

Effects of Di(2-ethylhexyl) Phthalate on the Gonadal Pathophysiology, Sperm Morphology, and Reproductive Performance of Male Rats

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Dietary exposure of adult male F344 rats to 0, 320, 1250, 5000, or 20,000 ppm DEHP for 60 consecutive days resulted in a dose-dependent reduction in total body, testis, epididymis, and prostate weights at 5000 and 20,000 ppm. Degenerative changes were observed in testis, along with decreased testicular zinc content, reduced epididymal sperm density and motility, and increased occurrence of abnormal sperm at 20,000 ppm. There was a trend towards reduced testosterone and increased luteinizing hormone and follicle stimulating hormone in serum at 5000 and 20,000 ppm. The mean percentage of fertile animals was unchanged and reduction in fertility parameters, although not marked in severity, were correlated with gonadal effects. Average litter size was reduced at 20,000 ppm, but initial pup weights and growth were unaffected. There were no grossly observed abnormalities in the offspring and the rate of neonatal deaths was similar in control and DEHP treated groups. Characteristic toxicity manifestations of DEHP included dose-dependent enlargement of liver and reduced sperm triglycerides and cholesterol. Additionally, serum albumin and total proteins were dose dependently increased upon treatment with DEHP. Cessation of exposure to DEHP initiated partial to complete recovery from toxicity in most cases. The magnitude of recovery were variable with that of the gonads being slower than other systems. These data suggest a lack of reproductive dysfunction in F344 male rats at DEHP doses below 20,000 ppm which produced measurable testicular degeneration and afflicted epididymal sperm morphology under the present experimental conditions.

Introduction

Prolonged dietary administration of di(2-ethylhexyl) phthalate (DEHP) and other phthalic acid esters (PAEs), commonly used plasticizers, cause testicular atrophy accompanied by selective depletion of testicular zinc, associated biochemical changes and increased urinary excretion of zinc in rodents (1,2). DEHP-induced testicular atrophy and zinc depletion may be related phenomena, since zinc is essential for maintaining the structure and function of gonads and its deficiency is known to produce testicular atrophy in man and animals (3,4). Mono(2-ethylhexyl) phthalate (MEHP) and other monoester metabolites of PAEs, produced by the hydrolytic activity of intestinal lipases, can reproduce the effects of their parent diesters and are suspected as causative agents (5,6). Inasmuch as gastrointestinal absorption of zinc does not

seem to be affected by these agents (7), it is suggested that polar metabolites of DEHP and other PAEs can selectively remove testicular zinc and thereby lead to tubular atrophy (5). Zinc deficiency affects production and release of gonadotrophins and testosterone (8), just as these hormones are known to maintain tissue zinc concentration and regulate uptake of zinc in testis and accessory sex organs (9). Perturbation of gonadotrophins or testosterone, therefore, may be responsible for DEHP gonadotoxicity due to afflicted hormonal regulation of gonadal physiology (10,11). We have demonstrated earlier that dietary zinc deficiency enhances the testicular atrophy in rats without affecting other systemic effects of DEHP such as liver enlargement or hypolipidemia, suggesting these effects to be organ-specific and probably a consequence of reduced transport of zinc into the testis (12). The phenomenon of gonadal toxicity and its mechanisms have been extensively studied, however, there is little information available on physiological effects such as reproductive performance following exposure to PAEs.

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The bulk of studies on reproductive dysfunction document decreased conception, embryo/fetotoxicity, and teratogenicity in exposed female animals (1,13), while reports of male antifertility were associated with the germ cell mutation effects (14,15) and lacked correlation with the magnitude of exposure and gonadotoxicity from PAEs. Therefore, the objectives of the present investigation were to compare the reproductive performance of male rats at the dose levels which caused (or did not cause) measurable gonadotoxicity, and to observe the patterns of recovery from toxicity upon discontinuance of exposure to DEHP. Observations on liver enlargement and hypolipidemia were included as characteristic toxicity indices of DEHP (16,17).

Materials and Methods

Animals and Chemicals

Sexually mature male (average age 15–16 weeks, 240 ± 10 g) and female (average age 12–13 weeks, 200 ± 10 g) F344 rats (Charles River, Kingston, RI) were maintained in a controlled environment with a 12 hr light/12 hr dark cycle, 22 ± 2°C, and 50 ± 20% relative humidity. The animals were housed in clear polycarbonate cages lined with hardwood chip bedding and were provided with ground food (Purina Rodent Chow, Ralston Purina Co., Richmond, IN) and fresh water *ad libitum*.

Di(2-ethylhexyl) phthalate (DEHP) was obtained from Hatco Chemical Co. (Fords, NJ). The purity of the preparation was >99% by GC and TLC analyses. Other chemicals used were reagent grade materials obtained from Fischer Scientific Co. (Fairlawn, NJ), Sigma Chemical Co. (St. Louis, MO), or Baker Instruments Corp. (Allentown, PA).

Treatment and Sample Preparation

The male rats (120 animals) were housed two per cage and acclimated on the normal diet for one week before dividing into five groups of 24 rats, each to be placed on one of the five dietary concentrations of DEHP (0, 320, 1250, 5000, or 20,000 mg/kg diet, ppm) for 60 consecutive days. The selection of DEHP doses was guided by the NTP chronic and subchronic studies in rats (18) and experimental reports regarding the gonadotoxicity of PAEs (5–7). Individual body weights and food consumptions for each cage were recorded weekly and the latter were expressed as grams of food consumed/day/rat. Blood samples were drawn from the retro-orbital sinus of eight rats (predesignated) of each group at desired intervals during the treatment and at the sacrifice. Serum prepared from these samples were used for various analyses as described below.

All animals were returned to normal diet (no DEHP) and housed individually with two sexually mature virgin females (untreated) on day 61 of the experiment. During a 5-day period of cohabitation, daily records of vaginal smears were made and mated females (confirmed by the

presence of sperm in the vagina) were housed separately in order to allow them to litter naturally. Unmated females at the end of cohabitation and those that mated but did not bear young were surgically examined for gross anatomical abnormalities which could have prevented pregnancy. From these experiments the effects of DEHP on the incidence of pregnancy, litter size, litter weight, and growth of pups up to 7 days of age were determined.

Eight male rats (predesignated) from each treatment group were sacrificed at the end of cohabitation. Testis, epididymis, prostate, seminal vesicles, and liver were removed, cleaned in normal saline, weighed, and fixed in 10% neutral buffered formalin or Bouin's solution for histopathological assessment. A sample of testicular tissue was stored frozen at -60°C for subsequent analysis of zinc. The remaining male rats (16 per group) were allowed to recover for additional 65 days and then mated again for the assessment of reproductive performance as described above. At the end of cohabitation all male rats were necropsied for organ weights and histopathological assessment as described above.

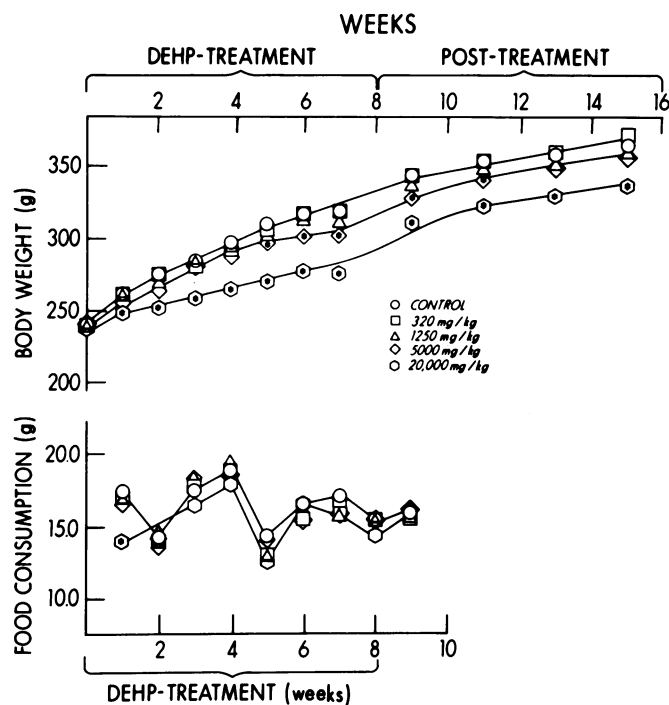


FIGURE 1. Effects of di(2-ethylhexyl) phthalate (DEHP) on the body weight gain and food consumption in rats. Sexually mature male F344 rats were acclimated on normal diet (no DEHP) for one week prior to placing them on one of the five treatment diets containing 0, 320, 1250, 5000, or 20,000 ppm DEHP. Dietary exposure to these concentrations of DEHP was continued for 60 consecutive days followed by a period of recovery for 70 days on normal diet (no DEHP). Body weight gains of all rats were recorded weekly and presented as g/week/rat. Food consumption in various DEHP dietary groups was recorded as g/3–5 days/2 rats, averaged and presented as g/day/rat on weekly intervals. Each value during the treatment is mean ± SE from 24 rats and that following recovery is mean ± SE from 16 rats in each dietary group. The asterisk (*) denotes values significantly different from the respective controls, $p < 0.05$.

Analyses

Serum samples collected from control and DEHP-treated rats were analysed for the total contents of triglycerides (19), cholesterol (20), albumin (21), and proteins (22) using an Encore Centrifuge Chemistry Analyser (Baker Instruments Corp., Allentown, PA). The concentrations of testosterone, follicle stimulating hormone (FSH), and luteinizing hormone (LH) were determined in serum by radioimmunoassay technique using reagent kits and procedure provided by the National Hormone and Pituitary Program of the National Institute of Arthritis, Metabolism, and Digestive Diseases (23). The testicular tissue from test animals was digested in nitric acid to obtain organic free aqueous solutions and measured for zinc content using an Inductively Coupled Plasma Emission Spectrometer (Jarrell-Ash, 1155A; analytical emission line = 213.8 nm). Lyophilized bovine liver (Standard Reference Material #1577a) containing 130 ± 13 mg/g zinc (certified by the National Bureau of Standards) was used for the standardization.

Evaluation of Sperm Density, Motility, and Morphology

Samples of mature sperm, capable of fertilization (24), were collected from the cauda region of the right epididymis by mincing it finely in Tyrode's buffer solution to a final volume of 3.0 mL at 37°C. A sample of this sperm suspension was immediately placed on a prewarmed hemocytometer to determine motility by counting all sperm in 20 fields (magnification 40×) and characterizing them motile or nonmotile by any movement versus no movement

(25). A 1.0-mL portion of sperm suspension was incubated with 50 μ L of 1% Eosin Y for 45 min, and a fine smear was examined under light microscope (magnification 400×) to evaluate sperm morphology, with classification of sperm as normal or abnormal (no hook, excessive hook, amorphous, pin-head, two heads or two tails, and short head) as described by Wyrobek and Bruce (26). To determine sperm density, a sample of sperm suspension was heated in boiling water bath for 30 sec (killing all sperm) and counted with a hemocytometer.

Histopathology

Tissue samples fixed in 10% neutral buffered formalin or Bouin's solution were washed, dehydrated with ethanol, and embedded in paraffin. Tissue sections of 5 to 7 μ m thickness were cut and stained with hematoxylin and eosin (H&E) for microscopic examination (27).

Statistical Evaluation

Results were evaluated by one-way analysis of variance and group means were compared by Student's *t* test (28). In all cases $p < 0.05$ was chosen as the criterion of significance.

Results

Dietary exposure to DEHP retarded body weight gain in a dose-dependent manner, with statistically significant reductions occurring in 5000 and 20,000 ppm groups (Fig. 1). At the 20,000 ppm dose, this effect was observed as early as week one, and it was progressive throughout

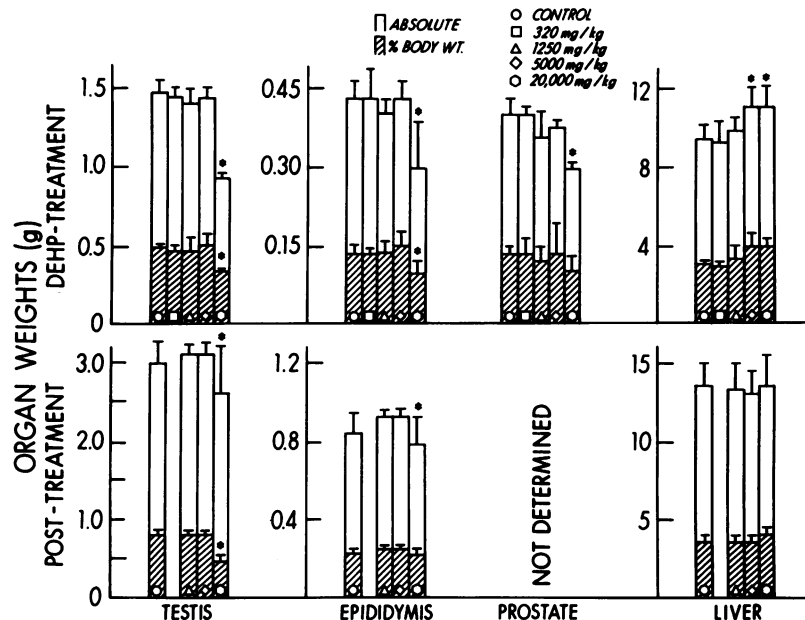


FIGURE 2. Effects of di(2-ethylhexyl) phthalate (DEHP) on the weights of testis, epididymis, prostate and liver (absolute and percent ratio to the body weight). Each value is mean \pm SE from 8 rats sacrificed at the end of cohabitation with females (for fertility assessment) following treatment with DEHP for 60 days and a mean \pm SE from 16 rats at the end of cohabitation following 70-days recovery from exposure to DEHP. Experimental conditions were same as described in Fig. 1. The asterisk (*) denotes values significantly different from the respective controls, $p < 0.05$.

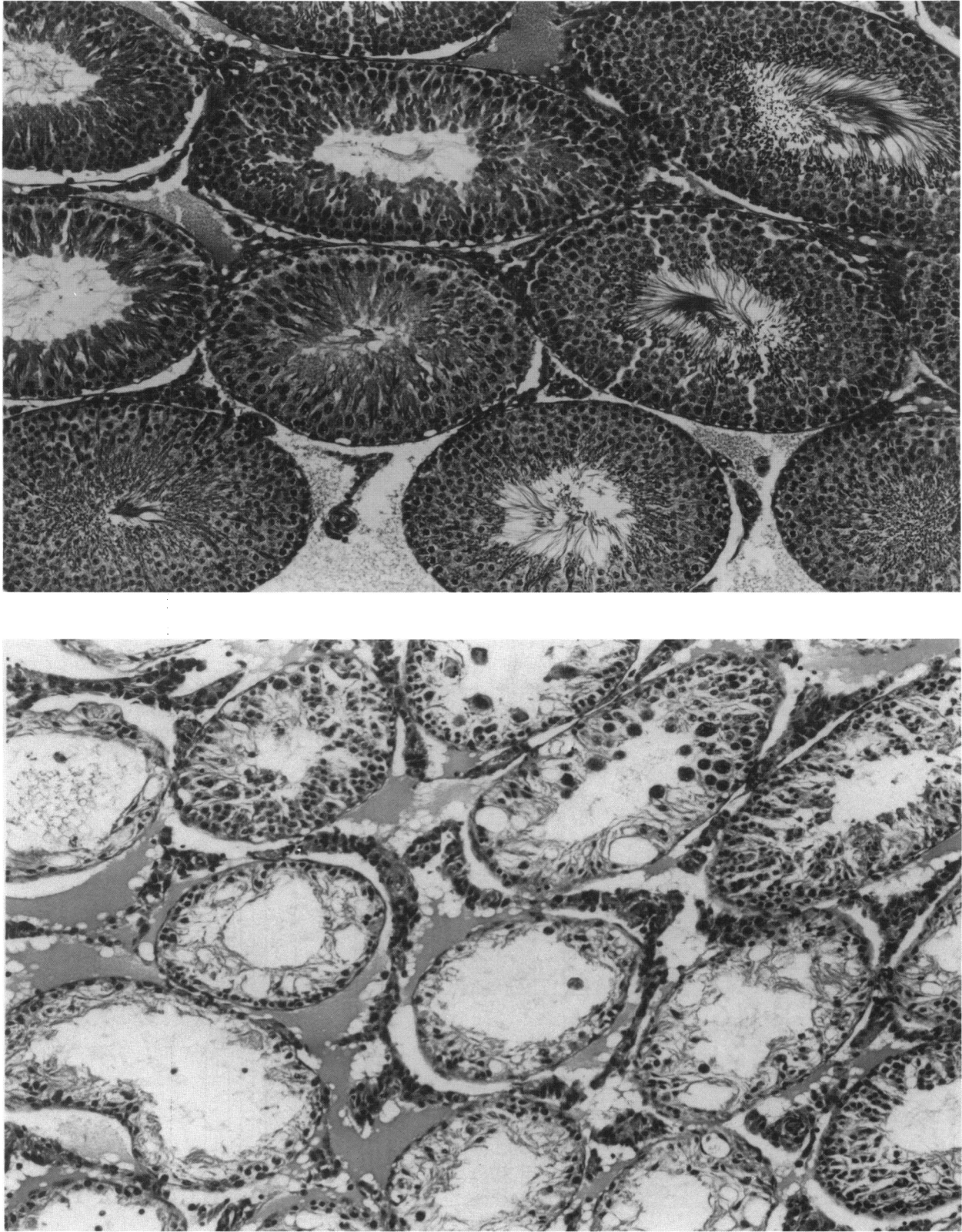


FIGURE 3. Effects of di(2-ethylhexyl) phthalate (DEHP) on the testicular morphology in rats: (a) normal seminiferous tubular structure and arrangement from a control rat (maintained on normal diet, no DEHP), normal spermatogenesis (magnification = $128\times$); (b) severe epithelial degeneration and atrophy of most seminiferous tubules with the loss of spermatogenesis from a rat maintained on DEHP dietary concentration of 20,000 ppm for 60 days (magnification = $128\times$).

Table 1. Histopathological scores on the DEHP-induced testicular atrophy in rats^a.

Atrophic seminiferous tubule, %	Severity score ^b	Incidence ^c				
		0 DEHP	320 ppm DEHP	1250 ppm DEHP	5000 ppm DEHP	20,000 ppm DEHP
<2 (normal)	0	4/8	3/6	4/8	4/8	0/8
2-25	1	4/8	3/6	4/8	4/8	1/8
26-75	2	0/8	0/6	0/8	0/8	6/8
>75	3	0/8	0/6	0/8	0/8	1/8

^a Characterized by a variable loss of spermatogenic cells in seminiferous tubules.

^b Relative scoring: 0 = normal; 1 = mild; 2 = moderate; 3 = severe.

^c Number of animals with specified lesions over the number of animals examined.

the DEHP treatment. On cessation of exposure to DEHP, rats from the 5000 ppm group recovered the lost weight gain, whereas those from the 20,000 ppm group started to gain weight similar to controls but still had lower body weights than controls, 70 days post-treatment, due to the slower growth during the treatment. Food consumption was largely unaffected except for a reduction during the first week in the 20,000 ppm group (Fig. 1). Mean food consumptions over the 60 days period of DEHP exposure were 16.5 ± 0.5 , $16.0 \pm .60$, 16.1 ± 0.6 , 16.2 ± 0.5 , and 15.4 ± 0.6 g/day/rat and amounted to an average DEHP intake of 0, 17.5, 69.2, 284.1, and 1156.4 mg/kg/day in DEHP dietary groups of 0, 320, 1250, 5000, and 20,000 ppm, respectively.

DEHP treatment significantly reduced absolute and relative weights of the testis and epididymis and absolute weight of the prostate at 20,000 ppm, while both absolute and relative liver weights were significantly increased at 5000 and 20,000 ppm and there was no significant change in the weights of seminal vesicles or pituitary at any of the doses tested (Fig. 2). Organ weights 70 days post-treatment suggested some but incomplete recoveries for testis and epididymis while liver weight had returned to normal (Fig. 2).

Histopathological evidence of tissue injury was limited to testis and characterized by severe atrophy of the seminiferous tubules and loss of spermatogenesis in rats fed 20,000 ppm DEHP diet (Fig. 3); the incidence and severity of the degenerative changes were markedly increased at 20,000 ppm as compared to other groups (Table 1). Testicular zinc analyses in control and two highest DEHP dose groups revealed a significant reduction at 20,000 ppm ($p < 0.01$) but no change at 5000 ppm; group mean \pm standard errors of testicular zinc concentrations for the control, 5000 and 20,000 ppm DEHP groups were

21.42 ± 0.34 , 21.31 ± 0.23 , and 14.20 ± 2.06 $\mu\text{g/g}$ fresh weight, respectively. The concentrations of FSH and LH in serum appeared to be increased at 20,000 ppm, while that of testosterone appeared to be decreased at 1250 to 20,000 (Fig. 4). However, these differences were not statistically significant, due to large internal variations except for FSH at 20,000 ppm.

Significant reductions in epididymal sperm density and motility were observed as well as increased percentages of morphologically abnormal sperm in rats on 20,000 ppm DEHP dose (Table 2). The incidence of pregnancy, mean litter weight on day 1, frequencies of stillbirths and neo-

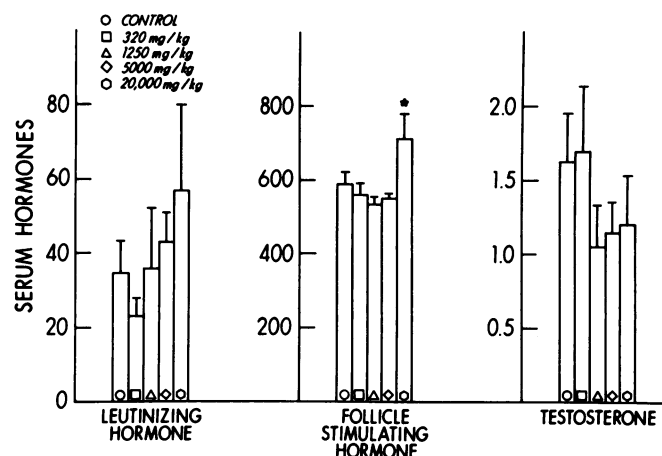


FIGURE 4. Effects of di(2-ethylhexyl) phthalate (DEHP) on the concentrations of gonadotrophins and testosterone in serum. All values are mean \pm SE of 8 rats from each of the dietary concentrations of DEHP, sacrificed at the end of cohabitation with females (for fertility assessment). Experimental conditions were the same as described in Fig. 1. The asterisk (*) denotes values significantly different from the respective controls, $p < 0.05$.

Table 2. Effect of DEHP on epididymal sperm density, motility, and morphology in rats.

DEHP, ppm	Density, spermatozoa/mg cauda epididymis $\times 10^4$	% Motile ^a	% Abnormal ^{a,b}
0 (control)	163 ± 16	60 ± 6	0.2 ± 0.1
320	146 ± 8	62 ± 11	0.1 ± 0.1
1250	142 ± 14	60 ± 10	0.1 ± 0.1
5000	170 ± 9	62 ± 7	0.0 ± 0.0
20,000	$102 \pm 17^*$	$31 \pm 11^*$	$1.3 \pm 0.6^*$

^a All values are mean \pm SE of 8 rats from each of the five DEHP dietary groups.

^b Cumulative representation of all abnormalities in sperm, e.g., no-hook, excessive-hook, amorphous, pin-head, two head/two tails, short sperm head, etc.

* Significantly different from respective controls, $p < 0.05$.

Table 3. Effect of DEHP on male fertility, litter size, pup growth, and survival.

DEHP, ppm	Recovery, days	Fertility ^a	Total live births	Total still births	Total neonatal deaths	Mean litter size ^b	Mean litter weight, g ^b	
							Day 1	Day 7
0 (control)	≥5	23/24	339	2	5	10.3 ± 1.4	5.2 ± 0.5	12.0 ± 1.0
320	≥5	20/22	263	3	1	9.4 ± 2.4	5.5 ± 0.5	12.2 ± 1.2
1250	≥5	14/24*	180	0	0	9.5 ± 1.8	5.6 ± 0.6	12.1 ± 1.5
5000	≥5	20/24	270	1	1	9.6 ± 1.6	5.5 ± 0.5	12.3 ± 1.0
20,000	≥5	19/24	237	0	1	8.8 ± 2.2*	5.5 ± 0.6	12.6 ± 1.5
0 (control)	≤70	13/16	147	1	2	9.3 ± 1.9	5.1 ± 0.2	10.8 ± 1.0
1250	≤70	11/16	112	0	1	8.6 ± 2.7	5.1 ± 0.2	10.2 ± 3.0
5000	≤70	14/16	160	1	1	8.8 ± 2.2	5.3 ± 0.3	10.5 ± 2.7
20,000	≤70	13/16	181	1	0	9.6 ± 1.6	5.2 ± 0.4	10.9 ± 0.6

^a Number of males with ability to impregnate one or both females over the number of males mated with two virgin females each for a 5-day period of cohabitation.

^b All values are mean ± SE from 19–33 litters (≥5 days of recovery) or 13–19 litters (≤70 days of recovery).

* Significantly different from respective controls, $p < 0.05$.

natal deaths, and mean litter growth up to 7 days of age were unaffected by DEHP treatment; however, mean litter size was significantly reduced at 20,000 ppm (Table 3).

Serum triglyceride concentrations were significantly and dose-dependently reduced throughout the period of

exposure to 1250, 5000 or 20,000 ppm DEHP, but returned to normal (or above-normal) levels following withdrawal from the DEHP diets (Fig. 5). Dose-dependent reductions in serum cholesterol were observed during the initial 2 weeks of exposure to 1250, 5000, or 20,000 ppm DEHP then declined to lesser magnitude with the continued exposure (Fig. 5). The hypocholesterolemic effect of DEHP was promptly reversed upon discontinuance of the exposure. Additionally, the concentration of albumin in serum was significantly increased by DEHP at all doses and in a dose-related manner (Fig. 6). This effect was most prominent during weeks 2 to 4 of exposure and less marked thereafter. A similar pattern, but of lesser severity, was observed for the total serum proteins at 5000 and 20,000 ppm (Fig. 6). Changes in serum albumin and total proteins were reversible on termination of exposure to DEHP.

Discussion

The objectives of this investigation were to assess the sensitivity of sexually mature rats to the toxic responses induced by the dietary administration of DEHP, to examine the reproductive performance of male rats following exposure to the gonadotoxic and subgonadotoxic dose levels, and to observe the patterns of recovery from toxicity upon the discontinuance of exposure to DEHP.

Dietary administration of DEHP in the present study produced phthalate ester characteristic toxicity in rats as indicated by reduced body weight gain, reduced testicular and accessory sex-organ weights, loss of testicular zinc, induction of seminiferous tubular atrophy, lowered serum triglycerides and cholesterol, and hepatomegaly (5–7,16,17). The toxic response to DEHP was dose-dependent and statistically significant with varying severities depending upon the target tissue.

Consistent with a previous report (16), reduced body weight gain upon exposure to DEHP was indicative of toxicity and not completely attributed to food consumption, as the latter was largely unaffected. The observations of DEHP-induced gonadotoxicity were also in agreement with the previous studies (5–7), though relatively milder since adult rats are less susceptible to

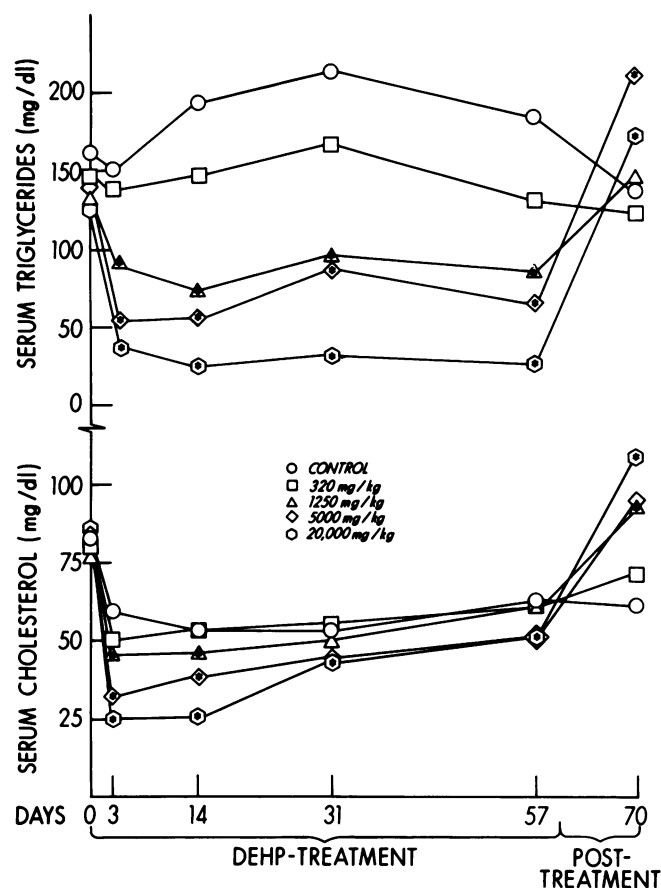


FIGURE 5. Effects of di(2-ethylhexyl) phthalate (DEHP) on the concentrations of serum triglycerides and cholesterol. Each value is mean ± SE of 8 rats from each of the five DEHP dietary concentrations. Experimental conditions were the same as described in Fig. 1. The asterisk (*) denotes values significantly different from the respective controls, $p < 0.05$.

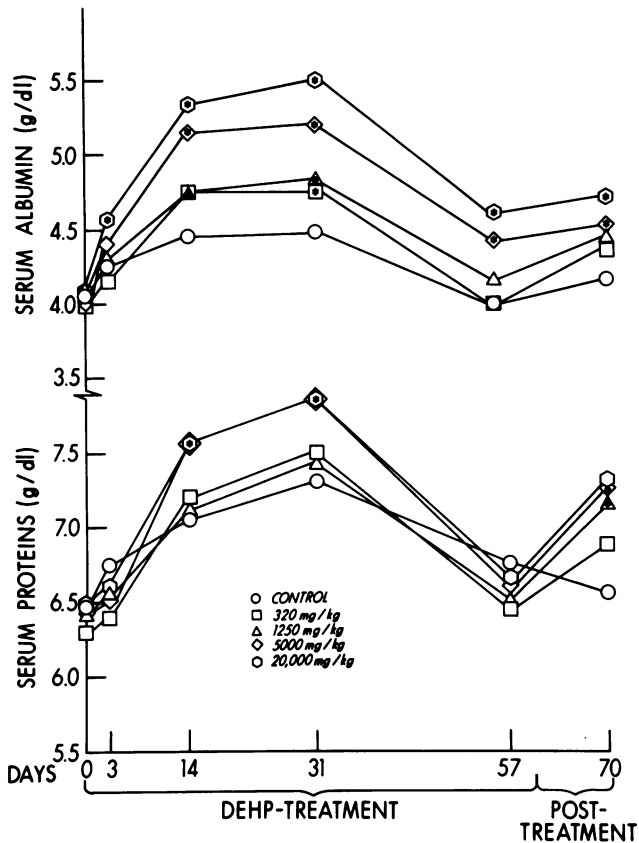


FIGURE 6. Effects of di(2-ethylhexyl) phthalate (DEHP) on the concentrations of serum albumin and total proteins. Each value is mean \pm SE of 8 rats from each of the five DEHP dietary concentrations. Experimental conditions were the same as described in Fig. 1. The asterisk (*) denotes values significantly different from the respective controls, $p < 0.05$.

these effects than young and sexually immature rats (12,29). There were trends towards increases in FSH and LH concentrations, and decrease in testosterone concentration of serum in DEHP-treated rats (observed in this study), and in CD-1 mice (30). Since a restricted release of testosterone into the blood would be expected to increase FSH and LH concentrations by a compensatory mechanism (11,31), present observations suggest that exposure to DEHP may alter circulating androgen release at the level of testis. This assumption is supported by a previous report of increased testicular testosterone and reduced serum testosterone in association with DEHP-induced testicular zinc depletion and seminiferous tubular atrophy in rats (10). Plasma testosterone concentration following DEHP-treatment was not lowered sufficiently, however, to significantly decrease seminal vesicle weight (indicative of hypoandrogenicity). Increased FSH at 20,000 ppm DEHP, in association with severe testicular atrophy and zinc depletion, may also have resulted from reduced output of the inhibin as a consequence of degenerative effects on the Sertoli cells (32,33). It is not clear, however, whether changes in these hormones precede or stem from testicular injury.

A DEHP dose (20,000 ppm) that caused frank testicular atrophy correspondingly reduced sperm density in

epididymal lumen, lowered motility, and induced structural abnormalities in sperm. The deterioration of seminiferous germinal epithelium or a spermicidal effect of DEHP and/or its metabolites may be responsible for such effects. A plausible mechanism for such effects may be the rapid depletion of zinc from spermatids (34), since high levels of zinc are essential for the maturation of sperm and maintenance of germinal epithelium (9). Male reproductive performance as assessed by fertility (ability to impregnate), litter weight, litter growth rate, still births, and neonatal survival was not affected despite obvious injury to testis and epididymal sperm. On the other hand, reduced litter size (number of live births) may be related to the reduced density, altered morphology, or a mutagenic event in sperm impairing fetal survival. The latter is supported by the reports that gross abnormalities in sperm (as observed with 20,000 ppm DEHP) are indicative of a mutagenic damage (35), and that treatment with DEHP can induce germ-cell mutations (dominant lethal) in male mice (14,15). In a continuous breeding study on CD-1 mice exposed to DEHP (1000 or 3000 ppm in feed for 7 days pre-mating and 98 days cohabitation periods), marked reduction in fertility and reduced litter size were observed in association with severe testicular atrophy and adversely affected epididymal sperm (30). These results indicate a clearcut correlation between gonadotoxicity and reproductive dysfunction, as well as greater sensitivity of CD-1 mice than F344 rats to such adverse biological effects of DEHP, based on the average DEHP intake of 142.1 or 396.7 mg/kg/day in mice on 1000 or 3000 ppm diets (30) as compared to the present 1156.4 mg/kg/day in rats on 20,000 ppm diet.

It is considered pertinent to mention here that DEHP in feed is efficiently absorbed from the gastrointestinal tract, rapidly biodistributed, and its metabolic products are extensively eliminated in urine and feces (36). The bulk of a single oral dose of DEHP in rats (> 97%) is excreted within 7 days, with the half-life in various tissues ranging from 1 to 5 days (36), whereas on prolonged exposure, metabolic disposition quickly reaches a steady state of body burden (within 4 days) independent of the administered dose (37). In the present investigation, therefore, increased body burden with the continued exposure or selective retention in specific tissues would not be expected to influence DEHP-induced gonadotoxicity and reproductive dysfunction, nor would it have affected the recovery from these effects.

Dose-dependent enlargement of the liver and hypolipidemia were observed in DEHP-treated rats at much smaller doses than those required to induce gonadotoxicity. In addition to these sensitive indices of toxic challenge from DEHP, serum albumin and total protein increased, probably as a secondary manifestation to hepatomegaly, although the biological significance of these changes is not understood.

In conclusion, the only functional reproductive consequence of exposure of male rats to the gonadotoxic concentrations of DEHP in diet was reduced litter size. This

effect was directly correlated with testicular atrophy, reduced epididymal sperm density and motility, and increased occurrence of morphologically abnormal sperm. At 70 days after treatment there was a partial or complete recovery in all parameters originally affected by DEHP. The completeness of recovery varied for different endpoints, with those of gonadal tissue being slower than for other systems.

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