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Reproductive Effects of Four Phthalic Acid Esters in the Mouse

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Reproductive Effects of Four Phthalic Acid Esters in the Mouse. LAMB, J. C., IV, CHAPIN, R. E., TEAGUE, J., LAWTON, A. D., AND REEL, J. R. (1987). *Toxicol. Appl. Pharmacol.* **88**, 255–269. These studies compared the reproductive toxicity of four phthalates by a continuous breeding protocol. Mice were given diets with diethyl phthalate (DEP) (0.0, 0.25, 1.25, or 2.5%), di-*n*-butyl phthalate (DBP) (0.0, 0.03, 0.3, or 1.0%), di-*n*-hexyl phthalate (DHP) (0.0, 0.3, 0.6, or 1.2%), or di(2-ethylhexyl) phthalate (DEHP) (0.0, 0.01, 0.1, or 0.3%). Both male and female CD-1 mice were dosed for 7 days prior to and during a 98-day cohabitation period. Reproductive function was evaluated during the cohabitation period by measuring the numbers of litters per pair and of live pups per litter, pup weight, and offspring survival. There was no apparent effect on reproductive function in the animals exposed to DEP, despite significant effects on body weight gain and liver weight. DBP exposure resulted in a reduction in the numbers of litters per pair and of live pups per litter and in the proportion of pups born alive at the 1.0% amount, but not at lower dose levels. A crossover mating trial demonstrated that female mice, but not males, were affected by DBP, as shown by significant decreases in the percentage of fertile pairs, the number of live pups per litter, the proportion of pups born alive, and live pup weight. DHP in the diet resulted in dose-related adverse effects on the numbers of litters per pair and of live pups per litter and proportion of pups born alive at 0.3, 0.6, and 1.2% DHP in the diet. A crossover mating study demonstrated that both sexes were affected. DEHP (at 0.1 and 0.3%) caused dose-dependent decreases in fertility and in the number and the proportion of pups born alive. A crossover mating trial showed that both sexes were affected by exposure to DEHP. These data demonstrate the ability of the continuous breeding protocol to discriminate the qualitative and quantitative reproductive effects of the more and less active congeners as well as the large differences in reproductive toxicity attributable to subtle changes in the alkyl substitution of phthalate esters. © 1987 Academic Press, Inc.

Phthalic acid esters are widely used as industrial plasticizers. By far, the major use is in polyvinyl chloride products, including numerous consumer items and medical supplies. In 1978, annual production of di-(2-ethylhexyl) phthalate (DEHP) was estimated at 400 million pounds (U.S. International Trade Commission, 1978). The biological activity of some phthalates is known and has been reviewed (Thomas *et al.*, 1978). Diethyl phthalate (DEP) has been shown to be inactive as a reproductive toxicant, while di-*n*-butyl phthalate (DBP), di-*n*-hexyl phthalate (DHP), and DEHP have been shown to adversely affect reproduction (Gangolli, 1982, for review).

The data on effects of the phthalate esters on female reproduction are limited. There are a few investigations on the teratogenicity

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of the phthalate esters, but little or no data on the effects of the phthalates on fertility in females. Both DBP and DEHP caused adverse effects on fetal development and increased resorptions when given in the diet of pregnant mice (Shiota and Nishimura, 1982). Both DBP and DEHP exposures were related to an increase in the incidence of external anomalies in the offspring (Shiota and Nishimura, 1982).

The developmental toxicity of DEP, DBP, DHP, and DEHP was studied with CD-1 mice in a short-term developmental toxicity protocol (NIOSH, unpublished data). In those studies, the compounds were administered by gavage at dose levels resulting in 0 to 10% maternal mortality. DEP caused 4% maternal mortality, but did not have any fetal effects; 10% of the DBP-exposed dams died and there were no viable litters; DHP-treated dams had 2% mortality and no viable litters; and DEHP caused no maternal mortality and there was a low incidence of viable litters (2/32).

Testicular toxicity has been demonstrated in the rat after exposure to DBP, diisobutyl phthalate, di-*n*-pentyl phthalate, DHP, and DEHP, but not dimethyl phthalate (DMP), DEP, dipropyl phthalate (DPP), di-*n*-heptyl phthalate, or di-*n*-octyl phthalate (Cater *et al.*, 1977; Foster *et al.*, 1980; Gray and Butterworth, 1980; Oishi and Hiraga, 1980). Monoesters of phthalic acid were similarly effective at inducing testicular atrophy (Foster *et al.*, 1981).

There are significant species differences in the testicular toxicity of the phthalate esters (Gray *et al.*, 1982). Rats and guinea pigs developed severe atrophy after treatment with DBP (2 g/kg/day for 7 or 9 days), mice developed only focal atrophy, and hamsters were not affected. Other investigators also demonstrated that the mouse is somewhat less sensitive to the testicular effects of DEHP and of mono-(2-ethylhexyl) phthalate than rats (Curto and Thomas, 1982). Adverse effects were not observed after DEP exposure in rats (Foster *et al.*, 1980), although the female rat has not been studied. The testicular toxicity of DHP in mice is similar to that observed in rats (Foster *et al.*, 1980).

DEP, DBP, DHP, and DEHP were tested by a continuous breeding protocol to evaluate their effects on fertility and reproduction in mice. This approach has been demonstrated to be useful in identifying effects on male and female reproductive function (Lamb, 1985; Reel *et al.*, 1985; Lamb *et al.*, 1985a,b). The protocol consists of a 14-week period during which male and female mice are cohabited as breeding pairs. The data collected during this period include the number of litters per pair, the number of pups per litter, the proportion of pups born alive, and the mean pup weight. Each pair may produce up to five litters, each of which is evaluated. The pairs are segregated at the end of 14 weeks so the females may deliver the final litter which can be used to evaluate reproductive effects on the second generation if adverse effects are not seen in the first generation. If reproduction is affected in the first generation, a crossover mating trial is conducted; that trial consists of cohabiting control males with treated females and treated males with control females to determine which sex has been affected. The first and second generation may be necropsied and target organs evaluated at the end of the respective mating trials.

The animals in these studies were exposed to dietary levels as high as 2.5% DEP, 1.0% DBP, 1.2% DHP, or 0.3% DEHP in the diet. Differences in the reproductive effects of the chemicals were measured and related to the alkyl substitution of the phthalic acid esters.

METHODS

Chemicals. DEP was obtained from Chemical Tech Industries (Kansas City, MO). Spectrophotometric and chromatographic analyses indicated that the test material was >99% pure and was stable in feed for 2 weeks at −20°C or at room temperature.

DBP was obtained from Chem Central (Kansas City, MO) and was determined to be >99% pure by thin-layer chromatography by two systems and gas chromatography by two systems. DBP was stable in the feed for up to

3 weeks at −20°C and at least 7 days when exposed to ambient room air and light.

DHP was synthesized by the Midwest Research Institute (Kansas City, MO) and was shown to be approximately 98% pure by thin-layer chromatography and gas chromatography by two systems. DHP was stable in the feed for up to 3 weeks at room temperature when stored in the dark and at least 7 days in room air and light.

DEHP was obtained from Hatco Chemical Corp. (Fords, NJ) and was found by chromatographic and spectrophotometric analyses to be >99% pure. DEHP was stable in the feed in room air and light for 7 days.

Animals. COBS Cr1:CD-1, (ICR)BR outbred albino mice (6 weeks of age) were purchased from Charles River Breeding Laboratories, Inc. (Kingston, NY). Two males and two females were sacrificed and their sera evaluated for antibodies against 11 mouse viruses (Microbiological Associates, Inc., Bethesda, MD). All sera were negative for viral antibodies. All study animals were individually identified and assigned to treatment groups using a stratified randomization procedure based on body weights.

Males and females were group housed by sex in solid-bottom polypropylene or polycarbonate cages with stainless-steel wire lids, during quarantine and the 1-week premating period. Subsequently, the animals were housed as breeding pairs or individually. Ab-Sorb-Dri bedding (Laboratory Products, Inc. Garfield, NJ) was used in all cages. Deionized/filtered water and ground rodent chow were provided *ad libitum*. Automatically controlled photoperiods were 14-hr light/10-hr dark (lights on 0700 to 2100 hr) and temperature was maintained at 23 ± 2°C. Cages were sanitized weekly using detergent and 180°F water.

Dosage formulation and analysis. Each concentration of each compound was mixed separately every week. A stock mixture of dosed feed was prepared by weighing an aliquot of test chemical and mixing it with a small amount of powdered feed in a beaker. DEP, DBP, and DHP diets were prepared with NIH-07 diet (Zeigler Bros., Gardners, PA) while DEHP diets were prepared with Purina certified chow (5002; St. Louis, MO). The stock mixture was then added to a preweighed portion of untreated ground feed and mixed in a Patterson-Kelly 8-quart stainless-steel V-type blender and blended for at least 20 min. Diets with DEP and DBP were stored at −20°C, and diets with DHP and DEHP were stored at 4°C until used. For each chemical, an aliquot of each formulation, and feed control, and the bulk material was sent to Midwest Research Institute (MRI) for referee analysis every 6 weeks. The measured concentrations were between 96 and 100% for DEP, 96 and 107% for DBP, 99 and 103% for DHP, and 86 and 107% for DEHP of target concentration.

General study design. The specific study design has been published (Reel *et al.*, 1985; Lamb *et al.*, 1985a,b). The 14-day dose-setting study for DEP utilized one control group and five groups of dosed animals (n = 8 males and 8 females per treatment level). The end points for this study were clinical signs, mortality, body weight gain, and consumption of food and water. The continuous breeding phase used a control group (n = 40 animals of each sex) and three dose groups (n = 20 animals of each sex). The mice were exposed to the chemical for a 7-day premating period and were then randomly grouped as mating pairs and cohabited and exposed for 98 days. Data were collected on all newborns during this period (body weight, proportion of males, number of litters per pair, number of live pups) within 12 hr of birth after which each litter was discarded. After the 98-day cohabitation, the pairs were separated for 21 days and exposed during which any final litters were delivered and kept for at least 21 days. For DEP, these final litters were allowed to mature while the mother continued to receive dosed feed until the pups were weaned at 21 days of age. When these F_1 animals were sexually mature (ca. 74 days), male offspring were mated to female offspring from the same treatment group (n = 20/group/sex), and the F_2 litters were examined for litter size, survival, sex, and pup weight. The F_1 animals were then necropsied.

When significant adverse effects were observed in the continuous breeding phase, a crossover mating trial was performed to determine whether F_0 males or females were more sensitive to the effects. For DBP, DHP, and DEHP, there were adverse effects noted on reproduction. Thus, at the end of the continuous breeding phase, the high-dose animals of each sex were mated to control mice of the opposite sex to determine the affected sex. The high-dose animals were selected to increase the possibility of detecting effects in the crossover mating. Three combinations of control and treated mice were selected: control males with control females, high-dose males with control females, and control males with high-dose females. The offspring of the crossover matings were analyzed as above, and the parents were necropsied. The crossover mating was not done for DEP, since significant effects not observed in the continuous breeding phase.

Necropsies were performed when there was evidence of an effect on reproduction or, at the least, in the second generation if there was no effect. End points examined for the females included organ weights and histology. At necropsy, the end points of target organ toxicity examined for the males included organ weights and histology, percentage of motile sperm, sperm concentration, and percentage of abnormal sperm (Wyrobek and Bruce, 1975). The right cauda epididymis was excised and weighed to the nearest 0.001 g. Sperm motility was assessed immediately following the removal of the cauda epididymis from the test animal. A small amount of seminal fluid was pressed from the cauda into a drop of Tyrode's solution on a prewarmed slide (37°C). The sample was coverslipped and examined for percentage of motile sperm, under 400 magnification with a stage warmer attached to the microscope. The data were reported as percentage of motile sperm per sample.

The entire cauda epididymis was placed in a petri dish with 2.0 ml sterile Tyrode's solution (pH 7.4) and gently chopped and the contents "milked out." The petri dish is swirled to disperse the sperm and incubated for 15 min. After the 15-min incubation, the sperm suspension is swirled several times and gently flushed through a Pasteur pipet.

Sperm count was performed as follows. A 0.5-ml aliquot of the original sperm suspension was placed in a test tube (15 × 100 mm) and diluted with 1.0 ml Tyrode's solution and mixed on a Vortex mixer. The sperm were killed by placing the test tube under hot running water for 1 min. The suspension was agitated and two aliquots were counted per sample in a hemocytometer (Fisher Scientific, Raleigh, NC). The data were reported as number of cauda epididymal sperm per milligram of tissue.

The procedures and criteria of Wyrobek and Bruce (1975) were used to evaluate sperm morphology. The remaining original sperm suspension (approximately 1 ml) was transferred to a test tube (10 × 75 mm) and one drop of 1% Eosin Y stain in water was added. The test tube was allowed to sit for approximately 45 min. The sperm were then resuspended. Four slides were made from each suspension, air dried overnight, and coverslipped. For each sample, 500 sperm were examined if possible at 400 magnification and scored as normal or abnormal. The data were reported as percentage of abnormal sperm per sample.

Statistical analysis. The Cochran–Armitage test (Armitage, 1971) was used to test for a dose-related trend in fertility. Pairwise comparisons involving mating and fertility indices were performed using Fisher's exact test.

The number of litters and the number of live pups per litter were computed on a per fertile pair basis and then treatment group means determined. The proportion of live pups was defined as the number of pups produced by each pair. The sex ratio was expressed as the proportion of male pups born to each fertile pair. Dose group means for these parameters were tested for overall differences by using the Kruskal–Wallis test (Conover, 1980) and for ordered differences by using Jonckheere's test (Jonckheere, 1954). Pairwise comparisons of treatment group means were performed by applying the Wilcoxon–Mann–Whitney U test (Conover, 1980).

Since the number of pups in a litter may affect the average weight of the litter, an analysis of covariance (Neter and Wasserman, 1974) was used to test for treatment differences in average pup weight, adjusting for average litter size (live and dead pups). Pairwise comparisons were done using a two-sided t test. A Kruskal–Wallis test was also performed.

For the organ weights, least-squares treatment group means were generated from an analysis of covariance (with body weight as the covariate) and were tested for overall equality using the F test, and for pairwise equality using a t test. All comparisons were two sided. The Kruskal–Wallis and Wilcoxon–Mann–Whitney U tests were also employed.

RESULTS

Diethyl Phthalate

The 14-day repeated-dose study included levels of 0.0, 0.25, 0.5, 1.0, 2.5, and 5.0% DEP in the feed. There were no clinical signs of toxicity or mortality in any group. The high-dose group gained significantly less weight than the controls, while animals in the 0.5% group gained more weight than animals in the 0.0, 2.5, or 5.0% groups.

Dose levels of 0.0, 0.25, 1.25, and 2.5% DEP were selected for the continuous breeding phase of the study. No treatment-related changes in physical appearance or demeanor were noted during this period. One male in the 1.25% group and two males and a female in the 2.5% DEP group died. At the beginning of the study, the control and high-dose groups weighed an average of 35.1 and 34.1 g, respectively. By Week 13, the controls weighed an average of 38.0 g, and the high-dose males weighed an average of 35.6 g; the 2.5% DEP animals weighed significantly less than controls at Week 13. The weight gain of females was not significantly affected. Analysis of feed consumption for the pairs showed that between 5.0 and 5.6 g of feed was consumed per mouse per day, regardless of treatment group.

Exposure to DEP did not alter the number of fertile pairs, the number of litters per pair, the number of pups per litter, the proportion of pups alive, or the live pup birth weight (Table 1).

Since fertility and reproductive performance were not affected in the parental mice (F_0 generation), the fertility and reproductive performance of the offspring were assessed once they reached sexual maturity. The final litters from the continuous breeding phase (F_1 generation) in the 0.0 and 2.5% DEP groups were weaned at 21 days of age. The F_1 animals had access to diet with the same levels of DEP as their parents until the end of the study. When these F_1 animals were approximately 10 weeks of age, pairs of control mice and pairs of 2.5% DEP-exposed mice were co-

TABLE 1

FERTILITY AND REPRODUCTIVE PERFORMANCE OF MATING PAIRS (F_0 GENERATION) DURING CONTINUOUS BREEDING

	% Diethylphthalate in diet			
	Control	0.25	1.25	2.5
No. fertile/No. cohabited[a]	40/40 (100)	20/20 (100)	19/19 (100)	18/18 (100)
Litters/pair[b] (*n*)	4.73 ± 0.16 (40)	4.90 ± 0.07 (20)	5.00 ± 0.00 (19)	5.00 ± 0.08 (18)
Live pups/litter[b] (*n*)	10.27 ± 0.48 (40)	12.64 ± 0.34 (20)[c]	11.73 ± 0.30 (19)[d]	10.61 ± 0.43 (18)
Proportion of pups born alive[b] (*n*)	0.96 ± 0.02 (40)	1.00 ± 0.00 (20)	1.00 ± 0.00 (19)	0.99 ± 0.00 (18)
Live pup weight[b] (*n*)	1.63 ± 0.02 (40)	1.59 ± 0.02 (20)	1.60 ± 0.02 (19)	1.64 ± 0.03 (18)
	% Di-*n*-butyl phthalate in diet			
	Control	0.03	0.3	1.0
No. fertile/No. cohabited (%)	39/39 (100)	20/20 (100)	18/18 (100)	15/20 (75)[c]
Litters/pair (*n*)	4.85 ± 0.09 (39)	5.00 ± 0.07 (20)	4.72 ± 0.18 (18)	1.80 ± 0.26 (15)[c]
Live pups/litter (*n*)	12.08 ± 0.38 (39)	12.27 ± 0.38 (20)	11.73 ± 0.45 (18)	1.72 ± 0.49 (15)[c]
Proportion of pups born alive (*n*)	1.00 ± 0.00 (39)	1.00 ± 0.00 (20)	0.99 ± 0.01 (18)	0.50 ± 0.11 (15)[c]
Live pup weight (*n*)	1.58 ± 0.02 (39)	1.56 ± 0.02 (20)	1.57 ± 0.02 (18)	1.64 ± 0.07 (10)
	% Di-*n*-hexyl phthalate in diet			
	Control	0.3	0.6	1.2
No. fertile/No. cohabited (%)	37/37 (100)	14/17 (82)[d]	1/19 (5)[c]	0/16 (0)[c]
Litters/pair (*n*)	4.89 ± 0.05 (37)	3.43 ± 0.34 (14)[c]	4.00 (1)	—
Live pups/litter (*n*)	12.29 ± 0.40 (37)	3.43 ± 0.48 (14)[c]	6.50 (1)	—
Proportion of pups born alive (*n*)	0.99 ± 0.00 (37)	0.84 ± 0.03 (14)[c]	0.79 (1)	—
Live pup weight (*n*)	1.60 ± 0.01 (37)	1.77 ± 0.05 (14)[c]	1.65 (1)	—
	% Di(2-ethylhexyl) phthalate in diet			
	Control	0.01	0.1	0.3
No. fertile/No. cohabited (%)	40/40 (100)	20/20 (100)	14/19 (74)	0/18 (0)[c]
Litters/pair (*n*)	4.65 ± 0.13 (40)	4.65 ± 0.18 (20)	3.07 ± 0.49 (14)[c]	—
Live pups/litter (*n*)	10.62 ± 0.32 (40)	9.92 ± 0.50 (20)	5.16 ± 1.13 (14)[c]	—
Proportion of pups born alive (*n*)	0.98 ± 0.01 (40)	0.99 ± 0.01 (20)	0.80 ± 0.09 (14)[c]	—
Live pup weights (*n*)	1.57 ± 0.02 (40)	1.58 ± 0.03 (20)	1.62 ± 0.04 (13)[c]	—

[a] Pairs were considered fertile if they produced one or more litters.
[b] Values are $\bar{x} \pm$ SE.
[c] Significantly different from control ($p < 0.01$).
[d] Significantly different from control ($p < 0.05$).

TABLE 2

FERTILITY AND REPRODUCTIVE PERFORMANCE OF PAIRS OF OFFSPRING (F_1 GENERATION): CONTINUOUS EXPOSURE TO DIETHYL PHTHALATE

	% Diethyl phthalate in diet	
	Control	2.5
No. with copulatory plugs/No. cohabited (%)	20/20 (100)	20/20 (100)
No. fertile/No. cohabited[a] (%)	19/20 (95)	19/20 (95)
Live pups per litter[b] (n)	11.53 ± 0.54 (19)	9.95 ± 0.57 (19)[c]
Proportion of pups born alive[b] (n)	0.99 ± 0.01 (19)	0.92 ± 0.05 (19)
Live pup weight in g[b] (n)	1.52 ± 0.03 (19)	1.47 ± 0.02 (19)

[a] A pair was judged fertile if it produced a litter of one or more live or dead pups.
[b] Values are $\bar{x}$ ± SE.
[c] Significantly different from control ($p < 0.05$).

habited to evaluate reproductive function. Body weight was decreased in the treated animals compared to controls; at weaning the male body weights were 8.22 ± 0.37 and 10.96 ± 0.64 g (n = 20, both groups, mean ± SE), respectively. At the start of cohabitation, these values for treated and control males were 29.13 ± 0.74 and 32.91 ± 0.81 g (n = 20 for each) for treated and control, respectively. Table 2 shows that exposure of the F_1 animals to DEP had no adverse effect on fertility, the proportion of pups born alive, and the live pup birth weight or sex, although the total number of live pups per litter (combined male and female) was significantly lower in the 2.5% DEP group.

The F_1 mice were necropsied at the conclusion of the offspring assessment. Table 3 contains data on organ weights and sperm parameters for these adult males at necropsy. Body and organ weights are given for the females in Table 4. Body weight was decreased by DEP in both sexes, while male prostate and female liver weight were increased, and female pituitary weight was decreased. Though sperm motility and the percentage of abnormal sperm were not affected by DEP exposure, the sperm concentration was decreased in the treated animals.

Di-n-butyl Phthalate

Sufficient data were available (NIOSH, unpublished data) to select DBP dietary levels without performing the 14-day repeated-dose study. The dose levels selected were 0.0, 0.03, 0.3, and 1.0% DBP.

There were no treatment-related signs of toxicity in the continuous breeding phase of the DBP study. Two control males died, and one male and one female from the 0.3% DBP group died. No animals from the 0.03 and 1.0% groups died during the study. Feed consumption was not affected by the addition of DBP to the diet. In Week 1 the body weights were 37.3 and 37.0 for the control and high-dose male mice and by Week 13 the control males weighed an average of 42.9 g and the high-dose males weighed 39.8 g. The female mice weighed 28.6 and 29.4 g at Week 1 and 39.8 and 38.1 g at Week 13 for the control and high-dose mice, respectively.

Only the high dose of DBP significantly affected reproduction, decreasing the fertility of breeding pairs, the number of litters per fertile pair, the average number live pups per litter, and the proportion of pups born alive compared to the control group (Table 1).

A crossover mating was conducted and while the proportion of detected matings was not affected, the proportion of fertile matings was significantly reduced in the control male with 1% DBP female group (Table 5). In addition, the number of live pups per litter, the proportion of pups born alive, and the live pup weight were signifi-

TABLE 3

MALE BODY WEIGHTS AND ORGAN WEIGHTS AND SPERM PARAMETERS OF OFFSPRING AT NECROPSY (F_1 GENERATION): DIETHYL PHTHALATE

	% Diethyl phthalate in diet	
	Control	2.5
Body weight (g)	34.16 ± 0.81 (20)[a]	30.20 ± 0.61 (20)
Liver (g)	1.83 ± 0.06 (20)	1.89 ± 0.05 (20)
Brain (g)	0.48 ± 0.00 (20)	0.48 ± 0.01 (20)
Pituitary (mg)	2.2 ± 0.1 (20)	2.1 ± 0.1 (20)
Left testis and epididymis (mg)	183 ± 5.7 (20)	167 ± 8.2 (20)
Right testis (mg)	137 ± 4.4 (20)	126 ± 3.9 (20)
Right epididymis (mg)	51 ± 1.3 (20)	51 ± 1.6 (20)
Prostate (mg)	25 ± 1.3 (20)	33 ± 2.3 (20)[b]
Seminal vesicles (mg)	340 ± 17.4 (20)	301 ± 10.8 (20)
% Motile sperm	60.20 ± 6.82 (20)	57.60 ± 6.61 (20)
Sperm concentration (No. sperm × 10^3/mg caudal tissue)	1021 ± 48 (20)	718 ± 36 (20)[b]
% Abnormal sperm[c]	3.97 ± 1.04 (20)	6.57 ± 2.08 (20)

[a] Values are $\bar{x}$ ± SE (n) (significance indicated if $p < 0.05$).
[b] Significantly different from control ($p < 0.01$).
[c] Tailless sperm not included in determination of percentage of abnormal sperm.

cantly decreased in this mating group compared to control pairs.

The control and 1.0% DBP-exposed F_0 mice were necropsied 26 days after the completion of the 7-day crossover mating trial.

TABLE 4

FEMALE BODY WEIGHTS AND ORGAN WEIGHTS OF OFFSPRING AT NECROPSY (F_1 GENERATION): DIETHYL PHTHALATE

	Diethyl phthalate in diet	
	Control	2.5
Body weight (g)	30.57 ± 0.61 (20)[a]	28.21 ± 0.51 (20)[b]
Liver (g)	1.84 ± 0.06 (19)	2.12 ± 0.07 (20)[b]
Brain (g)	0.48 ± 0.00 (20)	0.48 ± 0.00 (20)
Pituitary (mg)	3.5 ± 0.1 (20)	2.9 ± 0.1 (20)[b]
Ovary and oviduct (mg)	39 ± 2.1 (20)	38 ± 1.7 (20)
Uterus (mg)	340 ± 25.3 (20)	326 ± 23.9 (20)

[a] Values are $\bar{x}$ ± SE (n) (significance indicated if p < 0.05).
[b] Significantly different from control ($p < 0.01$).

Sperm assessment did not demonstrate any significant difference in the percentage of motile sperm, the sperm concentration, or the percentage of abnormal sperm in the cauda epididymis between male mice exposed to 0.0 or 1.0% DBP in the diet (Table 6). On the other hand, body weight was significantly decreased and the adjusted liver weight was significantly increased (2.16 ± 0.04 g for controls compared to 2.40 ± 0.05 g for 1.0% DBP; $p < 0.01$) in the male mice fed 1.0% DBP (Table 6). In the F_0 females, liver weight was significantly increased and uterine weight was significantly decreased in the 1.0% DBP-exposed group versus the control group (Table 7). No treatment-related gross or histopathologic lesions were noted for the testis, epididymis, prostate, or seminal vesicles in the male mice, or for the ovary, oviduct, uterus, or vagina in the female mice.

Di-n-hexyl Phthalate

No dose finding study was performed for DHP, since general toxicity data were avail-

TABLE 5

MATING TRIAL OF F_0 PAIRS TO DETERMINE THE AFFECTED SEX: DI-*n*-BUTYL PHTHALATE

	F_0 pairings		
	Control male × control female	1.0% DBP male × control female	Control male × 1.0% DBP female
No. with copulatory plugs/ No. cohabited (%)	16/19 (84)	18/20 (90)	12/19 (63)
No. fertile/No. cohabited[a] (%)	14/19 (74)	17/20 (85)	4/19 (21)[b]
Live pups/litter[c] (*n*)	7.71 ± 0.94 (14)	9.35 ± 0.87 (17)	0.75 ± 0.25 (4)[b]
Proportion of pups born alive[c] (*n*)	0.95 ± 0.05 (14)	0.99 ± 0.01 (17)	0.63 ± 0.24 (4)[d]
Live pup weight in g[c] (*n*)	1.83 ± 0.10 (14)	1.69 ± 0.04 (17)	1.41 ± 0.11 (3)[d]

[a] A pair was judged fertile if it produced a litter of one or more live or dead pups.
[b] Significantly different from controls ($p < 0.01$).
[c] Values are $\bar{x} \pm$ SE.
[d] Significantly different from control ($p < 0.05$).

able (NIOSH, unpublished data). Dietary amounts of 0.0, 0.3, 0.6, and 1.2% DHP were used in the continuous breeding phase of the study. In the control group three males died, in the 0.3% group three females died, in the 0.6% group one female died, and in the 1.2% group four females died. Food consumption was not affected by the addition of up to 1.2% DHP to the diet. Mean body weights for the male mice were 38.1, 38.7, 39.1, and 38.2 g

TABLE 6

F_0 MALE BODY WEIGHTS, ORGAN WEIGHTS, AND SPERM PARAMETERS AT NECROPSY: DI-*n*-BUTYL PHTHALATE

	% Di–butyl phthalate in diet	
	Control	1.0
Body weight (9)	42.53 ± 0.65 (37)[a]	39.00 ± 0.66 (20)[b]
Liver (g)	2.23 ± 0.05 (38)	2.27 ± 0.06 (20)
Brain (g)	0.48 ± 0.01 (38)	0.47 ± 0.01 (20)
Pituitary (mg)	2.3 ± 0.1 (36)	2.2 ± 0.1 (19)
Left testis and epididymis (mg)	204 ± 5.5 (38)	187 ± 7.6 (20)
Right testis (mg)	146 ± 3.8 (38)	138 ± 6.4 (20)
Right epididymis (mg)	61 ± 1.2 (38)	50 ± 2.0 (20)
Prostate (mg)	53 ± 3.4 (38)	45 ± 3.2 (20)
Seminal vesicles (mg)	443 ± 17.5 (38)	402 ± 13.6 (20)
% Motile sperm	55.58 ± 3.39 (38)	57.20 ± 5.71 (20)
Sperm concentration (No. sperm × 10^3/mg caudal tissue)	744 ± 26 (38)	713 ± 46 (20)
% Abnormal sperm[c]	5.50 ± 0.68 (38)	5.16 ± 0.65 (20)

[a] Values are $\bar{x} \pm$ SE.
[b] Significantly different ($p < 0.01$) from control group.
[c] Tailless sperm not included in determination of percentage of abnormal sperm.

TABLE 7

F_0 FEMALE BODY WEIGHTS AND ORGAN WEIGHTS AT NECROPSY: DI-*n*-BUTYL PHTHALATE

	% Di-*n*-butyl phthalate in diet	
	Control	1.0
Body weight (g)	39.17 ± 0.77 (39)[a]	37.86 ± 0.59 (19)
Liver (g)	2.08 ± 0.04 (39)	2.43 ± 0.05 (19)[b]
Brain (g)	0.50 ± 0.01 (39)	0.49 ± 0.00 (19)
Pituitary (mg)	3.8 ± 1.1 (39)	3.9 ± 0.2 (19)
Ovary and oviduct (mg)	41 ± 2.5 (39)	38 ± 1.4 (19)
Uterus (mg)	342 ± 18.3 (39)	245 ± 16.9 (19)[b]

[a] Values are $\bar{x}$ ± SE (n) (significance indicated if $p < 0.05$).

[b] Significantly different ($p < 0.01$) from the combined control group.

in Week 1 and 42.2, 41.5, 39.5, and 38.4 g in Week 13 for the control, 0.3, 0.6, and 1.2% groups, respectively. These data clearly demonstrate a dose-related decrease in body weight gain by DHP in the diet.

Exposure to DHP resulted in a dose-related decrease in the proportion of pairs able to produce even a single litter during the continuous breeding phase of the study (Table 1). Adverse effects were observed at levels below those exhibiting large effects on body weight. There were no litters in the 1.2% group and only 1 pair had a litter in the 0.6% group compared to all 37 pairs delivering at least one litter in the controls and 14 of the 17 pairs in the 0.3% group had at least one litter. The number of litters per pair, the number of live pups per litter, and the proportion of pups born alive were also significantly affected by DHP exposure. There were significant adverse effects on reproduction at the lowest dose, such that a no-observable-effect-level could not be determined in this particular study. Pup weight was greater in the 0.3% group than the controls, which was probably related to the smaller litter size in the 0.3% group. After adjusting statistically for litter size by an analysis of covariance (data not presented), there was no significant difference in pup weight.

A crossover mating study was performed. There was a significant decrease in the proportion of detected matings for the males receiving 1.2% DHP mated with control females (56%) compared to the controls (90%) and only 1 of the 18 treated males sired a litter (Table 8). Although there was no decrease in copulatory plugs detected when control males were mated with females receiving 1.2% DHP (88%) (Table 8), none of the DHP-treated females became pregnant, demonstrating that fertility was severely affected in both sexes.

The control and 1.2% DHP-exposed male and female F_0 mice were weighed and necropsied at the conclusion of the crossover mating study (Tables 9 and 10). Sperm assessment showed that the percentage of motile sperm and the sperm concentration in the cauda epididymis were significantly diminished in the 1.2% DHP-treated males compared to controls (Table 9). The percentage of abnormal sperm was unexpectedly lower in the treated males than in the control males, but only 3 of the 18 males in the 1.2% DHP-treated group had sufficient sperm to allow determination of the percentage abnormal sperm. There were significant decreases in the weights of the testis, epididymis, and seminal vesicles in the DHP-treated mice. Histological evaluation revealed extensive atrophy of the seminiferous tubules in mice given diets containing 1.2% DHP; i.e., the tubules were lined primarily by Sertoli cells and lacked evidence of normal spermatogenesis. Mature sperm were markedly diminished in the epididymides of mice receiving 1.2% DHP (as measured by hemocytometer counts). Microscopic changes were observed in the seminal vesicles of 3 of the 18 mice receiving 1.2% DHP. In contrast to the reproductive tract and gonadal effects for male mice, no treatment-related gross or microscopic lesions were detected in the ovaries, uterus, or vagina of the F_0 female mice.

TABLE 8

MATING TRIAL OF F_0 PAIRS TO DETERMINE THE AFFECTED SEX: DI-*n*-HEXYL PHTHALATE

	F_0 pairings		
	Control male × control female	1.2% DHP male × control female	Control male × 1.2% DHP female
No. with copulatory plugs/No. cohabited (%)	18/20 (90)	10/18 (56)[a]	15/17 (88)
No. fertile/No. cohabited[b] (%)	17/20 (85)	1/18 (6)[c]	0/17 (0)[c]
Live pups/litter[d] (*n*)	9.41 ± 0.92 (85)	14.00 (1)	—
Proportion of pups born alive[d] (*n*)	0.94 ± 0.04 (17)	1.00 (1)	—
Live pup weight in g[d] (*n*)	1.64 ± 0.04 (17)	1.58 (1)	—

[a] Significantly different from control ($p < 0.05$).
[b] A pair was judged fertile if it produced a litter of one or more live or dead pups.
[c] Significantly different from control ($p < 0.01$).
[d] Values are $\bar{x} \pm$ SE.

Body and kidney/adrenal weights were significantly decreased and liver weight was significantly increased in F_0 male and female mice fed 1.2% DHP in the diet compared to controls (Tables 9 and 10). Despite the organ weight changes, no treatment-related histopathologic differences were noted in the liver, kidney, or adrenal gland in the F_0 male or female mice.

Di(2-ethylhexyl) Phthalate

Doses for the continuous breeding phase were set at 0.0, 0.01, 0.1, and 0.3% DEHP by

TABLE 9

F_0 MALE BODY WEIGHTS, ORGAN WEIGHTS, AND SPERM PARAMETERS AT NECROPSY: DI-*n*-HEXYL PHTHALATE

	% Di-*n*-hexyl phthalate in diet	
	Control	1.2
Body weight (g)	40.33 ± 0.68 (37)[a]	36.19 ± 0.58 (18)[b]
Liver (g)	2.14 ± 0.05 (37)	2.74 ± 0.08 (18)[b]
Kidneys and adrenals (9)	0.86 ± 0.02 (36)	0.75 ± 0.03 (18)[b]
Left testis and epididymis (mg)	206 ± 5 (37)	93 ± 10 (18)[b]
Right testis (mg)	140 ± 3 (37)	42 ± 7 (18)[b]
Right epididymis (mg)	58 ± 2 (37)	42 ± 2 (18)[b]
Prostate (mg)	56 ± 5 (37)	46 ± 8 (18)
Seminal vesicles (mg)	499 ± 15 (37)	376 ± 21 (17)[b]
% Motile sperm	68.61 ± 3.89 (36)	14.83 ± 6.66 (6)[b,c]
Sperm concentration (No. sperm × 10^3/mg caudal tissue)	357 ± 25 (35)	25 ± 22 (18)[b]
% Abnormal sperm[d]	10.97 ± 0.87 (34)	8.02 ± 1.05 (3)[b,e]

[a] Values are $\bar{x} \pm$ SE (*n*) (significance indicated if $p < 0.05$).
[b] Significantly different ($p < 0.01$) from control group.
[c] Only 6 of 18 males had sufficient sperm counts for determining motility.
[d] Tailless sperm were not included in the determination of abnormal sperm.
[e] Only 3 of 18 males had a sufficient sperm count for determining abnormal and tailless sperm.

TABLE 10

F_0 FEMALE BODY WEIGHTS AND ORGAN WEIGHTS AT NECROPSY: DI-n-HEXYL PHTHALATE

	% Di-*n*-hexyl phthalate in diet	
	Control	1.2
Body weight (g)	37.06 ± 0.60 (38)[a]	34.94 ± 0.57 (17)[b]
Liver (g)	2.19 ± 0.05 (38)	2.70 ± 0.11 (17)[c]
Kidneys and adrenals (g)	0.64 ± 0.01 (38)	0.57 ± 0.02 (17)[c]

[a] Values are $\bar{x}$ ± SE (n).
[b] Significantly different ($p < 0.05$) from the control group.
[c] Significantly different ($p < 0.01$) from the control group.

weight in the feed. There were no treatment-related clinical signs of toxicity during the breeding phase of the study; one male from the 0.1% and two females from the 0.3% DEHP groups died. Analysis of feed consumption showed that the mice consumed between 4.8 and 5.4 g of food per day, regardless of treatment group. Mean body weights for male mice were 36.3 and 34.6 g for control and high-dose groups, respectively, at Week 1 and 36.9 and 37.4 g for control and high-dose groups, respectively, at Week 13.

Exposure to DEHP produced a dose-dependent decrease in the number of litters as well as the number and proportion of pups born alive and significant adverse effects were demonstrated in pairs exposed to 0.01% DEHP (Table 1).

Since fertility and reproductive performance were adversely affected by DEHP, a crossover mating trial was conducted with the F_0 mice. There was no apparent change in libido based on the proportion of copulatory plugs; however, there was a decrease in fertility for treated males and treated females compared to matings of control mice. Four litters were born to treated males × control females, and the proportion of pups born alive was decreased. There were no litters born to the control male × treated female pairs (Table 11).

After the crossover mating study, the mice were killed and selected organs were weighed (Tables 12 and 13). Although there were no differences in body weight of either sex, consumption of DEHP caused an enlargement of the liver, both absolute and relative to body weight. In addition, weights of the reproductive organs were reduced in both sexes.

While there were no DEHP treatment-related histologic lesions noted in the female mice, all but one of the high-dose male mice evidenced some degree of bilateral atrophy of the seminiferous tubules. In addition, 0.3% dietary DEHP reduced sperm motility and concentration and raised the incidence of abnormal sperm forms.

DISCUSSION

The continuous breeding study has been designed to improve the reliability and sensitivity of fertility assessment. In a standard multigeneration or mating trial study, fertility is measured on the basis of a 1- to 2-week mating trial which results in, at most, a single litter per breeding pair. At the conclusion of such a mating trial the individual pairs tested can be labeled only as either fertile or infertile. The historical data collected from control animals used in mating trial studies have demonstrated that fertility is a relatively insensitive end point of reproductive toxicity because of variations in control values (Schwetz *et al.*, 1980). The continuous breeding studies have been designed to decrease the variability of mating trials and to increase the information gained on fertility without substantially increasing study costs (Lamb, 1985).

Unlike the standard mating trial study, the continuous breeding study does not call for the separation of the male and female animals between mating and delivery of litters. The pairs are kept together for a 14-week period and multiple litters can be delivered to each pair. During the 14-week mating trial period the offspring are removed within 12 hr

TABLE 11

MATING TRIAL OF F_0 PAIRS TO DETERMINE THE AFFECTED SEX: DI(2-ETHYLHEXYL) PHTHALATE

Reproductive parameter	Treatment group: Control male × control female	0.3% DEHP male × control female	Control male × 0.3% DEHP female
No. with copulatory plugs/ No. cohabited (%)	18/20 (90)	16/20 (80)	13/16 (81)
No. fertile/No. cohabited[a] (%)	18/20 (90)	4/20 (20)[b]	0/16 (0)[b]
Live pups/litter[c] (*n*)	8.56 ± 0.82 (18)	6.50 ± 2.36 (4)	—
Proportion of pups born alive[c] (*n*)	0.91 ± 0.06 (18)	0.71 ± 0.24 (4)[b]	—
Live pup weight in g[c] (*n*)	1.64 ± 0.06 (17)[d,e]	1.73 ± 0.09 (3)[d,e]	—

[a] A pair was judged fertile if it produced a litter of one or more live or dead pups.
[b] Significantly different from control ($p < 0.05$).
[c] Values are $\bar{x} \pm$ SE.
[d] Significantly different from control ($p < 0.05$).
[e] All pups from one litter were dead.

of delivery and mating can occur at the first estrus postpartum. Information on fertility is collected in addition to that distinguishing fertile from non-fertile and actually gives some indices of subfertility. The number of litters per breeding pair is the measure in the protocol which can be used as an index of effects on fertility. The protocol also provides very stable information on reproductive effects because each pair produces as many as five litters and the data on the litters from each pair can be summed. This decreases the

TABLE 12

F_0 MALE BODY WEIGHT, ORGAN WEIGHTS AND SPERM PARAMETERS AT NECROPSY: DI(2-ETHYLHEXYL) PHTHALATE

	% Di(2-ethylhexyl) phthalate in diet: Control	0.3
Body weight (g)	38.25 ± 0.43 (35)[a]	39.01 ± 0.62 (19)
Liver (g)	2.23 ± 0.05 (36)	2.84 ± 0.07 (19)[b]
Right testis (mg)	135 ± 4.4 (36)	55 ± 7.9 (19)[a]
Right epididymis (mg)	58 ± 1.3 (36)	47 ± 1.9 (19)[b]
Prostate (mg)	70 ± 2.7 (36)	62 ± 4.1 (19)[c]
Seminal vesicles (mg)	369 ± 15.5 (36)	362 ± 15.1 (19)
% Motile sperm	87.47 ± 2.38 (36)	34.70 ± 13.41 (10)[b]
Sperm concentration (No. sperm × 10^3/mg caudal tissue)	473 ± 24 (36)	101 ± 50 (19)[b]
% Abnormal sperm[d]	2.01 ± 0.42 (36)	15.37 ± 5.50 (8)[b]

[a] Values are $\bar{x} \pm$ SE (*n*).
[b] Significantly different from control ($p < 0.01$).
[c] Significantly different from control ($p < 0.05$).
[d] Tailless sperm were not included in the determination of percentage of abnormal sperm.

TABLE 13

F_0 FEMALE BODY WEIGHTS AND ORGAN WEIGHTS AT NECROPSY: DI(2-ETHYLHEXYL) PHTHALATE

	% Di(2-ethylhexyl) phthalate in diet	
	Control	0.3
Body weight (g)	36.10 ± 0.45 (40)[a]	36.50 ± 0.40 (15)
Liver (g)	2.23 ± 0.04 (40)	3.03 ± 0.08 (15)[b]
Brain (g)	0.46 ± 0.01 (40)	0.46 ± 0.01 (15)
Pituitary (mg)	3.9 ± 0.2 (40)	3.6 ± 0.2 (14)
Ovaries and oviducts and uterus (mg)	399 ± 14.7 (40)	336 ± 28.8 (15)[c]

[a] Values are $\bar{x} \pm$ SE (n).
[b] Significantly different from the control group ($p < 0.01$).
[c] Significantly different from control group ($p < 0.05$).

ultimate variability of the data on parameters such as the number of live pups per litter, the proportion of pups born alive, and the live pup weight. It also allows the evaluation of data, such as the time between litters, and can identify whether or not certain effects on reproduction are only observable at specific times after the initial chemical exposure. In the case of the phthalate esters, the reproductive parameters were not time dependent (data not presented).

Although the continuous breeding study seems to improve significantly the data collected on fertility, there are certain trade-offs. First, since the male and female mice are housed as breeding pairs and the two sexes have such different body weights, it is not possible to determine accurately the relative dose of chemicals on a milligram per kilogram body weight basis. Such data can only be collected in either the range finding study or the separation period at the end of the 14-week mating trial. Second, a related problem is that multiple pregnancies are likely to have significant effects on the handling of the chemical by the female mouse. Third, the protocol produces large demands on data storage and analyses. Considering the importance of reliable data on the effects of chemicals on fertility, balanced with the relative imprecision of measurements of feed consumption in animal studies, the trade-off seems justified.

Beyond adding new insights into fertility assessment methods, these studies have provided new data on the effects of phthalate esters on fertility and reproductive function in both male and female mice. Previous studies have been limited to evaluating effects either on the testis or on development and have rarely evaluated fertility per se. These studies have demonstrated that fertility and reproduction are affected by exposure to DBP, DHP, and DEHP, but not DEP (Table 1).

At the top dose levels, all three phthalate esters (i.e., DBP, DHP, and DEHP) adversely affected the number of fertile matings. The top dose levels caused some decrease in body weight gain for DBP and DHP, but not DEHP. At dose levels where there were fertile matings, there was a decrease either in the number of live pups per litter or in the proportion of pups born alive. Although the developmental toxicity of DBP, DHP, and DEHP had been demonstrated (Shiota and Nishimura, 1982; NIOSH, unpublished data), the present investigations were the first to demonstrate effects on female fertility and reproduction (decreased litters or litter size) after exposure to the same compounds (Table 5, 8, and 11). Ideally in these studies the top dose group should cause some measure of general toxicity, such as a small decrease in body weight gain, to ensure that if effects are not produced it is not because the dose levels are too low. DEP did cause decreased body weight gain, but did not affect reproduction. The data from this study on DEP not only confirmed that the compound is not a developmental toxicant, but also demonstrated that it does not measurably affect fertility in the first generation; it was, however, associated with decreased litter size in the second generation.

These studies have also provided new data on the effects of phthalate esters on fertility in male mice. Both DHP and DEHP caused significant effects on fertility, epididymal sperm concentration, percentage of abnor-

mal sperm, and percentage of motile sperm. These new observations further expand the existing data demonstrating testicular atrophy after exposure to these phthalate esters (Cater *et al.*, 1977; Foster *et al.*, 1980; Gray and Butterworth *et al.*, 1980; Oishi and Hiraga *et al.*, 1980). The failure of DBP to cause significant adverse effects on fertility in male mice seems to be a species-specific response. It had been demonstrated that DBP does cause testicular atrophy in rats and guinea pigs, but mice were much less affected (Gray *et al.*, 1982). These continuous breeding studies show the lack of significant effects on the testis, epididymal sperm concentration, percentage of motile sperm, or percentage of abnormal sperm, in addition to the lack of effects on fertility and reproduction in male mice exposured to DBP.

In the continuous breeding studies, as in all reproductive toxicity tests, it is important to relate the findings in the reproductive system to the findings in other organ systems. The dose levels were selected such that severe systemic toxicity would not be caused at the high-dose level, to assure that effects on reproduction are not the result of severe effects on general health. The middle and low doses were selected to cause lesser or no adverse systemic effects, respectively. For all four compounds, the body weights (comparison based on male body weights) were within 3% for the high-dose groups and controls at the start of the studies. By the 13th week of the study, male body weights in the high-dose groups generally were lower than in the controls but were not suppressed more than 10%. An exception to this was DEHP, which did not significantly decrease body weight gain in the high-dose group, despite causing significant adverse effects on reproduction and fertility. Therefore, a common point of reference or at least a target for these studies is that the top dose group was selected to cause a 5–10% decrease in body weight gain by the 13th week.

These investigations have compared a comprehensive set of end points on fertility and reproduction in both male and female CD-1 mice. These data demonstrate that the number of litters per pair is a stable indicator of effects on fertility from study to study and across dose groups (Table 1). The compound DEP was given at sufficient levels to decrease body weight gain but did not affect fertility in the first generation, while the other phthalate esters, DBP, DHP, and DEHP, caused similar or lesser effects on body weight while severely affecting reproduction and fertility. The data on DEP help demonstrate that the model will tolerate significant effects on general health without causing changes in reproductive end points. The data from this protocol appear very reproducible and the protocol should be useful for the assessment of reproductive toxicity of a wide variety of chemicals.

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