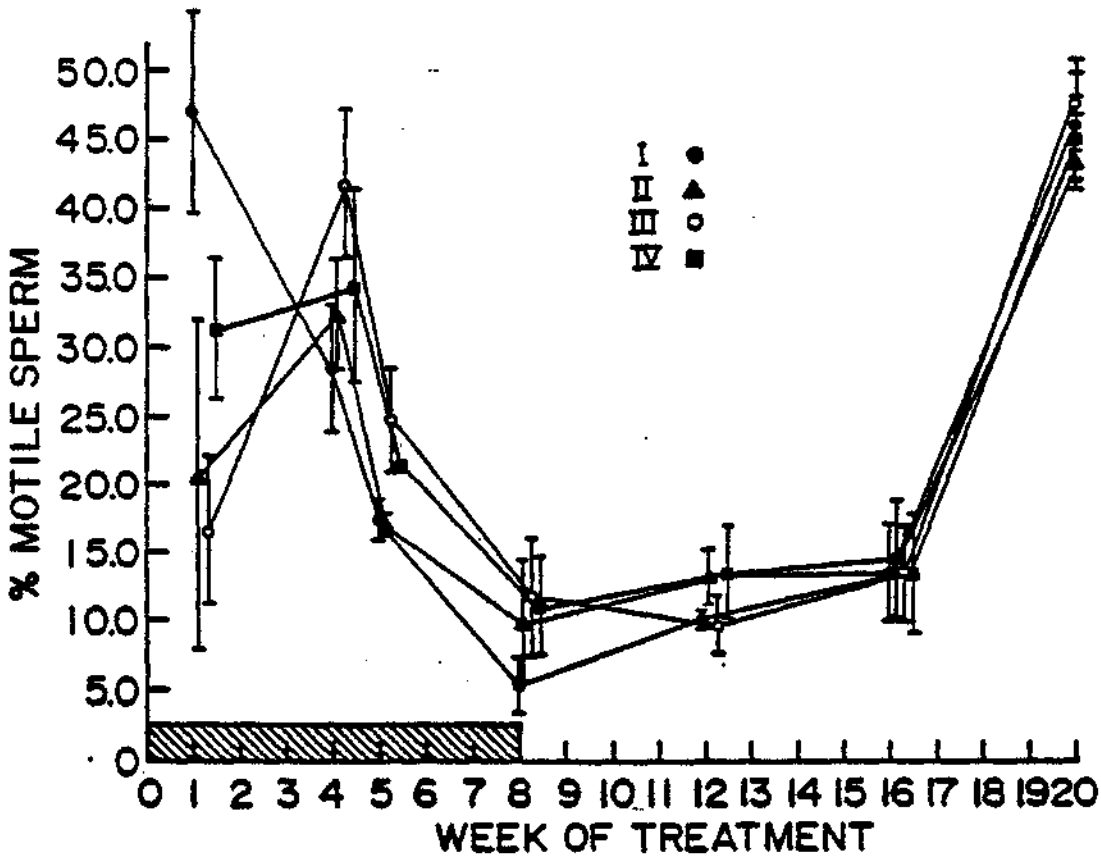


Figure 11. Average percent motile sperm. No significant change was observed in the percent motile sperm ($p<0.05$) when treated and control values were compared. The drop in motility seen from week 4 to week 16 may have resulted from not breeding those males; in contrast, the week 20 (mated) values were much higher. Values are mean $\pm$ S.E.M., n = 4 per group in weeks 1-16, n = 25 per group in week 20.

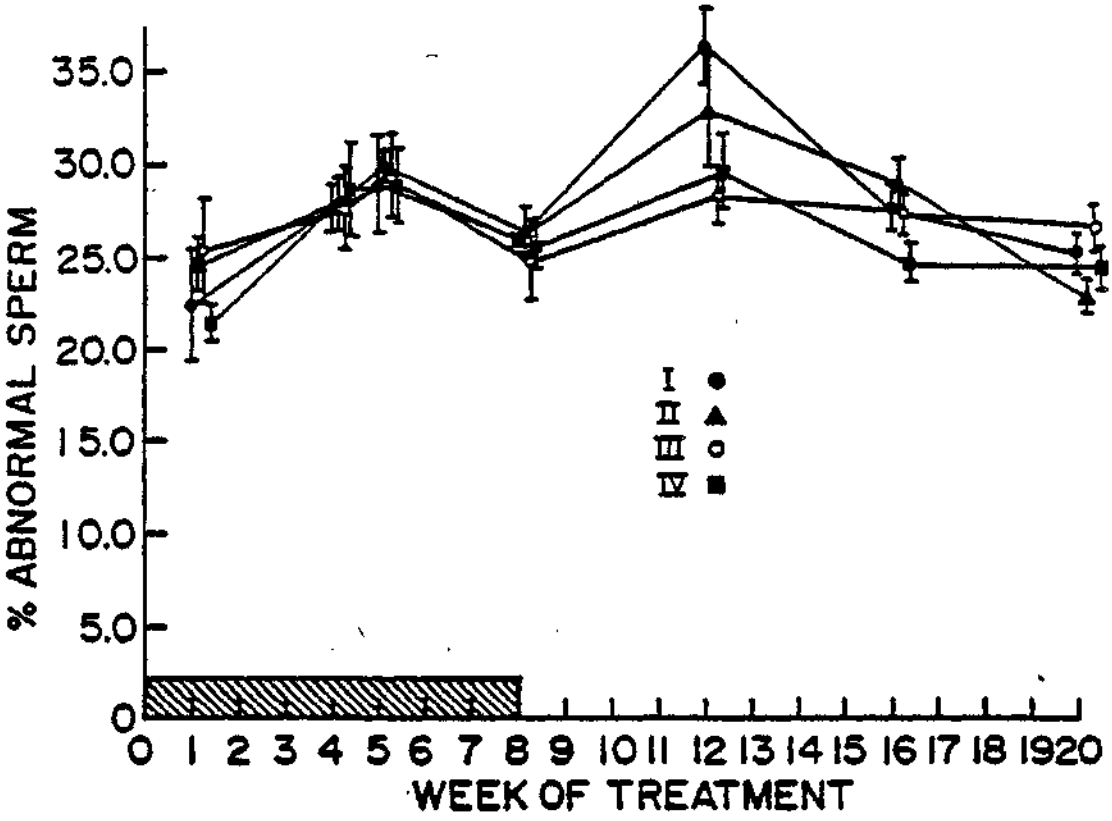


Figure 12. Average percent abnormal sperm. Treatment had no significant influence on the percent abnormal sperm ($p<0.05$). Values are mean $\pm$ S.E.M., n = 4 per group in weeks 1-16, n = 25 per group in week 20.

observed from weeks 16 to 20 of the study might be at least partly explained by the fact that the males whose sperm were checked on week 20 had been through an 8-week intensive mating program whereas the males monitored in the earlier weeks were virgins.

Teratological Examinations

The results of the teratology examination of the dams mated with treated or control males for each group by week are detailed in Tables 5-8. A comparison of the tables indicates that the average number of implants per litter, average number of resorptions per litter or average number live fetuses per litter (Tables 5-8) were unaffected by the male's chemical exposures. For example, the mean values for control and Group II (most heavily exposed) were 7.1 vs 7.4 implant sites per litter; also in Groups I and II there were 4.9 vs 5.0 live fetuses per litter and 2.18 vs 2.37 resorptions per litter. The average fetal weight was significantly ($p<0.05$) greater in all treatment groups as compared to controls. The total number of dead fetuses (i.e., offspring which weighed more than 0.3 gm; offspring weighing <0.3 gm were listed as a resorptions) was 1 in Group I, 2 in Group II, 1 in Group III and 0 in Group IV for the entire 8 week study. The ratio of male to female fetuses was also determined; no treatment group exhibited any significant change ($p<0.10$) in this ratio as compared to the control.

Congenital malformations were not significantly increased in the offspring of treated versus control males (Tables 5-10). Visceral malformations were observed with less frequency than external and skeletal malformations in all groups. The incidences of the most frequently observed malformations are summarized in Table 9: eye defects (anophthalmia and microphthalmia), jaw anomalies (agnathia, micrognathia), were observed in 1.4 to 2.4 percent and 1.2 to 1.6 percent of the fetuses,

Table 5

Effect of 2,4-D, 2,4,5-T and TCDD on Fetal Development
Group 1 Control

	Week of Study								Total 8 weeks
	1	2	3	4	5	6	7	8	
Number of females examined	19	22	22	25	23	19	22	19	171
Maternal weight gain	13.1 ± 1.0	12.8 ± 0.7	12.7 ± 0.7	12.0 ± 0.8	10.0 ± 0.7	10.1 ± 0.7	11.4 ± 0.8	12.1 ± 0.8	11.8 ± 0.3
Number of implants per litter	8.1 ± 0.5	7.7 ± 0.4	8.0 ± 0.4	7.4 ± 0.6	5.4 ± 0.5	5.8 ± 0.4	7.2 ± 0.5	6.6 ± 0.6	7.1 ± 0.2
Number of resorptions per litter	2.16 ± 0.38	2.41 ± 0.32	2.18 ± 0.32	2.16 ± 0.25	2.39 ± 0.25	2.16 ± 0.33	2.27 ± 0.3	1.68 ± 0.30	2.18 ± 0.11
Number of live fetuses per litter	5.9 ± 0.7	5.3 ± 0.5	5.9 ± 0.5	5.2 ± 0.6	3.0 ± 0.5	3.7 ± 0.4	4.9 ± 0.5	4.9 ± 0.6	4.9 ± 0.2
Average fetal weight per litter	1.04 ± 0.03	1.06 ± 0.02	1.05 ± 0.03	1.00 ± 0.02	1.03 ± 0.02	1.04 ± 0.03	0.99 ± 0.03	1.01 ± 0.03	1.03 ± 0.01
Male/female	59/52	47/68	63/64	69/60	38/32	32/38	52/53	42/52	404/419
Visceral Malformations:									
Number of fetuses examined	60	62	68	73	39	39	62	52	455
Number with visceral malformations	0	0	0	1	1	0	0	0	2
Skeletal and External Malformations:									
Number of fetuses examined	113	117	129	129	70	70	108	94	830
Number with malformations	2	3	4	4	4	0	4	5	26

Values are mean ± standard error of the mean.

Table 6

Effect of 2,4-D, 2,4,5-T and TCDD on Fetal Development
Group II

	Week of Study								Total 8 weeks
	1	2	3	4	5	6	7	8	
Number of females examined	19	18	21	17	22	20	17	14	148
Maternal weight gain	14.1 ± 0.5	12.7 ± 0.9	12.2 ± 0.8	11.5 ± 1.0	10.8 ± 0.7	10.4 ± 0.9	12.6 ± 0.9	12.0 ± 1.0	12.0 ± 0.3
Number of implants per litter	9.2 ± 0.4	7.4 ± 0.6	8.2 ± 0.4	6.2 ± 0.6	7.1 ± 0.4	6.2 ± 0.5	7.5 ± 0.4	7.0 ± 0.7	7.4 ± 0.2
Number of resorptions per litter	2.37 ± 0.27	1.94 ± 0.37	2.71 ± 0.40	1.76 ± 0.22	2.55 ± 0.36	2.30 ± 0.45	1.88 ± 0.38	3.43 ± 0.71	2.37 ± 0.14
Number of live fetuses per litter	6.8 ± 0.3	5.5 ± 0.7	5.6 ± 0.5	4.5 ± 0.7	4.5 ± 0.4	3.9 ± 0.6	5.6 ± 0.6	3.6 ± 0.7	5.0 ± 0.2
Average fetal weight per litter	1.00 ± 0.02	1.14 ± 0.03	1.08 ± 0.03	1.09 ± 0.02	1.02 ± 0.03	1.11 ± 0.03	1.02 ± 0.02	1.10 ± 0.03	1.08 ± 0.01
Male/female	62/67	39/60	61/48	36/39	45/54	43/34	42/54	28/20	356/376
Visceral Malformations:									
Number of fetuses examined	66	53	61	42	54	42	47	28	393
Number with visceral malformations	0	0	0	0	0	0	2	0	2
Skeletal and External Malformations:									
Number of fetuses examined	129	99	115	78	100	77	96	50	744
Number with malformations	2	6	4	0	5	1	7	1	26

Values are mean ± standard error of the mean.

Table 7

Effect of 2,4-D, 2,4,5-T and TCDD on Fetal Development
Group III

	Week of Study								Total 8 weeks
	1	2	3	4	5	6	7	8	
Number of females examined	18	15	21	17	19	22	21	12	145
Maternal weight gain	12.9 ± 0.9	12.9 ± 0.9	11.9 ± 0.7	12.0 ± 0.9	10.7 ± 0.8	10.9 ± 0.8	10.2 ± 0.6	12.2 ± 1.1	11.6 ± 0.3
Number of implants per litter	8.4 ± 0.6	7.6 ± 0.5	7.5 ± 0.4	6.6 ± 0.6	7.2 ± 0.5	6.6 ± 0.6	6.8 ± 0.4	7.0 ± 0.6	7.2 ± 0.2
Number of resorptions per litter	2.65 ± 0.58	2.27 ± 0.44	2.14 ± 0.35	1.65 ± 0.28	2.84 ± 0.37	2.14 ± 0.34	2.05 ± 0.30	2.33 ± 0.72	2/26 ± 0.1
Number of live fetuses per litter	5.7 ± 0.7	5.4 ± 0.6	5.3 ± 0.5	5.0 ± 0.5	4.3 ± 0.4	4.5 ± 0.5	4.8 ± 0.4	4.7 ± 0.7	4.9 ± 0.2
Average fetal weight per litter	1.06 ± 0.03	1.11 ± 0.02	1.15 ± 0.02	1.09 ± 0.03	1.09 ± 0.02	1.05 ± 0.03	1.00 ± 0.02	1.09 ± 0.03	1.08 ± 0.01
Male/female	47/50	41/39	58/53	33/50	46/35	51/47	51/49	29/26	356/349
Visceral Malformations:									
Number of fetuses examined	53	44	60	44	47	58	56	32	394
Number with visceral malformations	1	3	0	0	0	0	0	1	5
Skeletal and External Malformations:									
Number of fetuses examined	97	81	112	85	82	98	100	56	711
Number with malformations	2	1	4	2	5	0	5	1	20

Values are mean ± standard error of the mean.

Table 8

Effect of 2,4-D, 2,4,5-T and TCDD on Fetal Development
Group IV

	Week of Study								Total 8 weeks
	1	2	3	4	5	6	7	8	
Number of females examined	18	23	20	24	21	21	18	19	164
Maternal weight gain	11.6 ± 0.8	12.9 ± 0.8	11.5 ± 0.7	12.2 ± 0.8	9.9 ± 0.8	9.9 ± 0.8	11.9 ± 0.8	11.9 ± 0.9	11.5 ± 0.3
Number of implants per litter	8.9 ± 0.7	7.3 ± 0.6	7.0 ± 0.6	7.6 ± 0.5	6.0 ± 0.5	6.5 ± 0.5	7.5 ± 0.6	5.9 ± 0.7	7.0 ± 0.2
Number of resorptions per litter	4.00 ± 0.50	1.77 ± 0.29	1.30 ± 0.28	1.88 ± 0.30	1.95 ± 0.37	2.43 ± 0.35	2.39 ± 0.48	1.58 ± 0.30	2.11 ± 0.14
Number of live fetuses per litter	4.9 ± 0.6	5.6 ± 0.6	5.7 ± 0.5	5.8 ± 0.6	4.1 ± 0.5	4.1 ± 0.4	5.1 ± 0.6	4.3 ± 0.7	5.0 ± 0.2
Average fetal weight per litter	1.12 ± 0.04	1.16 ± 0.03	1.09 ± 0.02	1.11 ± 0.02	1.10 ± 0.03	1.07 ± 0.03	1.05 ± 0.03	1.07 ± 0.03	1.10 ± 0.01
Male/female	33/45	59/69	58/54	63/75	45/41	40/46	42/46	36/44	376/420
Visceral Malformations:									
Number of fetuses examined	41	67	62	77	50	50	50	46	443
Number with visceral malformations	0	2	1	1	1	0	0	0	5
Skeletal and External Malformations:									
Number of fetuses examined	78	129	113	139	86	86	92	82	805
Number with malformations	2	5	5	3	2	0	4	4	25

Values are mean ± standard error of the mean.

respectively. Cleft palate and heart or major blood vessel anomalies were observed in all groups in somewhat lower incidences (Table 9). The total percent malformed fetuses (Table 10) ranged from 3.1 to 3.6 percent and the weekly percentage did not show any treatment-related increases in congenital malformations. A comprehensive listing of malformations observed during the study has been included (Appendix Table 1).

Postnatal Litter Examinations

When females were allowed to carry their litters to term, the survival and development of their offspring were studied. The number of live pups and their mean body weight were compared in treated and control offspring (Tables 11-14). The lack of a toxic effect is graphically demonstrated by comparing values for control (Group I) animals to those for the animals exposed to the highest dose of phenoxy acid and TCDD (Group II). There was a marked reduction in the number of litters from day 0 to day 4 in all groups. In group I the number of litters fell from 80 to 44 and in group II from 90 to 60. This was accounted for in all groups by cannabalism by the mothers. After day 4, the loss of litters was greatly decreased. Other parameters show little difference between Groups I and II; on day 0, the number of live pups per litter were 4.40 and 4.19, number of dead pups per litter was 0.92 in both groups, and the average pup weights were 1.37 and 1.39. At day 21 the number of live pups per litter were 5.15 and 4.59 and the average pup weights were 7.48 and 7.60, respectively, for groups I and II. No effect on postnatal viability or growth could be attributed to the exposure of the adult male mice to phenoxy acids or to TCDD.

The pups were examined externally on day 0 for malformations (Tables 15 and 16). During the entire eight weeks the total malformation

TABLE 9

Summary of Most Frequently Occurring Defects in Fetuses Sired by Males Treated with 2,4-D, 2,4,5-T and TCDD

	Treatment Group			
	I	II	III	IV
Anopthalmia/ micropthalmia	1.4(12/830)	1.9(14/744)	2.0(14/711)	2.4(19/805)
Agnathia/micrognathia	1.3(11/830)	1.2(9/744)	1.4(10/711)	1.6(13/805)
Cleft Palate	0.6(5/830)	0.7(5/744)	0.7(5/711)	0.7(6/805)
Heart/Vessels Anomalies	0.2(1/455)	0.5(2/393)	1.0(4/394)	0.7(3/443)

Values are percent incidence of specific anomalies; no. specific malformations per no. observed is given in parentheses.

TABLE 10

Percent Malformed Fetuses Sired by Males Treated with 2,4-D, 2,4,5-T and TCDD

Week	Treatment Group I	II	III	IV
1	1.8(2/113)	1.6(2/129)	3.1(3/97)	2.6(2/78)
2	2.6(3/117)	6.1(6/99)	3.7(3/81)	5.4(7/129)
3	3.1(4/129)	3.5(4/115)	3.6(4/112)	5.3(6/113)
4	3.1(4/129)	0.0(0/78)	2.4(2/85)	2.2(3/139)
5	5.7(4/70)	5.0(5/100)	6.1(5/82)	3.5(3/86)
6	0.0(0/70)	1.3(1/77)	0.0(0/98)	0.0(0/86)
7	3.7(4/108)	8.3(8/96)	5.0(5/100)	4.3(4/92)
8	5.3(5/94)	2.0(1/50)	1.8(1/56)	4.9(4/82)
Total	3.1(26/830)	3.6(27/744)	3.2(23/711)	3.6(29/805)

Values are percent malformed fetuses, number malformed per number observed is given in parentheses.

No values are significantly different from controls ($p<0.05$).

Table 11

Effect of 2,4-D, 2,4,5-T and TCDD on Postnatal Development of Offspring of Treated Males
Group I Control

	Week of Study								Total 8 weeks
	1	2	3	4	5	6	7	8	
Day 0:									
Number of litters	17	15	15	10	5	10	5	3	80
Number live pups per litter	3.94 ± 0.70	4.67 ± 0.80	6.00 ± 0.62	2.60 ± 0.85	4.00 ± 1.10	3.70 ± 0.91	5.40 ± 0.51	5.00 ± 2.51	4.40 ± 0.32
Number dead pups per litter	1.47 ± 0.43	1.33 ± 0.45	0.20 ± 0.20	1.10 ± 0.41	1.00 ± 0.78	0.44 ± 0.18	0.20 ± 0.20	1.33 ± 1.33	0.92 ± 0.16
Average pup weight	1.39 ± 0.03	1.34 ± 0.02	1.37 ± 0.48	1.41 ± 0.02	1.36 ± 0.04	1.38 ± 0.05	1.33 ± 0.03	1.30 ± 0.03	1.37 ± 0.01
Day 4:									
Number of litters	8	5	10	4	4	6	5	2	44
Number live pups per litter	5.00 ± 1.20	6.60 ± 0.93	5.30 ± 0.75	5.50 ± 0.50	4.75 ± 0.75	4.00 ± 0.78	5.40 ± 0.51	7.50 ± 0.50	5.30 ± 0.33
Average pup weight	1.87 ± 0.10	2.22 ± 0.19	2.04 ± 0.15	2.43 ± 0.10	2.71 ± 0.24	2.32 ± 0.28	2.35 ± 0.19	2.25 ± 0.29	2.22 ± 0.07
Day 7:									
Number of litters	8	5	9	4	4	5	5	2	42
Number live pups per litter	4.29 ± 0.99	6.60 ± 0.93	5.78 ± 0.64	5.50 ± 0.50	4.75 ± 0.75	4.00 ± 0.95	5.40 ± 0.51	7.50 ± 0.50	5.32 ± 0.31
Average pup weight	3.13 ± 0.29	3.64 ± 0.33	3.60 ± 0.19	4.07 ± 0.18	4.71 ± 0.39	4.67 ± 0.26	4.03 ± 0.24	3.59 ± 0.35	3.83 ± 0.12
Day 21:									
Number of litters	8	4	9	4	4	5	5	2	41
Number live pups per litter	4.75 ± 0.98	6.00 ± 0.91	5.78 ± 0.64	5.25 ± 0.63	3.50 ± 1.04	4.00 ± 0.95	5.40 ± 0.51	7.50 ± 0.50	5.15 ± 0.32
Average pup weight	6.43 ± 0.51	7.02 ± 0.75	7.34 ± 0.32	8.74 ± 0.21	8.62 ± 0.54	7.71 ± 0.65	8.07 ± 0.51	6.41 ± 0.12	7.48 ± 0.21

Values are mean ± standard error of the mean.

Table 12

Effect of 2,4-D, 2,4,5-T and TCDD on Postnatal Development of Offspring of Treated Males
Group II

	Week of Study								Total
	1	2	3	4	5	6	7	8	8 weeks
Day 0:									
Number of litters	17	16	14	5	16	11	6	5	90
Number live pups per litter	4.76 ± 0.53	4.88 ± 0.68	5.29 ± 0.74	2.80 ± 1.20	3.55 ± 0.59	3.55 ± 0.74	3.67 ± 0.92	4.00 ± 1.41	4.19 ± 0.29
Number dead pups per litter	0.88 ± 0.40	1.06 ± 0.30	0.64 ± 0.27	1.00 ± 0.78	0.79 ± 0.26	1.25 ± 0.45	1.33 ± 0.80	1.00 ± 0.45	0.92 ± 0.14
Average pup weight	1.39 ± 0.03	1.39 ± 0.04	1.33 ± 0.02	1.47 ± 0.05	1.42 ± 0.03	1.42 ± 0.09	1.38 ± 0.07	1.36 ± 0.04	1.39 ± 0.01
Day 4:									
Number of litters	11	11	11	3	14	3	3	4	60
Number live pups per litter	4.63 ± 0.46	5.25 ± 0.56	5.36 ± 0.61	4.67 ± 0.67	4.64 ± 0.58	4.50 ± 0.50	3.67 ± 0.33	4.25 ± 1.44	4.79 ± 0.25
Average pup weight	2.21 ± 0.11	2.30 ± 0.14	2.11 ± 0.06	2.59 ± 0.19	2.47 ± 0.10	2.44 ± 0.18	2.36 ± 0.18	2.47 ± 0.21	2.33 ± 0.05
Day 7:									
Number of litters	11	11	11	3	14	3	3	4	60
Number live pups per litter	4.20 ± 0.44	4.73 ± 0.65	5.36 ± 0.61	4.67 ± 0.67	4.64 ± 0.58	3.67 ± 0.88	3.33 ± 0.68	4.25 ± 1.44	4.58 ± 0.25
Average pup weight	3.61 ± 0.19	3.65 ± 0.25	3.58 ± 0.10	4.12 ± 0.29	4.08 ± 0.22	3.03 ± 0.90	4.19 ± 0.21	4.04 ± 0.24	3.79 ± 0.09
Day 21:									
Number of litters	10	10	10	3	13	3	3	4	56
Number live pups per litter	4.00 ± 0.49	5.00 ± 0.58	5.10 ± 0.64	4.67 ± 0.67	4.92 ± 0.55	3.67 ± 0.88	3.33 ± 0.67	4.25 ± 1.44	4.59 ± 0.25
Average pup weight	7.28 ± 0.48	7.71 ± 0.24	7.20 ± 0.39	8.11 ± 0.45	8.43 ± 0.21	5.30 ± 0.14	8.43 ± 0.11	7.58 ± 0.52	7.60 ± 0.16

Values are mean ± standard error of the mean.

Table 13

Effect of 2,4-D, 2,4,5-T and TCDD on Postnatal Development of Offspring of Treated Males
Group III

	Week of Study								Total 8 weeks
	1	2	3	4	5	6	7	8	
Day 0:									
Number of litters	14	11	12	9	10	11	11	3	81
Number live pups per litter	5.64 ± 0.52	3.91 ± 1.01	5.67 ± 0.85	6.11 ± 1.03	3.80 ± 0.80	2.73 ± 0.75	3.82 ± 0.89	1.67 ± 1.67	4.44 ± 0.32
Number dead pups per litter	0.57 ± 0.34	1.55 ± 0.43	0.27 ± 0.14	0.11 ± 0.11	0.60 ± 0.34	0.50 ± 0.27	0.90 ± 0.41	1.00 ± 0.58	0.67 ± 0.13
Average pup weight	1.32 ± 0.02	1.39 ± 0.06	1.37 ± 0.27	1.36 ± 0.04	1.32 ± 0.06	1.39 ± 0.05	1.37 ± 0.02	1.38	1.36 ± 0.01
Day 4:									
Number of litters	12	3	8	4	8	5	7	1	48
Number live pups per litter	5.30 ± 0.47	4.33 ± 0.67	5.25 ± 0.10	5.25 ± 0.75	4.25 ± 0.49	4.60 ± 0.40	5.57 ± 0.53	5.00	4.91 ± 0.25
Average pup weight	2.03 ± 0.09	1.76 ± 0.89	2.19 ± 0.16	2.25 ± 0.16	2.50 ± 0.10	2.79 ± 0.08	2.73 ± 0.07	2.81	2.37 ± 0.06
Day 7:									
Number of litters	12	3	8	4	8	5	7	1	48
Number live pups per litter	5.00 ± 0.44	4.33 ± 0.67	5.13 ± 1.04	5.25 ± 0.75	4.25 ± 0.49	4.60 ± 0.40	5.57 ± 0.53	5.00	4.92 ± 0.24
Average pup weight	3.25 ± 0.21	2.79 ± 0.17	3.52 ± 0.93	3.67 ± 0.23	4.16 ± 0.13	4.67 ± 0.14	4.54 ± 0.13	4.66	3.82 ± 0.12
Day 21:									
Number of litters	12	3	7	4	8	5	6	1	46
Number live pups per litter	5.00 ± 0.44	4.00 ± 0.58	5.43 ± 0.97	5.00 ± 0.71	4.25 ± 0.49	4.60 ± 0.40	5.50 ± 0.62	4.00	4.87 ± 0.23
Average pup weight	6.83 ± 0.22	7.43 ± 0.64	7.12 ± 0.37	8.39 ± 0.25	8.15 ± 0.18	7.66 ± 0.24	8.59 ± 0.28	10.31	7.67 ± 0.15

Values are mean ± standard error of the mean.

Table 14

Effect of 2,4-D, 2,4,5-T and TCDD on Postnatal Development of Offspring of Treated Males
Group IV

	Week of Study								Total 8 weeks
	1	2	3	4	5	6	7	8	
Day 0:									
Number of litters	19	23	14	11	35	10	11	5	108
Number live pups per litter	4.84 ± 0.64	5.35 ± 0.68	5.29 ± 0.83	3.82 ± 1.06	3.67 ± 0.56	2.60 ± 1.19	4.45 ± 0.71	3.80 ± 1.66	4.41 ± 0.29
Number dead pups per litter	0.95 ± 0.29	0.74 ± 0.20	0.79 ± 0.38	1.09 ± 0.32	0.79 ± 0.28	1.50 ± 0.50	0.27 ± 0.14	0.80 ± 0.49	0.84 ± 0.11
Average pup weight	1.36 ± 0.02	1.36 ± 0.03	1.39 ± 0.03	1.44 ± 0.05	1.39 ± 0.03	1.43 ± 0.05	1.38 ± 0.03	1.43 ± 0.06	1.39 ± 0.01
Day 4:									
Number of litters	10	10	8	4	9	4	8	3	56
Number live pups per litter	5.57 ± 0.43	5.40 ± 0.67	6.00 ± 0.66	6.25 ± 1.03	3.33 ± 0.50	5.75 ± 1.49	4.88 ± 0.55	4.67 ± 0.88	5.13 ± 0.27
Average pup weight	2.24 ± 0.16	2.41 ± 0.08	2.60 ± 0.97	2.30 ± 0.13	2.40 ± 0.19	2.94 ± 0.50	2.54 ± 0.16	2.38 ± 0.28	2.41 ± 0.08
Day 7:									
Number of litters	10	10	8	4	9	4	8	3	56
Number live pups per litter	5.00 ± 0.47	5.40 ± 0.67	6.00 ± 0.66	6.00 ± 1.08	3.33 ± 0.50	5.75 ± 1.49	4.88 ± 0.55	4.67 ± 0.88	5.04 ± 0.26
Average pup weight	3.28 ± 0.31	4.08 ± 0.15	4.06 ± 0.18	3.88 ± 0.24	3.98 ± 0.41	3.80 ± 0.33	4.24 ± 0.25	3.99 ± 0.46	3.94 ± 0.10
Day 21:									
Number of litters	10	10	8	4	9	4	8	3	56
Number live pups per litter	5.00 ± 0.47	5.30 ± 0.63	5.75 ± 0.59	6.00 ± 1.08	3.33 ± 0.50	5.75 ± 1.49	4.75 ± 0.59	4.67 ± 0.88	4.96 ± 0.25
Average pup weight	6.99 ± 0.34	8.15 ± 0.33	7.53 ± 0.40	8.20 ± 0.09	8.53 ± 0.68	6.66 ± 0.74	8.34 ± 0.37	7.38 ± 0.97	7.80 ± 0.18

Values are mean ± standard error of the mean.

TABLE 15

Summary of Malformation Rates (%) in Postnatal Study[1]

Week	Treatment Group I	II	III	IV
1	5.4(5/92)	2.1(2/96)	1.1(1/87)	1.9(2/105)
2	2.2(2/90)	3.2(3/95)	5.0(3/60)	0.0(0/140)
3	1.1(1/93)	6.0(5/83)	6.6(5/76)	2.4(2/85)
4	0.0(0/37)	5.3(1/19)	8.9(5/56)	13.0(7/54)
5	4.0(1/25)	1.1(1/88)	4.5(2/44)	2.7(2/73)
6	2.2(1/45)	0.0(0/32)	2.1(1/48)	2.1(1/47)
7	7.1(2/28)	13.3(4/30)	3.8(2/53)	3.8(2/52)
8	5.3(1/19)	8.0(2/25)	0.0(0/8)	4.3(1/23)
Total	3.0(13/429)	3.8(18/468)	4.4(19/432)	2.9(17/579)

[1]All fetuses (live or dead) were assumed to be at risk.

TABLE 16

Summary of Specific Malformations in Postnatal Study

	I	II	III	IV
Anopthalmia/ microphthalmia	1.9(8/429)	2.6(12/468)	2.5(11/432)	1.7(10/579)
Agnathia/micrognathia	1.4(6/429)	1.3(6/468)	1.9(8/432)	1.4(8/579)
Cleft Lip/palate	0.2(1/429)	0.4(2/468)	0.0(0/432)	0.0(0/579)
All else	0.0(0/429)	0.0(0/468)	0.5(2/432)[a]	0.0(0/579)

[a]One exencephaly; one clubbed right hind limb.

rate in these mice was between 2.9 and 4.4 percent. The only individual values which approach statistical significance are the malformation rates for week four group IV (40 mg/kg/day phenoxy acid, 1.2 μg/kg/day TCDD) (Table 15) versus control. In that case the control animals had no malformations and the treated had 13% malformations; all of these were either eye or jaw anomalies.

As seen in the prenatal evaluations, eye and jaw malformations accounted for the majority of the defects noted (Table 16). Anopthalmia or micropthalmia were seen in 1.7 to 2.6 percent of the pups and agnathia or micrognathia were seen in 1.3 to 1.9 percent of the pups for all treatment groups.

DISCUSSION

In these studies we have evaluated the toxicity of three mixtures of 2,4-D, 2,4,5-T and TCDD on reproduction in male mice. Mating frequency, fertility, germ cell mutagenesis, and fetal or postnatal development were all considered in the selection of toxicological endpoints. Male mice were continuously exposed to high doses of 2,4-D plus 2,4,5-T mixed with 2 or 30 ppm TCDD in the phenoxy acid for 8 weeks. These TCDD levels represent average and high contamination levels within Herbicide Orange used in Vietnam (Young et al., 1978).

This study employed the free acids of 2,4-D and 2,4,5-T rather than the butyl esters which were components of Herbicide Orange because of the lower volatility of the acids. The esters are rapidly metabolized to the free acid in both plants and animals, and therefore, the systemic toxicity can be attributed to the free acid (Gehring and Betso, 1978) and should be comparable on a molar basis. The dose levels employed exhibited moderate to low direct toxicity in exposed male mice as evidenced by decreased body weight gain, changes in thymus and liver weights and morphologic changes in the liver. The mortality, however, was quite low. The severity of these toxic effects appeared to be primarily related to the TCDD content of the simulated "Herbicide Orange" mixture.

Despite the use of continuous, moderately toxic, chemical exposures throughout the complete period of spermatogenesis, no significant increase in reproductive abnormalities in the 2,4-D, 2,4,5-T or TCDD exposed groups were observed. TCDD has previously been demonstrated to alter spermatogenesis in C57BL/6 mice (McConnell et al., 1978), however, that study used a single high (lethal range) dose of TCDD. Additionally,

testicular lesions were only found in clinically ill animals, as opposed to those which survived exposure to similar doses (McConnell et al., 1978). Altered spermatogenesis has also been reported in rats (Kociba et al., 1976), guinea pigs (McConnell et al., 1978), and monkeys and chickens (Norback and Allen, 1973), although these were again toxic exposures. In the present study, morphological changes were not observed in the testis of treated mice. Throughout this study testis weight was not affected, nor was sperm motility or percent abnormal spermatozoa. Mean sperm concentration, however, was slightly reduced after five and eight weeks of dosing, although the effect was not statistically significant ($p>0.10$).

The levels of TCDD chosen for this study were within the range that had already been shown to result in cleft palate and kidney anomalies when given to pregnant C57BL/6 female mice (Moore et al., 1973). Mixtures of phenoxy acids plus specific levels of TCDD were used in order to better mimic human exposures to Herbicide Orange. Additionally, previous investigators (Neubert et al., 1973) showed that adding as little as 0.1 μg/kg TCDD to 2,4,5-T increased the teratogenicity in mice of 2,4,5-T in offspring of exposed mothers above that expected by a simple additive effect. The dose of TCDD in this study is at or above the 0.1 μg/kg/day level. Also, it should be emphasized that human exposures involved mixtures of 2,4-D, 2,4,5-T and TCDD (Herbicide Orange).

Certain chemicals, when given to adult males, can cause fetal death or alter normal development in offspring sired by those males (Joffe, 1979; Manson and Simons, 1980), however, such effects were not elicited in the experiments we report by exposing male mice to the 2,4-D, 2,4,5-T and TCDD mixtures. As evidenced by the numbers of implants and resorptions, neither embryo toxicity nor dominant lethal mutations could be

attributed to exposure to these chemicals. The unaffected values for percent abnormal sperm, which is a test for mutagenicity (Wyrobek, 1979) also leads one to the conclusion that the chemicals, as given, were not mutagenic towards the male germ cells. This correlates well with previous multigeneration studies (Murray et al., 1979) and dominant lethal assays (Khera and Ruddick, 1971).

The values for percent malformed fetuses also indicated that 2,4-D, 2,4,5-T and TCDD had no influence on the offspring of exposed males. The study was designed such that, if the overall percent malformed fetuses (3%; see Appendix Table 1) was doubled in one of the experimental groups, there was a 90% chance that it would have been detected. There was a 70-80% chance of detecting a four-fold increase in congenital defects in any one week. For any specific malformation or class of malformations the corresponding powers would be somewhat less. For example, for visceral defects (background rate 0.44%; see Table 5) the experiment had approximately a 90% chance of detecting an overall rate as high as 3% in any particular treatment group. The only variation which we could find elevated in treated versus control offspring, in the entire study, was the incidence of fused sternebrae (Appendix Table 2). In that case, we observed a statistically significant ($p<0.05$) increase in fused sternebrae in Group III at week 3 and in Group IV at week 4. The incidence in the controls at those times, however, was unusually low (0 in both weeks 3 and 4). This type of skeletal variation has been described as occurring as often as 5-15% or more in offspring from untreated pregnancies in mice and although it is frequently observed, the incidence is quite variable and this anomaly is considered a variation and not a malformation (Wilson, 1973).

APPENDIX TABLE 1

Summary of All Malformations Observed in Fetuses Sired by Males Treated with 2,4-D, 2,4,5-T and TCDD

	Treatment Group			
	I	II	III	IV
Visceral Malformations[1]				
Heart/Vessels Anomalies	0.2(1/455)	0.5(2/393)	1.0(4/394)	0.7(3/443)
Kidney Agenesis	0.2(1/455)	0.0(0/393)	0.3(1/394)	0.0(0/443)
Liver-two lobes only	0.0(0/455)	0.0(0/393)	0.0(0/394)	0.2(1/443)
Lung-lobes 2/3 normal size	0.2(1/455)	0.0(0/393)	0.0(0/394)	0.0(0/443)
Right kidney-1/2 normal size	0.0(0/455)	0.0(0/393)	0.0(0/394)	0.2(1/443)
Skeletal and External Malformations[1]				
Anopthalmia/micropthalmia	1.4(12/830)	1.9(14/744)	2.0(14/711)	2.4(19/805)
Agnathia/micrognathia	1.3(11/830)	1.2(9/744)	1.4(10/711)	1.6(13/805)
Cleft palate	0.6(5/830)	0.7(5/744)	0.7(5/711)	0.7(6/805)
Cleft lip/nose	0.0(0/830)	0.0(0/744)	0.3(2/711)	0.0(0/805)
Open eye	0.5(4/830)	0.0(0/744)	0.0(0/711)	0.0(0/805)
Exencephaly/hydrocephaly	0.2(2/830)	0.4(3/744)	0.1(1/711)	0.2(2/805)
No tongue	0.0(0/830)	0.0(0/744)	0.1(1/711)	0.0(0/805)
Umbilical hernia	0.1(1/830)	0.3(2/744)	0.3(2/711)	0.1(1/805)
Ribs fused/missing	0.1(1/830)	0.3(2/744)	0.0(0/711)	0.0(0/805)
Spinal centra doubled/misaligned	0.1(1/830)	0.0(0/744)	0.1(1/711)	0.0(0/805)
Spinal arches fused	0.0(0/830)	0.0(0/744)	0.1(1/711)	0.0(0/805)
Mandibles fused	0.0(0/830)	0.0(0/744)	0.1(1/711)	0.0(0/805)
Skull bones missing	0.0(0/830)	0.1(1/744)	0.1(1/711)	0.0(0/805)
Eye bones missing	0.0(0/830)	0.1(1/744)	0.0(0/711)	0.0(0/805)
Facial bones fused	0.2(2/830)	0.5(4/744)	0.4(3/711)	0.0(0/805)
Kinked Tail	0.1(1/830)	0.0(0/744)	0.0(0/711)	0.0(0/805)
Total Malformations[2]	5.2(43/830)	5.8(43/744)	6.6(47/711)	5.7(46/805)
Total Malformed Fetuses[2]	3.1(26/830)	3.6(27/744)	3.2(23/711)	3.6(29/805)

[1] Values are percent incidence of specific anomalies; no. specific malformations per no. observations is given in parentheses.

[2] Values are percent incidence of malformations or malformed fetuses; no. malformations or malformed fetuses per no. observations is given in parentheses.

APPENDIX TABLE 2

Incidence of Fused Sternabrae in Fetuses Sired by Males Treated with 2,4-D, 2,4,5-T and TCDD

Week	Treatment Group			
	I	II	III	IV
1	0.9(1/113)	1.6(2/129)	1.0(1/97)	1.3(1/78)
2	1.7(2/117)	3.0(3/99)	3.7(3/81)	1.6(2/129)
3	0.0(0/129)	0.9(1/115)	7.1(8/112)*	2.7(3/113)
4	0.0(0/129)	2.6(2/78)	2.4(2/85)	5.0(7/139)*
5	1.4(1/70)	2.0(2/100)	3.7(3/82)	2.3(2/86)
6	1.4(1/70)	5.2(4/77)	3.1(3/98)	3.5(3/86)
7	3.7(4/108)	2.1(2/96)	0.0(0/100)	2.2(2/92)
8	2.1(2/94)	2.0(1/50)	0.0(0/56)	1.2(1/82)
Total	1.3(11/830)	2.3(17/744)	2.8(20/711)	2.6(21/805)

*$p<.05$ vs. controls.

Certain anomalies are associated with embryotoxicity, not teratogenicity or mutagenicity. However, with chemical exposure only to the males, not pregnant females, embryotoxicity would not be expected in their offspring. If exposure of the embryo directly to the chemicals had occurred, via seminal plasma or sperm, one would expect to observe increased embryotoxicity in the first weeks of the study, when the chemical levels in the body (or ejaculate) were the highest. One would have also anticipated that during the first weeks of the teratology study germ cell toxicity would most likely have been detected, because the spermatozoa that were evaluated in the first week of mating had been exposed to the chemicals throughout all stages of the spermatogenic process. If the spermatogonia had been affected by the exposure, the effect would have been most apparent in the last weeks of the mating, because at that time, we were evaluating spermatozoa which were spermatogonia during the entire 8 week dosing period and only began to proceed through the spermatogenic cycle near the end of chemical exposure.

Thus, there does not appear to be a residual or transient effect of 2,4-D, 2,4,5-T and TCDD at the concentrations in this study, on the fertility of exposed male mice. In addition, exposure to these chemicals did not appear to influence the fetal or neonatal development or the viability of offspring sired by these mice.

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