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The Agricultural Group
800 N. Lindbergh Boulevard
St. Louis, Missouri 63167
Phone: (314) 694-1000

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December 12, 1995

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Office of Pollution Prevention and Toxic Substances
United States Environmental Protection Agency
401 M Street, SW
Washington, DC 20480



8EHQ-95-13553
INIT 12/13/95

ATTENTION 8(e) COORDINATOR

Dear Sir or Madam:

The information in this letter pertains to butyl benzyl phthalate and is being submitted to the U.S. Environmental Protection Agency in accordance with the EPA's interpretation of Section 8(e) of the Toxic Substances Control Act. Although we do not believe that this information constitutes a substantial risk for all of the reasons discussed below, the present submission is intended to discharge any 8(e) responsibilities that might exist and thus should be processed in accordance with the EPA's "substantial risk" procedures.

With this letter Monsanto submits to the TSCA 8(e) file a preprint to be published in *Environmental Health Perspectives* entitled "Gestational/neonatal exposure of rats to environmental estrogenic chemicals results in reduced testicular size and sperm production in adult life" by Sharpe, Fisher, Millar, Jobling and Sumpter. In this study the authors purport to show that treatment with butyl benzyl phthalate (and several other chemicals) during pre- and neonatal development results in reduced testicular size and in a concomitant reduction in sperm count in adult rats.



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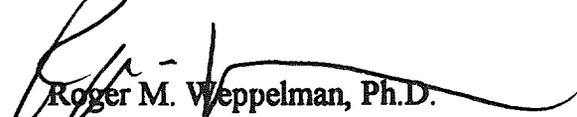
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We do not believe that these data represent a substantial risk because of limitations in the experimental design (the choice of negative control was inappropriate and only one dose level of butyl benzyl phthalate was tested) and because of the small magnitude of the changes reported with butyl benzyl phthalate (6.6-7.5% reductions in relative testicular wt. with concomitant reductions in sperm count per teste). Further, these findings appear inconsistent with results from other toxicity studies conducted with BBP under a number of different study, route and dosing conditions.

We also take this opportunity to note that Union Carbide has submitted to this office a copy of this same preprint because it contained data pertaining to 4-tert-octylphenol which were broadly similar to that described above for butyl benzyl pthalate.

Sincerely,


Roger M. Weppelman, Ph.D.
Manager, Regulatory Affairs.

Gestational Exposure of Rats to Xenoestrogens Results in Reduced Testicular Size and Sperm Production

Richard M. Sharpe,¹ Jane S. Fisher,¹ Mike M. Millar,¹ Susan Jobling,² and John P. Sumpter²

¹MRC Reproductive Biology Unit, Centre for Reproductive Biology, 37 Chalmers Street, Edinburgh EH3 9EW, Scotland; ²Department of Biology and Biochemistry, Brunel University, Uxbridge, Middlesex UB8 3PH UK

This study assessed whether exposure of male rats to two estrogenic, environmental chemicals, 4-octylphenol (OP) and butyl benzyl phthalate (BBP) during gestation or during the first 21 days of postnatal life, affected testicular size or spermatogenesis in adulthood (90–95 days of age). Chemicals were administered via the drinking water at concentrations of 10–1000 µg/l (OP) or 1000 µg/l (BBP); diethylstilbestrol (DES; 100 µg/l) and an octylphenol polyethoxyolate (OPP; 1000 µg/l), which is a weak estrogen or nonestrogenic *in vitro*, were administered as presumptive positive and negative controls, respectively. Controls received the vehicle (ethanol) in tapwater. In study 1, rats were treated from days 1–22 after birth; in studies 2 and 3, the mothers were treated for approximately 8–9 weeks, spanning a 2-week period prior to mating, throughout gestation and up until 22 days postnatal after giving birth.

With the exception of DES, treatment generally had no major adverse effect on body weight. In most instances, treated animals were heavier than controls at day 22 and at days 90–95. Exposure to OP, OPP or BBP at a concentration of 1000 µg/l resulted in a small (5–13%) but significant ($p < 0.01$ or $p < 0.001$) reduction in mean testicular size in studies 2 and 3, an effect that was still evident when testicular weight was expressed relative to body weight or kidney weight. The effect of OPP is attributed to its metabolism *in vivo* to OP. DES exposure caused similar reductions in testicular size but also caused reductions in body weight, kidney weight, and litter size. Ventral prostate weight was reduced significantly in DES-treated rats and to a minor extent in OP-treated rats. Comparable but more minor effects of treatment with DES or OP on testicular size were observed in study 1. None of the treatments had any adverse effect on testicular morphology or on the cross-sectional area of the lumen or seminiferous epithelium at stages VII–VIII of the spermatogenic cycle, but DES, OP and BBP caused reductions of 10–21% ($p < 0.05$ to $p < 0.001$) in daily sperm production. Humans are exposed to phthalates, such as BBP, and to alkylphenol polyethoxyolates, such as OP, but to what extent is unknown. More detailed studies are warranted to assess the possible risk to the development of the human testis from exposure to these and other environmental estrogens. **Key words:** butyl benzyl phthalate, daily sperm production, diethylstilbestrol, 4-octylphenol, Sertoli cell number, spermatogenesis. *Environ Health Perspect* 103:000–000 (1995)

The report by Carlsen et al. (1) that mean sperm counts in some men had declined by around 40–50% over the past 50 years, was greeted with a mixture of concern (2) and skepticism (3). The most recent data from a number of countries, which have charted changes in sperm counts in semen donors over the past 20–25 years, have, however, all reported a marked and significant downward trend (4–6). The most comprehensive of these studies, in Paris, concluded that sperm counts in fertile men have declined by around 2% per year over the past 23 years (6). Moreover, two of the cited studies (5,6) identified that the temporal decline in sperm counts appears to apply to men born from around 1950 onwards.

Two years ago, we hypothesized (7) that the reported decline in sperm counts might be related to an increasing incidence of other disorders of development of the male reproductive system (e.g., testicular cancer) and that this could have arisen because of increased exposure of the developing fetus to estrogens. One potential source of this

increased estrogen exposure was via environmental estrogenic chemicals, or “xenoestrogens,” the release of which has more or less coincided with the decline in sperm counts (8,9). Concern about such hormonally active pollutants, such as chlorinated pesticides, has been voiced for 10–20 years (10), but has become more acute recently because of the discovery of a range of new xenoestrogens, including bisphenol-A (11), certain alkylphenolic chemicals (12–14), certain phthalates (15), as well as a number of pesticides (16). Most of these estrogenic chemicals are ubiquitous in the environment, and humans are exposed to them daily by a number of routes (9). However, the risk to humans from these chemicals is currently theoretical because there are no data to show that these chemicals can cause any disorder of reproductive development or function in animals.

Pathways via which exposure of the developing male fetus or neonate to estrogenic chemicals could result in reduced testicular size and sperm production in adult

life have been identified (2,7,9), but there is no direct evidence to confirm whether this hypothesis has any factual basis. It is known that some phthalates are passed from the mother both across the placenta (17) and via milk (18), although comparable data on the transfer of alkylphenolic chemicals are lacking. The aim of the present studies was to evaluate whether exposure of the male rat fetus/neonate to either of two environmental estrogenic chemicals has any effect on testicular size and spermatogenesis in adult life.

Material and Methods

Animals and treatments. All rats used in these studies were of the Wistar strain and were bred in our own animal facility. They were maintained under standard, controlled conditions and had free access to food and water. Administration of chemicals was via the drinking water which was provided in a bottle per cage. A stock solution of each dose of chemicals was made by dissolving a weighed amount in ethanol such that addition of 0.5 ml of this stock to 5 l of tapwater resulted in the test dose; control animals had 0.5 ml ethanol/5 l added to their drinking water.

Study design. The most likely mechanism via which estrogenic chemicals could cause an irreversible reduction in testicular size and sperm production is by decreasing the number of Sertoli cells. In adult life, the number of Sertoli cells determines testicular size and sperm output in all animals that have been studied (19). In the male rat, Sertoli cells begin to proliferate soon after testicular differentiation (about day 15 of gestation) and continue until around day 15 of postnatal life, with perhaps minor proliferation until around day 21; after this time, no further Sertoli cell proliferation can occur (19). Thus, by day 22, the ultimate size to which the testis will grow in adulthood (90–95 days of age) has been predetermined (19–22).

Our studies were designed such that

Address correspondence to R.M. Sharpe, MRC Reproductive Biology Unit, Centre for Reproductive Biology, 37 Chalmers Street, Edinburgh EH3 9EW, Scotland.

We are grateful to Jim McDonald for expert help with the animals in these studies.

Received 7 June 1995; accepted 10 August 1995.

animals were exposed to chemicals for either the postnatal period (i.e., days 1–22 after birth) of Sertoli cell proliferation (study 1; see Fig. 1) or for the complete period of Sertoli cell proliferation (studies 2 and 3; see Fig. 1). In the later two studies, treatments were administered to adult female rats for 2 weeks before mating with a sexually experienced male, throughout mating, throughout gestation, and up until day 22 after giving birth (Fig. 1). This protocol of exposure was used to assess the possible effects of bioaccumulation. In all three studies, exposure of the male offspring to the test chemicals was thus largely indirect, via the placenta or milk.

In study 1, in which there was no prenatal exposure to the test chemicals, litters were culled to eight pups on the day of birth (day 1) by culling excess females. The same was done in study 2. In study 3, the full litter size was maintained from birth through day 22. The female offspring of test litters were not evaluated. Adult females used for mating in studies 2 and 3 were the same: when offspring of these females were weaned at the completion of study 2, the mothers were maintained on the same treatment for 2 weeks, then mated and exposed until the weaning of study 3 offspring (Fig. 1).

Test chemicals and doses. Three chemicals were selected for study based on the results of *in vitro* investigations suggesting that they were estrogenic (13–15). Of the three, 4-tert-octylphenol (OP; Aldrich Chemical Co., Gillingham, UK) and butyl benzyl phthalate (BBP; Chem Service, West Chester, Pennsylvania) were both estrogenic *in vitro*, whereas an octylphenol

polyethoxylate with a side-chain of five ethoxy groups (OPP; Igepal CO-520; Aldrich) was essentially devoid of estrogenicity *in vitro*. In study 1, nonylphenoxycarboxylic acid (NP1EC, K & K Labs, ION, Cleveland, Ohio), which is approximately 10-fold less estrogenic *in vitro* than OP (14), was also assessed at a single dose. Diethylstilbestrol (DES; Sigma Chemical Co., Poole, Dorset, UK), which is a potent nonsteroidal estrogen, was included as a positive control.

Little is known about the degree of exposure of humans to the chemicals used in the present studies, but concentrations of alkylphenolic compounds in the aquatic environment reportedly range up to hundreds of micrograms per milliliter (23,24), whereas human intake of phthalates is reportedly as high as 15 mg per day (200–300 µg/kg/day) (25). Therefore, we chose doses that would be mildly estrogenic based on *in vitro* analyses (14,15), but which remained within an order of magnitude of the possible environmental/human intake level. We tested OP at 1000 and 100 µg/l in all three studies (and also at 10 µg/l in study 1), whereas OPP and BBP were tested only at the single dose of 1000 µg/l in studies 2 and 3. DES was tested at 100 and 10 µg/l in study 1 and at 100 µg/l in studies 2 and 3. No formal confirmation that the test chemicals (or their metabolites) actually reached the male offspring, or in what amounts, was obtained, as the objective was simply to establish whether or not the chemicals exerted any biological effects. However, water intake, and thus the nominal intake of chemical/day, was assessed in

some of the treatment groups in study 3 by weighing water bottles every 48 hr.

Body and organ weights, litter size and composition. In studies 2 and 3, we recorded litter size and composition at birth. In all three studies, the male offspring were weighed at weaning (day 22), which was the day that treatment ceased. The male offspring were then maintained in their litters under standard conditions until 90–95 days of age, when they were killed by inhalation of CO₂, followed by cervical dislocation. Body weight was recorded and the right testis, left kidney, and ventral prostate were dissected out and weighed; the epididymis, seminal vesicles and left testis were also inspected macroscopically for any obvious abnormalities. We recorded kidney weight because the kidney varies according to body weight but was not expected to be affected by the experimental manipulations. The kidney was thus used as an internal "organ control" for the specificity of any effects observed on testis size.

Testicular morphology. At 90–95 days of age, representative animals from the various treatment groups were fixed with 3% glutaraldehyde in 0.2 M cacodylate buffer by perfusion via the dorsal aorta, as described elsewhere (26). The fixed testes were then cut transversely with a razor blade into 2-mm-thick sections and then into small blocks (1–2 mm²). After postfixation for 12–16 hr in the same fixative, the blocks were processed and embedded in plastic as described previously (26). Semithin sections (0.5 µm) were then cut, stained with toluidine blue, and examined using a Zeiss photomicroscope.

To provide a preliminary quantitative assessment of spermatogenesis, seminiferous tubules at stages VII–VIII of the spermatogenic cycle were subjected to image analysis (Cue-2, Olympus) to determine the cross-sectional area of the seminiferous tubule and seminiferous epithelium (27). We analyzed 10 round cross-sections for two or three rats per treatment group and calculated the mean and standard deviation for each animal. Stages VII–VIII were chosen for analysis because they contain representative germ cells from all steps of development (19).

Daily sperm production. In some of the animals from study 3, daily sperm production was determined by counting homogenization-resistant spermatids, using minor modifications of the techniques of Johnson *et al.* (28,29). A 500–700 mg portion of testicular tissue recovered at autopsy was immersion-fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer and kept at 4°C until used for daily sperm production determination within the next 6 weeks.

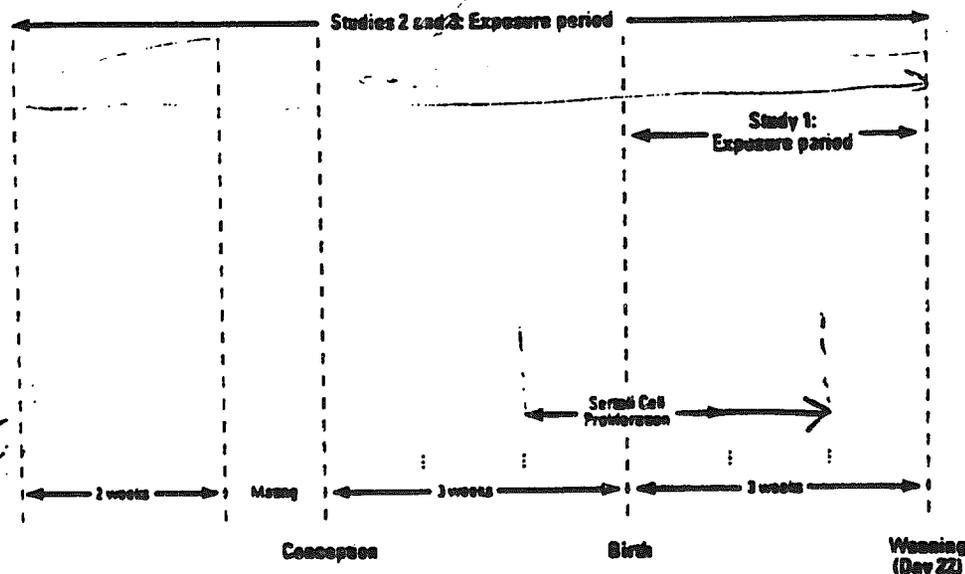


Figure 1. Experimental design of the present studies, indicating the periods of treatment in relation to the time of normal proliferation of Sertoli cells. After cessation of treatment (day 22 after birth) animals were maintained under normal conditions until they were killed at the age of 90–95 days.

The tissue was then removed, blotted, and weighed and two 50-mg portions cut with a scalpel, weighed, and homogenized separately in 5 ml 0.15 M NaCl, 0.05% Triton-X100, 0.025% sodium azide, using a Kinematica Polytron (PT-K/PCU-6) homogenizer at speed 5 for 60 sec (these conditions had previously been validated and optimized). Using a hemocytometer, homogenization-resistant step 18 and 19 spermatids were then counted separately in 3 aliquots of each of the 2 homogenates per sample and the mean of the 6 measurements calculated; the coefficient of variation for these replicates averaged 7% for all samples. This value was then corrected for sample weight and overall testis weight, and transformed to the daily sperm production by dividing by the appropriate time division (4.61) based on the proportional duration of stages VI-VIII in days, according to Leblond and Clermont (30). We confirmed that only step 18 and 19 spermatids were being counted by applying the same procedures to known lengths of seminiferous tubule isolated from normal adult rats by transillumination-assisted microdissection (31) at stages II-V (containing step 16 and 17 spermatids) and VI-VIII (containing step 18 and 19 spermatids).

Statistical analysis. In each of the three studies, each parameter in the different treatment groups was subjected to analysis of variance to determine whether there were significant effects of treatment. Where these were indicated, subgroup comparisons between means for the control and each treatment group were made using the variance from the study as a whole as the measure of error. All data were normally distributed, so no transformations were made and results are all reported as means \pm SD.

Results

Litter size and composition at birth were not evaluated in study 1, as there was no prenatal treatment of the mothers. In studies 2 and 3, in which the mothers were treated prenatally, there was no effect of treatment with OP, OPP or BBP, on litter size or composition. Exposure to DES (100 μ g/l) reduced average litter size by nearly half in study 3 and had a more minor effect in study 2 (Table 1). Curiously, the proportion of male offspring was increased significantly in the DES-exposed group in study 3 (Table 1).

At weaning, which corresponded to the cessation of treatment in all three studies, body weight of DES-exposed offspring was reduced significantly in studies 1 and 2 but was increased significantly in study 3, per-

haps because of the much smaller litter sizes (Table 1). Otherwise, exposure to any of the test chemicals had either no effect or, more commonly, resulted in a significant increase in body weight on day 22 (Table 1).

In study 1, mean body weight was generally higher in treatment groups, compared with controls, but only in the case of OP (100 μ g/l) did this reach statistical significance (Table 2). Average testis weight was reduced marginally, but significantly, in animals exposed to DES (100 μ g/l) and OP (1000 μ g/l) animals, and relative testis weight (i.e., relative to body weight or to kidney weight) was reduced significantly in these two groups and in animals exposed to the intermediate concentration (100 μ g/l) of OP (Table 2). Kidney weight was increased markedly in animals exposed to the two highest doses of OP and,

although this may have been due to some extent to the greater average body weight of the animals, a significant difference in kidney weight relative to body weight was still evident (Table 2).

In study 2, mean body weight in animals exposed to DES or OPP was reduced when compared to controls, whereas animals exposed to either dose of OP were increased in size by 8% or more (Table 3). Except for animals exposed to the lower dose of OP (100 μ g/l), all other treatment groups exhibited a highly significant decrease in absolute testis weight and in the ratio of testis/kidney size (Table 3); all treatment groups showed a significant decrease in relative testis weight. There were some minor, but significant, changes in absolute and relative kidney weight in some of the treatment groups. Ventral prostate weight was reduced by 16% in

Table 1. Litter size and composition at birth and body weight on day 22 (means \pm SD)

Study no.	Treatment group (μ g/l)	Litter size (no. of litters)	% Males at birth	Body weight (g) of males, day 22 (n)
1	Control	ND	ND	80 \pm 8 (70)
	DES (100)	ND	ND	53 \pm 7** (46)
	DES (10)	ND	ND	54 \pm 5† (48)
	OP (1000)	ND	ND	60 \pm 5 (56)
	OP (100)	ND	ND	65 \pm 6† (55)
	OP (10)	ND	ND	65 \pm 9** (34)
	Nonylphenol (100)	ND	ND	ND
2	Control	10.0 \pm 2.5 (5)	61 \pm 8	53 \pm 5 (29)
	DES (100)	8.2 \pm 1.7 (6)	64 \pm 14	46 \pm 8† (30)
	OP (1000)	12.0 \pm 1.1 (6)	46 \pm 14	61 \pm 8† (30)
	OP (100)	10.8 \pm 3.5 (6)	53 \pm 14	66 \pm 5† (33)
	OPP (1000)	10.6 \pm 3.2 (5)	63 \pm 15	54 \pm 7 (39)
	BBP (10000)	11.6 \pm 3.4 (5)	64 \pm 13	59 \pm 7** (36)
	Control	12.7 \pm 2.2 (6)	55 \pm 12	50 \pm 10 (36)
3	DES (100)	6.2 \pm 3.3 (5)**	80 \pm 20*	57 \pm 8** (27)
	OP (1000)	11.2 \pm 1.8 (5)	64 \pm 7	52 \pm 8 (39)
	OP (100)	10.8 \pm 2.1 (5)	57 \pm 13	54 \pm 7* (36)
	OPP (1000)	13.8 \pm 0.4 (6)	45 \pm 11	47 \pm 4 (34)
	BBP (1000)	13.4 \pm 1.5 (5)	57 \pm 3	57 \pm 8** (38)

Abbreviations: DES, diethylstilbestrol; OP, octylphenol; OPP, octylphenol polyoxyethylene; ND, not determined.

* $p < 0.05$, ** $p < 0.01$, † $p < 0.001$, compared to respective control value.

Table 2. Effect of exposure of male rats, from birth to day 22, to diethylstilbestrol, octylphenol, or nonylphenoxycetic acid added to the drinking water (μ g/l) on body weight, testis, and kidney weight (means \pm SD) at age 90-95 days (study 1)

Treatment group (μ g/l) ^a	N	Body weight (g)	Testis weight (mg)	Kidney weight (mg)	Testis/kidney weight ratio	Relative organ weight (mg/g body weight)	
						Testis	Kidney
Control	65	504 \pm 66	1988 \pm 163	1739 \pm 172	1.14 \pm 0.14	3.85 \pm 0.38	3.39 \pm 0.32
DES (100)	36	518 \pm 33	1894 \pm 218*	1797 \pm 124	1.06 \pm 0.16**	3.69 \pm 0.48*	3.49 \pm 0.20
DES (10)	44	506 \pm 40	1961 \pm 147	1732 \pm 131	1.16 \pm 0.09	3.89 \pm 0.34	3.37 \pm 0.24
OP (1000)	49	518 \pm 34	1898 \pm 130*	1883 \pm 223†	1.02 \pm 0.12†	3.68 \pm 0.23**	3.64 \pm 0.36**
OP (100)	29	556 \pm 37†	1930 \pm 126	1998 \pm 165†	1.02 \pm 0.10†	3.60 \pm 0.36**	3.54 \pm 0.23*
OP (10)	27	511 \pm 39	1940 \pm 132	1776 \pm 164	1.10 \pm 0.11	3.82 \pm 0.38	3.48 \pm 0.27
NPA (100)	33	522 \pm 45	1956 \pm 203	ND	ND	3.75 \pm 0.26	ND

Abbreviations: DES, diethylstilbestrol; OP, octylphenol; NPA, nonylphenoxycetic acid; ND, not determined.

^aLitters were culled to eight pups on the day of birth.

* $p < 0.05$, ** $p < 0.01$, † $p < 0.001$ compared to respective control value.

Table 3. Effect of exposure of male rats, throughout gestation and until postnatal day 22, to diethylstilbestrol, octylphenol, octylphenol polyethoxylate, or butyl benzyl phthalate added to drinking water ($\mu\text{g/l}$) on body weight and the weights of the testis, kidney, and ventral prostate (means \pm SD) at age 90–95 days (stud).

Treatment group ($\mu\text{g/l}$) ^a	N	Body weight (g)	Organ weights (mg)			Testis/kidney weight ratio	Relative organ weight (mg/g body weight)		
			Testis	Kidney	Ventral prostate		Testis	Kidney	Ventral prostate
Control	26	489 \pm 32	2014 \pm 155	1837 \pm 118	468 \pm 79	1.10 \pm 0.08	4.12 \pm 0.25	3.76 \pm 0.19	0.96 \pm 0.14
DES (100)	26	445 \pm 41 ^{**}	1750 \pm 180†	1759 \pm 158 ^{**}	393 \pm 44 ^{**}	1.00 \pm 0.09†	3.94 \pm 0.34 [*]	3.96 \pm 0.24 [*]	0.89 \pm 0.11
OP (1000)	27	530 \pm 53†	1899 \pm 123 ^{**}	1915 \pm 160 [*]	428 \pm 88	0.99 \pm 0.07†	3.61 \pm 0.32†	3.63 \pm 0.32	0.82 \pm 0.19 ^{**}
OP (100)	29	534 \pm 41†	2042 \pm 179	1880 \pm 112	442 \pm 82	1.09 \pm 0.10	3.94 \pm 0.38 ^{**}	3.53 \pm 0.23 ^{**}	0.84 \pm 0.16 [*]
OPP (1000)	32	461 \pm 32 [*]	1783 \pm 137†	1795 \pm 159	476 \pm 103	1.00 \pm 0.11†	3.88 \pm 0.30 ^{**}	3.90 \pm 0.35	1.03 \pm 0.20
BBP (1000)	35	476 \pm 28	1809 \pm 126†	1830 \pm 116	454 \pm 84	0.99 \pm 0.09†	3.81 \pm 0.34†	3.85 \pm 0.24	0.96 \pm 0.19

Abbreviations: DES, diethylstilbestrol; OP, octylphenol; OPP, octylphenol polyethoxylate; BBP, butyl benzyl phthalate.

^aLitters were culled to eight pups on the day of birth.

* $p < 0.05$, ** $p < 0.01$, † $p < 0.001$ compared to respective control values.

Table 4. Effect of exposure of male rats, throughout gestation and until postnatal day 22, to diethylstilbestrol, octylphenol, octylphenol polyethoxylate, or butyl benzyl phthalate added to drinking water ($\mu\text{g/l}$) on body weight and the weights of the testis, kidney, and ventral prostate (means \pm SD) at age 90–95 days (study 3)

Treatment group ($\mu\text{g/l}$) ^a	N	Body weight (g)	Organ weights (mg)			Testis/kidney weight ratio	Relative organ weight (mg/g body weight)		
			Testis	Kidney	Ventral prostate		Testis	Kidney	Ventral prostate
Control	36	479 \pm 26	1934 \pm 118	1749 \pm 169	522 \pm 84	1.13 \pm 0.14	4.09 \pm 0.28	3.86 \pm 0.33	1.08 \pm 0.16
DES (100)	23	466 \pm 34	1847 \pm 157 ^{**}	1792 \pm 183	387 \pm 83†	1.04 \pm 0.16 ^{**}	3.99 \pm 0.45	3.85 \pm 0.30 [*]	0.84 \pm 0.18†
OP (1000)	37	474 \pm 34	1696 \pm 140†	1621 \pm 121 ^{**}	461 \pm 63 ^{**}	1.05 \pm 0.07 ^{**}	3.57 \pm 0.23†	3.42 \pm 0.24†	0.98 \pm 0.11 ^{**}
OP (100)	34	461 \pm 22 [*]	1838 \pm 114 ^{**}	1699 \pm 121	470 \pm 76 [*]	1.09 \pm 0.09	4.00 \pm 0.31	3.69 \pm 0.22	1.03 \pm 0.20
OPP (1000)	34	460 \pm 32 [*]	1810 \pm 110†	1710 \pm 160	495 \pm 65	1.07 \pm 0.11 [*]	3.95 \pm 0.35 [*]	3.72 \pm 0.22	1.08 \pm 0.12
BBP (1000)	35	477 \pm 24	1819 \pm 119†	1830 \pm 133	486 \pm 63	1.01 \pm 0.08†	3.82 \pm 0.26†	3.79 \pm 0.20	1.03 \pm 0.12

Abbreviations: DES, diethylstilbestrol; OP, octylphenol; OPP, octylphenol polyethoxylate; BBP, butyl benzyl phthalate.

^aLitters not culled to a standard size at birth.

^bLitter size, etc., also affected by treatment (see Table 1).

* $p < 0.05$, ** $p < 0.01$, † $p < 0.001$ compared to respective control value.

DES-treated animals, though this effect largely disappeared when the size relative to bodyweight was evaluated (Table 3). Relative weight of the prostate was reduced significantly in animals exposed to either dose of OP.

In study 3, animals were noticeably smaller on average, both at weaning and at 90–95 days of age, than animals in studies 1 and 2 (Table 4). Under this regimen, no treatment group in adult life had a larger mean body weight than the control group, and two of the groups (OP at 100 $\mu\text{g/l}$ and OPP) showed a small but significant decrease in body weight relative to controls. In all treatment groups, testis weight was reduced significantly when compared to controls, though when expressed relative to body weight this difference disappeared for the groups exposed to DES or the lower dose of OP (Table 4). Kidney weight was reduced noticeably in animals exposed to 1000 μg OP/l, a difference still evident when expressed relative to body weight; however, the testis/kidney weight ratio in this group was still significantly lower than that observed in the control group (Table 4). As in study 2, ventral prostate weight was reduced significantly in DES-exposed animals but, in study 3, a significant reduction was also obvious in animals exposed to OP, particularly at the higher dose (Table

4).

Although exposure to the test chemicals throughout gestation and neonatal life resulted in fairly consistent reductions in testis size as adults, these decreases were only on the order of 5–13%. However, plotting the data for testis weight against body weight for four of the treatment groups from studies 2 and 3 (i.e., DES, OP at 1000 $\mu\text{g/l}$, BBP) shows that the treated animals come from a different population to the controls (Fig. 2). This is most evident by noting how few of the values for treated animals lie above the linear regression line plotted for the control group. Although a similar trend was evident in the DES-exposed animals, testicular weights were far more variable in this treatment group, probably because of the confounding effects of this treatment on litter size, etc.

Testicular morphology was indistinguishable in animals from the control and treatment groups, and no obvious abnormalities in the seminiferous tubules, interstitium, or vasculature were evident (Fig. 3). Image analysis confirmed this impression by demonstrating no adverse effect of treatment on the cross-sectional parameters of stage VII–VIII seminiferous tubules; indeed, for the most part, these parameters tended to be higher for the treated animals

distribution

than for the controls, though this is based on a small sample size (Table 5).

Daily sperm production in control animals from study 3 averaged $24.9 \pm 5.6 \times 10^6$ per testis per day (mean \pm SD, $n = 12$), which agrees closely with that determined morphometrically by Wing and Christensen (32). Animals exposed during fetal and neonatal life to DES, OP (1000 $\mu\text{g/l}$), or BBP in study 3 all showed significant reductions of 10–21% in the mean daily sperm production (Fig. 4), which were proportionately similar to the decrease in testis weight (Table 4); tissue from animals exposed to OPP was not evaluated.

The nominal intake of chemical was assessed in study 3 for animals in two of the treatment groups (OP at 1000 $\mu\text{g/l}$, BBP) based on water intake, and ranged from around 125 $\mu\text{g/kg/day}$ in the first 2 days after birth to 370 $\mu\text{g/kg/day}$ just before weaning (Table 6). As these calculations take no account of spillage, adsorption, or degradation of the chemicals (which were not evaluated), these values for intake can be viewed as over-estimates of the actual intake. The level of water intake was not affected by treatment (Table 6).

Discussion

The purpose of the present studies was to assess whether exposure of male rats to

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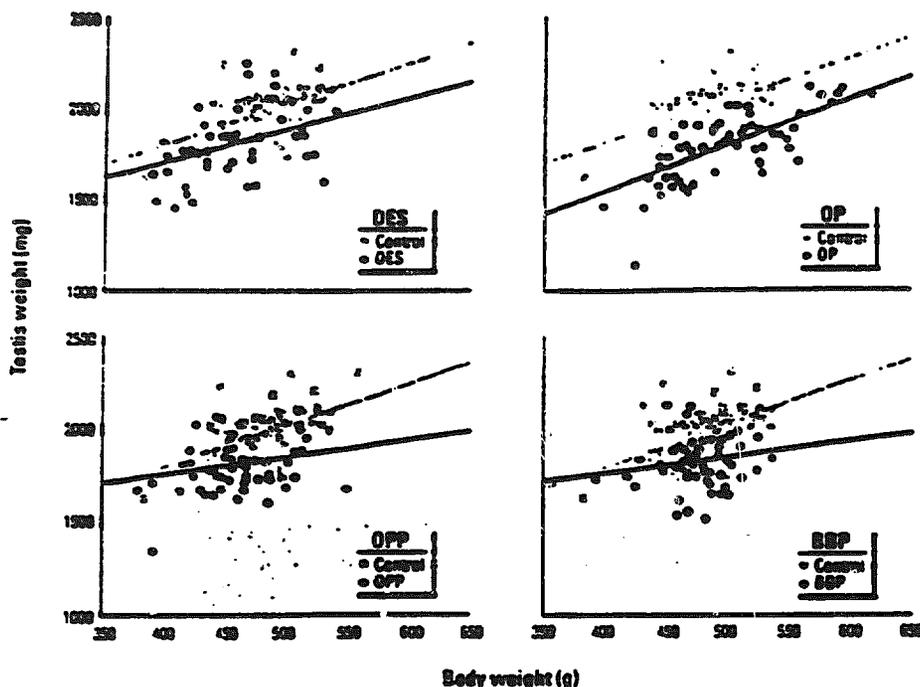


Figure 2. Scatter plots of testicular size versus body weight for animals from studies 2 and 3 combined. The data for controls are shown in each of the panels in comparison to that for animals exposed to diethylstilbestrol (DES), 1000 µg octylphenol/L octylphenol polyethoxylate, or butyl benzyl phthalate. Linear regression lines for the control and each treatment group are shown to aid comparison. Mean values for studies 2 and 3 are given separately in Tables 3 and 4.

from 5% to 21%, this effect occurred during a relatively short period of treatment and after exposure to relatively low levels of the chemicals. Previous data involving a similar protocol of exposure of rats to the estrogenic pesticide methoxychlor also reported a small reduction in adult testicular size and sperm counts (33), and a recent study in trout (34) has demonstrated inhibition of testis growth *in vivo* after exposure to estrogenic alkylphenolic chemicals.

Ultimate testicular size in all mammals that have been investigated is determined by the number of Sertoli cells present in the testis, despite the fact that it is the germ cells, rather than the Sertoli cells, which constitute the bulk of the testis (19,21). Each Sertoli cell can only support a fixed number of germ cells through their development into spermatozoa, and hence the more Sertoli cells present, the more germ cells present, and thus the larger the testis. The number of Sertoli cells can be increased or decreased experimentally in various animals by a number of treatments, with corresponding changes in testicular size and daily sperm production, and the same relationship appears to apply to men (19). Usually, when Sertoli cell number is altered, there is little change in the cross-sectional appearance or size of the seminiferous tubules because Sertoli cell number affects primarily the length, not the breadth, of the tubules (20,21). The number of Sertoli cells per testis is determined by the rate and duration of their proliferation, which usually occurs during a precisely timed period that begins in fetal life (shortly after testicular differentiation) and continues into neonatal life for a period that varies according to the species (19).

In the present studies, rats were exposed to estrogenic chemicals for either part (study 1) or all (studies 2 and 3) of the period when Sertoli cell proliferation occurs (Fig. 1). If these treatments had reduced the rate of Sertoli cell proliferation, we would expect that, in adult life, the testes would be smaller and daily sperm production will be reduced, but the cross-sectional appearance of the seminiferous tubules would probably be unchanged. Our findings are consistent with the treatments having reduced Sertoli cell number, though morphometric determination of Sertoli cell number will be necessary to determine whether this interpretation is correct; as the expected change in Sertoli cell number would only be of the order of 2-4%, measurements in large cohorts of animals will be necessary to demonstrate this. However, an earlier study (35) demonstrated that exposure of rats to di(2-ethylhexyl)phthalate for 5 days during

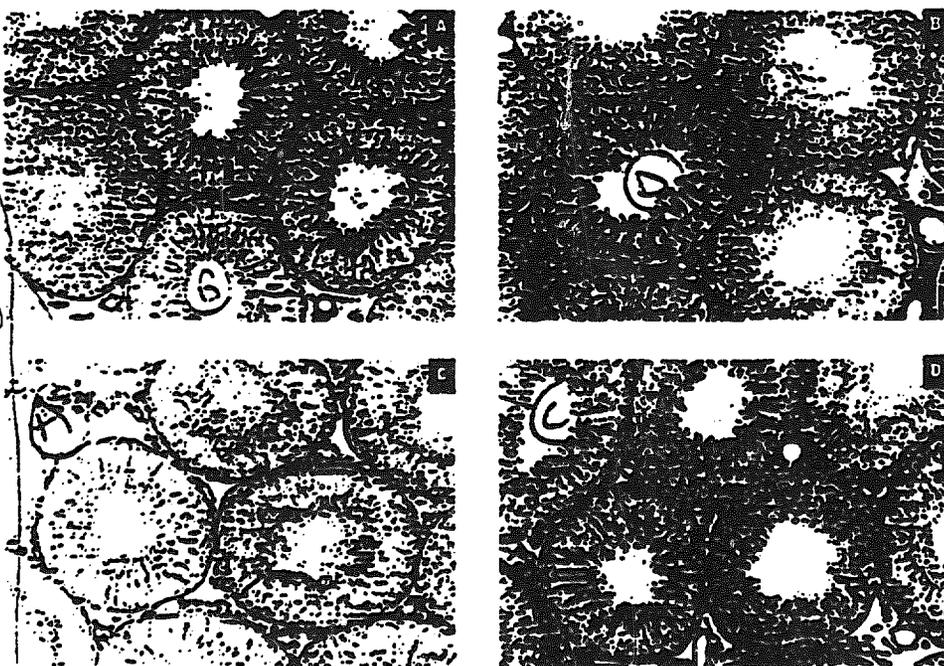


Figure 3. Representative testicular morphology in control animal (A) and in rats exposed during fetal/neonatal life to diethylstilbestrol (B), 1000 µg octylphenol (C), or butyl benzyl phthalate (D) in study 3. (A-D) × 120.

known estrogenic, environmental chemicals during gestation or neonatal life had any adverse effect on testicular size and spermatogenesis when these animals reached adulthood. The results are unequivocal in showing that exposure to such chemicals does cause a reproducible

and consistent decrease in ultimate testicular size and daily sperm production in rats, an effect which cannot be attributed to any obvious overt toxicity (judged by body weight and kidney weight). Although the chemical-induced decrease in testicular size and daily sperm production only ranged

Table 5. Quantitative analysis of the cross-sectional area of seminiferous tubules and the seminiferous epithelium at stages VII-VIII of the spermatogenic cycle in animals from study 3^a

Treatment group (µg/l)	Animal no.	Seminiferous tubule area (10 ³ µm ²)	Seminiferous epithelium area (10 ³ µm ²)
Control	1	77 ± 9	56 ± 6
	2	71 ± 9	51 ± 6
DES (1000)	1	94 ± 11	69 ± 6
	2	89 ± 12	68 ± 8
OP (1000)	1	79 ± 7	58 ± 5
	2	74 ± 6	56 ± 5
	3	76 ± 9	61 ± 6
OPP (1000)	1	83 ± 9	61 ± 8
	2	86 ± 12	63 ± 8
BBP (1000)	1	86 ± 11	62 ± 8
	2	86 ± 9	64 ± 7

Abbreviations: DES, diethylstilbestrol; OP, octylphenol; OPP, octylphenol polyethoxylate; BBP, butyl benzyl phthalate.

^aRats were exposed to chemical via drinking water throughout gestation and until postnatal day 22. Data are the means ± SD for 10 seminiferous tubules per animal and were based on analysis of paraffin-fixed tissue. Because of the small numbers of animals, no statistical analysis of these data was attempted.

... is metabolized such that the five phenoxy groups are cleaved, resulting in the formation of OP. This interpretation is supported by the observation that, whereas short-chain, alkylphenol polyethoxylates (such as OPP) do not bind to the estrogen receptor in cell-free systems (14), they are estrogenic in cell-based *in vitro* assays (13,14) and *in vivo* (34). It will be important in future studies to establish unequivocally whether only estrogenic chemicals are able to reduce testicular size and daily sperm production in the manner described here.

Irrespective of whether the reduction in testis size and daily sperm production caused by developmental exposure to OP or BBP resulted from their estrogenicity, the key question is whether these effects have relevance to humans. This is a complex issue which requires detailed dose-response data and measurement of the actual levels of the administered chemicals in the male rats. It would be appropriate to consider whether the nominal level of exposure of rats to OP and BBP in the present studies (which is presumed to be an overestimate of actual exposure levels) bears any relationship to the equivalent level of human exposure. There are little or no data specifically for OP, but there is some information on the environmental levels of the class of compounds to which OP belongs, namely, alkylphenol polyethoxylates, several of which have been shown to be estrogenic (14). Reported levels of these compounds in river water vary from the low micrograms per liter (44) to tens and hundreds of micrograms per liter (23,24), which approach the nominal levels of exposure in the present study. Even tapwater has been reported to contain estrogenic degradation products of both nonylphenol ethoxylate and octylphenol (37), although the combined concentration was only about 1 µg/l. However, as alkylphenol polyethoxylates are used widely in industrial and some household detergents and cleaners, in certain plastics, and in many other ways, human exposure via routes other than drinking water are likely.

In the case of BBP, there is more evidence for concern about the possible risk to human health. BBP and other phthalates are the most ubiquitous of all environmental contaminants, primarily because of their use as plasticizers, and human exposure is likely to be high (25,38,39). For example, a recent study reported levels of BBP alone as high as 47.8 mg/kg in some foil-wrapped butters (40), which would mean that ingestion of 50 g/day of such butter by a 60-kg woman would lead to an intake of approximately 40 µg/kg/day, which approaches the

... woman would lead to an intake of approximately 40 µg/kg/day, which approaches the

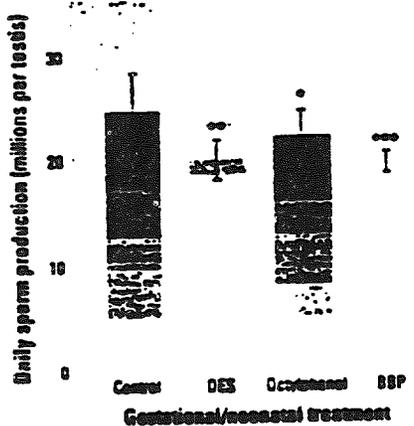


Figure 4. Daily sperm production (means ± SD) in representative control animals (n = 12) and in rats exposed during fetal/neonatal life to diethylstilbestrol (DES; n = 7), octylphenol (1000 µg/l; n = 18) or (BBP; n = 7) in study 3. *p < 0.05, **p < 0.01, ***p < 0.001 compared to control.

neonatal life resulted in a reduction in Sertoli cell number and some reduction in testis size and sperm production in adulthood; however, the level of phthalate exposure in this study was at least 1000-fold higher than in the present studies.

When animals are exposed to test chemicals, it is possible that toxic effects on organs (e.g., the liver) other than the testis or reproductive axis could lead, secondarily, to a reduction in testicular size and thus daily sperm production as a result of non-specific effects. Although this possibility cannot be excluded completely, the present data on body weight and kidney weight provide little evidence for any such effect—indeed, in most instances treated animals were larger than the controls. The exception was animals exposed to DES (positive controls), which showed consistent evidence of somewhat lower body weights. The explanation for this effect is not clear

Table 6. Estimation of the nominal intake of octylphenol (OP) and butyl benzyl phthalate (BBP) between birth and day 21 of postnatal life, based on water consumption in study 3 (means ± SD, N = 6)

Treatment group (µg/l)	Days postnatal	Water intake (ml/48 hr)	Nominal mean intake of chemical (µg/kg/day) ^a
Control	1 + 2	75 ± 22	—
	10 + 11	192 ± 55	—
	20 + 21	243 ± 72	—
OP (1000)	1 + 2	90 ± 36	129
	10 + 11	216 ± 42	309
BBP (1000)	1 + 2	88 ± 24	126
	10 + 11	192 ± 64	274
	20 + 21	256 ± 43	366

^aAssumes a body weight of 350 g for lactating females.

from the present studies, but it is likely that lactation may have been impaired by DES (36). If this is the case, it is somewhat puzzling why there was no evidence for such effects in animals exposed to any of the estrogenic chemicals, as these caused equal, or even larger, reductions in testicular weight than did DES exposure. This discrepancy could reflect differences in the pharmacokinetics of the chemicals compared with DES.

Although the estrogenic chemicals tested in the present studies exerted similar effects on testis size and daily sperm production, no evidence is provided that these effects resulted specifically from the estrogenicity of these compounds. The fact that treatment with OPP caused a similar reduction in testicular size as did treatment with OP, despite the fact that OP is non-estrogenic *in vitro* (14), could be interpreted as evidence against estrogenicity per se being a common causal mechanism. However, it is possible that, when ingested,

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tion of 50 µg/day of such butyryl by a 60-kg woman would lead to an intake of approximately 40 µg/kg/day, which approaches the nominal intake values in the present study. As the levels of total phthalates in other dairy produce can exceed 50 µg/kg (41,42), and there are many other possible sources of human exposure to these compounds, the present findings suggest that further studies of the estrogenicity of phthalates should be a priority. There is already a huge literature on the toxicity of phthalates, including their testicular toxicity, but few of the published studies have been able to detect developmental effects similar to those reported here. This is borne out by the reported no-observed effect level (NOEL) of BBi for testicular toxicity of 125–150 mg/kg/day (43,44). The fact that, in the present studies, nominal intake of 300-fold lower amounts than the NOEL resulted in around a 10% decrease in testicular weight in two separate studies with a commensurate fall in daily sperm production, argues further that the cause of these decreases differs from the previously reported toxic effects of these compounds on the testis.

The present data do not provide direct evidence of a link between human exposure to environmental estrogens and falling sperm counts in men. However, the findings do provide some preliminary, indirect evidence that exposure of rats to certain environmental estrogenic chemicals during gestation or neonatal life can result in reduced testicular size and sperm production in adulthood. As these effects occurred in rats after only 3–9 weeks of exposure, whereas in men the corresponding window of development (and Sertoli cell proliferation) spans several years, there is at least the theoretical possibility that similar effects in men might be of larger magnitude than those described here for the rat. However, considerably more work, particularly in establishing the likely level of human exposure to estrogenic chemicals, will be necessary if the risk to man from such exposure is to be assessed with any accuracy.

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