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# Reproductive Toxicity of Three Phthalic Acid Esters in a Continuous Breeding Protocol

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Reproductive Toxicity of Three Phthalic Acid Esters in a Continuous Breeding Protocol. HEINDEL, J. J., GULATI, D. K., MOUNCE, R. C., RUSSELL, S. R., AND LAMB, J. C., IV. (1989). *Fundam. Appl. Toxicol.* **12**, 508–518. A continuous breeding protocol was utilized to examine the reproductive toxicity of three phthalate esters. CD-1 mice were given diets with either di-*n*-propyl phthalate (DPrP: 0.0, 1.25, 2.5, or 5.0%), di-*n*-pentyl phthalate (DPP: 0.0, 0.5, 1.25, or 2.5%), or di-*n*-octyl phthalate (DOP: 0.0, 1.25, 2.5, or 5.0%). Both male and female mice (20 pairs per treatment group, 40 pairs of control animals) were dosed for 7 days prior to and during a 98-day cohabitation period. Reproductive function was evaluated during the cohabitation period by measuring number of litters per pair, live pups per litter, and pup weight. There was no apparent effect on reproductive function in the animals exposed to DOP at dose levels sufficient to cause a significant increase in liver weight. Both DPP and DPrP were toxic to the reproductive system as evidenced by a complete inhibition of fertility at 1.25 and 2.5% DPP or 5.0% DPrP, and reduced fertility (litters/pair and live pups/litter, 0.5% DPP; live pups/litter, 2.5% DPrP). Toxicity of DPP had a strong male component and female component, whereas DPrP was more toxic to the female than the male reproductive system. DPP and DPrP treatment was associated with decreased body weight, increased liver weight, decreased testis and epididymis weights, decreased epididymal sperm concentration, and elevated seminiferous tubule atrophy. A comparison of seven phthalate esters tested using this continuous breeding protocol indicates the relative order of reproductive toxicity as diethylhexyl, dihexyl, dipentyl, dibutyl, dipropyl; diethyl and dioctyl are nontoxic. © 1989 Society of Toxicology.

Esters of *o*-phthalic acid are used extensively as plasticizers to impart flexibility in many consumer products and medical devices such as polyvinylchloride bags. While, in general, acute toxicity of the most commonly encountered phthalates is low, some phthalates have been shown to be carcinogenic, to result in liver toxicity, and to cause testicular atrophy in rodents at high doses. [See Albro (1987) and Thomas and Thomas (1984) for reviews.] Specifically, testicular atrophy has been shown to result from the administration of dibutyl, dipentyl, dihexyl, and diethylhexyl phthalate to rats, whereas phthalate esters of shorter and longer chain length (e.g., diethyl, diheptyl, and dioctyl phthalate) do not affect reproduction *in vivo* (Foster *et al.*, 1980; Gray and Butterworth, 1980; Gangolli, 1982; Oishi and Hiraga, 1980; Gray *et al.*, 1982; Creasy *et al.*, 1987; Lindstrom *et al.*, 1988).

There are, however, few studies on the effects of various phthalate esters on fertility, especially with regard to structure–activity relationships and sexual specificity of the reproductive toxicity. We have recently re-

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ported the results of continuous breeding studies of diethyl, dibutyl, dihexyl, and diethylhexyl phthalate (Lamb *et al.*, 1987).

We now report the results of continuous breeding reproduction studies of dipropyl, dipentyl, and dioctyl esters of phthalate. In addition, we compare the structure of the seven phthalates with the data on fertility, affected sex, organ weights, and sperm parameters to obtain a structure–function relation of phthalate esters on reproductive function *in vivo*.

## METHODS

*General study design.* This study was conducted under the National Toxicology Program's Continuous Breeding protocol. The specific study design for the Reproductive Assessment by Continuous Breeding Protocol (RACB) has been published previously (Lamb, 1985; Reel *et al.*, 1985). The RACB protocol is divided into four tasks. Task 1 is a 14-day dose setting study that uses five doses and a control ($n$ = 8/sex/group). Endpoints for Task 1 are clinical signs, body weight, and food or water consumption. Task 2 is the continuous breeding phase, consisting of a control group (40 breeding pairs) and three dose groups (20 pairs/group). Ideally, Task 2 dose levels are set so that the highest dose will be expected to depress weight gain by approximately 10% and will permit >90% survival. The middle dose is selected to produce little or no systemic toxicity, whereas the low dose is designed to be a no-effect level. The animals are housed as breeding pairs for 98 days, following 7 days of premating consumption of dosed feed. Endpoints for Task 2 are clinical signs, parental body weight, fertility (number producing a litter/number of breeding pairs), litters per pair, live pups per litter, proportion of pups born alive, sex of live pups, pup body weights within 18 hr of birth, and food or water consumption. At the end of the 98 days, the pairs are separated and housed one animal/cage with continued dosing. Any litters born after the continuous breeding phase are reared by the dam until weaning, after which dosed feed is provided at the same concentration as during Task 2. These animals are used for assessment of second-generation fertility (Task 4, below).

When a positive effect on fertility is detected during Task 2, a 1-week crossover mating trial (Task 3) is performed on the parental animals to determine the affected sex. Task 3 is performed after the last Task 2 litter is weaned. The crossover mating trial consists of three groups of 20 pairs each: control males × control females, control males × high-dose females, and control females × high-dose males. Pairs are mated for 7 days or until a copulatory plug is detected whichever is first. Nondosed feed is provided for all animals during this week followed by continued dosing at the appropriate doses until necropsy. Endpoints for Task 3 are the same as for Task 2. At the end of Task 3, $F_0$ males and females are necropsied; the endpoints evaluated are selective organ weight (as shown in tables), body weight, epididymal sperm motility, morphology, number, and estrous cyclicity as monitored by vaginal lavage for the preceding 7 days. Selected organs are examined microscopically after fixation in 10% neutral buffered formalin (Bouin's fixative for testes) and embedding in paraffin; sections are stained with hematoxylin and eosin according to standard procedures.

Task 4, offspring assessment, is conducted if Task 2 shows no reproductive toxicity. In this phase, the last litter from Task 2 is reared, weaned, and kept to sexual maturity (74 ± 10 days) while housed by sex two or three per cage, consuming the same concentration of phthalate in the feed as their parents. At this time, males and females from different litters within the same treatment group are cohabited for 7 days or until a copulatory plug is detected, whichever is less, and then housed singly until delivery. The endpoints for this mating trial are the same as those in Task 3. At the end of Task 4, the $F_1$ mice are necropsied; the endpoints are the same as for the necropsy of the $F_0$ mice.

*Animals.* COBS Crl:CD-1 (ICR)BR outbred albino Swiss mice were purchased from Charles River Breeding Laboratories, Inc. (Kingston, NY) at 6 weeks of age. Mice were 11 weeks of age at the start of the continuous breeding phase of these studies. On receipt, 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. After a 2-week quarantine period some animals were used for the 14-day range finding studies. All study animals were individually identified by ear tag and assigned to treatment groups using a stratified randomization procedure based on body weights.

Male and female CD-1 mice were group-housed by sex during quarantine and the 1-week premating period in solid-bottom polypropylene or polycarbonate cages with stainless-steel wire lids. The animals were subsequently housed as breeding pairs or individually. Ad-Sorb-Dri (Laboratory Products, Inc., Garfield, NJ) bedding was used in all cages. Deionized/filtered water and ground rodent chow (NIH-07) were provided *ad libitum*. Automatically controlled photoperiods were 14 hr light/10 hr dark (lights on from 0700 to 2100 hr), and temperature was maintained at 23 ± 2°C. Cages were sanitized weekly using detergent and 180°F water. All animal care procedures conformed to the NIH *Guide for the Care and Use of Laboratory Animals* (NIH Publication 85-23).

*Chemicals.* Di-*n*-pentyl phthalate (DPP) and di-*n*-propyl phthalate (DPrP) were obtained from Eastman Kodak Company via Midwest Research Institute (Kansas City, MO). Di-*n*-octyl phthalate (DOP) was synthesized by Midwest Research Institute. Midwest Research Institute also checked the purity and stability of the chemi-

cals. All were shown to be greater than 99% pure by chromatographic and spectrophotometric analysis and to be stable (<5% loss) in feed at room temperature for at least 2 weeks.

*Dosage formulation and analysis.* Each dose level was independently blended into a small amount of ground NIH-07 diet (Zeigler Bros., Gardner, PA). This was then added to a preweighed portion of feed and mixed in a Patterson–Kelly 8-quart blender for 15 min with the intensifier bar in operation for the first 5 min. For each chemical, an aliquot of each formulation, the feed control, and the bulk chemical (Weeks 1, 6, 10, and 14 of study) were sent to Midwest Research Institute for analysis. Reference aliquots were within 85–113, 97–108, and 97–105% for DPrP, DPP, and DOP, respectively. Dose formulations were prepared every 2 weeks and stored at 4°C until added to feed cups at weekly intervals.

*Preliminary (Task 1) dose setting.* Di-*n*-pentyl phthalate was tested at 0.0 (control), 0.25, 0.5, 1.25, 2.5, and 5.0% (w/w) for the Task 1 dose range finding study. During this 2-week study, animals in the control, 0.25, 0.5, and 1.25% dose groups, on the average, gained 6–7% of their original weight. In the 2.5 and 5% dose groups, males lost 2 and 18% of their initial body weight, respectively, and females gained 3% and lost 11% of their initial body weight, respectively. Based on these data, the doses of DPP selected for Task 2 were 0 (control), 0.5, 1.25, and 2.5%.

DPrP was tested at concentrations of 0.0 (control), 0.25, 0.5, 1.25, 2.5, and 5.0% (w/w) during Task 1. There were no significant differences in the average daily food consumption; however, males in the 5.0% dose group gained only 1% of their initial weight compared to 7% for the controls. Similarly, the females in the control and 5% dose group gained 10 and 7% of their initial weight, respectively. No clinical signs of toxicity were noted at any dose level. Therefore, doses of 0.0 (control), 1.25, 2.5, and 5% were chosen for the continuous breeding phase (Task 2).

The 14-day dose range finding study was carried out with 0.0 (control), 0.50, 1.25, 2.50, 5.0, and 10% DOP in the diet. No clinical symptoms of toxicity were noted in the control or treated groups, with the exception of rough hair coat in a significant number (4–6 of 8) of animals in the 10.0% dose group. DOP had no effect on weight gain or daily feed consumption at any dose tested. Dose levels of 0, 1.25, 2.5, and 5.0% were selected for the continuous breeding study of DOP.

*Statistical analysis.* The Cochran–Armitage test (Armitage, 1971) was used to test for a dose-related trend in fertility (Task 2). In Task 3, where dose groups do not represent increasing dose levels (the animals are cross-mated), a $\chi^2$ test for homogeneity was used to test for an overall difference in fertility among groups. Pairwise comparisons between the control and dosed groups were made with Fisher's exact test (Fisher, 1934).

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 born alive, divided by the total number of pups produced by each pair. The sex ratio was expressed as the proportion of male pups born alive out of the total number of live pups born to each fertile pair. Dose group means for these parameters were tested for overall differences using the Kruskal–Wallis test (Kruskal and Wallis, 1952) and for ordered differences using Jonckheere's test (Jonckheere, 1954). Pairwise comparisons of treatment group means were performed by applying the Wilcoxon–Mann–Whitney *U* test (Mann and Whitney, 1947).

To remove the potential effect of the number of pups per litter on the average pup weight, an analysis of covariance (Neter and Wasserman, 1974) was performed. The covariate used was average litter size, including live and dead pups. Least-squares estimates of dose group means, adjusted for litter size, were computed and tested for overall equality using an *F* test and pairwise equality using a *t* test. To control for possible sex differences, these analyses were performed on males, females, and both sexes combined. An analysis of covariance was also used to adjust organ weights for total body weight. Unadjusted body and organ weights were analyzed using the Kruskal–Wallis and Wilcoxon–Mann–Whitney *U* tests. Dose-related trends were tested for by Jonckheere's test.

## RESULTS

### *Di-n-pentyl Phthalate*

During the 14-week continuous breeding phase (Task 2) male mice in the control, 0.5%, and 1.25% groups gained an average of 9, 10, and 3% of their original body weight, respectively, whereas the 2.5% group lost an average of 1% body weight. Group mean weights of the female mice varied with the gestational phase. The average daily feed consumption was similar for all groups except for the 2.5% group which was consistently higher by 8–35% than the control values. The average feed consumption and body weight data indicated that male mice in the 0.5, 1.25, and 2.5% dose groups received approximately 0.76, 2.16, and 4.79 g/kg body wt/day DPP.

All breeding pairs in the control group for DPP delivered at least one litter during the Task 2 continuous breeding phase with an average of 4.8 litters/pair (Table 1). Mice in the 1.25 and 2.5% dose groups were infertile; none of the breeding pairs delivered any lit-

TABLE 1

EFFECT OF DI-*n*-PENTYL, DI-*n*-PROPYL, AND DI-*n*-OCTYL PHTHALATE ON FERTILITY AND REPRODUCTIVE PERFORMANCE IN CD-1 MICE DURING CONTINUOUS BREEDING

| | % Di-*n*-pentyl phthalate in diet | | | |
|---|---|---|---|---|
| | 0.0 (Control) | 0.5 | 1.25 | 2.5 |
| No. fertile/No. cohabited | 37/37 | 4/19* | 0/19* | 0/20* |
| Litters/pair | 4.8 ± 0.1 (37) | 1.3 ± 0.3 (4)* | — | — |
| Live pups/litter | 11.1 ± 0.4 (37) | 1.1 ± 0.5 (4)* | — | — |
| Proportion of pups born alive | 0.98 ± 0.01 (37) | 0.68 ± 0.24 (4)* | — | — |
| Live pup weight (g) | 1.60 ± 0.02 (37) | 1.50 ± 0.08 (3) | — | — |
| | % Di-*n*-propyl phthalate in diet | | | |
| | 0.0 (Control) | 1.25 | 2.5 | 5.0 |
| No. fertile/No. cohabited | 37/37 | 20/20 | 18/18 | 0/18 |
| Litters/pair | 4.7 ± 0.1 (37) | 4.9 ± 0.1 (20) | 4.6 ± 0.2 (18) | — |
| Live pups/litter | 10.5 ± 0.5 (37) | 10.9 ± 0.4 (20) | 5.9 ± 0.6 (18)* | — |
| Proportion of pups born alive | 0.97 ± 0.02 (37) | 0.98 ± 0.01 (20) | 0.86 ± 0.04 (18) | — |
| Live pup weight (g) | 1.60 ± 0.02 (37) | 1.57 ± 0.02 (20) | 1.56 ± 0.03 (18) | — |
| | % Di-*n*-octyl phthalate in diet | | | |
| | 0.0 (Control) | 1.25 | 2.5 | 5.0 |
| No. fertile/No. cohabited | 36/37 | 20/20 | 18/18 | 20/20 |
| Litters/pair | 4.6 ± 0.2 (36) | 4.9 ± 0.1 (20) | 4.8 ± 0.1 (18) | 4.8 ± 0.1 (20) |
| Live pups/litter | 11.5 ± 0.4 (36) | 11.6 ± 0.5 (20) | 11.2 ± 0.7 (18) | 11.1 ± 0.6 (20) |
| Proportion of pups born alive | 1.0 ± 0.0 (36) | 1.0 ± 0.0 (20) | 0.98 ± 0.01 (18) | 0.98 ± 0.01 (20) |
| Live pup weight (g) | 1.60 ± 0.02 (36) | 1.58 ± 0.02 (20) | 1.60 ± 0.02 (18) | 1.61 ± 0.02 (20) |

*Note.* Pairs of animals were cohabited and dosed with appropriate control or phthalate-containing diet for 14 weeks. Pairs were considered fertile if they produced one or more litters. Except for No. fertile/No. cohabited, values are means ± SE. Number in parentheses denotes number of fertile pairs providing the data.

* Significantly different from the control group ($p < 0.05$).

ters during the 14-week study. In the 0.5% dose group, only 4/19 delivered a first litter and 2/19 delivered more than one litter (Table 1). In the 0.5% dose group, the only dose group that produced any litters, the number of litters and live pups/litter were significantly reduced (Table 1).

To determine the affected sex, the cross-over mating study was initiated utilizing controls and the 2.5% dose group. As shown in Table 2, the fertility of both males and females was completely inhibited by treatment with 2.5% DPP. In addition, the mating index (number with copulatory plug/number cohabited) for the 2.5% dose males × control females was significantly reduced, indicating that a significant number of males exposed to 2.5% DPP failed to even mate or that sperm

TABLE 2

FERTILITY AND REPRODUCTIVE PERFORMANCE OF DI-*n*-PENTYL PHTHALATE TREATED MICE AFTER A CROSSOVER MATING TRIAL

| Reproductive parameter | Treatment group | | |
|---|---|---|---|
| | Control male × Control female | 2.5% male × Control female | Control male × 2.5% female |
| No. with copulatory plug/No. cohabited (%) | 13/18 (72) | 7/19 (37)* | 16/20 (80) |
| No. fertile/No. cohabited (%) | 11/18 (61) | 0/19 (0)* | 0/20 (0)* |
| Live pups per litter | 7.8 ± 0.9[a] | — | — |
| Proportion of pups born alive | 1.00 ± 0.00 | — | — |
| Live pup weight (g) | 1.70 ± 0.06 | — | — |

*Note.* At the conclusion of the continuous breeding (Task 2), control animals were paired with either control or male or female treated animals for 7 days or until a copulatory plug was noted. All mice were fed the control diet during the mating trial and were then returned to the specific treatment diets after separation.

[a] Values are means ± SE for 11 pairs of control animals.

* Significantly different from the control group ($p < 0.05$).

number or sex accessory organ function was so diminished that no copulatory plug was detected.

Tables 3 and 4 show that at necropsy after the crossover mating, there was a significant decrease in body weight for both males and females (2.5% dose group) compared to the controls. In addition, the weight of the kidneys, epididymis, testis, and seminal vesicles was significantly decreased in the male. Epididymal sperm count decreased to the nondetectable level, whereas prostate weight remained normal and liver weight actually increased. When adjusted for body weight at necropsy, the weight of the liver remained elevated and the values for the testis, epididymis, and seminal vesicles remained significantly reduced ($p < 0.01$; data not shown). Similarly, when adjusted for reduced body weights, the group mean liver weight in the female treated mice was significantly increased and the kidney weight was significantly lower. There was no change in estrous cycle length due to DPP treatment.

Histopathologic examination of organs from the control and 2.5% groups revealed that at necropsy the testes were atrophic in 16/20 treated and 0/40 control males. Distinct treatment-related histopathologic changes were noted in 20/20 male treated mice. The lesions were seen mainly in the testis and epididymis and included severe degeneration of the seminiferous tubules (0% controls; 95% treated), interstitial cell hyperplasia (5% control; 100% treated), and reduction in sperm count and accumulation of fluid and degenerated cells in the epididymis (2.5% control; 100% treated).

DPP treatment produced no significant gross or histopathologic changes in the ovaries, oviduct, uterine horn, vagina, liver, or kidneys of the treated female mice.

### *Di-n-propyl Phthalate*

DPrP treatment caused a significant dose-related decrease in body weight gain during Task 2. Male mice in the 0.0 (control), 1.25, 2.5, and 5.0% DPrP groups gained an average of 15, 7, 5, and 2% of their original body weights, respectively, after 14 weeks of treatment (data not shown). Group mean body weights for the female mice varied with the gestational phase. Average daily consumption of control/dosed feed by pairs of animals in the control and three treatment groups was essentially the same throughout Task 2 (data

TABLE 3

EFFECT OF DI-*n*-PENTYL PHTHALATE ON BODY WEIGHT, ORGAN WEIGHTS, AND SPERM PARAMETERS OF TASK 2 MALE MICE AT NECROPSY

| | % *n*-Pentyl phthalate | |
|---|---|---|
| | 0.0 (Control) | 2.5 |
| Body (g) | 39.88 ± 0.67 | 35.84 ± 0.36* |
| Liver (g) | 1.98 ± 0.04 | 2.95 ± 0.08* |
| Kidneys (g) | 0.79 ± 0.02 | 0.55 ± 0.01* |
| Right epididymis (mg) | 54 ± 1 | 43 ± 1* |
| Right cauda epididymis (mg) | 20.5 ± 0.5 | 17.9 ± 0.6* |
| Right testis (mg) | 131 ± 3 | 29 ± 1* |
| Seminal vesicles (mg) | 650 ± 18 | 442 ± 17* |
| Prostate (mg) | 23 ± 1 | 22 ± 1 |
| Sperm concentration ($10^6$ sperm/g caudal tissue) | 1078 ± 43 | 0* |
| % Motile sperm | 94 ± 4 | — |
| % Abnormal sperm | 3.9 ± 0.4 | — |

*Note.* Animals were fed either control or treated feed continuously for the 14-week period of Task 2. The animals were then fed control diet during Task 3 cohabitation and returned to their respective control or treated diets until necropsy. Each value is the mean ± SE for 37 or 38 control and 19 treated mice.

* Significantly different from the control group ($p < 0.05$).

not shown). Feed consumption and body weight data indicated that the male mice in the 1.25, 2.50, and 5.0% dose groups received approximately 1.9, 4.1, and 8.6 g/kg body wt/day DPrP, respectively.

As shown in Table 1, all breeding pairs in the 0.0 (control), 1.25, and 2.5% dose groups delivered at least one litter. Mice in the 5% dose group were infertile; no litters were born (0/18). There was no significant effect of the 1.25% dose on any of the fertility and reproductive parameters measured. The results from the 2.5% dose group were intermediate: The animals in this dose group produced a mean of 4.6 ± 0.2 litters/pair with normal pup weight, but the number of pups per litter and the proportion of pups born alive were significantly reduced (Table 1). Since these results clearly show that DPrP is a reproductive toxicant at the 2.5 and 5% doses, a crossover mating trial was conducted to determine the affected sex. Both males and females fed 5% DPrP had a normal mating index during the crossover mating trial (Table 5). However, females that were treated with 5% DPrP and coinhabited with control males had 0% fertile matings compared to a control × control mating value of 72% and a 5% male × control female value of 67% ($p > 0.10$). That the treated males were not completely normal is evident from Table 5, which shows the detailed results of the crossover trial. Treated males mated to control females produced fewer live pups per litter, whereas live pup weight and proportion of pups born alive were not different from control × control matings.

At the conclusion of the mating trial, the male and female $F_0$ mice were necropsied; Tables 6 and 7 show that there was a de-

TABLE 4

EFFECT OF DI-*n*-PENTYL PHTHALATE ON BODY WEIGHT, ORGAN WEIGHTS, AND ESTROUS CYCLE LENGTH OF TASK 2 FEMALE MICE AT NECROPSY

| | % *n*-Pentyl phthalate | |
|---|---|---|
| | 0.0 (Control) | 2.5 |
| Body (g) | 34.99 ± 0.53 (37) | 31.89 ± 0.64 (20)* |
| Liver (g) | 2.04 ± 0.05 (37) | 2.96 ± 0.09 (20)* |
| Kidneys (g) | 0.57 ± 0.02 (37) | 0.46 ± 0.01 (20)* |
| Estrous cycle length (days) | 5.06 ± 0.15 (31)[a] | 4.50 ± 0.16 (16)[a] |

*Note.* Animals were fed either control or treated feed continuously for the 14-week period of Task 2. The animals were then fed control diet for the 7-day mating period and returned to their respective control or treated diets until necropsy. The number of animals providing the data is given in parentheses. Each value is the mean ± SE.

[a] In 6/37 control animals and 4/20 treated animals, estrous cycle length was not clear.

* Significantly different from the control group ($p < 0.05$).

TABLE 5

FERTILITY AND REPRODUCTIVE PERFORMANCE OF DI-*n*-PROPYL PHTHALATE-TREATED MICE AFTER A CROSSOVER MATING TRIAL

| Reproductive parameter | Treatment group | | |
|---|---|---|---|
| | Control male × Control female | 5% male × Control female | Control male × 5% female |
| No. with copulatory plug/No. cohabited (%) | 14/18 (78) | 15/18 (83) | 17/18 (94) |
| No. fertile/No. cohabited (%) | 13/18 (72) | 12/18 (67) | 0/17 (0)* |
| Live pups per litter | 9.0 ± 1.2[a] | 6.1 ± 0.8* | — |
| Proportion of pups born alive | 0.91 ± 0.08 | 0.94 ± 0.05 | — |
| Live pup weight (g) | 1.63 ± 0.04 | 1.74 ± 0.04 | — |

*Note.* At the conclusion of the continuous breeding (Task 2), control animals were paired with either control or male or female treated animals for 7 days or until a copulatory plug was noted. All mice were fed the control diet during the mating trial and were then returned to the specific treatment diets after separation.

[a] Each value is the mean ± SE of 12 or 13 fertile pairs.

* Significantly different from the control group ($p < 0.05$).

creased weight of the testis, epididymis, and seminal vesicles of the male mice along with a decreased number of sperm per cauda epididymis, decreased sperm motility, and increased numbers of morphologically abnormal sperm. Body and kidney weights were decreased, and liver weights were increased, for treated mice of both sexes. The chemical treatment did not alter the estimated estrous cycle length of the female mice.

Gross examination at necropsy revealed atrophic testes for 7/20 high-dose-treated animals compared to only 1/40 of the control mice. Distinct treatment-related histopathologic changes were noted in 19/20 male mice. The lesions were seen mainly in the testes and epididymis and included degeneration of seminiferous tubules (18% of control mice: 90% of high-dose-treated mice), interstitial cell hyperplasia (0% control:33% high-dose-treated), reduction in sperm count (5% control:35% high-dose-treated), and accumulation of fluid and degenerated cells in the epididymis (2.5% control:35% high-dose-treated). DPrP treatment did not cause any significant histopathologic changes in the ovaries, oviduct, uterine horn, vagina, liver, or kidneys of the female mice.

### *Di-n-octyl Phthalate*

There were no treatment-related changes in physical appearance, body weight gain, or food consumption during Task 2 (data not shown). The average feed consumption and mean body weight data from the first 14 weeks of Task 2 indicated that the mice in the 1.25, 2.5, and 5.0% dose groups received approximately 1.8, 3.6, and 7.5 g/kg body wt/day DOP, respectively. As shown in Table 1, DOP had no effect on fertility or reproductive performance as measured by number of litters produced per pair, number of live pups per litter, proportion of pups born alive, or live pup weight.

Since fertility and reproductive performance were not affected in the parental mice ($F_0$), the fertility and reproductive performance of the control and high-dose group offspring were assessed when they reached maturity (74 ± 10 days of age; Task 4). The final Task 2 litters ($F_1$) in the 0.0 and 5.0% dose groups were cohabited for 7 days or until a copulatory plug was found. The mating indexes (percentage plug positive/No. cohabited) for the control and treated $F_1$ mice were 80 and 95%, respectively. The fertility in-

TABLE 6

EFFECT OF DI-*n*-PROPYL PHTHALATE ON BODY WEIGHT, ORGAN WEIGHTS, AND SPERM PARAMETERS OF TASK 2 MALE MICE ($F_0$) AT NECROPSY

| | % *n*-Propyl phthalate in diet | |
|---|---|---|
| | 0.0 (Control) | 5 |
| Body (g) | 43.89 ± 0.70 | 37.41 ± 0.39* |
| Liver (g) | 2.16 ± 0.05 | 3.13 ± 0.07* |
| Kidneys (g) | 0.85 ± 0.02 | 0.61 ± 0.01* |
| Right epididymis (mg) | 56 ± 1 | 45 ± 1* |
| Right cauda epididymis (mg) | 20.8 ± 0.4 | 17.1 ± 0.5* |
| Right testis (mg) | 134 ± 3 | 86 ± 7* |
| Seminal vesicles (mg) | 649 ± 18 | 436 ± 13* |
| Prostate (mg) | 21 ± 1 | 19 ± 1 |
| Sperm concentration ($10^6$ sperm/g caudal tissue) | 1211 ± 45 | 713 ± 95* |
| % Motile sperm | 93 ± 2 | 86 ± 4* |
| % Abnormal sperm | 3.2 ± 0.2 | 4.8 ± 0.6* |

*Note.* Animals were fed either control or treated feed continuously for the 14-week period of Task 2. The animals were then fed control diet during Task 3 cohabitation and returned to their respective control or treated diets until necropsy. Each value is the mean ± SE of 40 (control) or 20 (treated) animals.

* Significantly different from the control group ($p < 0.05$).

dexes (percentage fertile/No. cohabited) for the control and treated pairs were 75 and 90%, respectively. No significant ($p > 0.05$) differences were noted with respect to the other reproductive parameters measured, including litter size, proportion of pups born alive, and pup weight (data not shown).

As shown in Table 8, DOP at the highest concentration tested had no effect on terminal body weight; testis, epididymis, or prostate weight; epididymal sperm concentration; percentage morphologically abnormal sperm; or percentage motile sperm of the $F_1$ males. Similarly, there was no change in terminal female body weight ($F_1$) or average estrous cycle length ($F_1$) (Table 9). However, it should be noted that DOP significantly increased liver weight in both males and females and increased kidney weight in females (Tables 8 and 9).

## DISCUSSION

The results of these continuous breeding studies show a relationship between alkyl chain length and reproductive toxicity of DPrP, DPP, and DOP. Of the three, DPP is the most toxic with effects on fertility at a concentration 0.5% in feed followed by DPrP at 2.5%. DOP was not a reproductive toxicant at concentrations up to and including 5% in the feed. Since there was no body weight depression at the 5% dose, we may not have reached a maximal tolerated dose. However, liver weight was increased in animals treated with DOP as well as those treated with DPrP and PPP. Also, others have shown that DOP

TABLE 7

EFFECT OF DI-*n*-PROPYL PHTHALATE ON BODY WEIGHT, ORGAN WEIGHTS, AND ESTROUS CYCLE LENGTH OF TASK 2 FEMALE MICE ($F_0$) AT NECROPSY

| | % *n*-Propyl phthalate in diet | |
|---|---|---|
| | 0.0 (Control) | 5 |
| Body (g) | 36.56 ± 0.50 (35) | 31.59 ± 0.50 (17)* |
| Liver (g) | 1.95 ± 0.04 (35) | 3.25 ± 0.07 (17)* |
| Kidneys (g) | 0.59 ± 0.02 (35) | 0.46 ± 0.01 (17)* |
| Estrous cycle length (days) | 4.86 ± 0.17 (22)[a] | 5.17 ± 0.21 (12)[b] |

*Note.* Animals were fed either control or treated diet continuously for the 14-week period of Task 2. The treated animals were then fed control feed for the 7-day mating period and again fed the treated diet until necropsy after the pups were born. The number of animals providing the data is given in parentheses. Each value is the mean ± SE.

[a] In 15/37 control animals, estrous cycle length was 7 days or not clear.

[b] In 5/17 treated animals, estrous cycle length was not clear.

* Significantly different from the control group ($p < 0.05$).

TABLE 8

EFFECT OF DI-*n*-OCTYL PHTHALATE ON BODY WEIGHTS, ORGAN WEIGHTS, AND SPERM PARAMETERS OF TASK 4 SECOND-GENERATION MALE MICE ($F_1$) AT NECROPSY

| | % *n*-Octyl phthalate in diet | |
|---|---|---|
| | 0.0 (Control) | 5 |
| Body (g) | 36.69 ± 0.86 | 35.5 ± 0.86 |
| Liver (g) | 1.96 ± 0.06 | 2.42 ± 0.08* |
| Kidneys (g) | 0.70 ± 0.02 | 0.70 ± 0.02 |
| Right epididymis (mg) | 49 ± 1 | 50 ± 1.6 |
| Right cauda epididymis (mg) | 19.1 ± 0.7 | 18.3 ± 0.4 |
| Right testis (mg) | 131 ± 5 | 131 ± 6 |
| Seminal vesicles (mg) | 429 ± 2 | 374 ± 9* |
| Prostate (mg) | 27 ± 2 | 28 ± 2 |
| Sperm concentration ($10^6$ sperm/g caudal tissue) | 1118 ± 64 | 1239 ± 78 |
| % Motile sperm | 94 ± 1 | 94 ± 1 |
| % Abnormal sperm | 5.0 ± 0.6 | 3.5 ± 0.4 |

*Note.* The fifth litter produced during Task 2 was allowed to grow until 74 + 10 days of age. They received either control or chemical treatment via lactation until weaning and then dosed feed until necropsy at 95 + 10 days of age. Each value is the mean ± SE of 20 animals.

* Significantly different from the control group ($p < 0.05$).

does not decrease testicular weight in the rat (Foster, 1980; Gray and Butterworth, 1980). Although both DPrP and DPP inhibited fertility, decreased testis and epididymal weights, and decreased sperm concentration, the crossover mating trial showed that the reproductive toxicity of DPrP was due primarily to a female effect, whereas that of DPP had both male and female components.

This paper is the second in a series delineating the reproductive toxicity of the phthalates utilizing the RACB protocol. This comprehensive study of seven phthalate esters of varying chain length has allowed an analysis of the structure–activity relationship with regard to reproductive toxicity. Table 10 shows a composite of the reproductive toxicity versus alkyl chain length. The most potent reproductive toxicants in the continuous breeding studies conducted in mice are the *n*-hexyl phthalates, with ethylhexyl phthalate being severalfold more potent than the nonsubstituted hexyl phthalate. As the side chain is lengthened to eight carbons (DOP) or shortened to two carbons (DEP), reproductive toxicity is lost. As the length of the *n*-alkyl substitution is shortened from six (DHP) to five (DPP) to four (DBP) to three (DPrP), there is a reduction in the potency of the reproductive effects. Minimal toxicity is detectable at 0.3, 0.5, 1, and 2.5% for DHP, DPP, DBP, and DPrP, respectively.

These data obtained from mice agree with the data obtained from rats as far as the phthalates that are testicular toxicants are concerned. However, the order of potency in the rat appears to be slightly altered in that DPP caused the greatest loss in testicular weight (Foster *et al.*, 1980). Fertility, however, was not measured in these studies. There is also a structure–activity relationship of these di-*n*-substituted phthalates with regard to affected sex. DBP was selective for the female and the female was more sensitive to

TABLE 9

EFFECT OF DI-*n*-OCTYL PHTHALATE ON BODY WEIGHT, ORGAN WEIGHT, AND ESTROUS CYCLE LENGTH OF TASK 4 SECOND-GENERATION FEMALE MICE ($F_1$) AT NECROPSY

| | % *n*-Octyl phthalate in diet | |
|---|---|---|
| | 0.0 (Control) | 5 |
| Body (g) | 30.03 ± 0.82 | 30.61 ± 0.54 |
| Liver (g) | 1.88 ± 0.06 | 2.34 ± 0.05* |
| Kidneys (g) | 0.479 ± 0.014 | 0.533 ± 0.011* |
| Estrous cycle length (days) | 4.63 ± 0.1 | 4.83 ± 0.2 |

*Note.* The $F_1$ generation received either control or chemical treatment through lactation until weaning and later via dosed feed until necropsy at 95 ± 10 days of age. Each value is the mean ± SE of 20 animals.

* Significantly different from the control group ($p < 0.01$).

TABLE 10

SUMMARY OF THE REPRODUCTIVE TOXICITY OF DI-*n*-PHTHALATES IN A CONTINUOUS BREEDING STUDY OF MICE

| Phthalate ester | Fertility | Affected sex | Testis weight | Sperm concentration | Mating index | Estrous cycle length |
|---|---|---|---|---|---|---|
| Diethyl | — | — | — | — | — | — |
| Dipropyl | ↓ | ♀/♂ | ↓ | ↓ | — | — |
| Dibutyl | ↓↓ | ♀ | — | — | ↓♀ | 0 |
| Dipentyl | ↓↓↓ | ♀/♂ | ↓↓ | ↓↓↓ | ↓♂ | — |
| Dihexyl | ↓↓↓↓ | ♀/♂ | ↓↓ | ↓↓ | ↓♂ | |
| Diethylhexyl | ↓↓↓↓↓ | ♀/♂ | ↓↓ | ↓ | — | 0 |
| Dioctyl | — | — | — | — | — | — |

*Note.* With regard to fertility, increasing number of arrows indicates a lower dose which resulted in a detectable effect in the RACB protocol. Affected sex pertains to results of crossover mating trial, i.e., fertility. The number of arrows indicates the relative effect. —, No change from control values: 0, not evaluated.

DPrP in the crossover mating trials, whereas DEHP, DPP, and DHP affected both male and female fertility. The data for DEHP, DPP, and DHP do not allow for an analysis of possible differential sensitivity of the sexes. Nonetheless, the data suggest that the female mouse reproductive system may be more sensitive to the toxicity of some phthalates than that of the male.

These *in vivo* reproduction studies also suggest possible sites of action of the phthalate toxicity. Since sex accessory gland weights in the male and estrous cycle length in the female were never consistently altered, even with the most potent phthalates, it is unlikely that alteration of steroid hormone levels or gonadotropins is a major component of the reproductive toxicity of the phthalates. Thus, these results suggest an action of phthalates either on the production or function of the gametes (which appears as decreased sperm concentration for males treated with dipropyl, dipentyl, dihexyl, and diethylhexyl phthalate) or on the supporting Sertoli/granulosa cells. While there are no data regarding the effects of phthalates on granulosa cell function per se, there are numerous reports of *in vivo* studies of the development of lesions (Creasy *et al.*, 1983, 1987) or *in vitro* studies (Gray and Gangolli, 1986; Gray and Beamand, 1984), which indicate the Sertoli cell as a site of action of phthalates. This has recently been supported by *in vitro* data. For example, MEHP, the active metabolite of DEHP, has been shown to inhibit the ability of FSH to stimulate cAMP accumulation, to stimulate basal lactate production, to inhibit pyruvate secretion, and to decrease ATP levels in cultured rat Sertoli cells (Chapin *et al.*, 1988; Foster and Lloyd, 1987; Heindel and Chapin, 1988; Moss *et al.*, 1988).

These data on the *in vivo* reproductive toxicity of seven phthalates which delineate their structure–activity profile and suggest possible sites of action should be helpful for the further analysis of the specific mechanism of phthalate-induced reproductive toxicity.

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