Normal Reproductive Organ Development in CF-1 Mice Following Prenatal Exposure to Bisphenol A

S. Z. Cagen, Shell Chemical Co., Houston, TX, USA
J. M. Waechter, Jr, The Dow Chemical Co., Midland, MI, USA
S. S. Dimond, General Electric Co., Pittsfield, MA, USA
W. J .Breslin, MPI Research, Mattawan, MI, USA
J .H. Butala, Consultant to Aristech, Pittsburgh, PA, USA
F. W. Jekat, Bayer AG, Wuppertal, Germany
R. L. Joiner, General Electric Co., Pittsfield, MA, USA
R. N. Shiotsuka, Bayer Corp., Stilwell, KS, USA
G. E. Veenstra, Shell Chemicals Ltd., London, England
L. R. Harris, The Society of the Plastics Industry, Inc., Washington, DC, USA

ABSTRACT

Bisphenol A (BPA) is a monomer used in the manufacture of a multitude of chemical products, including epoxy resins and polycarbonate. The objective of this study was to evaluate the effects of BPA on male sexual development. This study, performed in CF-1 mice, was limited to the measurement of sex organ weights, daily sperm production (DSP), epididymal sperm count, and testis histopathology in the offspring of female mice exposed to low doses of BPA (0, 0.2, 2, 20, or 200 μg/kg/day) by deposition in the mouth on gestation days 11-17. Male sexual development determinations were made in offspring at 90 days of age. Since this study was conducted to investigate and clarify low-dose effects reported by Nagel *et al.* (1997) and vom Saal *et al.* (1998), the study protocol purposely duplicated the referenced studies for all factors indicated as critical by those investigators. An additional group was dosed orally with 0.2 μg/kg/day of diethylstilbestrol (DES), which was selected based as the maternal dose reported to have the maximum effect on the prostate of developing offspring (vom Saal *et al*., 1996; 1997). No treatment-related effects on clinical observations, body weight, or food consumption were observed in adult females administered any dose of BPA or DES. Similarly, no treatment-related effects on growth or survival of offspring from dams treated with BPA or DES were observed. The total number of pups born per litter was slightly lower in the 200 μg/kg/day BPA group when compared to controls, but this change was not considered treatment-related since the litter size was within the normal range of historical controls. There were no treatment-related effects of BPA or DES on testes histopathology, daily sperm production or sperm count, or on prostate, preputial gland, seminal vesicle, or epididymis weights at doses previously reported to affect these organs or at doses an order of magnitude higher or lower. In conclusion, under the conditions of this study, the effects of low doses of BPA reported by Nagel *et al*. (1997) and vom Saal *et al*. (1998) or of DES reported by vom Saal *et al*. (1997) were not observed. The absence of adverse findings in the offspring of dams treated orally with DES challenges the "low-dose hypothesis" of a special susceptibility of mammals exposed perinatally to ultra low doses of even potent estrogenic chemicals. Based on the data in the present study and the considerable body of literature on effects of BPA at

similar and much higher doses, BPA should not be considered as a selective reproductive or developmental toxicant.

INTRODUCTION

Bisphenol A (BPA) is an important chemical used principally as a monomer in the manufacture of a multitude of chemical products, including epoxy resins and polycarbonate. BPA has been extensively evaluated for toxicity in a variety of tests in rodents, including developmental toxicity, reproductive toxicity, and carcinogenicity (Morrisey *et al*., 1987; Morrisey *et al*., 1989; NTP, 1982). Generally, BPA has shown a low order of toxicity, was not a selective developmental or reproductive toxicant, and was not carcinogenic to rats or mice. Several *in vitro* and *in vivo* genotoxicity studies have been conducted, all with negative results (BIBRA, 1989).

Although it has been known for several decades that BPA is weakly estrogenic in specialized protocols (Dodds and Lawson, 1936; Bitman and Cecil, 1970), recently there has been renewed attention to the estrogenicity of BPA. In 1993, Krishnan and coworkers discovered that BPA leaching from autoclaved polycarbonate flasks was confounding studies to determine if *S. cerevisiae* produced estrogens. More recently, Gaido *et al*. (1997) confirmed the weak estrogenicity of BPA *in vitro*, showing BPA to be approximately 15,000 times less potent than 17β-estradiol, and Kuiper *et al*. (1997) demonstrated that BPA could interact with both the α- and β-estrogen receptors.

The *in vivo* estrogenic potential of BPA was originally demonstrated in short-term uterotrophic assays in rodents using parenteral administration (Dodds and Lawson, 1936; Bitman and Cecil, 1970). However, recent studies have demonstrated a clear route-dependency in the magnitude of the uterotrophic response to BPA. Significantly larger oral doses were required to produce an estrogenic response than those required subcutaneously (Laws and Carey, 1997; Twomey, 1998a; Twomey; 1998b). No uterotrophic responses to BPA were observed at oral doses ranging from 2 to 100,000 μg/kg/day, whereas the no observed effect levels (NOELs) following subcutaneous administration ranged from 2 to 1,000 μg/kg/day (Twomey, 1998a; Twomey, 1998b). This route-dependency in uterotrophic response is consistent with the more extensive metabolic clearance of BPA via the oral route as significantly lower BPA concentrations in blood were present in orally exposed rats when compared to the concentrations in rats dosed intraperitoneally or subcutaneously (Pottenger *et al*., 1996). These data suggest that the estrogenic properties of BPA have not been manifest in previously conducted oral toxicity studies because of the relatively rapid metabolism and elimination of orally administered BPA and the low estrogenic potency of BPA.

Recently, however, experiments by Nagel *et al*. (1997) and vom Saal *et al*. (1998) reported that administration of low oral doses of BPA to pregnant female mice (n = 5-7) on gestation days (GD) 11-17 produced statistically significant increases in the weights of the prostate and preputial glands and a decrease in epididymis weights and the efficiency of sperm production in their male offspring. These results were not consistent with the

previously reported absence of reproductive or developmental effects following oral BPA exposures.

Therefore, the objective of the present study was to repeat the experiments of Nagel *et al*. (1997) and vom Saal *et al*. (1998) using a larger number of animals per treatment group in a rigorously controlled environment.

MATERIALS AND METHODS

Study Design

Since the present study was specifically undertaken to repeat the experiments of Nagel *et al.* (1997) and vom Saal *et al*. (1998), the study was designed to duplicate the procedures detailed in those reports as closely as possible with the following exceptions: 1) larger numbers of mice were used in all groups to increase the statistical power and hence sensitivity of the study, 2) four BPA doses instead of two were used, 3) two methods were used for determination of sperm count, 4) male offspring were sacrificed at 90 days instead of 180 days because effects on male sex accessory organs were reportedly driven by the *in utero* BPA exposure and sacrifice time after puberty was not critical (vom Saal and Thayer, 1997), and 5) males were individually housed from the time of weaning because group housing of males (in triads) is known to significantly affect the weight of sex accessory organs, including the prostate and preputial glands with dominant males having significantly larger organ weights than subordinate males (Bartos and Brain, 1993). In addition, food consumption and growth in pregnant dams and their offspring were measured throughout the study, as well as delivery and litter data.

Test Materials

Bisphenol A (BPA) used in this study, with a purity of >99%, was obtained from The Dow Chemical Company (Midland, MI). Diethylstilbestrol (DES), with a purity of 99%, was obtained from Sigma Chemical Co. (St. Louis, MO). Tocopherol-stripped corn oil (ICN Biomedicals Inc., Aurora, OH) served as the vehicle and control substance.

Dose Preparation and Analysis

Appropriate amounts of BPA and DES were mixed with tocopherol-stripped corn oil to achieve the desired concentrations. Fresh solutions were prepared weekly for each concentration and stored in glass containers. Based on the expected body weight of 40 grams for pregnant CF-1 mice at the midpoint of dosing (GD14), 0.75mL/kg was administered to each mouse. The dose solutions from each weekly preparation were analyzed prior to dosing to determine that the BPA and DES concentrations were within ∀10% of targeted concentrations.

Animals and Treatment

Time-mated CF-1 mice were obtained from Charles River Laboratories (Portage, MI). Upon receipt at the laboratory, the mice were housed individually. During the 11-day acclimation period, all mice were weighed on GD 0 and GD 10, observed daily for any clinical signs of disease, and given a detailed physical examination prior to the start of the

study. Animals gaining >4.5 grams in body weight during the GD 0-10 pre-exposure interval were randomized into seven groups on GD 10, using a stratified (by weight) block randomization procedure, until 28 mice/treatment group were assigned (Table 1). The weight gain criterion reduced placement of non-pregnant females on the study.

On GD 11-17, the mice were dosed orally with BPA (0, 0.2, 2, 20, or 200 μg/kg/day) or DES (0.2 μg/kg/day) in a tocopherol-stripped corn oil vehicle by deposition into the mouth using a micropipetter as reported by Nagel *et al*. (1997). All doses were adjusted daily, based on body weight, to provide constant dose levels (Table 1).

Throughout the study, all mice were kept in an environmentally controlled room with temperature and relative humidity maintained between 69°-75°F (21°-24°C) and 43-65%, respectively. Fluorescent lighting provided illumination 12 hr/day via an automatic timer and lighting levels were maintained below 18 ft-candles (measured 1 meter off the floor and approximately 1-6 inches in front of the cages on each side of the rack). Low-volume music was played in the animal rooms to provide background noise (vom Saal and Thayer, 1997).

Diet (Certified Rodent Chow #5002, PMI Feeds, Inc., St. Louis, MO) and drinking water were available *ad libitum*. Certification analysis of each lot of diet was performed by the manufacturer. The same lots of diet were provided to animals from all groups at the same time during the course of the study to control across groups for possible variation in the content of the diet. Water was available via glass bottles with Teflon seals during the exposure period.

Females were housed individually throughout the study except during lactation when they were housed with their litters. Adult females and weanling males were individually housed in polypropylene plastic tubs with stainless steel lids and corncob bedding. Males to be retained to 90 days were individually housed following weaning in suspended, stainless steel, wire-mesh cages.

In-Life Observations

All mice were observed at least twice a day, seven days a week, for morbidity, mortality, and signs of injury. During treatment (GD 11-17), a detailed clinical examination of each mouse was performed once daily and weekly after treatment stopped. Each weaned pup was given a detailed clinical examination on the day of weaning, daily for the 4 consecutive days following weaning, and weekly thereafter until study termination.

Food consumption in time-mated females was recorded during the intervals of GD 0-7, 7-10, 10-11, and 11-17. Following selection of females to be placed on study, food consumption was measured during GD 11-17. After parturition, food consumption was recorded twice during the first and second weeks of lactation and at 2- to 3-day intervals during the last week of lactation. For post-weaning males that were retained until 90 days of age, food consumption was recorded weekly at the time each body weight was recorded.

Time-mated females were weighed on GD 0, 10, and 11-18. Females that delivered litters were weighed on postnatal days (PND) 1, 4, 7, 14, and 21. Male and female pups were weighed individually on PND 1, 4, and 22. Each male pup to be maintained to 90 days of age was weighed on PND 22 and weekly thereafter.

Table 1. Assignment to Study

		Number of Animals	
Group	**Dose (µg/kg/day)**	**Dams[a]**	**Selected F_1 Males[b]**
1	Control	28	65
2	Control	28	94
3	0.2 DES	28	80
4	0.2 BPA	28	84
5	2 BPA	28	75
6	20 BPA	28	70
7	200 BPA	28	80

[a] 28 females/group were dosed on GD 11-17.

[b] At weaning, the surviving males (maximum of 4 per litter) from each litter were continued on study, untreated, until 90 days of age.

Litter Data

All litters were examined as soon as possible after delivery. The day of parturition (PND 0) was recorded for each litter. The number of live and dead pups and sex and weight of each pup were recorded for each litter on PND 1, 4, and 22. In addition, any visible physical abnormalities or demeanor changes during lactation were recorded for pups in each litter. To reduce the variation in the growth of pups, litters were culled on PND 4 to a total of 8 pups (8 males when possible) using a computer-generated randomization procedure. If fewer than 8 males were available, the appropriate number of females was retained to achieve a total of 8 pups. Litters with 8 or fewer pups were not culled. Preferential culling of runts was not performed. Culled pups were examined externally for abnormalities and euthanized.

Weaning of all litters were performed on PND 22. A maximum of 4 male weanlings per litter was randomly selected, using a computer-generated procedure, to continue on test to 90 days of age. Weanlings not held were examined externally for abnormalities and euthanized.

Table 2. Natural Delivery and Litter Data Summary

	0 µg/kg/day Corn Oil	0.2 µg/kg/day DES	0.2 µg/kg/day BPA	2 µg/kg/day BPA	20 µg/kg/day BPA	20 µg/kg/day BPA

Females on study (n)	56	28	28	28	28	28
Females pregnant (n)	51	27	27	25	27	27
Females with Liveborn (n)	48	25	27	24	24	25
Gestation Index (%)	94	93	100	96	89	93
Duration of gestation (days)	19.22 ∀ 0.73[a] (51)[b]	19.56 ∀ 0.97 (27)	19.7 ∀ 0.38 (27)	19.52 ∀ 0.77 (25)	19.48 ∀ 0.94 (27)	19.33 ∀ 0.92 (27)
Total pups born/litter (n)	12.37 ∀ 3.02 (51)	10.44 ∀ 4.09 (27)	10.78 ∀ 2.78 (27)	10.84 ∀ 4.36 (25)	10.19 ∀ 3.91 (27)	9.60 ∀ 3.85* (27)
Stillborn (%)	7 (51)	14 (27)	5 (27)	9 (25)	16 (27)	8 (27)
Pups surviving 1 day (%)	96 (48)	99 (25)	97 (27)	99 (24)	93 (24)	100 (25)
Pups surviving 4 days (%) (viability index)	89 (48)	98 (25)	90 (27)	98 (24)	90 (24)	98 (25)
Pups surviving 22 days (%) (lactation index)	88 (47)	90 (25)	91 (25)	92 (24)	81 (24)	91 (25)
Live pups/litter[c]						
Day 0	11.37 ∀ 4.09 (51)	9.63 ∀ 4.73 (27)	10.26 ∀ 2.99 (27)	10.36 ∀ 4.93 (25)	9.22 ∀ 4.49 (27)	9.37 ∀ 4.11 (27)
Day 1	11.56 ∀ 3.35 (48)	10.32 ∀ 3.90 (25)	10.00 ∀ 3.29 (27)	10.67 ∀ 4.46 (24)	9.63 ∀ 4.06 (24)	10.08 ∀ 3.16 (25)
Day 4 preculling	10.91 ∀ 3.48[d] (45)	10.16 ∀ 3.82 (25)	9.26 ∀ 3.9 (27)	10.54 ∀ 4.38 (24)	9.33 ∀ 4.00 (24)	9.84 ∀ 3.08 (25)
Day 22	6.77 ∀ 4.54 (48)	6.36 ∀ 2.50 (25)	6.19 ∀ 2.91 (27)	6.33 ∀ 2.73 (24)	6.04 ∀ 3.13 (24)	6.76 ∀ 2.17 (25)
Male sex ratio - Day 0 (%)	49.8	52.3	55.2	61.8	50.2	51.4

[a] Mean ∀ S.D.

[b] Number in parentheses represents litters per group.

[c] Dams that delivered an entire litter with only stillborn pups are excluded from Days 1, 4, and 22 statistics.

[d] Three litters were inadvertently culled on Day 3 and have been excluded from the mean.

* Significantly different from the control group mean (p#0.01)

Necropsy and Organ Weights

A complete necropsy at 90 ∀ 2 days of age was performed on all retained male offspring. Dissection of the male reproductive organs was conducted using the technique described below as used by Nagel *et al*. (1997) and documented by vom Saal and Thayer (1997). The dissections were conducted with the laboratory technicians blind to the treatment group of each animal.

The preputial glands were carefully dissected free of fat and connective tissue to the point where they entered the urethra and placed on and covered with a paper towel. The fluid content of the glands was expressed prior to weighing by placing them under a 5000-g weight for approximately 30 seconds (∀5 seconds).

The testes with attached epididymides were removed. The epididymides were carefully trimmed free from the testes and each testis (left and right) was weighed separately. The right testis was frozen at approximately -70°C for the measurement of daily sperm

production. The left testis was fixed in 1.5% gluteraldehyde/4% formaldehyde for microscopic examination. The right caudal epididymis was removed, weighed, and frozen at approximately -70°C for epididymal sperm count. The left epididymis and the remainder of the right epididymis were fixed in 10% neutral buffered formalin.

The abdominal cavity was then opened to expose the abdominal sex accessory organs. The coagulating glands were carefully separated from the seminal vesicle by blunt dissection and collected. The seminal vesicles were carefully separated from the ventral prostate by teasing the prostate back from the seminal vesicle to the point where the seminal vesicle entered the urethra, being careful not to rupture it. The seminal vesicles were placed on and covered with a paper towel and the fluid content was expressed prior to weighing by placing them under a 5000-g weight for approximately 30 seconds (∀5 seconds). Both the coagulating glands and seminal vesicles were fixed in 10% neutral buffered formalin.

The ventral and dorsal lobes of the prostate were carefully teased free of fat, connective tissue and urethra prior to removal of the entire prostate. The prostate was then weighed and placed in 10% neutral buffered formalin. A separate study showed the ability of the prosection technique to detect an increase of prostate weight in young CF-1 mice induced by methyl testosterone (data not shown).

The brain, liver, and kidneys of all 90-day old male offspring were removed and weighed. Both absolute and relative (to body weight and to brain weight) organ weights are reported.

Sperm Parameters

Daily sperm production. Daily sperm production (DSP) was determined following the general procedures outlined by Blazak *et al*. (1993), as revised by Buchanan (1997). The right testis was decapsulated, placed in a measured volume of physiologically buffered saline (PBS), and homogenized. An aliquot of the homogenate was evaluated with a hemocytometer for the number of homogenization-resistant step 17-19 sperm heads. In the mouse, developing spermatids typically spend 4.84 days in steps 17-19 (Blazak *et al*., 1993). Thus, DSP can be estimated by dividing the number of spermatids per testis by 4.84.

Epididymal sperm count. The right cauda epididymis was weighed, placed in a measured volume of PBS, and homogenized. An aliquot of the sperm/saline mixture was then counted in a hemocytometer. The hemocytometer count was multiplied by appropriate volume and dilution factors to give a total cauda epididymal sperm count. A sperm count per gram of epididymal tissue was obtained by dividing the total count by the gram weight of the cauda epididymis.

Table 3. Males: Summary of Organ Weight Values

	0 µg/kg/day Corn Oil	0.2 µg/kg/day BPA	0.2 µg/kg/day BPA	2 µg/kg/day BPA	20 µg/kg/day BPA	20 µg/kg/day BPA

Parameter Measured (grams)	(44)[a]	(23)	(23)	(22)	(18)	(24)
Body Weight	34.680 ∀ 2.3037[b]	34.689 ∀ 2.4290	36.311 ∀ 2.5686	35.702 ∀ 2.3254	36.989 ∀ 2.6023**	36.466 ∀ 3.2988*
Brain	0.461 ∀ 0.0156	0.458 ∀ 0.0194	0.460 ∀ 0.0171	0.465 ∀ 0.0189	0.467 ∀ 0.0240	0.460 ∀ 0.0166
Epididymis	0.094 ∀ 0.0080	0.095 ∀ 0.0075	0.096 ∀ 0.0069	0.095 ∀ 0.0051	0.096 ∀ 0.0082	0.095 ∀ 0.0080
Right caudal epididymis	0.012 ∀ 0.0020	0.012 ∀ 0.0017	0.012 ∀ 0.0015	0.012 ∀ 0.0016	0.013 ∀ 0.0021	0.012 ∀ 0.0022
Kidney	0.828 ∀ 0.0616	0.823 ∀ 0.0767	0.897 ∀ 0.0842	0.855 ∀ 0.1017	0.871 ∀ 0.0553	0.843 ∀ 0.0787
Liver	2.222 ∀ 0.2059	2.193 ∀ 0.1930	2.332 ∀ 0.2285	2.317 ∀ 0.2693	2.339 ∀ 0.1985	2.348 ∀ 0.2871
Preputial gland	0.102 ∀ 0.0150	0.095 ∀ 0.0168	0.104 ∀ 0.0145	0.100 ∀ 0.0150	0.104 ∀ 0.0123	0.098 ∀ 0.0180
Prostate	0.039 ∀ 0.0067	0.039 ∀ 0.0050	0.042 ∀ 0.0090	0.039 ∀ 0.0081	0.041 ∀ 0.0069	0.040 ∀ 0.0077
Seminal vesicle	0.107 ∀ 0.0145	0.105 ∀ 0.0095	0.110 ∀ 0.0115	0.105 ∀ 0.0106	0.113 ∀ 0.0165	0.110 ∀ 0.0149
Testis, left	0.116 ∀ 0.0120	0.115 ∀ 0.0097	0.114 ∀ 0.0089	0.127 ∀ 0.0078	0.116 ∀ 0.0101	0.116 ∀ 0.0110
Testis, right	0.123 ∀ 0.0121	0.120 ∀ 0.0110	0.121 ∀ 0.0091	0.123 ∀ 0.0084	0.123 ∀ 0.0110	0.122 ∀ 0.0119

[a] Number in parentheses represents litters per group

[b] Mean ∀ S.D.

* Significantly different from the control group mean (p#0.05).

** Significantly different from the control group mean (p#0.01).

Statistics

Two full groups (each with n = 28) of vehicle-exposed mice (control groups) were used in this study (Table 1). Prior to statistical evaluation of potential treatment-related effects, statistical analyses were performed on these two groups. Because the two groups were not statistically different, potential treatment-related effects were evaluated against the combined control values.

Descriptive statistics (means and standard deviations) were reported for food consumption. Body weights, gestation/lactation body weight gains, organ weights, sperm counts, sperm production, and litter size were analyzed using the litter as the experimental unit (Haseman and Kupper, 1979). These data were first analyzed by the Levene's test (Milliken and Johnson, 1992). If the Levene's test was not significant ($p>0.01$), an analysis of variance (ANOVA) was conducted. If the ANOVA was significant ($p<0.05$), a Dunnett's test (Dunnett, 1955) was performed. If the Levene's test was significant ($p<0.01$), a rank transformation was performed on these data and an ANOVA was conducted. If the ANOVA was significant ($p<0.05$), the Wilcoxon Rank-Sum Test (Connover, 1980) with Bonferroni's correction was performed.

Fertility indices were analyzed by the Fisher exact probability test (Agresti, 1990) and Bonferroni's correction was used for multiple testing of groups in comparison to a single control. Evaluation of the neonatal sex ratio was performed by the binomial distribution test (Gill, 1978). Survival indices and other incidence data among neonates were analyzed using the litter as the experimental unit by a non-parametric ANOVA. If the ANOVA was significant, the Wilcoxon Rank-Sum Test with Bonferroni's correction was performed. The nominal alpha level used was 0.05.

RESULTS

Test Articles Dose Verification

Concentration analyses for BPA and DES in dose solutions were conducted each week prior to the use of dose solutions for treatment. The average concentrations ranged from 93 to 109% of the nominal values throughout the study.

Animal Health Status Evaluations - Dams

No differences in clinical observations were observed between the control and the DES or BPA groups. A total of 8 dams died (2 in the controls, 1 in the DES group, 3 in the 0.2 μg/kg/day BPA group, 1 in each of the 2 and 20 μg/kg/day BPA groups, and one was euthanized *in extremis* in the 0.2 μg/kg/day BPA group during the lactation period). The cause of death or moribundity of these animals could not be determined.

No consistent differences in food consumption during gestation or lactation were observed in the DES- or BPA-treated animals when compared to the control group.

Gestation and lactation body weights are summarized in Figure 1 and Figure 2. No statistically significant differences in body weights or body weight changes were observed between the control group and the DES or BPA groups.

Animal Health Status Evaluations - F_1 Male Offspring

No treatment-related effects on the incidence of clinical observations were observed in post-weaning males. Food consumption was similar between the control group and the DES and BPA treatment groups during the post-weaning period.

Body weights of male offspring are summarized in Figure 3. No statistically significant differences in body weights were observed between the control group and the DES group during the post-weaning period. When compared to control values, statistically significantly higher body weights were observed in the 0.2 μg/kg/day BPA dose group on PND 57, 2 μg/kg/day BPA dose group on PND 36, and 20 μg/kg/day BPA dose group on PND 36, 50, 57, and 64 (Figure 3). Body weights at sacrifice on day 90 were found to be significantly higher for the 20 and 200 μg/kg/day BPA dose groups (Table 3). Body weight differences were not attributed to treatment since there was neither a consistent trend within or across dose groups or evidence of a dose-response relationship, whether inverse or proportional to dose.

Litter Data
No statistically significant differences in the fertility index were observed between the control group and the DES or BPA groups (data not shown). Delivery and litter data are shown in Table 2. No statistically significant differences in the gestation index, duration of gestation, total live born index, viability index, lactation index, number of live pups per litter on PND Days 0, 1, 4, or 22, or pup sex ratio were observed in the DES- or BPA-treated animals when compared to the control group. The total number of pups born per litter was statistically lower in the 200 μg/kg/day BPA dose group when compared to the control group. The control value for this endpoint was higher than typically observed in this strain of mouse (9-10 pups/litter; Charles River, 1998) and the value for the 200 μg/kg/day BPA group was the same as the historical controls; therefore, this change was not considered treatment-related. No statistically significant differences in pup weights on PND 1, 4, or 22 were observed between the control group and the DES or BPA dose groups (Figure 3). No physical abnormalities or demeanor changes were observed in pups of any litter.

Organ Weights
Organ weights are summarized in Table 3. There were no treatment-related effects of BPA on prostate, preputial gland, seminal vesicle, or epididymis weights (either absolute or relative to brain or body weight) for any treatment groups. In addition, there were no treatment-related effects upon any of the absolute organ weights for the male offspring of DES- or BPA-treated dams. A few statistically significant changes were found in the relative organ weights in the offspring of BPA-treated mice; these differences could not be attributed to treatment (data not shown). The relative brain weight was slightly decreased (~5%) in the 0.2, 20, and 200 μg/kg/day BPA groups. These differences are explained by the slightly higher body weights found in these groups relative to the negative controls (~4-6%). In addition, the kidney to brain ratio for the 0.2 μg/kg/day BPA group was higher than controls; this was spurious and had no relationship to test article administration since no other BPA-treated group had differences in this ratio.

Cauda Epididymal Sperm Concentration, Daily Sperm Production, and Efficiency of Sperm Production
Cauda epididymal sperm concentration, daily sperm production and the efficiency of sperm production are summarized in Figure 4. No statistically significant differences in cauda epididymis sperm concentration, daily testis sperm production or the efficiency of sperm production (DSP/g testis) were observed between the control group, DES group or any of the BPA groups. Of the groups examined, the mean values for cauda epididymis sperm concentration and testes sperm production were lowest in the male offspring of dams given 200 μg/kg/day of BPA, although these values were not statistically different from control values.

Pathology
Macroscopic. There were no treatment-related macroscopic or microscopic changes observed in adult females, pre-weaning pups, or post-weaning males.

Microscopic. There were no treatment-related effects observed microscopically. Rarely, focal to diffuse atrophy of the seminiferous tubules was noted. Affected tubules were smaller than normal, and, generally, were lined by Sertoli cells with absence of the spermatogenic epithelium. Occasionally, some tubules which appeared otherwise normal, contained spermatid giant cells (giant cells, syncytial). Although stage-specific frequency histograms were not prepared, all testes appeared to have all spermatogenesis stages evident with normal maturation and spermatid release. The changes noted microscopically were considered incidental and spontaneous and were typical of those seen in this age and strain of laboratory mouse.

DISCUSSION

The present study was limited in scope because it was designed specifically to replicate the findings in the recent reports of Nagel *et al*. (1997) and vom Saal *et al*. (1998). These investigators reported that exposure of pregnant mice to extremely low doses of BPA (2 or 20 μg/kg/day) was causally related to an increase in prostate and preputial gland weights and a decrease in epididymal weights and the efficiency of sperm production in their adult male offspring. Although every attempt was made to control all factors deemed critical by the original investigators to the replication of their findings, the observations of Nagel *et al*. (1997) and vom Saal *et al*. (1998) were not confirmed in the present investigation.

Although the "maximum effect" oral dose of DES was used in this study as previously reported and recommended (vom Saal,1996, vom Saal *et al*., 1997), no effects on prostate weight due to DES treatment were observed in this study. It should be noted that this dose of DES was nearly three orders of magnitude below that reported by other researchers to produce effects on the male offspring of dams dosed by subcutaneous injection (100 μg/kg/day; McLachlan, 1981; Newbold, 1995). The no observed effect level (NOEL) for male offspring in the studies of McLachlan and Newbold was 10 μg/kg/day, which was nearly two orders of magnitude higher than the dose used in the current study. Pylkkanen and coworkers (1996) have reported that the subcutaneous injection of 2 μg per pup DES into 1- to 3-day old male mice neonates resulted in an increase in prostate weight, however, this direct subcutaneous dose to the males was about 727 μg/kg/day (about 3600 times higher than the oral dose to the dams used in the current study). Further, the NOEL expected for oral exposure to DES would be even higher than the 10 μg/kg/day NOEL determined by Newbold and McLachlan because of the extensive conjugative metabolism of DES in both the intestinal wall and liver (Metzler, 1981). Therefore, although the absence of any effect of DES was inconsistent with the findings reported by vom Saal *et al*. (1997), these findings were consistent with the previously reported NOELs for DES effects to male offspring.

The absence of DES-induced effects on maternal and litter parameters at a dose of 0.2 μg/kg/day is also consistent with the published literature. Studies in rats with DES have shown significant effects on fetal survival (34% incidence of resorptions versus 3% in controls) at 45 μg/kg/day (dams gavaged on GD 6-18); higher doses (200 μg/kg/day)

produced 100% embryolethality (Cornwall *et al*., 1984). Decreased fertility as an effect of DES was also observed in female mice whose mothers received subcutaneous doses from 0.01 to 100 μg/kg/day (McLachlan *et al*., 1982) but this occurred only after several weeks of continuous breeding at the doses below 2.5 μg/kg/day. Another continuous breeding protocol with dietary DES produced significant effects in parental males, including lower sperm concentration and fertility, at 50 ppb (~8 μg/kg/day), but no effects at 10 ppb (~1.5 μg/kg/day) (Reel *et al*., 1985a).

No treatment-related effects on clinical observations, body weight, or food consumption were observed in adult females administered BPA at doses of 0.2, 2, 20, or 200 μg/kg/day. Similarly, no treatment-related effects on growth or survival of offspring from dams were observed at any BPA dose. The total number of pups born per litter was statistically lower in the 200 μg/kg/day BPA dose group when compared to the control group, but this change was not considered treatment-related since the slightly lower value for litter size was within the normal historical range for this strain of mouse (9-10 pups/litter; Charles River, 1998). In addition, previous studies have not shown BPA effects on litter size in mice at higher doses (Morrissey *et al*., 1987). There was an increase in the percentage of litters with resorptions at BPA doses of 1250, but not 500, 750, or 1000 mg/kg/day following treatment on GD 6-15. At the 1250 mg/kg/day BPA dose, there was ample evidence of systemic toxicity, such as effects on maternal body weights and liver weights. Similarly, Reel *et al*., (1985b) reported a reduction in the number of pups per litter at BPA doses in the feed of 0.5 or 1.0% (~875 or 1750 mg/kg/day), but not at 0.25% (~437 mg/kg/day) in a mouse reproductive toxicity study using a continuous breeding design. The highest dose also caused maternal body, liver, and kidney weight effects (Reel *et al*., 1985b).

The lack of effect on daily sperm production (DSP) or epididymal sperm count following BPA administration in the present study is consistent with the previous report of vom Saal *et al*. (1998), who reported no effects of BPA on DSP but did not determine sperm count as derived from the cauda epididymis. These observations are also consistent with the absence of any histopathological effects in the testes and the absence of any decrease in testicular or epididymal weights in the male offspring of BPA-treated dams.

However, the lack of an effect on the efficiency of sperm production (DSP/g testis) observed in this study is in contrast with the results from vom Saal *et al*. (1998). Since vom Saal reported that the mice treated with 20 μg/kg/day of BPA had the highest testes weight and lowest DSP in their offspring (although neither were statistically different from control), the statistically lower value of efficiency derived from these two measurements could have been due to chance alone. Furthermore, there were no treatment-related effects of BPA on absolute or relative weights of preputial glands, seminal vesicles, or epididymis at any dose in the present study. The absolute organ weights for preputial glands and seminal vesicles in the present study were approximately twice that found by vom Saal and coworkers, which could be explained by the difference in housing of the male weanlings. Vom Saal *et al*. caged the weanling males together in group housing for five months before separating them until sacrifice at six months,

whereas in this study the male offspring were individually caged from the day of weaning. Individually housed mice are not subject to the influence of group housing which is known to significantly affect the weight of sex accessory organs, including the prostate and preputial glands with dominant males and individually housed males having significantly larger organ weights than subordinate males (Bartos and Brain, 1993). If the effect of group housing on sex accessory organs that occurred during the five-months of group housing in the prior studies (Nagel *et al*., 1997; vom Saal *et al*., 1997, 1998) was not fully reversed in the one month when the animals were housed individually, then the inadvertent random selection of subordinate versus dominant males across treatment groups may account for the previous findings.

In conclusion, under the conditions of this study, there were no effects on the weights of the prostate, preputial glands, epididymis, or other reproductive organ weights in the offspring of BPA- or DES-treated dams. In addition, sperm parameters of male offspring from dams exposed to very low doses of BPA or DES during gestation were not statistically different from controls. The absence of adverse findings in the offspring of dams treated orally with DES challenges the "low-dose hypothesis" of a special susceptibility of mammals exposed perinatally to ultra low doses of even potent estrogenic chemicals. Based on the data in the present study and the considerable body of literature on effects of BPA at similar and much higher doses, BPA should not be considered as a selective reproductive or developmental toxicant.

REFERENCES

Agresti, A. (1990). *Categorical Data Analysis*. John Wiley and Sons, New York.

Bartos, L. and Brain, P. F. (1993). Physiological responses to social status and housing conditions in male mice subject to food competition tests. Boll. Zool. 60: 293-296.

BIBRA (1989). *Toxicology Profile Bisphenol A*. The British Industrial Biological Research Association, Carshalton, Surrey, U. K.

Bitman, J. and Cecil, H. C. (1970). Estrogenic activity of DDT analogs and polychlorinated biphenyls. J. Agr. Food Chem. 18(6): 1108-1112.

Blazak, W.F., Treinen, K. A. and Juniewicz, P. E. (1993). Application of Testicular Sperm Head Counts in the Assessment of Male Reproductive Toxicity. In *Methods in Toxicology*, Vol. 3, Part A, Male Reproductive Toxicology. Ed: R. E. Chapin and J. J. Heindel. Academic Press, Inc., San Diego.

Buchanan, D. L. (1997). Personal communication, May 1997. Daily Sperm Production Procedure received from P. S. Cooke to J.M. Waechter, Jr.

Charles River Laboratories (1998). Personal communication to W. J. Breslin.

Conover, W.J. (1980). Practical Nonparametric Statistics, 2nd Edition, John Wiley & Sons, Inc. New York.

Cornwall, G. A., Carter, M. W., and Bradshaw, W. S. (1984) The relationship between prenatal lethality or fetal weight and intrauterine position in rats exposed to diethylstilbestrol, xeranol, 3,4,3N,4N-tetrachlorophenyl, or cadmium. Teratol. 30: 341-349.

Dodds, E. C. and Lawson, W. (1936). Synthetic estrogenic agents without the phenanthrene nucleus. Nature 137: 996.

Dunnett, C.W. (1955). A multiple comparison procedure for comparing several treatments with a control. J. Amer. Stat. Assoc. 56: 52-64.

Gaido, K. W., Leonard, L. S., Lovell, S., Gould, J. C., Babai, D., Portier, C. J., and McDonnell, D. P. (1997). Evaluation of Chemicals with Endocrine Modulating Activity in a Yeast-Based Steroid Hormone Receptor Gene Transcription Assay. Toxicol. Appl. Pharmacol. 143: 205-212.

Gill, J. L. (1978). *Design and Analysis of Experiments in the Animal and Medical Sciences*. The Iowa State University Press, Ames, Iowa.

Haseman, J. K. and Kupper, L. L. (1979). Analysis of dichotomous response data from certain toxicological experiments. Biometrics 35: 281-293.

Krishnan, A. V., Stathis, P., Permuth, S. F., Tokes, L., and Feldman, D. (1993). Bisphenol A: An estrogenic substance is released from polycarbonate flasks during autoclaving. Endocrinol. 132: 2279-2286.

Kuiper, G. G. J., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilsson, S., and Gustafsson, J-A. (1997). Comparison of the Ligand Binding Specificity and Transcript Tissue Distribution of Estrogen Receptors α and β. Endocrinol. 138(3): 863-870.

Laws, S. C. and Carey, S. A. (1997). Comparison of the estrogenic activity of 4-tertoctylphenol, nonylphenol, bisphenol A and methoxychlor in Long Evans female rats. Toxicol. 36: 359.

McLachlan, J. A., Newbold, R. R., Shah, H. C., Hogan, M. D., and Dixon, R. L. (1982). Reduced fertility in female mice exposed transplacentally to diethylstilbestrol (DES). Fertil. Steril 38: 363-371.

McLachlan, J. A. (1981). Rodent models for perinatal exposure to diethylstilbestrol and their relation to human disease in the male. Chapter 11, pp. 148-157, In *Developmental Effects of Diethylstilbestrol in Pregnancy*. Ed: A. L. Herbst and H. A. Bern, Thieme-Stratton, New York.

Metzler, M. (1981). The metabolism of diethylstilbestrol. CRC Crit. Rev. Biochem. 10: 171-213.

Milliken, G.A. and Johnson, D.E. (1992). *Analysis of Messy Data*. Chapman and Hall, London.

Morrissey, R.E., George, J.D., Price, C.J., Tyl, R.W., Marr, M.C., and Kimmell, C.A. (1987). The developmental toxicity of bisphenol A in rats and mice. Fund. Appl. Toxicol. 8: 571-582.

Morrissey, R. E., Lamb, J. C., Morris, R. W., Chapin, R. E., Gulati, D. K., and Heindel, J. J. (1989). Results and Evaluations of 48 Continuous Breeding Reproduction Studies conducted in Mice. Fund. Appl. Toxicol. 13: 747-777.

Nagel, S. C., vom Saal, F. S., Thayer, K. A., Dhar, M. G., Boechler, M., and Welshons, W. V. (1997). Relative binding affinity-serum modified access (RBA-SMA) assay predicts the relative in vivo bioactivity of the xenoestrogens bisphenol A and octylphenol. Environ. Health. Perspec. 105: 70-76.

Newbold, R. R. (1995). Cellular and molecular effects of developmental exposure to diethylstilbestrol: Implications for other environmental estrogens. Environ. Health Perspec. 103 (Suppl. 7): 83-87.

NTP (1982). Carcinogenesis bioassay of bisphenol A (CAS No. 80-05-7) in F344 rats and B6C3F1 mice (feed study). National Toxicology Program, Technical Report Series No. 215.

Pottenger , L. H., Domoradzki, J. Y., Markham, D. A., Hansen, S. C., and Waechter, Jr., J. M. (1997). Bioavailability of ^{14}C-bisphenol A (BPA) in F344 rats following oral (po), subcutaneous (sc) or intraperitoneal (ip) administration. Toxicol. 36: 143.

Pylkkanen, L., Mäkelä, S., and Santti, R. (1996). Animal models for the preneoplastic lesions of the prostate. Euro. Urol. 30: 243-248.

Reel, J. R. Lawton, A. D., Wolkowski-Tyl, R., Davis, G. W., and Lamb, J. C. (1985a). Evaluation of a new reproductive toxicology protocol using diethylstilbestrol (DES) as a positive control compound. J. Am. Coll. Toxicol. 4: 147-162.

Reel, J. R., Tyl, R. W., Lawton, A. D., and Lamb, J. C. (1985b). Bisphenol A: Reproduction and fertility assessment in CD-1 mice when administered in the feed. Report to the National Toxicology Program from Research Triangle Institute, Research Triangle Park, N.C. [NTIS PB86-103207].

Twomey, K. (1998a). Bisphenol A: Uterotrophic assay in immature rats (subcutaneous dosing). Report to the Bisphenol A Global Industry Group from Central Toxicology Laboratory, Cheshire, U.K. [CTL Report No. CTL/P/5943].

Twomey, K. (1998b). Bisphenol A: Uterotrophic assay in immature rats (oral dosing). Report to the Bisphenol A Global Industry Group from Central Toxicology Laboratory, Cheshire, U.K. [CTL Report No. CTL/P/6029].

vom Saal, F. S. (1996). Personal Communication. Letter to L. R. Harris. August 12, 1996.

vom Saal, F. S., Timms, B. G., Montano, M. M., Palanza, P., Thayer, K. A., Nagel, S. C., Dhar, M. D., Ganjam, V. K., Parmigiani, S., and Welshons, W. V. (1997). Prostate enlargement in mice due to fetal exposure to low doses of estradiol or diethylstilbestrol and opposite effects at high doses. Proc. Natl. Acad. Sci. 94: 2056-2061.

vom Saal, F. S. and Thayer, K. A. (1997). Personal Communication, MPI Research staff prostate prosection training. University of Missouri. April 25, 1997.

vom Saal, F. S., Cooke, P. S., Buchanan, D. L., Palanza, P., Thayer, K. A., Nagel, S. C., Parmigiani, S., and Welshons, W. V. (1998). A physiologically based approach to the study of bisphenol A and other estrogenic chemicals on the size of reproductive organs, daily sperm production, and behavior. Toxicol. Indust. Health 14(1/2): 239-260.