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Estrogenic Activity of Octylphenol, Nonylphenol, Bisphenol A and Methoxychlor in Rats

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Considerable attention has recently been focused on environmental chemicals that disrupt the reproductive system by altering steroid receptor function. Although numerous *in vitro* and *in vivo* methods have been shown to be useful approaches for identifying chemicals that can disrupt reproduction through a direct interaction with the estrogen receptor, it is imperative that the protocols selected be capable of detecting chemicals with a broad range of estrogenic activity. Here we evaluate the reliability of the 3-day uterotrophic assay for detecting chemicals with strong or weak estrogenic activity in both prepubertal and ovariectomized adult Long Evans rats. These data were compared to additional measures of estrogenic activity, which included the age of vaginal opening, the induction of cornified vaginal epithelial cells in ovariectomized adult rats, and estrous cyclicity in intact adult rats. Test chemicals selected for these studies included 17-β-estradiol, ethynyl estradiol, methoxychlor, 4-tert-octylphenol, 4-nonylphenol and bisphenol A. Data from *in vitro* receptor binding assays compared the ability of the test chemicals to compete with [^{3}H]-estradiol or [^{3}H]-promegestone for binding to estrogen or progesterone receptors. As expected, the binding affinities for the estrogen receptor ranged from high to low, as reflected by Ki concentrations of 0.4 nM for 17-β-estradiol and ethynyl estradiol, and 0.05–65 μM for 4-tert-octyphenol, 4-nonylphenol, and methoxychlor. Although none of the test chemicals demonstrated a high affinity for binding to the progesterone receptor, 4-tert-octylphenol and 4-nonylphenol exhibited a weak affinity, with Ki concentrations ranging from 1.2 to 3.8 μM. *In vivo* studies indicated that the 3-day uterotrophic assay in prepubertal rats was the best method for detecting estrogenic activity when compared with all other end points, based upon the dose-response data for ethynyl estradiol (0.01–0.1 mg/kg), 4-tert-octylphenol (50–200 mg/kg, oral), and 4-nonylphenol (25–100 mg/kg, oral). Although oral doses of ethynyl estradiol (0.01 mg/kg) and 4-nonylphenol (50 mg/kg) induced a significant increase in uterine weight in the prepubertal rats, these doses were ineffective for stimulating a similar response in ovariectomized adult rats. The age of vaginal opening was advanced following oral exposure from postnatal days 21–35 to ethynyl estradiol (0.01 mg/kg), methoxychlor (50 mg/kg), 4-tert-octylphenol (200 mg/kg), and 4-nonylphenol (50 mg/kg). Although bisphenol A (200 mg/kg, oral) induced a significant uterotrophic response within 3 days in prepubertal rats, doses up to 400 mg/kg failed to advance the age of vaginal opening. Monitoring changes in the vaginal epithelium of ovariectomized adult rats was the least effective method for detecting estrogenic activity for 4-tert-octylphenol and bisphenol A. The number of 4–5 day estrous cycles was reduced during a 25-day exposure to ethynyl estradiol (0.01 mg/kg), methoxychlor (50 mg/kg), 4-tert-octylphenol (200 mg/kg), 4-nonylphenol (100 mg/kg), and bisphenol A (100 mg/kg) by oral gavage. Although long periods of extended diestrus (7–14 days) were generally correlated with exposure to ethynyl estradiol and 4-tert-octylphenol, the cycling patterns following exposure to methoxychlor, 4-nonylphenol and bisphenol A were not as clearly defined, with shorter periods of extended diestrus (4–7 days) and/or estrus (3–5 days) intermittently observed throughout the exposure period. Together these data provide a comparison of the 3-day uterotrophic assay with alternative measures of estrogenic activity for a group of test chemicals with a broad range of affinities for the estrogen receptor. These data can be useful during the assessment and validation of methods for screening environmental chemicals for endocrine disrupting activity.

Key Words: **female reproductive toxicology; endocrine disruptors; ethynyl estradiol; 4-tert-octylphenol; 4- nonylphenol; bisphenol A; methoxychlor; rat; uterotrophic assay; estrous cyclicity; vaginal opening assay.**

Environmental chemicals that disrupt endocrine function have been linked to adverse effects on the reproductive system in wildlife and humans. Although there are several mechanisms through which environmental chemicals might alter the endocrine system, chemicals that mimic steroid hormones through an interaction with the estrogen receptor continue to receive considerable attention. Numerous environmental chemicals have been identified that can bind to

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the estrogen receptor and initiate transcription of the estrogen receptor-regulated genes *in vitro* (Bolger *et al*., 1998; Bulger *et al*., 1978a; Danzo, 1997; Gaido *et al*., 1997; Gould *et al*., 1998). Many of these chemicals also stimulate estrogen receptor-mediated physiologic responses *in vivo* (Carney *et al*., 1997; Cook *et al*., 1997; Cooper and Kavlock, 1997; Gray *et al*., 1996; Gray and Ostby, 1998; Rudel, 1997; Shelby *et al*, 1989). Although environmental exposure to these chemicals has the potential to disrupt reproductive function, their actual impact on reproductive health has not been defined thoroughly.

In 1996, through the Food Quality Protection Act (Public Law 104–170, August 3, 1996, *http://www.epa.gov/oppfead1/fqpa/backgrnd.htm*) and an amendment to the Safe Drinking Water Act (Public Law 104–182), the U.S. Congress issued a mandate to the U.S. Environmental Protection Agency (U.S. EPA) to develop and initiate a screening program to identify chemicals that could disrupt endocrine function. Working toward this goal, the U.S. EPA has submitted an Endocrine Disruptor Screening Program: Proposed Statement of Policy and has invited public comment on the technical and policy aspects of the program (Federal Register, 1998). The program, based largely upon recommendations from the Endocrine Disruptor Screening and Testing Advisory Committee (U.S. EPA, 1998), is designed to detect chemicals that alter the estrogen, androgen, and thyroid systems in human, fish, and wildlife.

Part of the proposed Tier 1 screening battery includes tests to evaluate the effects of chemicals on the female reproductive system (Federal Register, 1998). Included in the screen are *in vitro* estrogen receptor binding and transcriptional activation assays, as well as a rodent 3-day uterotrophic assay and a 20-day pubertal female protocol that determines the age at vaginal opening. It is imperative during the validation of these protocols to understand potential technical issues that might compromise the intra- and interlaboratory reproducibility of the protocols. In addition, it is important to continue to compare these assays with other methods to ensure that the screening battery includes the most relevant assays (ICCVAM, 1999).

The studies reported here evaluate the reliability of the 3-day uterotrophic assay for detecting environmental chemicals that possess weak estrogenic activity in both prepubertal and ovariectomized adult rats. Environmental chemicals tested were methoxychlor, 4-tert-octylphenol, 4-nonylphenol, and bisphenol A. Several technical issues relevant to the 3-day uterotrophic assay were addressed by comparing dosing routes (e.g., subcutaneous injection and oral gavage) and two postexposure observation times (e.g., 6 and 24 h following the last dose) for each test chemical. Data from the 3-day uterotrophic assays were compared with other end points of the Tier 1 screening battery, which included the age at vaginal opening in the prepubertal rat and the *in vitro* estrogen receptor binding assay.

Additional end points not included in the Tier I screening battery were evaluated to determine their usefulness in detecting endocrine disrupting activity that may or may not be mediated via an interaction with the estrogen receptor. *In vitro* progesterone receptor binding assays were conducted to determine if this steroid receptor might also be a target for the chemicals evaluated. In addition, changes in the vaginal cytology of adult ovariectomized (e.g., cornification of epithelial cells) and intact (e.g., a measure of estrous cyclicity) rats were monitored. The appearance of cornified epithelial cells in vaginal smears of ovariectomized adult rats within 48–72 h following exposure to estrogen is well documented (Allen and Doisy, 1924; Del Castill and Di Paola, 1942) and has been promoted as a reliable test for estrogenic activity (Reel *et al*., 1996). However, the usefulness of this end point for detecting weak estrogenic activity has not been thoroughly investigated nor has its sensitivity been compared directly with the 3-day uterotrophic assay.

An evaluation of estrous cyclicity in intact adult rats was included in this series of experiments for several reasons. First, estrous cyclicity provides a method for evaluating the endocrine disrupting activity of each test chemical under physiologic conditions where endogenous concentrations of estrogen vary. Second, it is known that exposure to 17-β-estradiol can disrupt the normal 4- to 5-day estrous cycle in adult female rats by inducing an extended period of diestrus consistent with pseudopregnancy within 5–7 days after the exposure (Gilmore and McDonald, 1969). This is due to the estrogen-dependent increase in prolactin that rescues ovarian corpora lutea and the subsequent synthesis and release of progesterone (Cooper and Goldman, 1999; Lu *et al*., 1980). Therefore, it is possible to detect estrogenic activity by monitoring estrous cyclicity during a relatively short exposure period. Also, monitoring estrous cyclicity provides a means to identify alterations in ovarian and neuorendocrine function that are mediated through nonestrogenic as well as estrogenic mechanisms (Blasberg *et al*., 1997; Clark *et al*., 1998; Cooper *et al*., 1996).

The environmental chemicals used in these studies were selected because they have been reported to stimulate estrogenic activity *in vitro* (Bolger *et al*., 1998; Danzo, 1997; Gaido *et al*., 1997; Routledge and Sumpter, 1997; Soto *et al*., 1995) and *in vivo* (Ashby and Tinwell, 1998; Bicknell *et al*., 1995; Bulger *et al*., 1978a,b; Cook *et al*., 1997; Cummings, 1997; Cunny *et al*., 1997; Gray *et al*., 1988, 1989; Gray and Ostby, 1998; Gould *et al*., 1998; Jobling and Sumpter, 1993; Lech *et al*., 1996; Lee and Lee, 1996; Nimrod and Benson, 1997; Steinmetz *et al*.,1997; White *et al*., 1994). Positive controls used for the studies included 17-β-estradiol and ethynyl estradiol, both of which exhibit an high affinity for the estrogen receptor. Thus, the relative binding affinities of all the test chemicals for the estrogen receptor ranged from 100 for 17-β-estradiol to less than 0.0004 for the environmental chemicals.

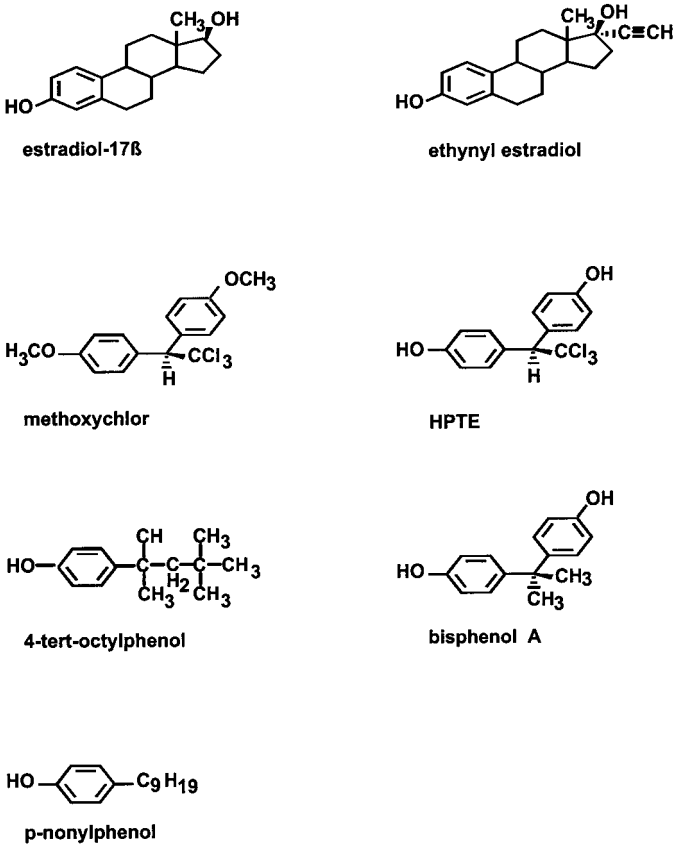

FIG. 1. Chemical structures of test chemicals: 17-β-estradiol; ethynyl estradiol; methoxychlor; 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE); 4-tert-octylphenol; 4-nonylphenol, and bisphenol A.

MATERIALS AND METHODS

Chemicals

Chemicals were purchased from the following sources: 17-β-estradiol-3-benzoate, diethylstilbestrol, ethynyl estradiol, and methoxychlor (purity 95%) were obtained from Sigma Chemical Company, St. Louis, MO; 4-tert-octylphenol (purity 97%) and bisphenol A (purity 99%) were obtained from Aldrich Chemical Company, Inc., Milwaukee, WI; 4-nonylphenol (a mixture of branched side chains containing 85% p-isomers) was from Fluka Chemical Corp., Ronkonkoma, NY. Chemical structures for the test chemicals are shown in Figure 1. 2,2-Bis(p-hydroxyphenyl)-1,1,1-trichlorethane (HPTE) was a gift from Dr. William R. Kelce, Monsanto Company, St. Louis, MO. 17β-[2,4,6,7–^{3}H]-estradiol (^{3}H-E; 111 Ci/mmol), [17α-methyl-^{3}H]-promegestone (^{3}H-R5020); 86 Ci/mmol) and radioinert R5020 (purity was $>$ 98% by HPLC prior to use) were obtained from DuPont-New England Nuclear, Wilmington, DE).

Animals

Long Evans rats [14-day timed pregnant or adult (60 days)] were obtained from Charles Rivers Laboratories, Raleigh, NC, and were maintained under controlled temperature (20–24°C), humidity (40–50%) and light (14 h light/10 h dark) conditions with Purina Laboratory Rat Chow (5001) and water available *ad libitum.* Pregnant dams were allowed to deliver their pups naturally; 3 days postpartum all litters were culled to 8 pups, with at least 1 male per litter. Pups were weaned at 20 days, weight-ranked, and placed into treatment groups such that the mean body weight ± S.D. for all groups were comparable. During the remainder of the study, all animals were housed under conditions identical to thoses described for the timed-pregnant rats. Adult animals were ovariectomized under ketamine (87 mg/ml) and xylazine (13 mg/ml) anesthesia at a dose of 0.1 ml/kg and allowed to recover for 21 days prior to use in the uterotrophic or vaginal cytology studies.

Dosing Solutions and Procedures

A stock solution of ethynyl estradiol (10 mg/ml) was prepared in 95% ethanol and stored at –20°C. Dosing solutions for the prepubertal animals were prepared in corn oil at concentrations of 0.002–80 mg/ml and used at a dosing volume of 0.5 ml/100 g BWT. Dosing solutions for the adult animals were prepared in corn oil at concentrations of 0.01–400 mg/ml. The highest dose group for bisphenol A used with the adult animals was prepared as a suspension of 100 mg/ml corn oil, as suspensions of higher concentrations were not homogeneous. Dosing volume for the adult animals was 0.1 ml/100 g BWT.

Experimental Design

Uterotrophic assays, vaginal opening assays, and the evaluation of estrous cyclicity were conducted as separate experiments for each test chemical using a complete block design that included a control and test chemical dose response. Data for each parameter were replicated in the highest treatment group for each test chemical in a separate experiment. Experiments that compared the uterotrophic response 6 or 24 h after the last dose or via different dosing routes were conducted as complete blocks for each test chemical.

3-Day Uterotrophic Assays, Prepubertal and Ovariectomized Adult Rats

Prepubertal 21-day-old females (intact) and 60-day-old females (ovariectomized 3 weeks before) were used to compare the uterotropic effects of ethynyl estradiol, methoxychlor, 4-tert-octylphenol, 4-nonylphenol, bisphenol A, or the corn oil vehicle. All animals were dosed once per day for 3 days by either oral gavage or subcutaneous injections and were killed either 6 or 24 h after the last dose. Specifically, one group of prepubertal animals was used to compare dosing by oral gavage and subcutaneous injection and were killed 6 h after the last dose (e.g., methoxychlor 50 mg/kg; 4-tert-octylphenol 50, 100, 200 mg/kg; 4-nonylphenol 25, 50, 100, 200 mg/kg; or bisphenol A 200 mg/kg). Another group of prepubertal rats was used to compare uterine weights 6 and 24 h after the last oral dose (e.g., ethynyl estradiol 0.01, 0.1 mg/kg; methoxychlor 50 mg/kg; 4-tert-octylphenol 200, 400 mg/kg; 4-nonylphenol 50, 100 mg/kg; or bisphenol A 100, 200, 400 mg/kg). All adult ovariectomized animals were dosed by oral gavage and killed 6 h after the last dose with the exception of 17-β-estradiol, which was administered by subcutaneous injection (e.g., ethynyl estradiol- 0.01, 0.1 mg/kg; methoxychlor 25, 50, 100 mg/kg; 4-tert-octylphenol 50, 100, 200 mg/kg; 4-nonylphenol 25, 50, 100 mg/kg; or bisphenol A 100 mg/kg). Uteri were removed and adhering fat was trimmed away. Uteri were weighed with and without the fluid and frozen for progesterone receptor assays.

Age at vaginal opening, prepubertal rats. Females were dosed with ethynyl estradiol (0.01 mg/kg), methoxychlor (50 mg/kg), 4-tert-octylphenol (50, 100, 200 mg/kg), 4-nonylphenol (25, 50, 100 mg/kg), bisphenol A (50, 100, 200, 400 mg/kg), or the corn oil vehicle by oral gavage beginning at 21 and continuing through 35 days of age. Animals were monitored daily for vaginal opening.

Changes in vaginal cytology, intact and ovariectomized adult rats. Changes in vaginal epithelial cells were monitored by taking daily vaginal smears as described by Cooper *et al*., (1993) in both cycling and ovariectomized adult rats following exposure to the test chemicals by oral gavage. For the cycling studies, all animals were monitored for 3 weeks prior to treatment; only those animals displaying consistent 4- to 5-day estrous cycles were used in the study. Cycling animals were dosed for 25 days by subcutaneous injection with 17-β-estradiol (0.005 mg/kg) or by oral gavage with ethynyl estradiol (0.01, 0.1 mg/kg), 4-tert-octylphenol (50, 100, 200 mg/kg), 4-nonylphenol (25, 50, 100 mg/kg), methoxychlor (50 mg/kg), and bisphenol A (100 mg/kg).

Vaginal smears were obtained daily and evaluated under a low-power light microscope for the presence of leukocytes, nucleated epithelial cells, or cornified cells. The vaginal smears were classified as diestrus (presence of leukocytes), proestrus (presence of nucleated epithelial cells), or estrus (presence of cornified epithelial cells), as characterized by Everett (1989). Extended estrus was defined as exhibiting cornified cells with no leukocytes for at least 3 consecutive days and extended diestrus as the presence of leukocytes for at least 4 days (Cooper and Goldman, 1999). If a female displayed an extended diestrus smear for 7 or more days, a tail blood sample was obtained for a progesterone assay in order to futher identify the underlying ovarian status (e.g., pseudopregnancy or anestrus). Vaginal smears from the ovariectomized animals were monitored 5 days prior to treatment to confirm a persistent diestrus state. These animals were dosed for 11 days by subcutaneous injection with 17-β-estradiol (0.005 mg/kg) or oral gavage with ethynyl estradiol (0.001, 0.01, 0.1 mg/kg), methoxychlor (50 mg/kg), 4-tert-octylphenol (50, 100, 200 mg/kg), 4-nonylphenol (25, 50, 100 mg/kg), and bisphenol A (100 mg/kg). Vaginal smears were evaluated daily for the presence of cornified epithelial cells indicative of an estrogenlike response.

Progesterone Radioimmunoassay

Serum progesterone concentrations were determined using radioimmunoassay (RIA) kits obtained from Diagnostic Products Corporation (Los Angeles, CA). The detection limit for the assay was 0.02 ng/ml.

In vitro Estrogen and Progesterone Receptor Competitive Binding Assays

The apparent affinities of each test chemical for the estrogen and progesterone receptors were measured by their ability to inhibit [^{3}H]-estradiol or [^{3}H]-promegestone (i.e., a progesterone agonist) binding *in vitro* as described earlier (Laws *et al*., 1994,1996b). Briefly, cytosolic extracts for the estrogen and progesterone receptor competition binding assays were prepared using uteri obtained from adult ovariectomized (11 days) rats injected with corn oil (for estrogen receptor assays) or 17-β-estradiol (0.1 mg/kg, sc, 48 h, for progesterone receptor assays). Uterine cytosolic extracts for estrogen receptor assays were incubated for 30 min at 30°C using 1 nM [^{3}H]-estradiol and for progesterone receptor assays 18 h at 4°C using 1 nM [^{3}H]-promegestone (R5020) in the presence or absence of increasing concentrations (0.0001–1000 μM) of each test chemical in a total volume of 0.5 ml. Nonspecific binding of [^{3}H]-estradiol or [^{3}H]-promegestone was assessed by adding 100 molar excesses of radioinert diethylstilbestrol or R5020, respectively. Bound [^{3}H]-ligand for all receptors was isolated using hydroxyapatite (i.e., 250 μl of 60% HAP slurry) extraction and quantified in ethanol extracts by scintillation counting as previously described (Laws *et al*., 1994). Specific binding was calculated by subtracting nonspecific binding from total binding. Data were analyzed using a nonlinear regression program for fitting one-site competitive binding curves (GraphPad Prism; Graphpad Software, Inc., San Diego, CA) and estimates of IC_{50} concentrations (e.g., competitor concentration required to inhibit 50% of ^{3}H-estradiol or ^{3}H-promegestone) were generated. IC_{50} values obtained from the competitive binding curves were converted to Ki values using the equation $Ki = IC_{50}/(1 + L/Kd)$ (Cheng and Prusoff, 1973), where L is the radioligand concentration and Kd is the equilibrium dissociation constant for the receptor.

Statistics

For statistical analysis the data were grouped by experiment, as indicated in the tables and figures. Data were tested by Bartlett's test for homogeneity of variance (GraphPad InStat, GraphPad Software, San Diego, CA) and ANOVA [General Linear Models (GLM) procedure; Statistical Analysis System (SAS), SAS Institute, Inc., Cary]. When significant ($p < 0.05$) treatment differences were indicated by ANOVA, the Dunnett Multiple Comparison Test was used to compare each treatment group with the control (GraphPad InStat, GraphPad Software, San Diego, CA). Where heterogeneity of variance was evident from the Barlett's test, the Mann-Whitney test (two treatment groups/block) or the Kruskal-Wallis Nonparametric Anova Test/Dunn's Multiple Comparison Test (three or more treatment groups/block) were used to compare treatment means with the control ($p < 0.05$; GraphPad InStat, GraphPad Software, San Diego, CA).

RESULTS

3-Day Uterotrophic Assays, Prepubertal Rats

Postexposure observation time. Figure 2 compares the uterotrophic effects of ethynyl estradiol, methoxychlor, 4-tert-octylphenol, 4-nonylphenol, and bisphenol A in prepubertal Long Evans rats 6 and 24 h after receiving the last of three daily doses by oral gavage. Data are reported as percent of controls, as uterotrophic assays were conducted as separate experiments for each test chemical. Control uterine wet weights for each experiment ranged from 26.8 to 33.5 mg (6 h) and 29.0 to 34.6 mg (24 h). Raw data from each experiment were analyzed separately. Significantly different values noted in Figure 2 are based on the analysis of the mean uterine weight (mg) $\pm$ SE for each treatment. Uterine weight 6 h following the last of three doses of ethynyl estradiol (0.01–0.1 mg/kg), methoxychlor (50 mg/kg), 4-tert-octylphenol (200–400 mg/kg), 4-nonylphenol (50–100 mg/kg), and bisphenol A (200–400 mg/kg) were significantly higher than controls. However, the increase in uterine weight was not maintained in all of the treatment groups at 24 h after the last dose. Although uterine weights following exposure to ethynyl estradiol, methoxychlor, and 4-nonylphenol continued to be significantly elevated 24 h after the last dose, this was not the case for 4-tert-octylphenol and bisphenol A. In the latter two treatment groups, uterine weight had returned to control levels by 24 h after the last exposure.

Subcutaneous injection versus oral gavage as dosing route. Figure 3 compares the uterotrophic response as percent of controls following exposure to the test chemicals via subcutaneous injection or oral gavage. A 3-day exposure regimen was used in these studies, with uterine weight evaluated 6 h after the last dose. The magnitude of the uterotrophic response was greater following exposure via a subcutaneous injection to 4-tert-octylphenol (200–400 mg/kg) and bisphenol A (200 mg/kg) as compared with exposure by oral gavage. In contrast, oral exposure to methoxychlor (50 mg/kg) or 4-nonylphenol (50, 100, and 200 mg/kg) resulted in a greater increase in uterine weight as compared with exposure by subcutaneous injection.

3-Day Uterotrophic Assays, Ovariectomized Adult Rats

The effects of the test chemicals in adult ovariectomized Long-Evans rats were evaluated to determine whether the age and endocrine status of the animal might alter the uterotrophic response during exposure. Results from the 3-day uterotrophic assay in ovariectomized adult rats are shown in Figure 4. 17-β-estradiol (0.005 mg/kg, sc), ethynyl estradiol (0.1 mg/kg,

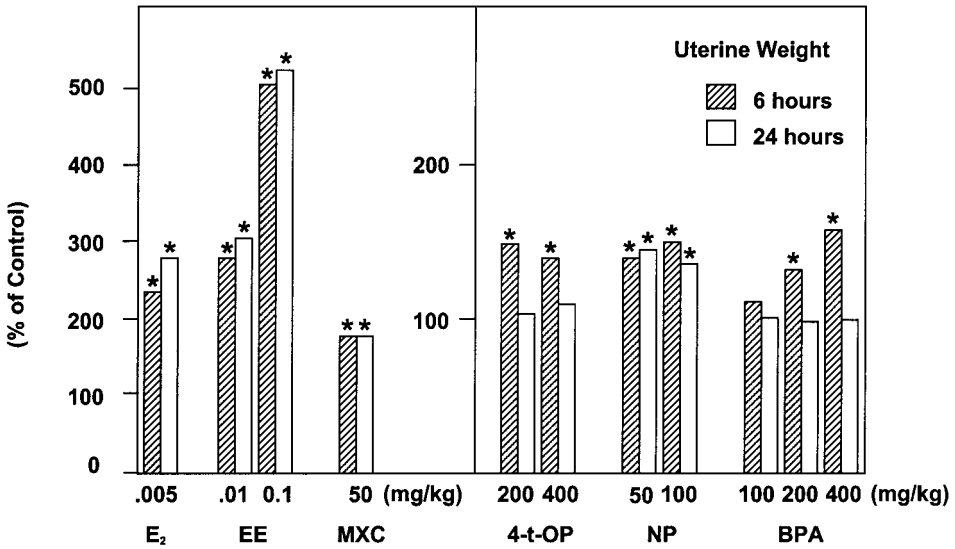

FIG. 2. 3-day uterotrophic assays in prepubertal rats: comparison of effects 6 and 24 h after the last dose. Wet uterine weight expressed as percent of control following exposure to 17-β-estradiol by sc injection, or to ethynyl estradiol (EE), methoxychlor (MXC), 4-tert-octylphenol (4-t-OP), 4-nonylphenol (NP), or bisphenol A (BPA) by oral gavage. Animals received a single daily dose from 21 to 23 days of age and were killed 6 or 24 h after the last dose. Control uterine weights [mean ± S.E (n)[for 17-β-estradiol (sc injection): 39.1 ± 2.7 (8) for 6 h and 33.5 ± 2.4 (6) for 24 h. Control uterine weights (mean ± S.E. [n = 6]) for each treatment group receiving dose by oral gavage: 27.7 ± 2.2 mg (EE, 6 h); 33.5 ± 2.7 mg (MXC, 4-t-OP, 6 h); 23.3 ± 1.9 mg (NP, 6 h); 26.8 ± 1.6 mg (BPA, 6 h); 29.0 ± 5 mg (EE, 24 h); 33.5 ± 2 mg (MXC, 24 h); 34.6 ± 1.7 mg (4-t-OP, 24 h); 33.8 ± 2 mg (NP, 24 h); 33.5 ± 2 mg (BPA, 24 h). For statistical analyses the data were grouped by experiment (e.g., test chemical) and postexposure time (6 or 24 h). *Significant treatment effect by ANOVA or the Kruskal-Wallis Nonparametric Anova Test (p = 0.05) with comparison to the control by Dunnett's Multiple Comparison Test, Mann-Whitney test, or Dunn's Multiple Comparisons Test ($p < 0.05$).

oral), methoxychlor (50–100 mg/kg, oral), 4-tert-octylphenol (100–200 mg/kg, oral), and 4-nonylphenol (100 mg/kg, oral) induced a significant increase in uterine weight as compared with controls. However, bisphenol A (100 mg/kg, oral) did not alter uterine weight. A comparison of these data with that obtained from the prepubertal rats suggests that the uterotro-

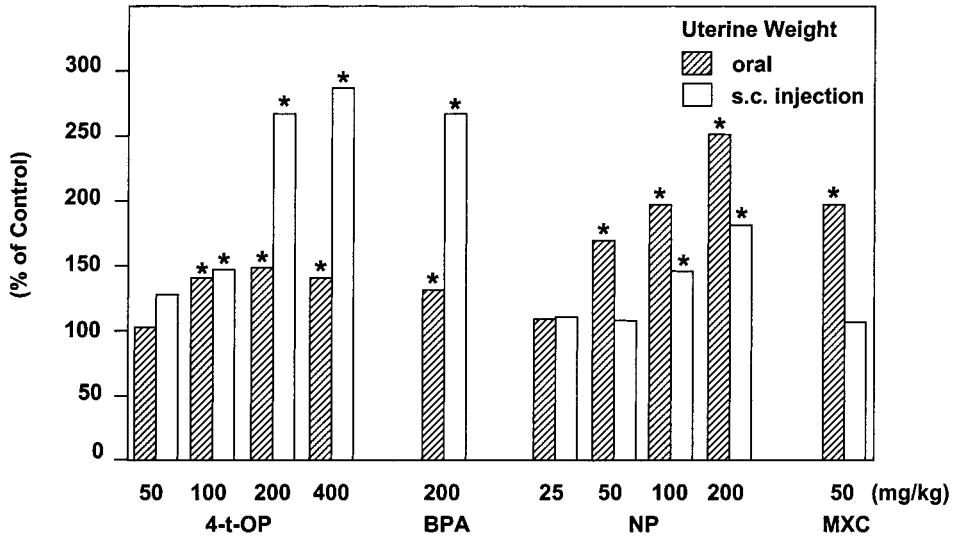

FIG. 3. 3-day uterotrophic assays in prepubertal rats: comparison of the effects of sc injection or oral gavage dosing routes. Wet uterine weight expressed as percent of control following exposure to ethynyl estradiol (EE), methoxychlor (MXC), 4-tert-octylphenol (4-t-OP), 4-nonylphenol (NP), or bisphenol A (BPA). Animals received a single dose each day from 21 to 23 days of age by sc injection or oral gavage and were killed 6 h after the last dose. Control uterine weight [mean ± S.E. (n = 6)] for each treatment group were as follows: 27.7 ± 2.2 mg (EE, oral); 33.5 ± 2.7 mg (MXC, 4-t-OP, oral); 23.3 ± 1.9 mg (NP, oral); 26.8 ± 1.6 mg (BPA, oral); 28.5 ± 2.0 (4-t-OP; sc); 33.7 ± 2.7 (MXC, NP, sc); 26.5 ± 1.3 (BPA, sc). For statistical analyses the data were grouped by experiment (e.g., test chemical) and route of exposure. * Significant treatment effect by ANOVA or the Kruskal-Wallis Nonparametric Anova Test (p = 0.05) with comparison to the control by Dunnett's Multiple Comparison Test, Mann-Whitney test, or Dunn's Multiple Comparisons Test ($p < 0.05$).

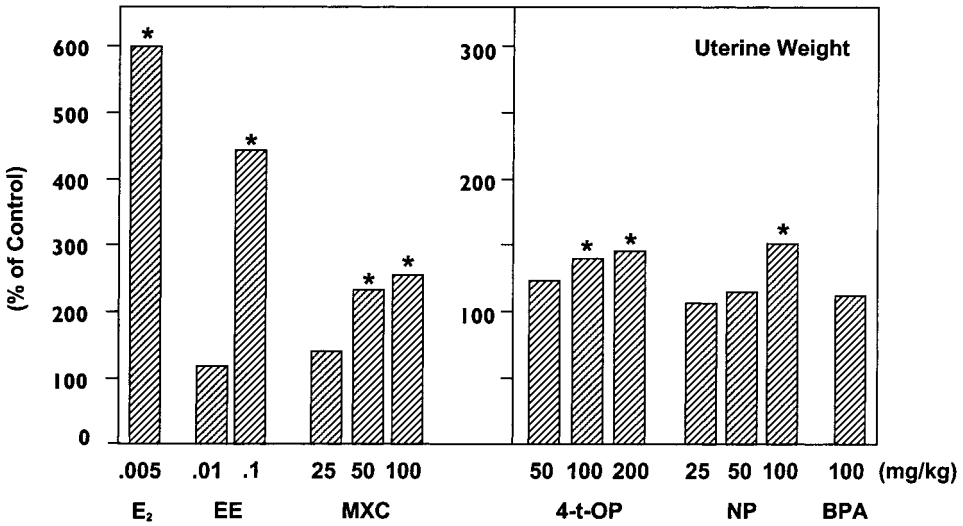

FIG. 4. 3-day uterotrophic assays in adult ovariectomized rats. Wet uterine weight expressed as percent of control following sc exposure to 17-β-estradiol (E_2) or oral exposure to ethynyl estradiol (EE), methoxychlor (MXC), 4-tert-octylphenol (4-t-OP), 4-nonylphenol (NP), or bisphenol A (BPA). Animals received a single daily dose for 3 days and were killed 6 h after the last dose. Control uterine weights [mean $\pm$ S.E. ($n = 6$)] for each treatment group were as follows: 93 $\pm$ 1.8 mg (EE, MXC); 95 $\pm$ 3.5 mg (E2, 4-t-OP); 94 $\pm$ 2.9 mg (NP); 109 $\pm$ 5.5 mg (BPA). For statistical analyses the data were grouped by experiment (e.g., test chemical). *Significant treatment effect by ANOVA or the Kruskal-Wallis Nonparametric Anova Test ($p = 0.05$) with comparison to the control by Dunnett's Multiple Comparison Test, Mann-Whitney test, or Dunn's Multiple Comparisons Test ($p < 0.05$).

phic assay may be less sensitive in the adult ovariectomized animals, as doses of ethynyl estradiol (0.01 mg/kg) and 4-nonylphenol (50 mg/kg) that did not alter uterine weight in the adult animals (Fig. 3) were capable of inducing a significant uterotrophic response in the younger rats (Fig. 2). This was not the case following treatment with methoxychlor (50 mg/kg) or 4-tert-octylphenol (50 mg/kg), where the uterotrophic responses were similar in each age group.

Age at Vaginal Opening, Prepubertal Rats

Alterations in the time of vaginal opening in prepubertal Long Evans female rats following daily exposure by oral gavage to a single test compound from 21–35 days of age are reported in Table 1. During the treatment period, none of the test compounds had any significant effects on body weights. Ethynyl estradiol, methoxychlor, 4-tert-octylphenol, and 4-nonylphenol each significantly advanced the age at vaginal opening. The dose of methoxychlor or 4-nonylphenol (50 mg/kg) that advanced vaginal opening was 5,000 times higher than that of ethynyl estradiol. An even higher dose of 4-tert-octylphenol (200 mg/kg; 20,000 times that of ethynyl estradiol) was required to accelerate vaginal opening by about 3.2 days. In contrast, oral exposure to bisphenol A with doses up to 400 mg/kg/day (40,000 times that of ethynyl estradiol) was ineffective in altering the time of vaginal opening in Long Evans rats.

The age at vaginal opening following exposure to 17-β-estradiol or methoxychlor by subcutaneous injection was also evaluated. As expected, 17-β-estradiol (0.005 mg/kg) advanced the age of vaginal opening. However, methoxychlor (50 mg/kg) did not alter the age of vaginal opening when administered by sc injection [e.g., control: 32.2 $\pm$ 0.7 days (8); E2: 26.6 $\pm$ 0.4 (10); methoxychlor: 31.5 $\pm$ 0.5 (8)].

Vaginal Cytology, Adult Ovariectomized Rats

Table 2 shows the changes in vaginal cytology during an 11-day exposure period to a single test chemical in ovariectomized rats. As expected, exposure to 17-β-estradiol (0.005 mg/kg/day, sc) and ethynyl estradiol (0.1 mg/kg/day, oral) resulted in a persistent estrous endocrine status, which was reflected by the appearance of cornified epithelial cells in vaginal washes from ovariectomized rats within 3 days following the initial dose. Cornified epithelial cells were also observed following exposure to a lower dose of ethynyl estradiol (0.01 mg/kg/day, oral) after 8 days of treatment, whereas the lowest dose of ethynyl estradiol (0.001 mg/kg/day, oral) did not cause a significant change in the appearance of the vaginal cytology. Although methoxychlor (50 mg/kg/day, oral) was capable of inducing cornified epithelial cells within 7 days of exposure in all animals in this treatment group, neither 4-tert-octylphenol nor bisphenol A were effective in altering vaginal cytology with any of the doses tested. One-half of the animals treated with the highest dose of 4-nonylphenol (100 mg/kg, oral) displayed a single day with cornified vaginal epithelial cells on the last day of treatment. As compared to changes in vaginal cytology in the ovariectomized animals, uterine weight was a better indicator of the ability of 4-tert-octylphenol to stimulate an estrogenic response (Fig. 4). Whereas no oral doses of 4-tert-octylphenol altered vaginal cytology during 11 days of exposure, this chemical was capable of inducing a

TABLE 1
Age at Vaginal Opening in Prepubertal Long-Evans Rats following Exposure by Oral Gavage Beginning at 21 Days of Age

Treatment (mg/kg/day)	Age at vaginal opening[a] (days)	Days vaginal opening advanced[a]	Body weight (g)[a]	
			21 days of age[b]	33 days of age[c]
Experiment 1				
Control (corn oil)	30.6 ± 0.42	—	43.4 ± 1.8	114 ± 3.2
Ethynyl estradiol				
0.01	24.6 ± 0.18*	6.0 ± 0.18	44.1 ± 1.4	111 ± 2.7
4-tert-Octylphenol				
50	31.1 ± 0.55	—	43.4 ± 1.8	112 ± 3.1
100	29.7 ± 0.65	0.88 ± 0.65	44.0 ± 2.1	113 ± 2.8
200	27.5 ± 0.57*	3.18 ± 0.57	43.4 ± 1.9	108 ± 3.0
Bisphenol A				
200	30.8 ± 0.67[d]	—	44.3 ± 2.1[d]	113 ± 3.4[d]
400	30.5 ± 0.57	—	43.9 ± 2.2	113 ± 3.5
Experiment 2				
Control (corn oil)	33.4 ± 0.63	—	44.6 ± 2.6	125 ± 4.7
Methoxychlor				
50	25.0 ± 0.33*[,e]	8.4 ± 0.33	45.7 ± 2.6	119 ± 4.7
4-Nonylphenol				
25	31.8 ± 0.64	1.5 ± 0.64	46.2 ± 2.0	132 ± 4.2
50	28.1 ± 0.64*	5.3 ± 0.64	45.1 ± 2.1	126 ± 4.0
100	26.6 ± 0.52*	6.8 ± 0.18	45.1 ± 2.1	124 ± 5.5
Experiment 3				
Control (corn oil)	33.7 ± 0.53	—	40.2 ± 0.85	120 ± 2.0
Bisphenol A				
50	34.8 ± 0.23	—	40.2 ± 0.74	120 ± 1.8
100	34.2 ± 0.37	—	40.9 ± 0.81	120 ± 1.4
400	34.7 ± 0.59	—	40.4 ± 0.63	113 ± 2.1

[a] Mean ± SE ($n = 8$ unless indicated otherwise).
[b] Body weight on the first day of treatment.
[c] Body weight on the last day of treatment.
[d] $n = 7$.
[e] The age at vaginal opening [mean ± SE (n)] following exposure to methoxychlor (50 mg/kg) by subcutaneous injection was not significantly different from control [control: 32.2 ± 0.7 (8); methoxychlor: 31.5 ± 0.5 (8)].
* Significant treatment effect within each experiment by ANOVA ($p < 0.01$); significantly different from the control by the Dunnett Multiple Comparison Test ($p < 0.01$).

significant uterotrophic effect following a 3-day exposure to the same doses as those used for the vaginal cytology study.

Estrous Cyclicity, Intact Adult Rats

Estrous cyclicity in adult Long Evans rats was monitored during 25 days of exposure to evaluate the ability of each test chemical to alter ovarian and neuroendocrine function (Table 3). 17-β-estradiol (0.005 mg/kg/day, sc) and ethynyl estradiol (0.01 and 0.1 mg/kg/day, oral) significantly reduced the number of 4- to 5-day cycles during the exposure period. Most animals in these treatment groups initially displayed an extended diestrous vaginal smear. Many of these animals remained in diestrus for 10–14 days, suggesting that they were pseudopregnant (Cooper and Goldman, 1999; Gilmore and McDonald, 1969; Lu *et al*., 1980). Continued exposure for longer than 18–20 days resulted in periods of extended estrus in some of the animals. Oral exposure to 4-tert-octylphenol (200 mg/kg/day, oral) induced a similar response. The number of 4- to 5-day cycles in the animals in this treatment group was significantly decreased, whereas the number of days of diestrus was increased. Serum progesterone concentrations (mean ± SE) in animals displaying extended diestrus for 7–10 days were elevated (50.89 ± 5.6 ng/ml as compared with controls on the first day of diestrus, 3.73 ± 0.28 ng/ml). While 4-nonylphenol disrupted cyclicity, the specific alteration in the cycle differed from that of 4-tert-octylphenol. Although there were some shorter periods of extended diestrus (i.e., 4–6 days) following exposure to 4-nonylphenol (25 mg/kg, oral), the higher dose of 4-nonylphenol (100 mg/kg, oral) caused periods of extended estrus (e.g., 3–5 days with cornified epithelial cells). Methoxychlor (50 mg/kg, oral) and bisphenol A (100 mg/kg, oral) also significantly reduced the total number of 4- to 5-day estrous cycles. In the methoxychlor group, periods of extended diestrus

TABLE 2
Incidence of Cornified Vaginal Epithelial Cells in Ovariectomized Adult Long Evans Rats during an 11-Day Treatment Period

Treatment	Dose (mg/kg/d)	Route	Vaginal cytology[a,b]		
			Animals with 80–100% cornified epithelial cells ($n = 6$)	Days of treatment until cornified epithelial cells observed[c]	Days with cornified epithelial cells[c]
Control (corn oil)	0	Oral	0	—[d]	—[d]
17-β-Estradiol	0.005	sc	6	3 ± 0	8 ± 0
Ethynyl estradiol	0.001	Oral	1	10	1
	0.01	Oral	6	8 ± 1.0	1.8 ± 0.7
	0.1	Oral	6	3 ± 0	8 ± 0
Methoxychlor	50	Oral	6	7.2 ± 1.6	2.2 ± 0.8
4-tert-Octylphenol	50	Oral	0	—	—
	100	Oral	0	—	—
	200	Oral	1	7	1
4-Nonylphenol	25	Oral	0	—	—
	50	Oral	1	7	1
	100	Oral	3	8.7 ± 1.5	0.5 ± 0.3
Bisphenol A	100	Oral	0	—	—

[a] Vaginal cytology was monitored for 5 days prior to treatment. All animals exhibited persistent diestrous vaginal smears prior to treatment.
[b] Animals were dosed once daily by oral gavage for 11 consecutive days. Changes in vaginal cytology were evaluated daily.
[c] Mean ± SE when number of animals with cornified epithelial cells was greater than 1.
[d] (—) Indicates that no cornified epithelial cells were observed in any of the animals in this treatment group during the 11-day treatment period.

and estrus were evident throughout the 25-day observation time. Of the bisphenol A animals, seven animals displayed normal cycles, six animals displayed periods of extended diestrus, and two animals displayed extended estrous during at least one cycle.

In vitro Estrogen and Progesterone Receptor Competitive Binding Assays

A summary of the *in vitro* estrogen and progesterone receptor competitive binding assays are presented in Table 4. These data are reported as equilibrium dissociation constants for the inhibition of receptor binding (Ki) which were calculated from estimates of the IC_{50} concentrations for each test chemical. The Ki concentration (μM) is useful when comparing the ability of competitors to inhibit *in vitro* binding to multiple steroid receptors because it takes into account both the radioligand concentration and the Kd of each receptor type. Ki concentrations in the nM range indicate a high affinity for the receptor (i.e., E2, DES, EE for ER; and P, R5020 for PR) with lesser affinity reflected by Ki values in the μM range (i.e., HPTE, 4-t-OP, NP, and BPA for ER). These data also show that 4-tert-octylphenol and 4-nonylphenol exhibit similar affinities for the estrogen and progesterone receptors, with Ki concentrations of 0.7 μM (estrogen receptor) and 1.2–3.8 μM (progesterone receptor). These values are compared with those of two organochlorine pesticides, chlordecone and o,p-DDT, which traditionally have been classified as environmental estrogens but also exhibit a similar affinity for the progesterone receptor.

DISCUSSION

All of the test chemicals evaluated were capable of inducing an uterotrophic response within 3 days in the prepubertal Long Evans rats. Based upon the dose-response data for this assay and molar equivalent doses for each test chemical, the relative potencies of the test chemicals following oral exposure were as follows: ethynyl estradiol >>> methoxychlor = 4-nonylphenol > bisphenol A = 4-tert-octylphenol. The effective molar equivalent doses ranged from 4,000 to 5,800 (methoxychlor and 4-nonylphenol) and 13,000 to 14,000 (bisphenol A and 4-tert-octylphenol) times higher than that required of ethynyl estradiol to induce a significant uterotrophic response in prepubertal rats. In addition, based upon the dose-response data for 4-tert-octylphenol and 4-nonylphenol, the 3-day uterotrophic assay in the prepubertal rats was the best indicator of estrogenic activity when compared with the age at vaginal opening in prepubertal rats, estrous cyclicity in intact adult rats or changes in vaginal cytology (cornification of vaginal epithelial cells), and the 3-day uterotrophic assay in ovariectomized adult rats (Table 5).

The data from the 3-day uterotrophic assays in prepubertal rats also demonstrated the importance of study design. Factors such as the time the animals were killed following the last dose and the route of exposure significantly influenced the outcome of our studies. The importance of observation time following exposure became apparent in our initial studies with 4-tert-octylphenol when no uterotrophic effects were detected in the intact sexually immature rats killed 24 h following the last of

TABLE 3
Effects on Estrous Cyclicity in Adult Long-Evans Rats during 25 Days of Treatment

Treatment	Dose (mg/kg/day)	Route	Days of diestrus	Days of estrus	Number of 4–5 day cycles[c]	Change in cycle[e]
Block 1						
Control (corn oil)	0	sc	12.2 ± 0.3 (6)[a,b]	7.2 ± 0.4 (6)[a]	4.8 ± 0.3 (6)[a]	—
17-β-Estradiol	0.005	sc	18.7 ± 1.7 (7)*	4.8 ± 1.1 (7)	0.1 ± 0.1 (7)*[,d]	D, E
Block 2[f]						
Control (corn oil)	0	Oral	13.0 ± 0.2 (13)	6.1 ± 0.3 (13)	5.2 ± 0.2 (13)	
Ethynyl estradiol	0.01	Oral	14.4 ± 0.6 (15)	5.7 ± 0.5 (15)	3.4 ± 0.4 (15)*	D
	0.1	Oral	12.3 ± 1.4 (6)	10.1 ± 1.5 (7)*	0.4 ± 0.3 (7)*	D, E
4-tert-Octylphenol	50	Oral	12.4 ± 0.4 (7)	6.7 ± 0.4 (7)	5.1 ± 0.3 (7)	—
	100	Oral	13.3 ± 0.7 (7)	6.4 ± 0.5 (7)	4.6 ± 0.5 (7)	D
	200	Oral	15.6 ± 0.7 (14)*	5.8 ± 0.5 (14)	2.2 ± 0.4 (14)*	D
4-Nonylphenol	25	Oral	12.6 ± 0.5 (7)	7.3 ± 0.5 (7)	3.4 ± 0.6 (7)	D, E
	50	Oral	12.6 ± 0.2 (7)	7.4 ± 0.6 (7)	3.7 ± 0.7 (7)	D, E
	100	Oral	12.8 ± 0.3 (16)	7.1 ± 0.3 (16)	3.4 ± 0.3 (16)*	D, E
Block 3						
Control (corn oil)	0	Oral	12.8 ± 0.4 (9)	6.4 ± 0. (9)	4.7 ± 0.4 (9)	
Methoxychlor	50	Oral	14.8 ± 0.7 (10)	6.4 ± 0.6 (10)	2.7 ± 0.4 (10)*	D, E
Block 4						
Control	0	Oral	12.7 ± 0.3 (9)	6.0 ± 0.2 (9)	5.2 ± 0.2 (9)	
Bisphenol A	100	Oral	13.7 ± 0.5 (15)	6.3 ± 0.4 (15)	3.7 ± 0.3 (15)*	D, E

[a] Mean ± SE (n).

[b] All animals exhibited at least three consecutive 4 or 5-day cycles prior to treatment.

[c] Number of complete 4 or 5-day cycles during the 25-day treatment period.

[d] Although no animal in this group exhibited a single 4–5 day cycle after treatment began, for statistical analysis one animal was recorded as completing one cycle.

[e] Diestrus (D): extended diestrus for greater than 3 days. Estrus (E): extended estrus of 3 days or more.

[f] A second incomplete block was conducted in this experiment for control, ethynyl estradiol (0.01 mg/kg), 4-t-octylphenol (200 mg/kg), and 4-nonylphenol (100 mg/kg). Because there was no significant block effect, the data have been combined for statistical analysis.

* Significant treatment effect within block by Mann-Whitney test ($p < 0.01$; two treatment groups) or Kruskal-Wallis test [($p < 0.05$: more than two treatment groups; significantly different from control by Dunn's Multiple Comparison Test ($p < 0.05$)].

3 doses by oral gavage (Laws *et al*., 1996a). Time-course studies reported here showed increased uterine wet weight at 6 but not at 24 h following the last of three doses of 4-tert-octylphenol. The increase in uterine weight at 6 h was not due totally to water imbibition, as uterine dry weights were also significantly increased at 6 but not at 24 h postexposure (data not shown). Similar data for uterine weight 6 and 24 h post-exposure were also obtained following oral exposure to bisphenol A. This may have been a factor in studies reported by Gould *et al*. (1998), where no change in uterine weight was observed in sexually immature rats when evaluated 20 h after the last of three doses of bisphenol A (5–150 mg/kg). Studies reported by Ashby and Tinwell (1998) indicate that observation time may not be as critical with higher oral doses of bisphenol A. These authors report significant changes in uterine weight 24 h following the last exposure to high oral doses of bisphenol A (400–800 mg/kg).

It is also important to note that uterine weight remained significantly elevated 24 h after the last dose of ethynyl estradiol, methoxychlor, and 4-nonylphenol. This suggests that oral exposure to 4-tert-octylphenol and bisphenol A induce weaker, short-term estrogenic effects such as those seen following exposure to estriol (Clark and Mani, 1994), whereas methoxychlor and 4-nonylphenol induce longer-term effects similar to that of 17-β-estradiol. Data reported by Lee and Lee (1996) support our observation of a longer-term uterotrophic response following exposure to 4-nonylphenol, albeit these authors used an intraperitoneal injection as the exposure route for their time-course studies.

It is well known that differences in the metabolic activation and/or elimination of the test chemicals varies according to exposure route (Klaassen, 1986). The influence of dosing route on the uterotrophic response was obvious in the present studies. Because methoxychlor is a proestrogen that must undergo hydroxylation in the liver to produce its estrogenic metabolite HPTE (Bulger *et al*., 1978b), oral exposure produced the greater uterotrophic response. Whereas oral exposure to 50 mg/kg methoxychlor induced a significant increase in uterine weight in prepubertal rats, sc exposure to the same dose of methoxychlor had no effect. Our data also show that the magnitude of the uterotrophic response following oral exposure to 4-nonylphenol was greater than that following sc injection. Conversely, exposure to 4-tert-octylphenol or bisphenol A by sc injection produced the greatest changes in uterine

TABLE 4
Ki Concentrations Reflecting *in Vitro* Binding Affinity for Estrogen and Progesterone Receptors

Chemical	Estrogen receptor K_i[a,b] (μM)	Progesterone receptor K_i[a] (μM)
17β-Estradiol (E2)	0.0004 ± 0.00001[c]	2.3 ± 0.3[c]
Diethylstilbestrol (DES)	0.0002 ± 0.00002	11 ± 0.8
Ethynyl estradiol (EE)	0.0004 ± 0.00005	1.04 ± 0.1
Progesterone (P)	> 1000	0.005 ± 0.0002
R5020	> 1000	0.001 ± 0.0001
Chlordecone	1.5 ± 0.2	2.3 ± 0.4
o,p-DDT	3.2 ± 0.2	4.5 ± 0.2
Methoxychlor (MXC)	65 ± 9	137 ± 8
HPTE (MXC metabolite)	0.053 ± 0.01	12 ± 2
4-tert-Octylphenol (4-t-OP)	0.781 ± 0.25	3.8 ± 0.4
4-Nonylphenol (NP)	0.672 ± 0.10	1.2 ± 0.3
Bisphenol A (BPA)	1.57 ± 0.18	60 ± 5

[a] $K_i = IC_{50}/(1 + L/Kd)$ where L = radioligand concentration and Kd = equilibrium dissociation constant of radioligand. IC_{50} estimates were generated using GraphPad InPlot, Scientific Graphics and Curve Fitting, San Diego, CA. (ER: L = 1.0 nM; Kd = 0.6 nM; PR: L = 1.0 nM, Kd = 0.5 nM).

[b] The means of the estrogen receptor binding data were used as part of a training set of chemicals to construct a three-dimensional QSAR model and were reported in Waller *et al.* (1996) as the negative log of K_i (μmol).

[c] Mean ± SE ($n = 2$).

weight as compared with those observed following oral exposure. These results are in agreement with studies reported by Gray and Ostby (1998), where the uterotrophic response in sexually immature rats was greater following sc injection as compared to oral exposure to 4-tert-octylphenol (200 mg/kg). Similar results comparing oral and sc exposures to high doses of bisphenol A (400–800 mg/kg) have been reported by Ashby and Tinwell (1998). Cook *et al*. (1997) have proposed using intraperitoneal injections as the preferred route of exposure when testing chemicals for endocrine disrupting activity, suggesting that this route would maximize sensitivity and facilitate potency comparison by eliminating the variability in gut absorption rate. This group has shown that high doses of methoxychlor (750 mg/kg × 4 days) or bisphenol A (500 mg/kg × 4 days) administered by intraperitoneal injections significantly increased uterine weight in young ovariectomized rats. However, because these animals showed a significant loss in their final body weights as compared with that of controls, using such high doses would not meet the criteria for selecting doses at or just below the maximum tolerated dose (MTD) (Weideman, 1993; Maloney, 1993) that may be recommended in some screening programs.

Our studies of the test chemicals using ovariectomized adult rats show that the uterotrophic assay in these animals was

TABLE 5
Comparison of Effects following Exposure to Test Chemicals in Rats of Different Age and Endocrine Status

			Sexually immature Long-Evans rats		Adult Long Evans rats		
					Intact	Ovariectomized	
Treatment	Dose (mg/kg/d)	Route	Age at vaginal opening	Uterine weight[a]	Estrus cyclicity (No. of cycles)[b]	Vaginal cell cornification[c]	Uterine weight[a]
17-β-Estradiol	0.005	sc	↓	↑	↓	+	↑
Ethynyl estradiol	0.01	Oral	↓	↑	↓	+	—
	0.1	Oral	↓	↑	↓	+	↑
Methoxychlor	50	Oral	↓	↑	↓	+	↑
4-tert-Octylphenol	50	Oral	—	—	—	—	—
	100	Oral	—	↑	—	—	↑
	200	Oral	↓	↑	↓	—	↑
4-Nonylphenol	25	Oral	—	—	—	—	—
	50	Oral	↓	↑	—	—	—
	100	Oral	↓	↑	↓	—	↑
Bisphenol A	100	Oral	—	—	↓	—	—
	200	Oral	—	↑	ND	ND	ND
	400	Oral	—	↑	ND	ND	ND

Note. ND, no data collected.

[a] Based upon the wet uterine weight 6-hours following the last of 3 daily doses.

[b] Based upon the number of complete 4–5 day cycles during the 25-day treatment period.

[c] Based upon 50% of animals exhibiting cornified epithelial cells for 1 or more days during an 11-day treatment period.

↓ Indicates a statistically significant ($p < 0.05$) decrease.

↑ Indicates a statistically significant ($p < 0.05$) increase.

— Indicates no statistically significant ($p < 0.05$) effect.

slightly less reliable as an indicator of estrogenic activity as compared with the prepubertal rat. The higher oral doses of ethynyl estradiol (10-fold increase) and 4-nonylphenol (2-fold) required to detect a significant uterine weight change in the ovariectomized adult as compared with the prepubertal rat may be a reflection of differences in metabolism/excretion of the test chemicals and/or the age and endocrine status of the animal.

Although monitoring changes in the vaginal cytology of ovariectomized adult rats continues to be regarded as a reliable test for detecting estrogenic activity (Allen and Doisy, 1924; Carney *et al*., 1997; O'Connor *et al*., 1996; Reel *et al*., 1996), we found this to be a poor assay for detecting the potential estrogenic activity of the test chemicals used in this study. While cornified epithelial cells appeared in vaginal smears within 72 h following exposure to 17-β-estrogen and ethynyl estradiol (0.01–1.0 mg/kg), neither the lowest dose of ethynyl estradiol (0.001 mg/kg) nor any of the oral doses of 4-tert-octylphenol or bisphenol A induced a change in the vaginal cytology. Gray and Ostby (1998) have reported vaginal mucification and vaginal histologic alterations in ovariectomized rats after a 10-week exposure to 4-tert-octylphenol (200 mg/kg). Thus, longer exposure periods, along with vaginal histologic evaluation, may be required in order to detect changes in vaginal cytology during exposure to environmental chemicals with weak estrogenic activity.

It is clear from the data presented here that each of the test compounds can reduce the number of regular 4- to 5-day estrous cycles during the 25-day treatment period. However, the manner in which the cycle was altered varied depending upon the test chemical and the dose. Although ethynyl estradiol and 4-tert-octylphenol generally induced long periods of extended diestrus, the alterations in the cycling patterns for methoxychlor, 4-nonylphenol, and bisphenol A were not as clearly defined. Shorter periods of extended diestrus (e.g., 5–7 days) and/or estrus (e.g., 3–5 days) were intermittently observed during the exposure period in animals dosed with methoxychlor, 4-nonylphenol, and bisphenol A. Although we were unable to detect significant correlations between the specific day of the cycle when the initial exposure occurred and onset of these irregular cycles, some of the changes observed may have been due to an alteration in the timing of the surge of luteininzing hormone (LH) rather than an estrogen-like induction of a pseudopregnancy. Goldman *et al*. (1994) and Stoker *et al*. (1996) have shown that exposure to the fungicides metam sodium and thiram during critical periods on the day of proestrus delays the LH surge and ovulation through their action at the level of the central nervous system. Such delays are typically associated with a cycle displaying 2–3 days of estrus (Cooper *et al*., 1994). Additional studies with dosing initiated on a specific day of the estrous cycle are needed to clarify whether the effects of methoxychlor, 4-nonylphenol, and bisphenol A on estrous cyclicity are due to their estrogenic activity or due to other mechanisms that may become apparent only when exposure occurs during critical periods.

Several laboratories have also evaluated the effects of 4-nonylphenol, methoxychlor, and 4-tert-octylphenol on estrous cyclicity in rats. Whereas Cunny *et al*. (1997) noted no treatment-related changes in estrous cycle patterns during week 8 of a 90-day treatment period in rats fed diets containing 4-nonylphenol (e.g., yielded dietary intakes of 15–150 mg/kg/day), a multigenerational study of 4-nonylphenol (2000 p.p.m.) showed an increase in the length of the estrous cycles in the F_1 and F_2 generations (NTP, 1997). Additionally, exposure to methoxychlor (50 mg/kg, oral) during the perinatal and juvenile developmental periods resulted in a disruption of the adult estrous cycle (Chapin *et al*., 1997). Blake and Ashiru (1997) reported alterations in estrous cyclicity in adult Sprague-Dawley rats following sc injections of 4-tert-octylphenol (40 mg/rat) three times weekly. In these studies, persistent estrus was observed in 16 of 21 rats, with the appearance of cornified cells in vaginal smears occurring within 3 days in animals treated daily with 40 mg 4-tert-octylphenol. The rapid occurrence of persistent estrus in these animals is not consistent with sc exposure to 17-β-estradiol, which has been reported to initially cause an extended period of diestrus (Gilmore and McDonald, 1969), suggesting that perhaps the 4-tert-octylphenol may be capable of altering estrous cyclicity via multiple mechanisms. The fact that data reported by Blake and Ashiru (1997) do not agree with our results for 4-tert-octylphenol may be due to different dosing regimens or animal strains, or to subtle differences in the methods for evaluating the vaginal smears between the two labs. In the studies reported here we also observed elevated serum progesterone concentrations in animals displaying an extended diestrus for 7 or more days, which was consistent with pseudopregnancy.

The relative potencies of methoxychlor, HPTE, 4-tert-octylphenol, 4-nonylphenol, and bisphenol A in our *in vitro* competitive estrogen receptor binding assay are in general agreement with those reported by White *et al*. (1994), Shelby *et al*. (1996) and Danzo (1997). The more novel observation from the estrogen and progesterone receptor binding data presented here is that some of the test chemicals were capable of binding to both steroid receptors. For example, similar concentrations of 4-tert-octylphenol and 4-nonylphenol, as well as the estrogenic pesticides chlordecone and o,p-DDT, inhibited both estrogen and progesterone receptor binding in our *in vitro* binding assays. Although *in vitro* transcriptional activation studies and *in vivo* studies of progesterone receptor function were not included in the series of experiments reported here, such data could provide a more thorough understanding of the mechanism(s) of action for these chemicals.

The likelihood that environmental chemicals can interact with multiple steroid receptors is further reinforced by the recent finding that HPTE, the estrogenic metabolite of methoxychlor, also inhibits androgen receptor–dependent transcriptional activity in HepG2 human hepatoma cells (Maness *et*

al.,1998). Sohoni and Sumpter (1998) reported that bisphenol A is antiandrogenic and nonylphenol is a weak androgen agonist in an *in vitro* yeast-based assay. Similarly, chemicals that competitively bind to both the estrogen receptor and the TCDD-related aryl hydrocarbon receptor (AhR) exhibit both estrogenic and AhR-mediated antiestrogenic activities in MCF-7 human breast cancer cell lines (Liu *et al*, 1994; Nesaretnam *et al*, 1996). Collectively, these observations support the contention that multiple receptor systems are likely targeted by certain environmental chemicals and that *in vitro* screening protocols to detect endocrine disrupting chemicals should evaluate multiple receptors.

In summary, the studies reported here compare the 3-day uterotrophic response in prepubertal and ovariectomized adult rats following oral exposure to 17-β-estradiol, ethynyl estradiol, methoxychlor, 4-tert-octylphenol, 4-nonylphenol, and bisphenol A. They demonstrate that lower doses of ethynyl estradiol and 4-nonylphenol can induce an uterotrophic response in prepubertal rats as compared with ovariectomized adult rats. In addition, these data show that dosing route and the observation time are critical factors that can influence whether a significant uterotrophic response will be observed. By including additional end points in our studies, the relative sensitivity of the 3-day uterotrophic assay with vaginal opening assay in prepubertal rats, cornification of vaginal epithelial cells in adult, ovariectomized rats, and estrous cyclicity in intact adult rats were compared. Based upon the dose-response data for ethinyl estradiol, 4-tert-octylphenol, and 4-nonylphenol, the 3-day uterotrophic assay in the prepubertal rats was the best indicator of estrogenic activity as compared with the other end points (Table 5). Monitoring changes in vaginal cytology of ovariectomized adult rats was a poor indicator of estrogenic activity for 4-tert-octylphenol and bisphenol A. Finally, examining estrous cyclicity may be a useful screening method for endocrine disrupting activity that can expand the scope of the study to include chemicals that target ovarian and neuroendocrine function, and in general has the potential to detect both estrogenic and/or nonestrogenic mechanisms of action. These data, along with the technical issues discussed regarding the 3-day uterotrophic assay, can be useful in the current assessment and validation of methods for screening chemicals for endocrine disrupting activity.

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