Abstract

Brominated naphthalenes have been identified as toxic contaminants of the polybrominated biphenyl mixture Firemaster, which was found to be responsible for the 1973 Michigan livestock feed contamination incident. Extensive adult and embryo/fetal toxicity was associated with consumption of the contaminated feed. In order to characterize the embryotoxic and teratogenic properties of hexabrominated naphthalenes (HBNs), the most prevalent of the brominated naphthalenes in Firemaster, pregnant C57Bl/6N mice were treated on gestation days 6-15 with 0, 0.5, 1.0, 2.5, 5.0, 7.5 and 10.0 mg HBN/kg body weight/day (p.o.) and sacrificed on gestation day 18. Maternal and fetal toxicity were characterized and a complete teratological evaluation was performed. Dose-related effects on maternal body and thymus weight, and liver to body weight ratios were seen. Dose-related increases were observed for fetal subcutaneous edema, involution of lymphatic organs, delayed cranial ossification and fetal mortality. A steep dose response curve was shown for cleft palate, with 4.8% and 98.6% of the fetuses per litter affected at 1.0 and 2.5 mg/kg, respectively. Kidney lesions, best described as apparent hydronephrosis, were an even more sensitive indicator of fetal toxicity with 100% of the fetuses having bilateral dilated renal pelves at 1.0 mg/kg and 90% having at least a unilateral dilated renal pelvis at 0.5 mg/kg.

A pilot teratology study conducted by dosing animals between gestation days 10-13 with 0, 10, 100 or 1000 mg HBN/kg body

weight/day revealed that 100% of the fetuses had cleft palate at each of the HBN treatment levels.

Results of single and multiple (10 day) dosing toxicity studies with adult, female C57Bl/6N mice suggest that HBN is much more toxic when administered in multiple smaller doses than as a single large dose. Singly-dosed animals exposed at levels as high as 1000 mg/kg failed to show signs of toxic stress during the course of a 35-day follow-up, while animals that received multiple smaller doses of as low as 5.0 mg/kg/day exhibited overt signs of toxicity (wasting, lethargy and bleeding) at 7 days of dosing. Dose-related hepatic and thymic effects were also seen in multiply-dosed but not singly-dosed animals.

Thus, HBN is a potent toxic, teratogenic and fetotoxic agent that produces a spectrum of teratogenic and toxic lesions that is similar to TCDD and other structurally-related halogenated aromatic hydrocarbons.

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Table of Contents

Introduction

Polybrominated biphenyls (PBBs) are halogenated aromatic hydrocarbons that were originally synthesized for uses as flame retardants in thermoplastics, coatings and lacquers and polyurethane foam (Brinkman and deKok, 1980). Prior to 1973, the biological activity of PBBs had not been extensively studied due to minimal potential human exposures to these compounds and relatively low production levels. PBBs contributed to less than 1% of the total fire retardant market sales during 1970-1974, their period of greatest use and production (Brinkman and deKok, 1980). However, in May of 1974, it was determined that a commercial mixture of PBBs known as Firemaster had been inadvertently incorporated into livestock feed at a feed mill near Battle Creek, Michigan, where it was apparently mistakenly substituted for a magnesium oxide feed supplement (Kay, 1977; Carter, 1976). Subsequent distribution and consumption of the contaminated feed over a period of at least 9 months led to extensive livestock toxicity and mortality throughout the state. Eventually, about 37,000 livestock (29,800 cattle, 5,920 hogs and 1,470 sheep) and 1,600,000 poultry became contaminated at levels necessitating their destruction (Dunckel, 1975). Additionally, to prevent further primary and secondary contamination, 788 tons of animal feed, 3,000 lb of butter, 18,000 lb of cheese, 34,000 lb of dry milk products and approximately 5,000,000 eggs were disposed of by burial (Dunckel, 1975).

It has been estimated that hundreds of thousands, if not millions, of people were exposed to these PBBs by ingestion of contaminated meat and dairy products (DiCarlo, et al, 1978). The nature and magnitude of human health effects due to PBB exposure are to this date unclear.

Firemaster was produced and marketed in two forms. The major components of both forms were: hexabrominated biphenyls (60-70%), heptabrominated biphenyls (approximately 30%) and pentabrominated biphenyls (approximately 4%). Firemaster FF-1 was in a pulverized form and contained 2% calcium polysilicate as an anticaking additive. It was produced only in limited quantities between 1971 and 1972. Firemaster BP-6 was not pulverized and contained no anticaking additives. It was produced from 1970 until late 1974, when it was removed from the market following the disclosure that Firemaster FF-1 had been involved in the feed contamination incident. In addition to the major components of Firemaster, at least another 23 compounds have been stated to be present as contaminants (Brinkman and deKok, 1980).

In 1978, it was determined that the brominated naphthalenes comprise the largest class of trace contaminants present in Firemaster (Hass, et al, 1978; O'Keefe, 1979). It has been estimated that the bromonaphthalenes are present at approximately 200 ppm, of which 150 ppm are hexabrominated naphthalenes (HBNs). The remainder is penta- and tetrabrominated isomers (Birnbaum, et al, 1982; Tondeur, et al, 1984). Numerous studies have investigated the toxicity of PBB mixtures (NTP Technical Report No. 244, 1983; Corbett, et al, 1975; Gupta and Moore, 1979;

Gupta, et al, 1981) and individual PBB isomers (Render, et al, 1982; Bairstow, et al, 1978) but only a few have examined the toxicity of the bromonaphthalenes.

There are several lines of reasoning that suggest that some of the toxicity of the Firemaster mixture may be due to the presence of the brominated naphthalenes. A potentially analogous situation exists for some commercial polychlorinated biphenyl mixtures. The toxicity of these PCB mixtures has been correlated with the levels of PCDF contaminants (Vos, et al, 1970). Firemaster has been shown to contain no bromodibenzofurans or bromodibenzodioxins as contaminants (Hass, et al, 1978). Yet it is almost as toxic as some of the Aroclor mixtures (Kimbrough, 1981). One possible explanation is that some of the toxicity of the PBB mixture might be due to the presence of certain toxic contaminants. If this is the case, then the brominated naphthalenes should be considered as potential contributors to Firemaster toxicity.

The polybrominated naphthalenes (PBNs) are structurally-related to the polyhalogenated biphenyls, dibenzofurans and dibenzodioxins. Consequently, exposure to PBNs might be expected to produce a spectrum of biochemical alterations and toxicological lesions similar to these other toxic halogenated aromatic hydrocarbons. In a limited number of studies, this has been shown to be the case. PBN toxicosis in guinea pigs has been characterized by an almost immediate, dose-related decrease in body weight gain or actual weight loss, a decrease in food

consumption, listlessness, marked thymic involution, adrenal cortical hemorrhage, testicular atrophy and hyperplasia of the transitional epithelium lining the urinary tract from the renal pelvis to the urinary bladder (McKinney, et al, 1982). Additionally, HBN has been shown to be chloracneigenic in humans (Kaufman and Djerassi, 1979) and can cause centrolobular hepatic accumulation of lipid in rats (Kohli, et al, 1981). Many of these same lesions are associated with exposure to other toxic halogenated aromatic hydrocarbons (McKinney and McConnell, 1982).

It is of interest to evaluate the teratogenicity and embryotoxicity of HBN because fetal wastage was among the symptoms of the toxic syndrome seen in cattle shortly after the feed contamination incident is hypothesized to have begun (September 1973). Cattle that consumed the contaminated feed exhibited hyperkeratosis of the skin, lameness, increased frequency of urination and lacrimation, marked weight loss, decreased milk production and decreased feed consumption. Some of the animals that lost weight did so irreversibly and subsequently died. Animals in the early stages of pregnancy returned to estrus (embryonic resorption was suspected but not confirmed), and some of those in the later stages gave birth to stillborn calves. Complications of pregnancy were common (in particular, dystocia due to failure of the pelvic ligaments to relax, metritis, and retained placenta) and calves that were alive at birth often were large and sometimes died shortly after parturition (Jackson and Halbert, 1974).

PBBs however, have variously been reported to be teratogenic and embryotoxic (Beaudoin, 1977; Corbett, et al, 1975), nonteratogenic (Fiscor and Wertz, 1976; Preache, et al, 1976) and nonembryotoxic (Aftosmis, et al, 1972; Harris, et al, 1978) depending upon the dose level and test species examined.

Additionally, hyperkeratosis of the skin in farm animals had previously been associated with exposure to chlorinated naphthalenes (Brinkman and Reymer, 1976; Kover, 1975) and not with exposure to other halogenated aromatics (Kay, 1977). This also raises the possibility that some of the toxicity seen in livestock following consumption of the contaminated feed was due to the presence of brominated naphthalenes.

Results of microsomal enzyme induction studies with various constituents of Firemaster also suggest that the toxicity of this PBB mixture may be in part due to some of the minor components.

There are two major classes of inducible, microsomal drug-metabolizing enzymes, those that are induced by exposure to 3-methylcholanthrene (3-MC) and those that are induced by exposure to phenobarbital (PB). For several classes of structurally-related halogenated aromatic hydrocarbons, there is a strong correlation between the toxicity of an individual congener and its ability to induce the microsomal enzymes that are associated with exposure to 3-MC (Poland and Knutson, 1982; Parkinson and Safe, 1981). These enzymes include the cytochrome P-448-dependent mono-oxygenase, benzo(a)pyrene hydroxylase (Poland and Knutson, 1982). Exposure to the less toxic and non-toxic congeners is associated either with enzyme induction

characteristics that are typical of PB exposure, for example the induction of the cytochrome P-450-dependent mono-oxygenase, aminopyrine N-demethylase (Goldstein, 1979), or with a lack of enzyme-inducing ability (Goldstein, et al, 1977). The toxic congeners may induce both types of enzyme activities, with the magnitude of the P-448 component being correlated with toxicity.

The Firemaster mixture is capable of producing mixed microsomal enzyme induction (i.e. both P-448 and P-450 induction) (Dent, et al, 1976; Render, et al, 1982; Robertson, et al, 1982). Greater than 80 percent of the mixture is known to be associated with purely P-450 induction while less than 20 percent is associated with mixed induction (Render, et al, 1982; Robertson, et al, 1981). The total amount of the P-448 induction by Firemaster is not accounted for by the contributions of the known PBB congeners in the 3-MC-type inducing component. It is possible that since synthetic mixtures of polybrominated naphthalenes (Robertson, et al, 1984) and a tetrabromonaphthalene (Goldstein, 1979) have been shown to be inducers of P-448, that the brominated naphthalene component of Firemaster may contribute to the cytochrome P-448 induction capabilities of the mixture. It would follow then, that brominated naphthalenes may contribute to the overall toxicity of Firemaster.

A complicating factor to be considered is the fact that disposition studies in rats have revealed that the synthetic HBN used in the guinea pig toxicity studies and shown to produce fatty livers in rodents is actually a mixture of two closely-related isomers: 1,2,3,4,6,7-HBN and 2,3,4,5,6,7,-HBN (Figure 1)

present in approximately a 65:35 ratio (Birnbaum, et al, 1983). These two isomers have very different pharmacokinetic and toxic properties. The major isomer is readily metabolized, is excreted in the feces and is much more toxic than the persistent, minor isomer (Birnbaum, et al, 1983; Birnbaum and McKinney, 1985). There is reason to believe that a similar isomeric ratio might exist in Firemaster, but this has not been experimentally verified.

This is the first study to characterize the teratogenic and fetotoxic potential of brominated naphthalenes. The C57Bl/6N mouse teratology system has been used extensively to examine the toxicity of other structurally-related halogenated aromatic hydrocarbons (Birnbaum, et al, 1985; Weber, et al, 1984; Neubert, et al, 1973; Courtney and Moore, 1971). Information generated from this study can serve as the basis for establishing the toxic potency of HBN relative to other halogenated aromatics.

This study was not intended to produce any quantitative statements about the relative role played by HBN in the overall cumulative toxicity of Firemaster. It has been suggested that there may be potentiating or synergistic interactions between brominated naphthalenes and non-planar brominated aromatic hydrocarbons present in Firemaster (McKinney, et al, 1982; McKinney, et al, 1982a). It has also been suggested that these structurally-related halogenated aromatics express their toxicities in an additive manner (Birnbaum, unpublished observations). Reconstruction experiments with various components of Firemaster would be expected to clarify this issue.

This study does not address this point but rather serves to answer several fundamental questions regarding the toxic properties of HBN. Specifically:

(1) How toxic is HBN to adult female C57Bl/6N mice when administered as a single dose or as repetitive, multiple doses?

(2) If toxicity is observed in either of these dosing regimens, how is it expressed in terms of overt symptomology and gross pathology? (i.e. Is there any similarity to the toxic syndrome seen previously following exposure to other toxic, structurally-related halogenated aromatics (ex: PBBs, PCBs, TCDD)?

(3) How potent, relative to other toxic halogenated aromatic hydrocarbons, is HBN in eliciting fetotoxic and teratogenic effects in the offspring of C57Bl/6N mice?

(4) Is the spectrum of teratogenic effects and developmental anomalies induced by maternal exposure to HBN similar to that which has been documented for maternal exposure to other toxic halogenated aromatics?

The first two questions serve as a basis from which the second two questions can be addressed. As will be referred to later, if teratogenicity is to be properly demonstrated, it must be done at dose levels lower than that which produce signs of maternal toxicity.

The answers to all of these questions and their implications are presented herein.

Experimental Rationale

The aim of this study was to characterize the teratogenic and fetotoxic properties of HBN. It was first necessary to define appropriate dose levels that could be used in the complete teratogenicity study. To demonstrate teratogenicity, dose levels should be selected that do not produce any signs of maternal toxicity. In this manner, for compounds that cross the placenta, the observed fetal toxicity or teratogenicity can be attributed to the toxic agent itself and not to potential secondary effects that may be mediated through maternal toxicity.

Generally, doses required to produce embryotoxicity and teratogenicity are lower than those required to produce maternal toxicity (Manson, et al, 1982), but there are no standard ratios for these values. Obviously, at lower exposure levels, there may still be some subtle alterations of maternal metabolism that could somehow influence embryonic and fetal development, but by judiciously selecting a range of dose levels, one should be able to cover exposure levels that will elicit no maternal or fetal effects, at the low end, and embryolethality or perhaps measurable maternal toxicity, at the high end. The exposure levels of teratological interest then, are those in the intermediate range above any no-effect level (if any exist) and below the embryolethal/maternally toxic doses.

Knowledge of the acute oral toxicity of a compound is often used as a starting point in the process of dose selection for many types of toxicity studies including evaluation of teratogenicity. For HBN, however, there was no published information regarding any aspect of toxicity in mice. Consequently, our first preliminary study was directed towards evaluating acute oral toxicity in the test species that would eventually be used to investigate HBN teratogenicity. All of the studies to be described were conducted using C57Bl/6N mice. This species has been used in ongoing studies with TCDD and structurally-related compounds and is the prototypical "responsive" strain for many of the biological effects associated with exposures to this class of chemicals (Poland and Glover, 1980). As such, it serves as a good model system to characterize the potential teratogenic effects of HBN.

To establish appropriate dose levels to evaluate HBN acute toxicity in mice, several qualitative and quantitative comparisons were made between some known and estimated values of various toxic endpoints for HBN and another toxic halogenated aromatic hydrocarbon 2,3,7,8-tetrachlorodibenzofuran (TCDF). The toxicity of TCDF had been evaluated in the male Hartley guinea pig lethality system (McKinney and McConnell, 1982) (single dose LD50 $\approx$ 7 ug/kg) and in the C57Bl/6N mouse teratology system (with dosing on gestation days 10-13, ED50 for cleft palate induction was approximately 50-100 ug/kg) (Weber, et al, 1984). An LD50 at 30 days for TCDF in C57Bl/6N mice had been estimated at between

10 and 100 mg/kg (Birnbaum, personal communication). As an approximation, TCDF is 1,000 to 10,000 times more toxic to guinea pigs than to mice, and the ED50 for cleft palate induction in mice (dosing on GD10-13) was about 10 times greater than the single dose LD50 value in guinea pigs.

HBN toxicity had previously only been evaluated in the guinea pig lethality model (LD50 ≈ 460 ug/kg) and was found to be between 10 and 100 times less toxic than TCDF (McKinney and McConnell, 1982). If a similar ratio of relative toxicities exists for HBN as for TCDF, it would be expected that HBN would have an acute, oral LD50 of greater than 500 mg/kg in mice and an ED50 for cleft palate induction in mice (dosing on GD10-13) of between 5 and 10 mg/kg. The dose levels used in the acute toxicity evaluation were based upon this analogy but were limited by maximum solubility.

A pilot teratology study was performed as the next preliminary experiment. This involved dosing the pregnant dams on gestation days 10-13, the period of greatest sensitivity for cleft palate induction by TCDD (Neubert, et al, 1973). Cleft palate is a highly sensitive, easily detectable and quantifiable indicator of exposure to toxic halogenated aromatics. From this type of study one can make quantitative comparisons with other toxins based on cleft palate incidence. Exposure levels for the teratology pilot were based upon the TCDF analogy.

Results from these two preliminary experiments were used to determine the appropriate dose levels for the definitive teratology study. As will be referred to later, the exposure levels

ultimately chosen were in the correct range to evaluate the spectrum of teratogenic lesions and developmental anomalies induced by HBN exposure.

When planning the complete teratology study, which was to involve dosing on gestation days 6 through 15, it was realized that some of the females that were "plug positive" would turn out to be nonpregnant. Weight gain on gestation day 6 is a fairly unreliable indicator of pregnancy due to the relatively small amount of growth that has taken place in utero by this time. It was decided to continue dosing and monitoring the body weights of the nonpregnant animals, and at sacrifice to obtain selected organ to body weight ratios. This was done to make optimal use of the experimental animals and to obtain information regarding the toxic effects of repeated dosing of HBN in nonpregnant C57Bl/6N mice of comparable age and nutritional status to those in the concurrent teratology study.

Materials and Methods

For all of the experiments to be described, 6 week-old, virgin female C57Bl/6N mice, weighing about 20 g, were obtained from Charles River Breeding Laboratories, Portage, MI, and were allowed to acclimate in the NIEHS animal facility for at least 2 weeks prior to being put on study. The temperature, relative humidity and photocycle were maintained at approximately 22°C, 50% and 12 h light/12 h dark, repectively. Food (NIH Open Formula No. 31) and glass distilled water were provided ad libitum.

For the acute single dose toxicity study, the acclimated, 8 week-old female mice were housed 5 per cage and received a single dose of HBN in corn oil by gavage (10 ml/kg). The dose levels administered were 0, 3, 10, 30, 100, 300 and 1000 mg HBN/kg body weight. Animals were weighed every Monday, Wednesday and Friday for 5 weeks postexposure, and were examined for overt signs of toxicity twice daily (once daily on weekends). The mice were sacrificed 36 days after dosing. Body, liver and thymus weights were measured at necropsy.

For the definitive teratology study, the mice were cohabited overnight with proven adult male C57Bl/6N mice (2 females per 1 male). The following morning, the females were checked for the presence of a vaginal plug as evidence of mating. Detection of a vaginal plug indicated day 0 of gestation. Plug-positive females were removed from cohabitation with the males, weighed, uniquely identified with a numbered metal eartag, and housed 3 animals per cage.

Weight gain on gestation day 6 was used as the criteria for determining the likelihood of pregnancy. Likely pregnant animals were randomly assigned to treatment groups and received 10 daily doses of 0, 0.5, 1.0, 2.5, 5.0, 7.5 or 10.0 mg HBN/kg body weight, by gavage in corn oil (10 ml/kg) on gestation days 6 through 15. Dams were weighed daily and checked twice daily for signs of overt toxicity.

On day 18 of gestation, the dams were weighed and sacrificed by decapitation. Fetuses were removed by caesarean section, examined for gross abnormalities, weighed individually and randomly assigned for either skeletal analysis by Alizarin Red staining (Dawson, 1926) or soft tissue analysis by fixation in Bouin's Solution (Wilson, 1965). Maternal liver and thymus weights and fetal mortality were also recorded.

For the evaluation of multiple dose toxicity, the females that were ultimately determined to be nonpregnant, but were included in the dosing schedule for the teratology study were monitored for signs of toxicity. Body weights were obtained daily and animals were checked twice daily for overt signs of toxicity. Surviving animals were sacrificed on the thirteenth day after exposure had begun (i.e. 3 days after the last dose had been received). At necropsy, body, liver and thymus weights were determined and observations of gross liver pathology were made. The nonpregnant status of these animals was confirmed by uterine examination (compression of the uterus between 2 acrylic plates to visualize potential implantation sites). Animals that did not survive the duration of the study were removed from the cages

and necropsied as rapidly as possible to minimize cannibalism and the potential effects due to tissue autolysis. Due to the variable time lapses between death and necropsy for these animals, their body and organ weights were obtained but were not included in any of the data analysis.

The pilot teratology study was performed under the same experimental conditions as the definitive teratology study with the following exceptions: animals were dosed with 0, 10, 100 and 1000 mg HBN/kg body weight/day on gestation days 10 through 13; the only teratogenic endpoint evaluated was cleft palate; and no maternal thymus weights were obtained at necropsy.

The HBN used in these studies was synthesized in the Laboratory of Environmental Chemistry at NIEHS by the direct bromination of naphthalene catalyzed by iron powder in refluxing methylene bromide (McKinney, et al, 1981). This method has been recently shown to produce a mixture of two closely-related isomers: 1,2,3,4,6,7-HBN and 2,3,4,5,6,7-HBN in approximately a 65:35 ratio (Birnbaum, et al, 1983). High field proton NMR was employed to determine the isomeric ratio and GC-MS was used to verify the composition of brominated naphthalene.

For the definitive teratology study, Kruskal Wallis analysis of variance procedures were employed to test the statistical significance of the dose-related changes (Siegel, 1956). Multiple pairwise comparisons among treatment means were made by the Wilcoxon Two-Sample Test (Noether, 1967). Dixon's Outlier's Test (Dixon and Massey, 1969) was used to show that a single control litter was an outlier at the 0.01 levels of significance.

Body weight data for multiple dose toxicity were analyzed by William's multiple comparison procedure (Williams, 1972) and Koziol's nonparametric method for analysis of growth curves (Koziol, et al, 1981).

Statistical significance of organ to body weight data, liver pathology scores and the pilot teratology data was evaluated with Student's T-test (Hoel, 1971).

Results and Discussion

Single Dose Toxicity Study

The results of the single-dose (p.o.) acute toxicity study suggest that HBN has a low acute toxicity in adult female C57Bl/6N mice. Animals that received a single dose as high as 1000 mg/kg failed to show any signs of toxic stress during the course of a 36-day follow-up. There was a slight suppression of weight gain in animals that received 1000 mg/kg but this was not statistically significant (Figure 2). The liver to body weight ratios determined at necropsy (Table 1) were significantly elevated over control levels at all dose levels exceeding 10 mg/kg ($p < 0.01$). Thymus to body weight ratios did not show any significant dose-related changes, although at the highest exposure levels (100, 300 and 1000 mg/kg) values for this parameter were depressed relative to controls (Table 1).

For comparative purposes, it would have been useful to generate an LD_{50-30} value. However, since the acute toxicity of HBN was too low to cause any lethality, this was not possible. One of the objectives of evaluating acute toxicity was to be able to better plan subsequent experiments. It was decided to use this same exposure range (0, 10, 100 and 1000 mg/kg) in the next preliminary experiment, the pilot teratology study. No toxicity was evident when animals had been dosed singly at levels as high as 1000 mg/kg, but it was considered possible that we would encounter toxicity when animals received four daily doses of as high as 1000 mg/kg/day. Some of these structurally-related

compounds are known to be more toxic when administered in multiple, smaller doses than as a single, large dose (Gupta and Moore, 1979). The range of exposure levels selected for the pilot teratology study would be able to detect maternal toxicity regardless of how much more toxic HBN was when administered in four multiple doses and would also be able to detect the anticipated ED_{50} for cleft palate induction (between 5 and 10 mg/kg, based on the TCDF analogy).

Teratology Pilot Study

Table 2 is a summary of experimental results for the teratology pilot study. There were no signs of overt maternal toxicity at any dose level, but maternal liver to body weight ratios were significantly elevated ($p < 0.01$) over control levels at all of the doses administered. Maternal weight gain was actually increased at the highest exposure level. This may be partially related to the increased liver weight and/or maternal edema.

Fetal mortality was significantly increased relative to controls at 100 and 1000 mg/kg ($p < 0.01$). Correspondingly, the average number of live fetuses per litter was significantly decreased relative to controls at these exposure levels ($p < 0.01$). The mean live fetal weight exhibited no dose-related changes. Cleft palate was induced in all of the fetuses from the treated dams except for a single fetus at 10 mg/kg that had agnathia. The presence of this abnormality probably precluded cleft palate formation as a result of the altered facial development. A single fetus, also at 10 mg/kg, had a complete

medial facial cleft in addition to the cleft palate. Fetal subcutaneous edema was evident at all of the dose levels and two fetuses in a single litter at 1000 mg/kg had focal hemorrhages around the neck that were not due to excessive handling or tactile stresses during dissection. The most important point to be made regarding the implications of the data in Table 2 is that even at the lowest dose level administered, 10 mg/kg, we were able to induce cleft palate in essentially 100% of the fetuses without seeing any signs of overt toxicity in the dams. Therefore, it was decided that exposure levels in the subsequent definitive teratological evaluation would not exceed 10 mg HBN/kg.

Complete Teratology Study

The results of the definitive teratological evaluation are given in Figures 3, 5 and 7, and Tables 3, 4 and 5. Overt maternal toxicity was evident at 5.0, 7.5 and 10.0 mg/kg/day. Typically, this was indicated by wasting, listlessness, decreased weight gain or actual weight loss, vaginal bleeding and outright mortality. Liver to body weight ratios exhibited a dose-related increase as shown in Table 3. At all of the exposure levels, liver to body weight ratios were significantly elevated relative to the controls (at 0.05 mg/kg, $p < 0.05$; at 1.0, 2.5, 5.0, 7.5, and 10.0 mg/kg, $p < 0.01$). The thymus to body weight ratios exhibit a less well-defined, dose-related trend (data not shown) that actually reversed at 7.5 and 10.0 mg/kg due to maternal weight loss.

At 5.0, 7.5 and 10.0 mg/kg, fetal mortality was significantly increased over controls ($p < 0.01$) (Table 3). At the two highest

dose levels, fetal mortality was 100% and was accompanied by overt maternal toxicity and actual maternal weight loss. At 5.0 mg/kg there was 86% fetal mortality. This was also accompanied by overt maternal toxicity but it was not as severe or as frequent as that seen at 7.5 and 10.0 mg/kg. Dams exposed to 5.0 mg/kg did not show any actual maternal weight loss.

A significant increase in the incidence of fetal soft tissue (Table 4) and skeletal (Table 5) anomalies was observed after maternal exposure to HBN. The spectrum of teratogenic lesions and developmental abnormalities associated with HBN administration is very similar to that produced by exposure to other toxic halogenated aromatic hydrocarbons such as 2,3,7,8-TCDD (Neubert, et al, 1973; Moore, et al, 1973; Courtney and Moore, 1971) and 2,3,7,8-TCDF (Weber, et al, 1984; Hassoun, et al, 1984). This includes hydronephrosis, cleft palate, involution or atrophy of lymphatic organs (particularly the thymus and spleen), subcutaneous edema and a general delay of ossification. The various toxic endpoints evaluated differed in sensitivity and therefore in the degree of response at each dose level.

The soft tissue abnormalities are summarized in Table 4 with the litter as the experimental unit and in Figure 3 as a percentage of the total number of fetuses at each dose level. Hydronephrosis is the most sensitive soft tissue abnormality associated with HBN exposure, reaching 100% at 1.0 mg/kg. Kidneys with bilateral, severely dilated renal pelves are shown compared with normal, control kidneys in Figure 4. In Table 4,

hydronephrosis is tabulated on the basis of the presence or absence of a dilated renal pelvis (and corresponding reduction of the renal papilla). At 0.5 mg/kg, approximately 90% of the fetuses examined per litter had hydronephrotic kidneys. At 1.0 mg/kg, all of the fetuses examined had some degree of hydronephrosis. In Figure 5, kidney changes are graded according to the severity of hydronephrosis and coded on the basis of which of the kidneys were affected. It should be noted that as previously reported for other halogenated aromatics (Weber, et al, 1984; Moore, et al, 1973), when the kidney changes are not evenly distributed bilaterally, the right kidney is affected to a greater extent than the left. This is the case for HBN exposure. Uneven lateral distribution of blood flow to the kidneys may, in part, account for this effect. At the higher dose levels tested, both kidneys were severely affected. Hydronephrosis has previously been observed to be the most sensitive soft tissue abnormality induced by exposure to TCDD and related compounds (Courtney and Moore, 1971; Moore, et al, 1973).

Cleft palate is also produced in response to maternal HBN exposure. Maximal frequency occurs at 2.5 and 5.0 mg/kg. Both cleft palate and hydronephrosis exhibit extremely steep dose responses to HBN administration that are typical of exposure to TCDD-like compounds (Weber, et al, 1984; Neubert, et al, 1973). A control and HBN-treated palate are shown in Figure 5.

Involution or atrophy of fetal spleen and thymus as determined by gross observation during soft tissue analysis (a modified Wilson slice technique) showed dose-related changes. The

thymus appeared to be slightly more sensitive to toxic insult than the spleen. Significant thymic effects were noted at dose levels greater than or equal to 1.0 mg/kg ($p < 0.01$). Significant differences for splenic atrophy were observed at 2.5 and 5.0 mg/kg ($p < 0.01$ relative to controls). Involution of fetal lymphatic organs has been noted previously in response to maternal exposure to TCDD (Neubert, et al, 1973).

The incidence of fetal subcutaneous edema was significantly increased ($p < 0.05$ relative to controls) at 2.5 and 5.0 mg/kg. This lesion, which should not be considered as a true teratogenic response, but rather as a manifestation of fetal toxicity, has been previously reported to be associated with maternal exposure to halogenated aromatics (Schwetz, et al, 1973; Moorhead, et al, 1977) and was correlated with the increase in mean live fetal weight shown in Table 3.

Umbilical hernias were observed in some fetuses at 2.5 and 5.0 mg/kg. The data are suggestive of a dose-related trend but are not statistically significant. This lesion had not been previously reported as a consequence of exposure to halogenated aromatic hydrocarbons. Fetal diaphragmatic hernias have been reported to occur subsequent to maternal exposure to PBBs (Beaudoin, 1977), but the occurrence of umbilical hernias has not been documented.

The frequency of skeletal anomalies is summarized in Table 5 with the litter as the experimental unit and in Figure 7 with the fetus as the experimental unit. A general delay of ossification was observed at 1.0, 2.5 and 5.0 mg/kg. This was most evident

for the nasal and cranial bones. The criteria for assessing the degree of nasal bone ossification was the presence or absence of a central region devoid of ossification with the edges surrounding this region being diffuse and clearly not completely ossified. No fetuses were observed to have complete ossification of the nasal bones while retaining any central opening. The degree of cranial ossification was evaluated by measuring the width of the medial fissure between the frontal skull bones (the metopic fontanel) and assessing the density and integrity of the edges of the parietal and frontal bones.

The conformation of sternabrae (i.e. splitting and misalignment) and the degree of sternabral ossification were evaluated. Typically, these parameters are highly variable and unreliable as indicators of fetal toxic stress. However, significant increases were observed in the frequency of split sternabrae at 1.0, 2.5 ($p < 0.05$) and 5.0 mg/kg ($p < 0.01$), and misaligned sternabrae at 2.5 ($p < 0.05$) and 5.0 mg/kg ($p < 0.01$). No dose-related trends were evident for the degree of sternabral ossification. One previous study (Schwetz, et al, 1973) reported that maternal HCDD (hexachlorodibenzo-p-dioxin) exposure was associated with a dose-related decrease in the extent of fetal sternabral ossification.

Multiple Dose Toxicity Study

HBN was found to be much more toxic when administered in multiple, smaller doses than by a single, large dose. Animals participating in this portion of the study received 10 daily doses of between 0 and 10 mg/kg. Animals in the acute toxicity

evaluation received a single dose of between 0 and 1000 mg/kg. Overt toxicity was evident for some animals receiving multiple doses of as low as 5.0 mg/kg/day after seven days of exposure, while no overt signs of toxicity were observed for animals dosed singly at 1000 mg/kg.

The body weight trends of the multiply-dosed animals exhibit dose-related depressions (Figure 8). When expressed as a percent change from original body weight (pre-dosing weight for the first day on study), animals receiving 5.0, 7.5 and 10.0 mg HBN/kg body weight/day showed highly significant ($p < 0.01$) reductions in body weight (relative to controls) by 7, 6 and 5 days of dosing, respectively (Figure 9). Some of the animals that received the highest dose levels exhibited reductions in body weight and showed signs of overt toxicity. These included lethargy, wasting and a characteristic ocular protrusion. Some of these same HBN-treated animals subsequently died prior to termination of the study (Table 6).

Liver to body weight ratios were significantly elevated over control levels for all of the experimental treatment groups. At the higher dose levels (5.0, 7.5 and 10.0 mg/kg/day) in which the experimental animals lost weight, the increase of liver to body weight ratios was enhanced by body weight loss concurrent with an increase in absolute liver weight. The observation of increased liver to body weight ratios is typical of exposure to toxic halogenated aromatic hydrocarbons and is attributable to microsomal enzyme induction and hyperproliferation of endoplasmic reticulum in the hepatocytes (Fowler, et al, 1973; Norback and

Allen, 1972). Fatty infiltration (centrolobular accumulation of lipid) may also be a contributing factor in HBN-induced liver enlargement (Kohli, et al, 1981). At necropsy, qualitative evaluations of liver surface color and texture were made and a composite score was derived for each liver based on the severity of discoloration ("paleness") and the intensity of a lobular surface pattern (best described as surface "mottling"). The mean values for this composite score are shown in Table 7. A definite dose-related increase is evident at 2.5, 5.0, 7.5 and 10.0 mg/kg ($p<0.01$). This suggests that, as for other toxic halogenated aromatic hydrocarbons (Kover, 1975; Render, et al, 1982; Kimbrough, 1974) the liver is a target organ for HBN toxicity. Although not substantiated by histochemical analysis, fatty infiltration, centrolobular necrosis, cytoplasmic vacuolization and bile duct hyperplasia would be suspected. These changes have been associated with exposure to compounds that are structurally related to HBN and some of these lesions would be consistent with the gross pathological observations noted at necropsy.

The thymus to body weight ratios exhibited a dose-related decrease that was statistically significant at 1.0 mg/kg ($p<0.005$) (Table 6). At dose levels above 1.0 mg/kg, the absolute thymus weights continued to decrease until the lower limit of detection for our experimental balance (10 mg) was reached (i.e. the thymuses continued to decrease in size and weight but we could not document the values below this point). Also, the absolute values for thymus weight exhibit too high a degree of variability to serve as meaningful indicators of toxicity without being standardized to body weight. At the three

highest dose levels, the experimental animals lost body weight at a greater relative rate than they lost thymus weight. Therefore, the thymus to body weight ratio trends were observed to reverse at the higher exposure levels. Thymic atrophy has been reported to be associated with TCDD exposure (Harris, et al, 1973; Gupta, et al, 1973).

Conclusions

The primary aim of this study was to characterize the teratogenic and fetotoxic properties of hexabrominated naphthalenes in C57Bl/6N mice. In order to assess these properties, a preliminary study was conducted that generated information about acute toxicity, and a study performed concurrently with the teratology experiment yielded information regarding multiple dose toxicity.

The data presented herein indicate that HBN is a potent teratogenic and fetotoxic agent, causing fetal malformations at dose levels as low as 0.5 mg/kg and fetal mortality at 5.0 mg/kg. HBN has a relatively low acute toxicity in female C57Bl/6N mice, but is much more toxic when administered in multiple doses. When exposed to a single p.o. dose, animals treated with 1000 mg/kg failed to show any indications of toxicity during the course of a 36-day follow-up. However, when animals were treated for 10 days with doses as low as 5.0 mg/kg/day, overt signs of toxicity were evident by the seventh day of dosing.

Clearly, HBN is a toxic halogenated aromatic hydrocarbon that is capable of producing a spectrum of toxic and teratogenic lesions similar to TCDD. However, it is much less potent. It has been suggested that HBN does in fact conform to the structural requirements for the "dioxin receptor" although much less efficiently than the prototypical compound TCDD (McKinney and McConnell, 1982). This may partially explain its reduced potency for eliciting the biological responses associated with binding to and activating this receptor. Also, the major, relatively toxic isomer in the HBN mixture is susceptible to

chemical oxidation (Brady, et al, 1982), suggesting that it is readily metabolized and excreted. This may also contribute to HBN's reduced toxic potency relative to TCDD, which is a highly persistent and toxic compound (McKinney and McConnell, 1982; Poland and Knutson, 1982).

Consistent with the above argument is the fact that animals that died after multiple exposures to HBN exhibited symptoms of the characteristic "wasting syndrome" previously associated with TCDD administration (McConnell, et al, 1978; Moore, 1978). Although not conclusive evidence, the fact that HBN produces similar lesions with parallel dose response curves to TCDD is in agreement with the hypothesis that HBN would act by a similar mechanism as TCDD.

Among the better mechanistically characterized toxic responses to halogenated aromatics is the teratogenic end point, cleft palate. TCDD is known to produce cleft palate by preventing the terminal differentiation (programmed cell death) of the medial edge epithelial cells of the palatal shelves, thereby blocking fusion of the shelves. As the head expands during subsequent development, the palatal shelves move apart, forming a cleft (Pratt, et al, 1980). It is probable that HBN produces cleft palate by this same mechanism. The mechanism of TCDD-induced kidney changes, which are best described as apparent hydronephrosis, is poorly understood at this time as is the nature of the causal events associated with the "wasting syndrome".

Recent experiments in this laboratory (Birnbaum, unpublished observations) suggest that many of these structurally related compounds that are thought to elicit their toxic effects by binding to and activating the dioxin receptor can be considered to act in an additive manner and, therefore, their relative potencies can be expresssed as "TCDD-equivalents". In our GD10-13 teratology test system, HBN is approximately 1000 times less toxic than TCDD and in the guinea pig lethality system, HBN is about 230 times less toxic than TCDD (McKinney and McConnell, 1982). This would mean that a unit of HBN would be "equivalent" in terms of toxic potency to between .001 and .004 units of TCDD. This same analogy can be applied to other test systems used for evaluating the toxicity of halogenated aromatic hydrocarbons, for example, AHH induction studies. When expressed in this manner, it is apparent that relative to TCDD, HBN is not as toxic. However, it must be kept in mind that TCDD is one of the most toxic and highly teratogenic agents ever known to man and that by conventional standards, HBN is highly toxic and teratogenic. This is not intended to be a highly quantitative convention, but rather to illustrate the relative toxic potencies of these compounds under the assumption that they are acting in an additive manner. It is also likely that the relative potencies may vary considerably depending upon the species and test system being employed to evaluate toxicity.

Firemaster contains many compounds that are structurally related to TCDD. An important point to be made here is that

although each of these may be described as being relatively nontoxic when considered as individual compounds, the sum total of "TCDD-equivalents" present may have been great enough to elicit the toxic responses that were seen. When the individual compounds in Firemaster are expressed as "TCDD-equivalents" per unit weight, the brominated naphthalenes are among the most toxic. They are, however, also among the least prevalent in the PBB mixture. Therefore, the brominated naphthalene contribution per se, to the overall toxic properties of Firemaster is probably minor, unless potentiation or synergistic interactions are involved between the brominated naphthalenes and some of the other components of Firemaster. We have not investigated this possibility directly as it applies to the Firemaster mixture, but the "TCDD-equivalent" concept would seem to argue against the likelihood of potentiation or synergism being involved. This question may serve as the basis for further studies.

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Table 1. Toxicity of mice exposed to a single oral dose of HBN and monitored for 35 days post-exposure. Selected parameters at necropsy.

DOSE mg/kg	(N)	MEAN BODY WEIGHT (BW) [a,c]	LIVER WEIGHT (LW) [a,c]	$\frac{LW}{BW} \times 100$ [a,d]	THYMUS WEIGHT (TW) [a,c]	$\frac{TW}{BW} \times 100$ [a,d]
0	(6)	22.8 ± 0.8	1.3 ± 0.1	5.6 ± 0.3	0.035 ± 0.016	0.155 ± 0.072
3	(6)	22.2 ± 0.9	1.3 ± 0.1	5.9 ± 0.6	0.032 ± 0.009	0.143 ± 0.036
10	(6)	22.6 ± 1.2	1.3 ± 0.1	5.6 ± 0.1	0.037 ± 0.004	0.166 ± 0.020
30	(6)	22.5 ± 0.7	1.4 ± 0.1	6.2 ± 0.3[b]	0.039 ± 0.013	0.172 ± 0.058
100	(6)	23.8 ± 1.7	1.5 ± 0.1[b]	6.3 ± 0.4[b]	0.034 ± 0.009	0.141 ± 0.034
300	(6)	22.4 ± 1.7	1.4 ± 0.2	6.4 + 0.6[b]	0.030 ± 0.007	0.134 ± 0.023
1000	(6)	22.0 ± 1.3	1.4 ± 0.1	6.4 ± 0.6[b]	0.027 ± 0.009	0.127 ± 0.040

[a] Data expressed as $\bar{x} \pm$ S.D.

[b] Significantly different from controls ($p<0.01$).

[c] In grams.

[d] Expressed as percent of body weight.

Table 2. Effects of HBN administration during gestation days 10-13 on maternal and embryo/fetotoxicity. Selected parameters at necropsy.

DOSE mg/kg LEVEL (N)	MATERNAL WEIGHT GAIN[a]	MATERNAL LIVER/BODY WEIGHT RATIO[f] (x 100)	NUMBER LIVE FETUSES PER LITTER[f]	% FETAL MORTALITY[b]	LIVE FETAL WEIGHT[e]	%CLEFT PALATE PER LITTER[b]
0 (7)	2.41 ± 0.27	7.21 ± 0.11	7.30 ± 0.40	7.10 ± 3.70	1.14 ± 0.02	0.00
10 (11)	2.58 ± 0.38	9.31 ± 0.24[d]	6.45 ± 0.49	13.50 ± 4.00	1.17 ± 0.02	98.80 ± 1.1[d]
100 (10)	2.08 ± 0.83	10.18 ± 0.12[d]	4.90 ± 0.64	37.37 ± 7.84[d]	1.14 ± 0.03	100.00[d]
1000 (11)	3.56 ± 0.62[d]	10.45 ± 0.53[d]	5.72 ± 0.74	31.55 ± 8.41[d]	1.16 ± 0.02	100.00[d]

[a] Gestation days 10-18, expressed in grams ($\bar{x}$ ± S.D.).

[b] Data expressed as mean percentage per litter ($\bar{x}$ ± S.D.).

[c] Significantly different from controls ($p<0.05$).

[d] Significantly different from controls ($p<0.01$).

e. Data expressed in grams ($\bar{x}$ ± S.D.)

f. Data expressed as $\bar{x}$ ± S.D.

Table 3. Maternal and fetal toxicity following HBN administration to pregnant dams during gestation days 6-15. Selected parameters at necropsy.

DOSE LEVEL mg/kg/day	(N)	MATERNAL WT. GAIN[a]	MATERNAL LIVER WT. / BODY WT (x 100)[f]	NUMBER OF LIVE FETUSES[e]	PERCENT FETAL MORTALITY[b]	NUMBER OF DEAD PLUS RESORBED FETUSES[b]	LIVE FETAL WEIGHT[e]
0.0	(8)	4.9 ± 0.4	6.9 ± 0.2	7.3 ± 0.5	9.5 ± 4.2	0.8 ± 0.3	1.17 ± 0.02
0.5	(4)	4.7 ± 0.7	7.7 + 0.2[c]	7.0 ± 0.6	10.5 ± 4.8	0.8 ± 0.4	1.17 ± 0.02
1.0	(8)	4.6 ± 0.5	8.2 ± 0.1[d]	6.6 ± 0.8	12.6 ± 6.2	0.8 ± 0.3	1.19 ± 0.03
2.5	(11)	4.6 ± 0.5	9.5 ± 0.3[d]	6.5 ± 0.5	15.3 ± 4.5	1.2 ± 0.4	1.28 ± 0.02[d]
5.0	(10)	6.1 ± 0.9	10.6 ± 0.3[d]	0.9 ± 0.5[d]	86.0 ± 7.1[d]	6.4 ± 0.7[d]	1.28 ± 0.02[d]
7.5	(9)	-0.4 ± 1.2[d]	10.0 ± 0.2[d]	0.0 ± 0.0[d]	100.0 ± 0.0[d]	7.3 ± 0.3[d]	----------
10.0	(9)	-1.2 ± 1.3[d]	10.0 ± 0.2[d]	0.0 ± 0.0[d]	100.0 ± 0.0[d]	6.7 ± 0.5[d]	----------

a Gestation days 6-18, expressed in grams ($\bar{x}$ ± S.D.).

b Data expressed as mean percentage per litter ($\bar{x}$ ± S.D.).

c Significantly different from controls ($p < 0.05$).

d Significantly different from controls ($p < 0.01$).

e. Data expressed as mean value per litter ($\bar{x}$ ± S.D.).

f. Data expressed as $\bar{x}$ ± S.D.

Table 4. Soft tissue abnormalities in offspring of C57Bl/6N mice receiving daily doses of HBN during gestation days 6-15.

DOSE LEVEL mg/kg/day	(N)	CLEFT PALATE[a]	UNDERSIZED THYMUS[a]	UNDERSIZED SPLEEN[a]	SUBCUTANEOUS EDEMA[a]	UMBILICAL HERNIA[a]	HYDRO-NEPHROSIS[a]
0	(8)	0.0 ± 0.0	6.3 ± 6.3	3.1 ± 3.1	0.0 ± 0.0	0.0 ± 0.0	12.5 ± 35.4
0.5	(5)	0.0 ± 0.0	10.0 ± 10.0	5.0 ± 5.0	0.0 ± 0.0	0.0 ± 0.0	90.0 ± 22.4[c]
1.0	(8)	4.8 ± 2.4	57.1 ± 11.6[c]	11.5 ± 8.5	2.5 ± 2.5	0.0 ± 0.0	100.0 ± 0.0[c]
2.5	(11)	94.6 ± 2.4[c]	93.2 ± 4.9[c]	31.8 ± 4.5[c]	28.3 ± 7.0[c]	9.6 ± 4.8	100.0 ± 0.0[c]
5.0	(4)	75.0 ± 25.0[b]	100.0 ± 0.0[c]	87.5 ± 12.5[c]	85.0 ± 15.0[c]	37.5 ± 23.9	100.0 ± 0.0[c]

[a] Data expressed as the mean percentage of fetuses affected per litter ($\bar{x}$ ± S.D.).

[b] Significantly different from controls ($p<0.05$).

[c] Significantly different from controls ($p<0.01$).

Table 5. Skeletal abnormalities in offspring of C57Bl/6N mice receiving daily doses of HBN during gestation days 6-15.

DOSE LEVEL mg/kg/day	(N)	DELAYED CRANIAL OSSIFICATION[a]	DELAYED OSSIFICATION OF NASAL BONES[b]	DELAYED STERNABRAL OSSIFICATION[a]	SPLIT STERNABRAE[a]	MISALIGNED STERNABRAE[a]
0	(8)	7.3 ± 4.8	0.3 ± 0.1	22.9 ± 8.9	0.0 ± 0.0	26.0 ± 9.5
0.5	(5)	31.7 ± 18.3	0.3 ± 0.1	5.0 ± 5.0	0.0 ± 0.0	57.7 ± 8.9
1.0	(8)	40.6 ± 9.9[b]	1.2 ± 0.2[d]	29.0 ± 7.0	45.2 ± 11.1[d]	42.7 ± 11.9
2.5	(11)	81.2 ± 10.5[d]	1.9 ± 0.1[d]	49.2 ± 12.1	79.2 ± 6.7[d]	90.6 ± 5.3[d]
5.0	(2)	100.0 ± 0.0[c]	2.0 ± 0.0[c]	0.0 ± 0.0	100.0 ± 0.0[c]	100.0 ± 0.0[c]

[a] Data expressed as mean percentage of fetuses affected per litter ($\bar{x}$ ± S.D.).

[b] Data expressed as a mean composite score per litter based on severity of delay (0: no delay, 1: slight delay, 2: marked delay).

[c] Significantly different from controls ($p<0.05$).

[d] Significantly different from controls ($p<0.01$).

Table 6. Toxicity of mice exposed to 10 consecutive daily doses of HBN and sacrificed 3 days after the last exposure. Selected parameters at necropsy.

DOSE LEVEL mg/kg	(N)	$\frac{\text{LIVER WT.}}{\text{BODY WT.}}$[a] x 100	$\frac{\text{THYMUS WT.}}{\text{BODY WT.}}$[a] x 100	PERCENT MORTALITY
0	(11)	5.60 ± 0.51	0.24 ± 0.03	0.0
0.5	(17)	6.44 ± 0.28[c]	0.21 ± 0.04	0.0
1.0	(12)	7.26 ± 0.49[c]	0.16 ± 0.06[c]	0.0
2.5	(8)	8.16 ± 1.21[c]	<.07[d]	0.0
5.0	(8)	9.48 ± 0.79[c]	<.07[d]	0.0
7.5	(12)	9.47 ± 0.71[c]	<.07[d]	25.0[e]
10.0	(10)	10.38 ± 0.55[c]	<.07[d]	30.0[f]

[a] Mean + S.D.

[b] Significantly different from controls ($p<0.05$).

[c] Significantly different from controls ($p<0.005$).

[d] See text for explanation.

[e] Mortality was on experimental days 10, 12, and 13.

[f] Mortality was on experimental days 10, 11, and 12.

Table 7. Gross liver pathology for mice exposed to 10 consecutive daily doses of HBN and sacrificed 3 days after the last exposure.

DOSE LEVEL mg/kg/day	COMPOSITE Score [a,b]
0.0	0.1 ± 0.3
0.5	0.1 ± 0.3
1.0	0.5 ± 0.3
2.5	2.0 ± 0.0[c]
5.0	4.3 ± 0.7[c]
7.5	4.6 ± 1.2c
10.0	5.0 ± 0.6[c]

[a] Mean ± S.D.

[b] Composite score based on the sum of scores for severity of surface discoloration (paleness) and abnormal texture (mottling).
Color: 0 = normal; 4 = extremely pale. Texture: 0 = normal, 3 = very mottled.

[c] Significantly different from controls ($p<0.01$).

Figure Legends

Figure 1. Structures of HBN isomers produced by direct bromination of naphthalene.

Figure 2. Body weights of mice following a single oral dose of HBN. Animals were sacrificed on the 35th day of exposure.

Figure 3. Dose response curves of selected soft tissue abnormalities associated with HBN exposure of pregnant dams during gestation days 6-15. Data expressed as percent of total number of fetuses at each dose level. --□-- cleft palate, --0-- subcutaneous edema, --△-- umbilical hernia, --▽-- undersized thymus, --◇-- undersized spleen.

Figure 4. Control (left) and HBN-treated (right) kidneys at gestation day 18. Note that in the treated animal the right kidney is more severely affected than the left kidney.

Figure 5. Histogram of kidney changes in offspring of HBN-treated mice. Dilation of the renal pelvis is graded as normal (0), mild (1), or severe (2) and is expressed as the effect observed on the left kidney/right kidney.

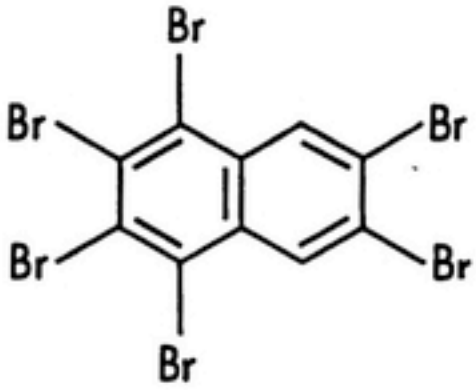
Figure 6. Control (left) and HBN-treated (right) palates of mouse fetuses at day 18 of gestation.

Figure 7. Dose response curves of selected skeletal abnormalities associated with HBN administration to pregnant dams during gestation days 6-15. Data expressed as percent of total number of fetuses at each dose level. --□-- delayed sternabral ossification, --○-- split stern sternabrae, --△-- misaligned sternabrae, --◇-- delayed cranial ossification (combined cranial and nasal bones).

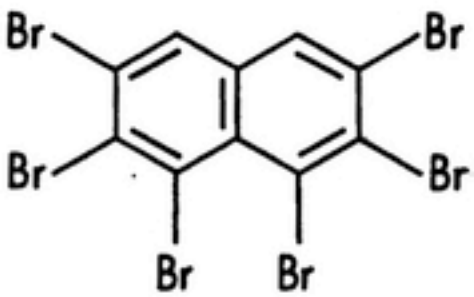
Figure 8. Average body weights of mice during and after repeated exposures to HBN (10 daily doses followed by 3 days of observation prior to sacrifice). Data are expressed as absolute body weight in grams.

Figure 9. Relative body weight changes during and after repeated exposures to HBN (10 daily doses followed by 3 days of observation prior to sacrifice). Data are expressed as percent change of body weight from the pre-dosing weight on day 1 of study.

Figure 1



1,2,3,4,6,7-HBN



2,3,4,5,6,7-HBN

Figure 2

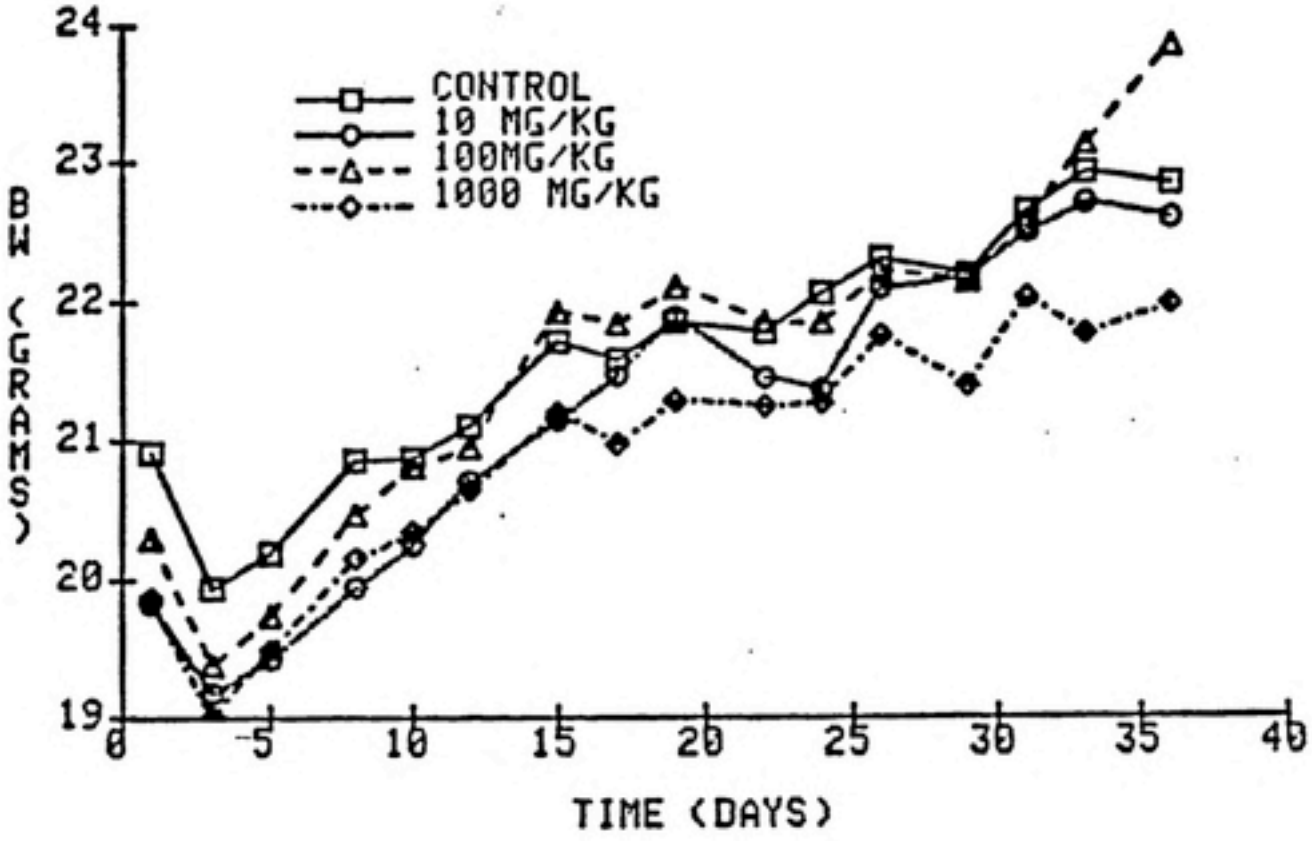

CONTROL
10 MG/KG
100MG/KG
1000 MG/KG
BW (GRAMS)
TIME (DAYS)
24
23
22
21
20
19
0
5
10
15
20
25
30
35
40

Figure 3

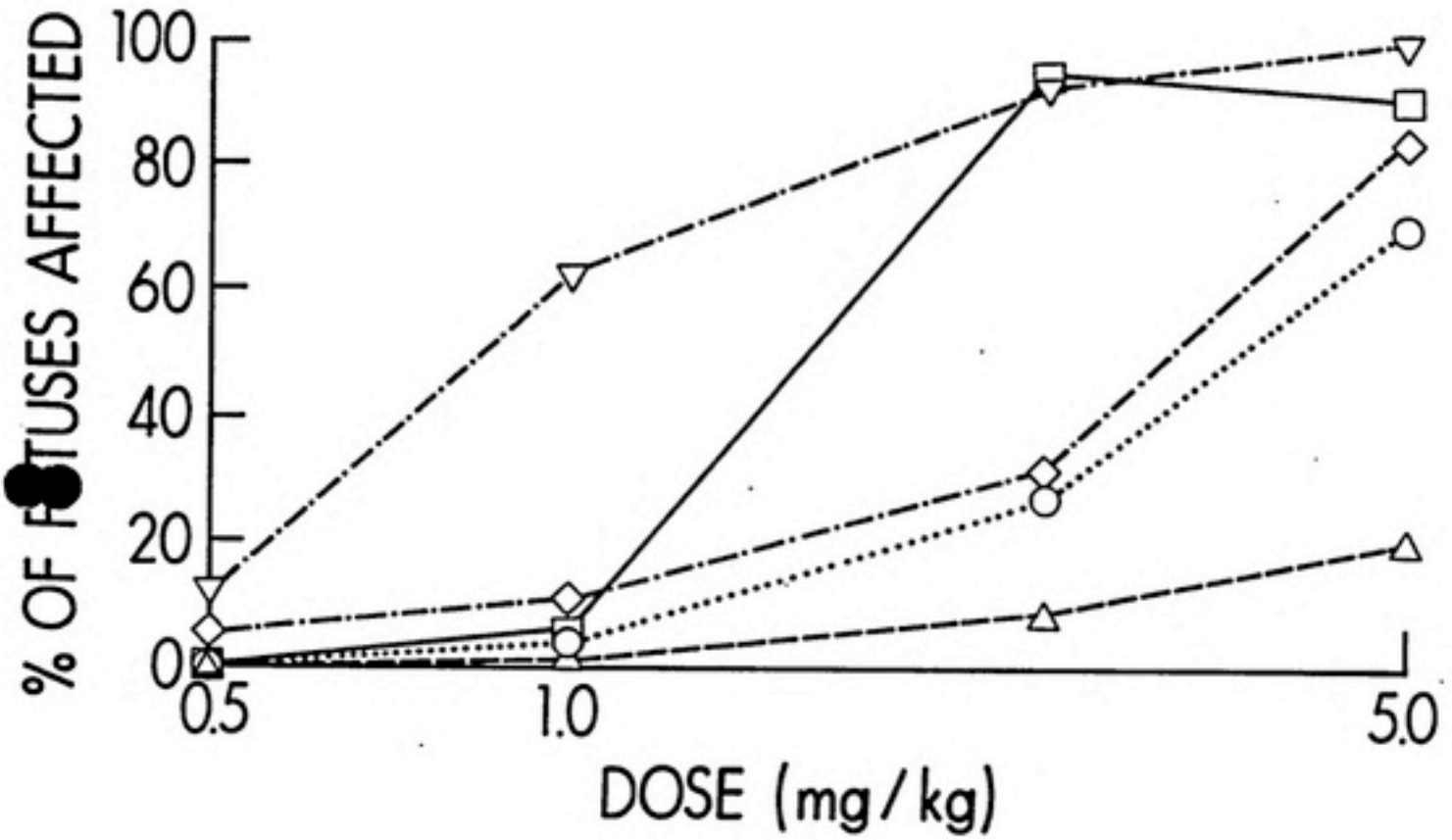


Figure 4

Figure 5

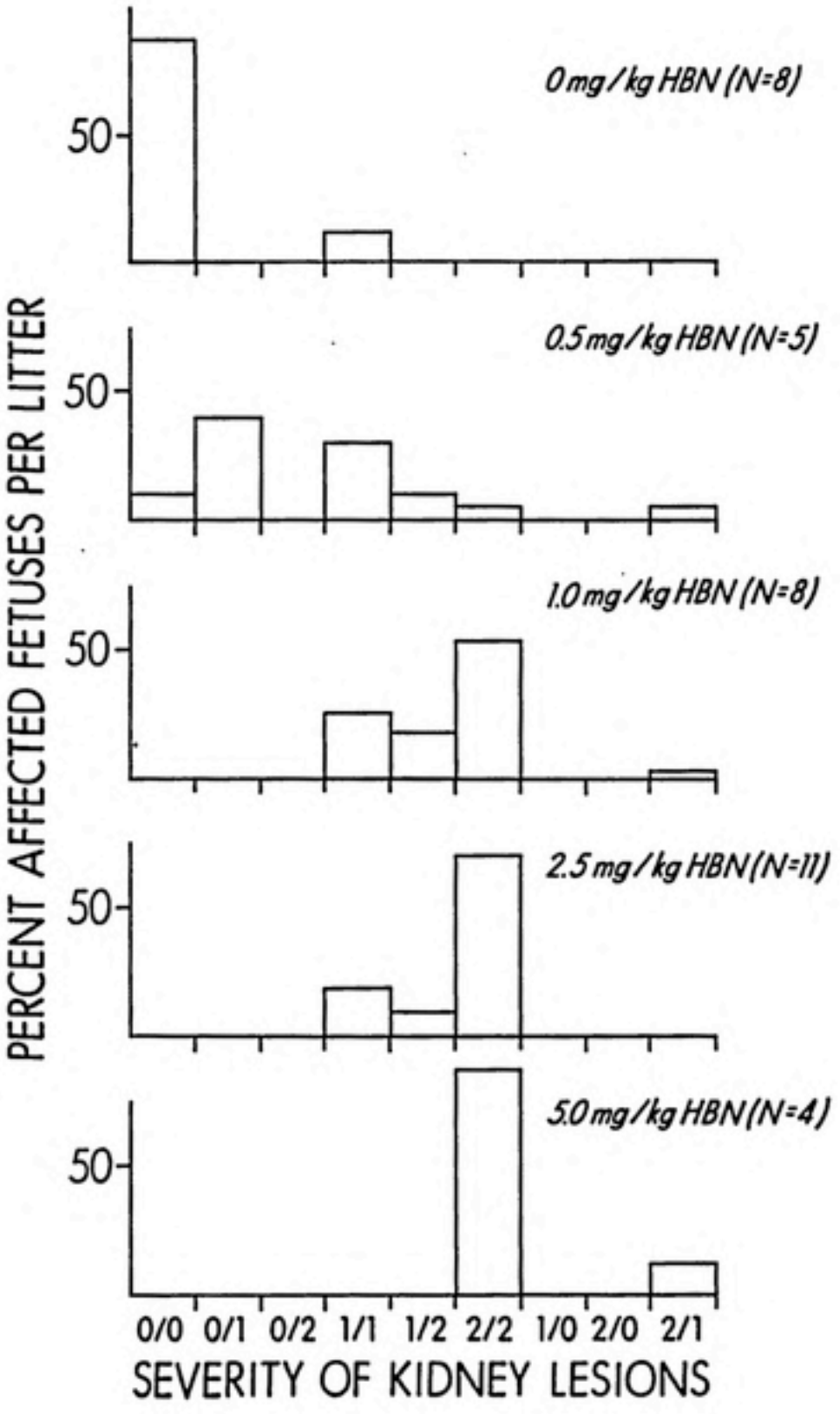

0 mg/kg HBN (N=8)
0.5 mg/kg HBN (N=5)
1.0 mg/kg HBN (N=8)
2.5 mg/kg HBN (N=11)
5.0 mg/kg HBN (N=4)
50
PERCENT AFFECTED FETUSES PER LITTER
0/0 0/1 0/2 1/1 1/2 2/2 1/0 2/0 2/1
SEVERITY OF KIDNEY LESIONS

Figure 6

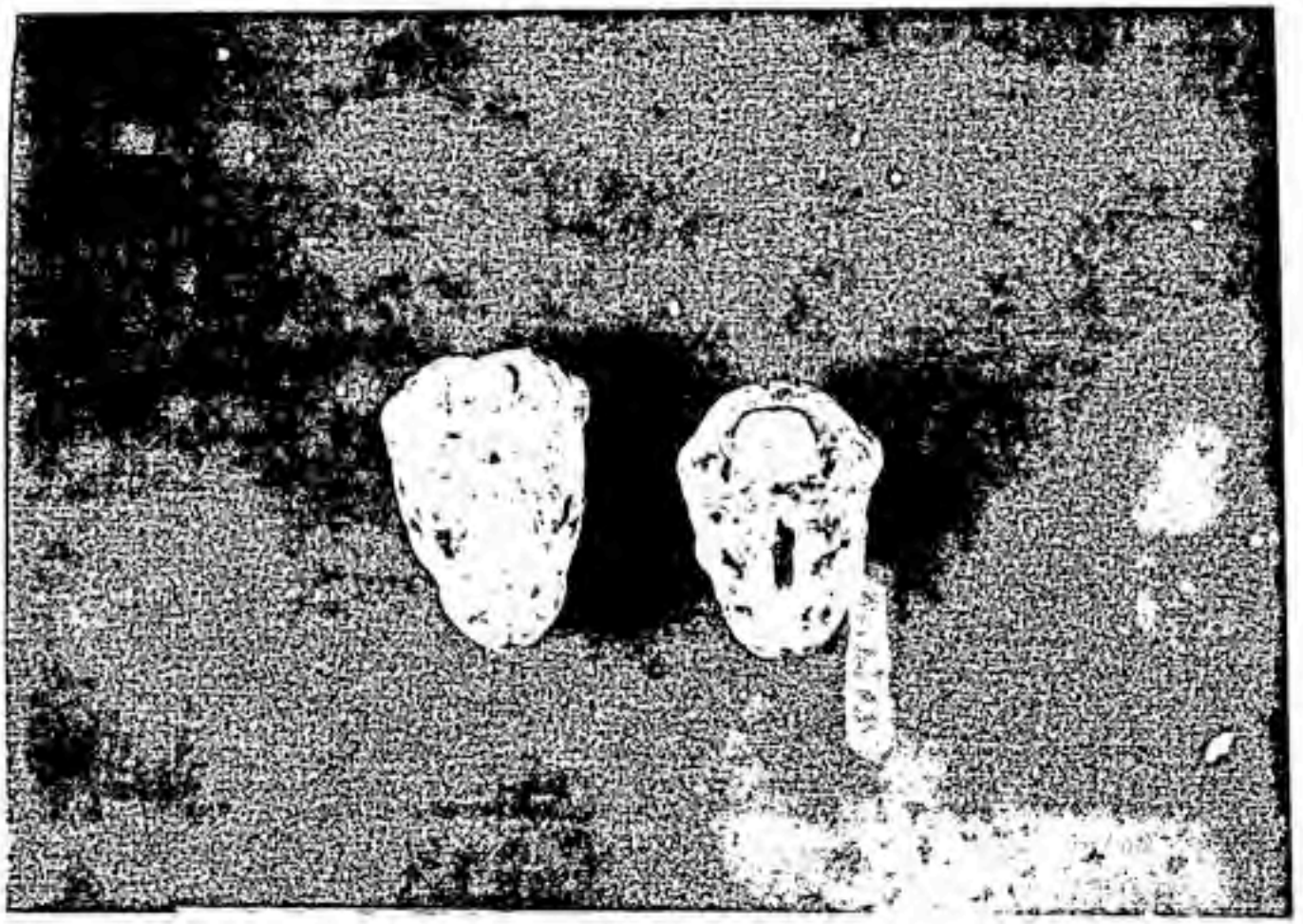


Figure 7

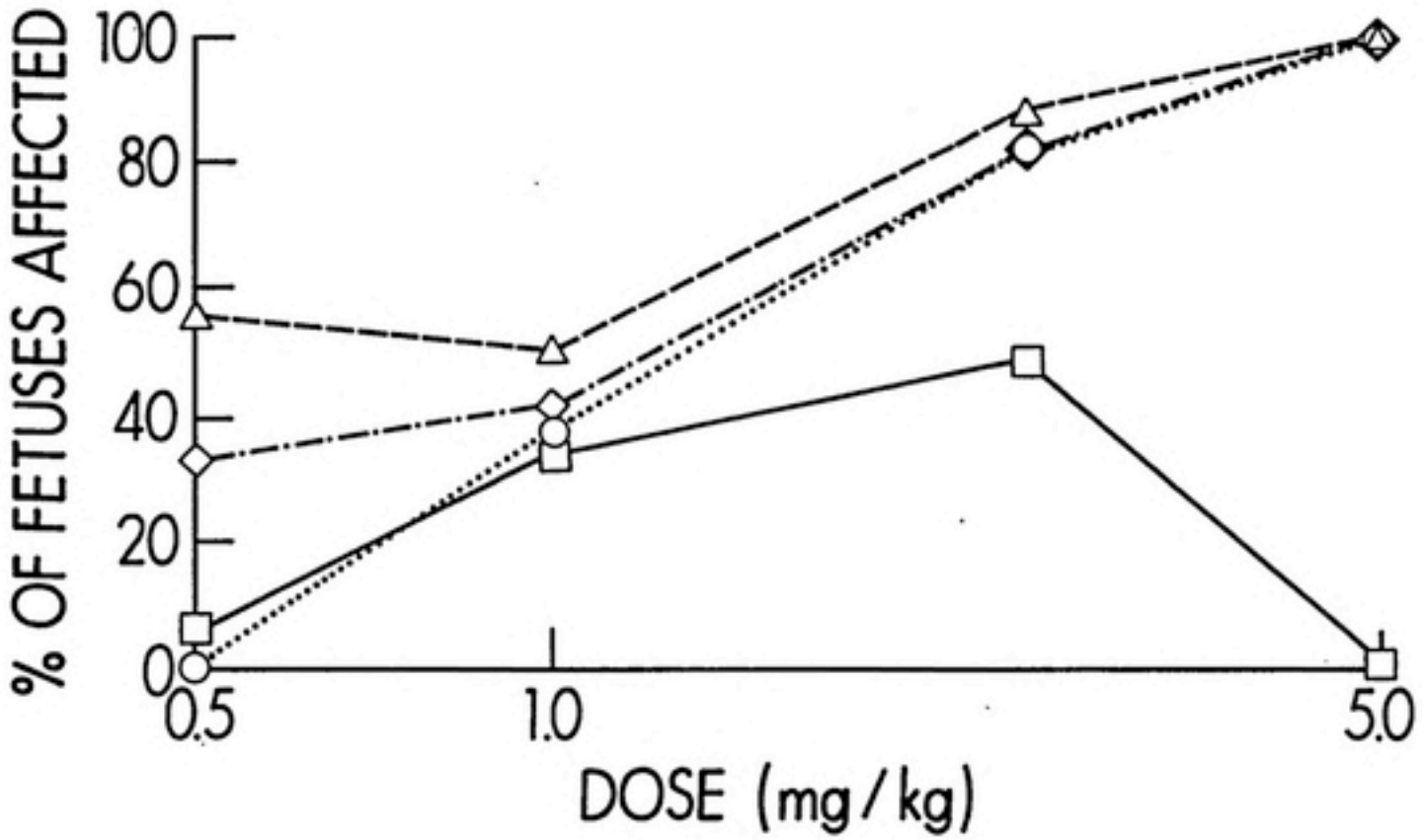


Figure 8

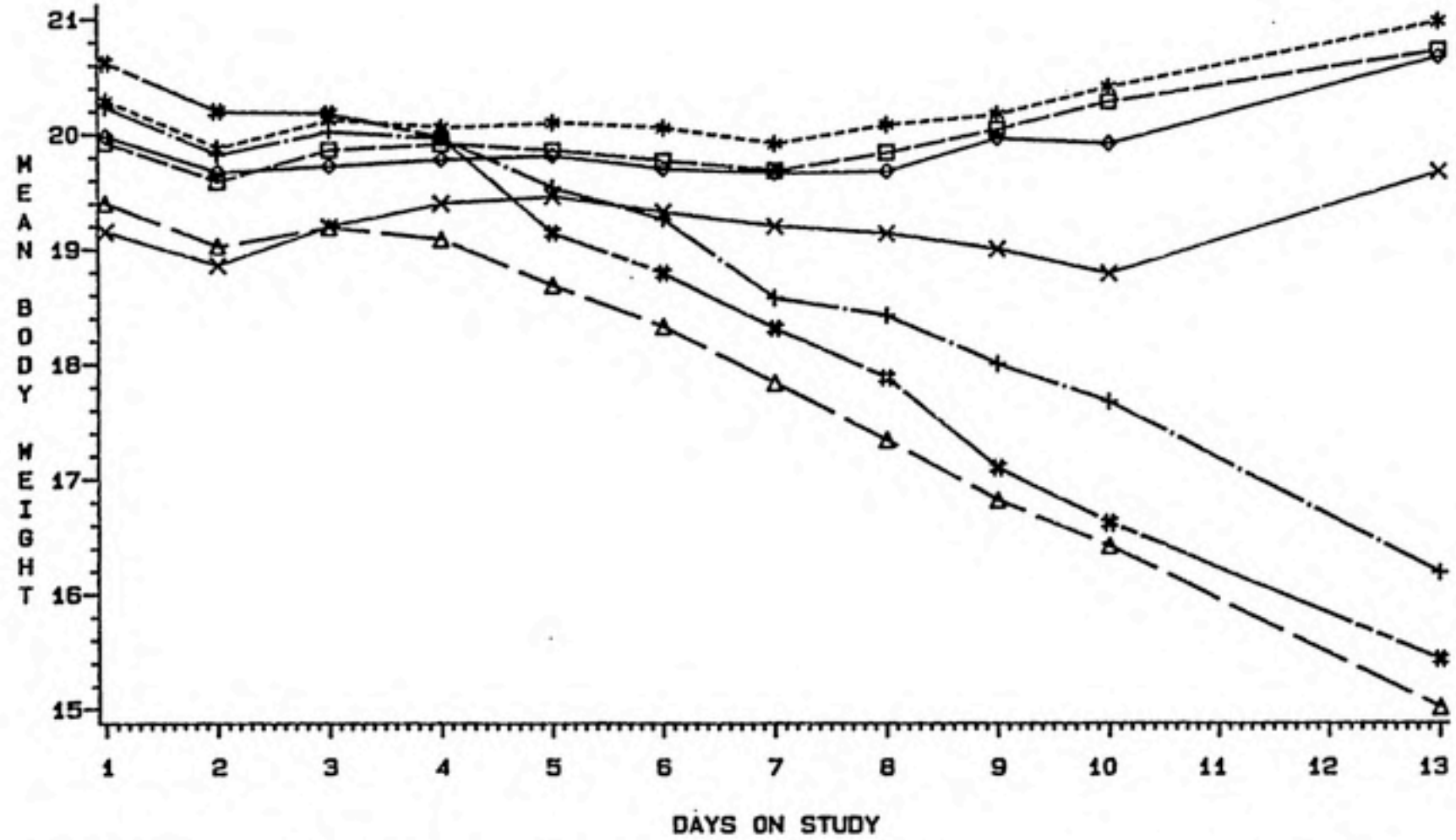
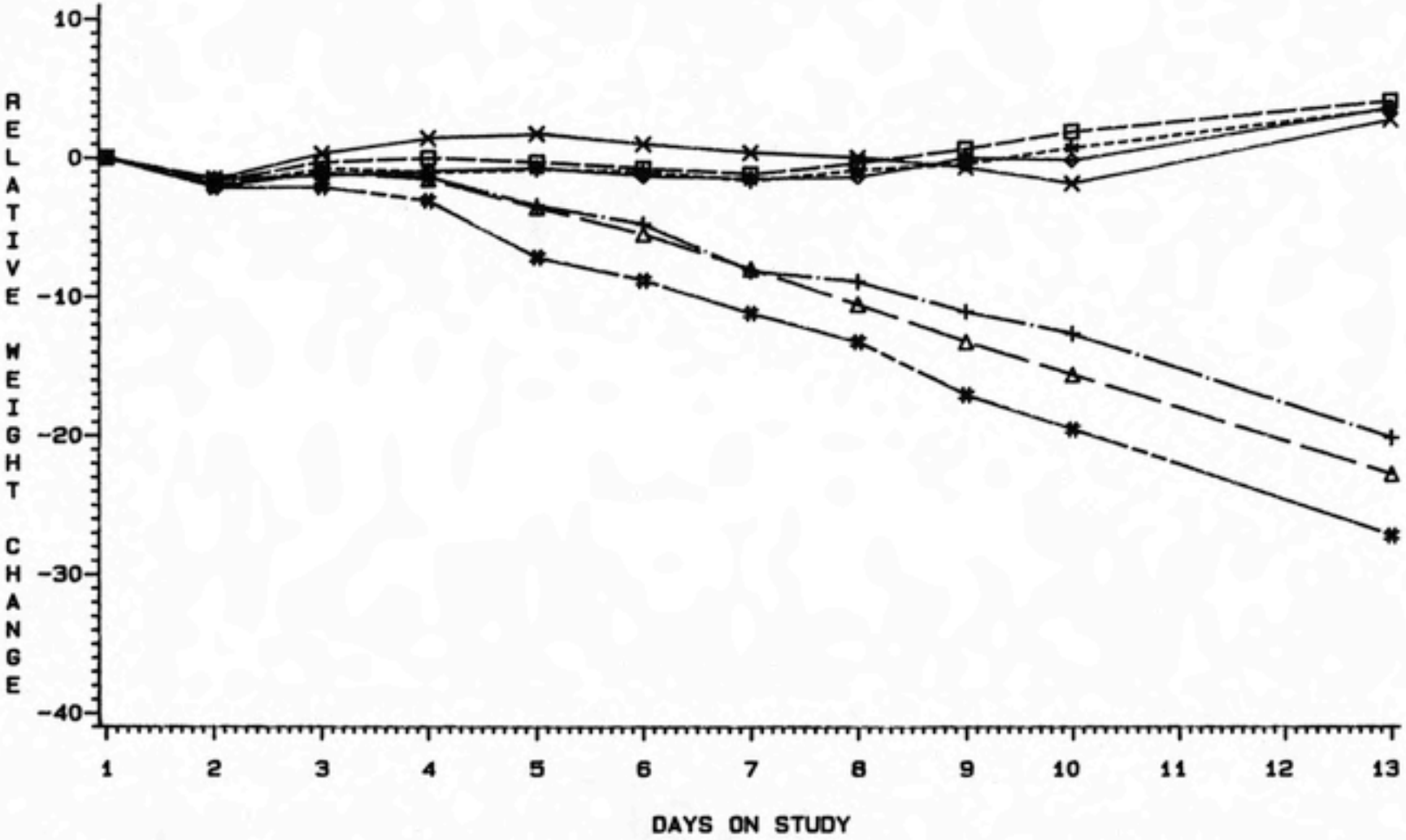

MEAN BODY WEIGHT
21
20
19
18
17
16
15
1
2
3
4
5
6
7
8
9
10
11
12
13
DAYS ON STUDY
LEGEND: DOSE
UNTREATED CONTROL
0.5 mg/kg
1.0 mg/kg
2.5 mg/kg
5.0 mg/kg
7.5 mg/kg
10.0 mg/kg

Figure 9


RELATIVE WEIGHT CHANGE
10
0
-10
-20
-30
-40
1
2
3
4
5
6
7
8
9
10
11
12
13
DAYS ON STUDY
LEGEND: DOSE
UNTREATED CONTROL
2.5 mg/kg
10.0 mg/kg
0.5 mg/kg
5.0 mg/kg
1.0 mg/kg
7.5 mg/kg