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TITLE: One-Generation Extension Study of Vinclozolin and Di-n-Butyl Phthalate Administered by Gavage on Gestational Day 6 to Postnatal Day 21 in CD® (Sprague-Dawley) Rats

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PROPOSED STUDY IN-LIFE DATES: August - November 2001

AMENDMENTS:

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1.0 BACKGROUND AND OBJECTIVES

The Food Quality Protection Act of 1996 requires the EPA to develop and implement a screening program for determining the potential in humans for estrogenic (and anti-estrogenic) effects from pesticides. This program has been expanded on the advice of an advisory committee to include androgenic (and anti-androgenic) effects and effects from thyroid-hormone (TH)-like (or anti-TH) substances. One of the tests being considered for inclusion in this screening program is a mammalian, two-generation reproductive toxicity test. The basic two-generation test is described by the EPA Office of Prevention, Pesticides, and Toxic Substances' Health Effects Test Guideline 870.3800: Reproduction and Fertility Effects (U.S. EPA 1998).

Although the basic two-generation study design was developed to provide information on insult to the reproductive tract, there is concern that certain effects may be missed, simply because the reproductive tract has not had sufficient time to develop before the observations are made. In the standard two-generation test, most F1 animals are sacrificed and examined at postnatal day (pnd) 21; only one animal per sex per litter is usually allowed to continue to maturity. These animals are used to breed the F2 generation. The study design being tested through this work assignment will examine whether or not allowing more of the F1 generation males to continue through puberty to adulthood will provide additional information in detecting endocrine-mediated effects.

The objectives of this one-generation extension study are to determine the following:

1. Whether some of the effects from perinatal exposure to Di-n-Butyl Phthalate (DBP) or to vinclozolin that can be easily detected after puberty are missed in weanling animals of the F1 generation.

2. Whether some of these effects occur at an incidence that would go undetected if only one male per litter is retained past puberty and examined at adulthood.

In summary, the hypothesis being tested is that adverse reproductive effects will be detected if three or more F1 males per litter are examined at or after puberty but will be missed if most of the F1 males are examined only at weaning, and only one male/litter is retained to adulthood. The hypothesis will be tested using two known and well-characterized anti-androgens, DBP and vinclozolin, each at two doses, one a known effect level and one a LOAEL (lowest observable effect level).

2.0 MATERIALS AND METHODS

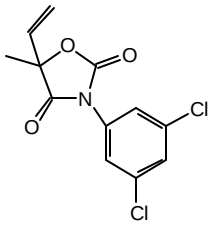
2.1 Test Substances

2.1.1 Vinclozolin

Chemical Name: 3-(3,5-Dichlorophenyl)-5-ethenyl-5-methyl-2,4-oxazolidinedione

CAS Number: 5-0471-44-8

Chemical Structure:



Supplier: BASF

Manufacturer's Batch No.:

Appearance:

Molecular Formula: $C_{12}H_9Cl_2NO_3$

Molecular Weight: 286.114

Storage Conditions:

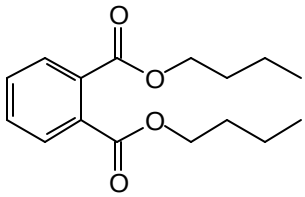
Dosing Suspensions:

2.1.2 Di-n-butyl phthalate

Chemical Name: Dibutylphthalate (DBP)

CAS Number: 84-74-2

Chemical Structure:



Supplier:

Manufacturer's Batch No.:

Appearance:

Molecular Formula: $C_{16}H_{22}O_4$

Molecular Weight: 278.35

Storage Conditions:

Dosing Suspensions:

2.2 Chemical Safety and Handling

See MSDSs of both chemicals in Attachment.

2.3 Dose Formulation and Analysis

The dosing suspensions will be prepared at a frequency determined by stability tests performed prior to the start of the study. Suspensions will be prepared at Battelle Chemical Repository, Sequim, WA, and stored in wide-mouth, amber bottles. They will be shipped via 24-hour express delivery and logged into the Materials Handling Facility prior to transfer to the Reproductive and Developmental Toxicology Laboratory for dosing. The test materials will be suspended in Mazola® corn oil (CAS No. 8001-30-7), with the concentration determined by the following formula:

$$\text{Concentration (mg/ml)} = \frac{\text{Dose per time (mg/kg)}}{\text{Dosage volume per time (5.0 ml/kg)}}$$

An aliquot of each dose level per formulation will be analyzed by Battelle. The dosing bottles will be identified at RTI by a five-digit random number Rx code, and a color code. Personnel, other than the Laboratory Supervisor, Project Toxicologist, and Study Director, will not be informed of the test chemicals or formulation concentrations until all laboratory work is completed (i.e., the study technicians will be "blind" for chemical and dose). Aliquots from the dosing bottles will be collected on the first day of dosing (the first gestational day [gd] 6), and on the first pnd 0, 7, 14, and 21, which will be shipped to Battelle Chemical Repository, Sequim, WA, for analysis.

2.4 Animals

2.4.1 Species and Supplier

The proposed test animals will be the Sprague Dawley Derived Outbred Albino Rat Crl:CD®(SD) IGS BR supplied by Charles River Laboratories, Inc., Raleigh, NC.

2.4.2 Live Animals and Species Justification

The use of live animals has been requested by the Sponsor. Alternative test systems are not available for the assessment of effects of chemicals on reproduction and development in intact mammals for determining the potential risk for humans from endocrine-mediated effects of pesticides and other chemicals. The Charles River CD® rat has been the subject of choice on reproductive and developmental toxicology contracts at RTI since 1976, and has been used for other reproductive toxicology studies with this test material. Large historical data bases for reproductive performance and prevalence of spontaneous malformations in control rats are available from studies conducted at RTI (currently based on over 300 control litters) as well as from the supplier (Charles River, 1988). This study does not unnecessarily duplicate any previous study.

2.4.3 Total Number, Age, and Weight

One hundred seventy (170) nulliparous female rats, nine to ten weeks of age and approximately 200-225 grams upon arrival, will be purchased for this study. One hundred (100) male rats of the same strain from the RTI breeding colony, originally from the same supplier, will be used to generate timed-mated females. Female rats will be approximately 10 to 11 weeks of age and approximately 200-300 grams in weight on gd 0. One hundred twenty-five (125) sperm-positive female rats, designated the F0 generation, will be used in this study (i.e., five groups of 25 sperm-positive dams each).

2.4.4 Quality Control

The shipment of females will be quarantined on arrival, and quality control evaluation will be initiated within one day after receipt. Within one day after receipt, five female rats will be chosen from the shipment, sacrificed, and blood collected for assessment of viral antibody status. Heat-inactivated serum will be sent to BioReliance (Rockville, MD) for their Level 1 Rat Antibody Screen. The viral screen will consist of evaluation for the presence of antibodies against the following: Toolan H-1 virus (H-1), Sendai virus, Pneumonia virus of mice (PVM), rat coronavirus/sialodacryoadenitis (RCV/SDA), Kilham rat virus (KRV), CAR Bacillus, and Mycoplasma pulmonis (*M. Pul*.). In addition, fecal samples from representative animals will be externally examined for intestinal parasites.

2.4.5 Sentinels

After the selection of study and quality control animals, four additional female rats will be randomly selected, eartagged, and designated as sentinels. They will be singly housed in the study room(s), with feed and water available *ad libitum* (as described below). They will be examined once daily by cageside observation for morbidity or mortality at the same time as the clinical observations or morbidity/mortality checks for the study animals. The clinical condition of sentinel animals will be recorded only in the event that an animal is moribund or found dead. If a sentinel animal is terminated moribund, blood will be collected at termination and serum samples frozen. During the F0 and F1 adult necropsies, a maximum of one or two surviving sentinel females will be terminated, blood samples collected, and serum samples prepared. All sentinel serum samples will be submitted to BioReliance (Rockville, MD) for serological evaluation (see above section on Quality Control).

2.4.6 Quarantine

The animals will be quarantined for approximately one week prior to the start of treatment. They will be observed daily for general health status and ability to adapt to the Animal Research Facility's (ARF) husbandry conditions. They will be released from quarantine, if suitable for use (based on QC results), by the attending ARF veterinarian or his designate.

2.5 Animal Husbandry

2.5.1 Housing, Feed, and Water

During a seven-day quarantine period, animals will be randomly assigned to cages. Males will be housed singly in solid-bottom, polycarbonate cages (8"x19"x10.5"). Nonmated females will be group housed (maximum three per cage), and mated females and F1 male weanlings will be singly

housed in solid-bottom, polycarbonate cages (8"x19"x10.5") fitted with stainless steel wire lids (Laboratory Products, Rochelle Park, NJ),. Sani-Chip® cage bedding (P.J. Murphy, Forest Products, Inc., Montville, NJ) will be used in all cages. Pelleted feed (No. 5002 Purina Certified Rodent Chow®) and tap water from the Durham, NC water system, in plastic bottles with stainless steel sipper tubes, will be available *ad libitum* for the initial study females during quarantine, and for the F0 females and retained F1 males to the end of the study. Breeding colony males will be on *ad libitum* feed and water. The water for the males is provided by an automatic watering system (Edstrom Industries, Inc., Waterford, WI); the parental females during the initial mating period will also be on the automatic watering system. The analysis of the rodent chow for chemical composition and possible chemical contamination, and analysis of Durham City water will be provided by the suppliers and maintained in the study records. It is anticipated that contaminant levels will be below certified levels for both feed and water and will not affect the design, conduct, or conclusions of this study. Rat chow will be stored at approximately 60-70°F, and the period of use will not exceed six months from the milling date. At all times, animals will be housed, handled, and used according to the NRC Guide (NRC, 1996).

2.5.2 Environmental Conditions

Environmental conditions in the ARF will be continuously monitored, recorded, and controlled during the course of the study by an automated system (Siebe/Barber-Colman Network 8000 System with Version 4.4.1 Signal® software (Siebe Environmental Controls (SEC)/ Barber-Colman Company, Loves Park, IL). Animal rooms used for this study will be maintained on a 12:12 hour light:dark cycle. Target conditions for temperature and relative humidity in the animal rooms will be between 64-79°F (18-26°C) and 30-70%, respectively, with 10-15 air changes per hour (NRC, 1996). Temperature and/or relative humidity excursions will be documented in the study records and the final report.

2.5.3 Animal Identification

All F0 maternal rats will be individually identified by ear tag after arrival at RTI. In addition, each sperm-positive female will receive a dam study number. All retained postweanling F1 males will also be uniquely identified by eartag at weaning, as well as receiving a male study number. All data generated during the course of this study will be tracked by these numbers.

2.5.4 Limitation of Discomfort

Some adult toxicity may be caused by exposure at the high doses of each test material. Discomfort or injury to animals will be limited, in that if any animal becomes severely debilitated or moribund, it will be humanely terminated by CO_2 inhalation. All necropsies will be performed after terminal CO_2 asphyxiation. F1 pnd 4 culled pups will be euthanized by decapitation.

2.5.5 Breeding

For breeding, individual females will be placed in the home cage of singly-housed males (i.e., one male and one female). On the following morning and each morning thereafter, the females will be examined for the presence of vaginal sperm or a vaginal copulation plug (Hafez, 1970). The day on which vaginal sperm or plugs are found will be designated as gd 0. These females are presumed pregnant. The initial sperm-positive females (dams), designated the F0 generation, will be housed individually or with their litters until scheduled sacrifice. Sperm-negative females will be retained in the same male's cage and checked for sperm or vaginal plug on successive mornings until insemination occurs or the treatment groups are filled, whichever comes first. When all treatment groups are filled, remaining sperm-negative females will be sacrificed by asphyxiation with CO_2. The fate of all animals will be fully documented.

3.0 EXPERIMENTAL DESIGN

3.1 Study Design

The study will be conducted with two treatment groups per test material (two test materials) and one vehicle control group, each comprised initially of 25 presumed pregnant (sperm-positive) rats (Table 1). A graphical representation of the study design is presented in Figure 1 below.

Table 1. One-Generation Extension Study Design and Target Doses

Group No.	No. Animals Dosed	No. Days Exposure	Dosing Period (gd-pnd)	Dose (mg/kg/day)	Dosing Concentration (mg/ml)	Dose Volume (ml/kg)
1	25	36-38	6-21	0	0	5
2	25	36-38	6-21	50 VIN	10 VIN	5
3	25	36-38	6-21	100 VIN	20 VIN	5
4	25	36-38	6-21	100 DBP	20 DBP	5
5	25	36-38	6-21	500 DBP	100 DBP	5

VIN = Vinclozolin
DBP = Di-n-butyl phthalate

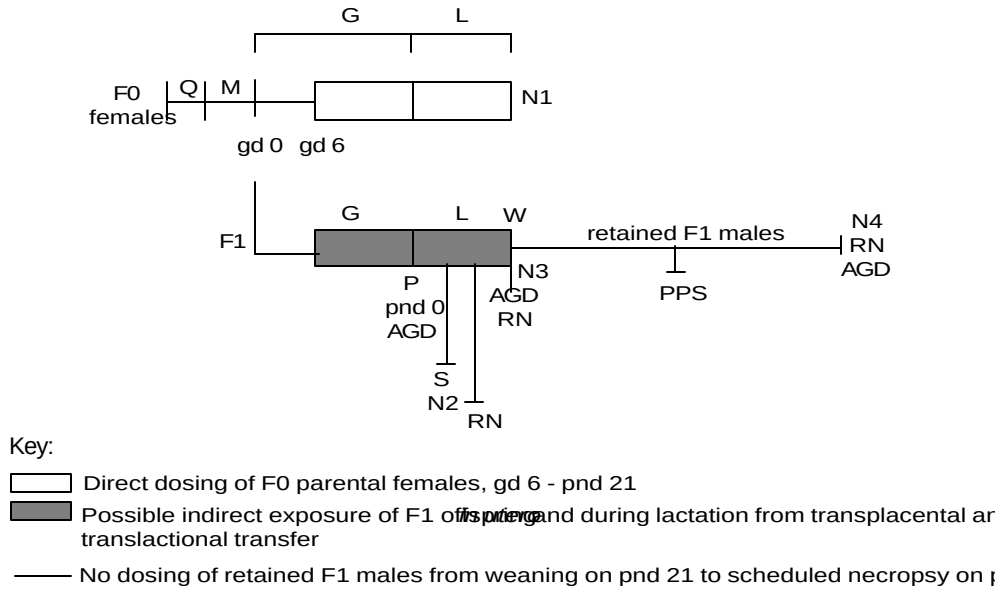


Figure 1. One-Generation Extension Study Design

Q	=	quarantine (one week)	L	=	lactation (three weeks)
M	=	mating (one week)	pnd	=	postnatal day
G	=	gestation (~three weeks)	P	=	parturition (date of birth, pnd 0)
gd	=	gestational day	AGD	=	anogenital distance
W	=	wean on pnd 21	PPS	=	acquisition of preputial separation

S = standardize litters to 10 (with maximum number of males) on pnd 4

RN = examination of males for retained nipples

N1 = necropsy of F0 parental females at weaning of F1 litters

N2 = necropsy culled females to confirm sex

N3 = necropsy any remaining F1 females (and confirm sex), and necropsy three F1 males per litter at weaning on pnd 21

N4 = necropsy of retained F1 males at pnd 95 ± 5

Tentative Study Dates[a]

F0 females arrive at RTI:
F0 females paired with breeding colony males:
F0 gd 0:
Dosing (gd 6 - pnd 21):
Parturition of F1 offspring (pnd 0):
Weaning of F1 offspring (pnd 21):
Sacrifice of F0 dams:
F1 postwean holding period (minimum 70 days):
Sacrifice of F1 males:
Submission of nonaudited draft final report:
Submission of audited draft final report:

[a] The end dates are tentative and will depend on the duration of mating of F0 females and the gestation and lactation of F1 offspring.

3.2 Dosage Selection

DBP is used as a coalescing aid in latex adhesives, as a plasticizer in cellulose plastics, and as a solvent in dyes. It is no longer used in poly-vinyl chloride (PVC) plastics (NTP, 2000). Exposure during gestation results in developmental toxicity, and DBP crosses the placenta in rats (Saillenfait et al., 1998; Ema et al., 1993, 1994, 1995a, 1998). It also results in reproductive toxicity in offspring from in utero/lactational exposure (Gray et al., 1998, 2000; Mylchreest et al., 1998a,b, 1999) or from continuous exposure (Wine et al., 1997). The intestinal metabolite, mono-n-butyl phthalate, also causes developmental toxicity in rats; this is most likely the proximate toxicant (Ema et al., 1995b). Although DBP binds to the androgen receptor, the effects on the male offspring reproductive tract are not considered to be mediated by receptor binding to cause the antiandrogenic effects (Mylchreest et al., 1998a).

DBP apparently acts by inhibiting fetal testicular testosterone biosynthesis *in vitro* and *in vivo* (Mylchreest et al., 1999). In adult rats, it also causes testicular toxicity but, as expected, no malformations (Cater et al., 1977). Daily oral (gavage) administration of DBP to dams, during gestation and lactation of 100 mg/kg/day through 750 mg/kg/day, results in dose-related reproductive malformations in male offspring, with approximately 75% of the male offspring affected at 750 mg/kg/day. The male malformations include shortened anogenital distance, small flaccid testes,

agenesis of portions (caput, corpus, cauda) of or the entire epididymis, delayed puberty, retained nipples and areolae, etc. (Gray et al., 1998; 2000). An oral dose of 50 mg/kg/day has been defined as the NOAEL (no observable adverse effect level) by Mylchreest et al. (1998a,b, 1999). Therefore, for this study, DBP in corn oil will be administered by oral gavage once daily on gd 6 through pnd 21 at 0 (vehicle control), 100 mg/kg/day (the LOAEL; lowest observed adverse effect level), and at 500 mg/kg/day (an obvious effect level).

Vinclozolin is a systemic dicarboximide fungicide used on grapes, other fruit, vegetables, hops, ornamental plants and turf (Kelce et al., 1997). Multigeneration studies in rats indicate that *in utero*/lactational exposure results in demasculinized male offspring (van Ravenzwaay, 1992). Gray et al. (1994) confirmed and extended these findings by administering vinclozolin in corn oil by gavage once daily on gd 14 through pnd 3 in rats at 0, 100, or 200 mg/kg/day. Male offspring exhibited dose-related incidences and severities of male reproductive tract malformations (see below) and renal system malformations, including hydroureter, hydrophrosis, and urinary bladder stones. *In vitro* studies indicate that the two vinclozolin metabolites bind to the androgen receptor (Kelce et al., 1994a) and, acting as antiandrogens, inhibit subsequent androgen receptor-dependent transcriptional activation (Wong et al., 1995). This mechanism has been confirmed *in vivo* (Kelce et al., 1997) with exposure to 200 mg/kg/day vinclozolin, resulting in alteration of the expression of androgen-dependent genes. The adverse effects of vinclozolin are mediated by its metabolites (Kelce et al., 1994b). The adult rat is also responsive to exposure to vinclozolin, but reproductive tract malformations are, as expected, not produced (Anderson et al., 1995).

In one dose-response study (Hellwig et al., 2000), Wistar and Long-Evans rats were orally dosed with vinclozolin from gd 14 to pnd 3 at 200, 12, 6, 3, 1, or 0 mg/kg/day. The high dose (200 mg/kg/day) was maternally toxic, and male offspring from both strains exhibited reduced anogenital distance; retained nipples/areolas; hypospadia; penile hypoplasia; development of a vaginal pouch; hypoplasia and chronic inflammation of the epididymides, prostate, seminal vesicles, and coagulating glands; testicular tubule atrophy; and chronic inflammation of the urinary bladder. At 12 mg/kg/day, retained nipples/areolas were present in both strains in preweanling males but persisted only in Long-Evans rats. Long-Evans rats (but not Wistar) also exhibited a low incidence of hypoplasia of accessory sex organs. The NOAELs were therefore 12 mg/kg/day in Wistar rats and 6 mg/kg/day in Long-Evans rats (there were no effects in either strain at 3 or 1 mg/kg/day).

In another dose response publication (Ostby et al., 1997), maternal rats were dosed by gavage on gd 14 through pnd 3 with vinclozolin at 0, 100, and 200 mg/kg/day (first study), with expected male offspring reproductive malformations observed at both doses. In a second study, the doses were 0, 3.125, 6.25, 12.5, 25, 50, and 100 mg/kg/day. In offspring males, reduced anogenital distance was observed at $\geq$3.125 mg/kg/day, retained areolas were observed at $\geq$6.25 mg/kg/day, and ventral

prostate weight was reduced and hypospadia observed at 50 mg/kg/day. Ectopic testes were only observed at 100 and 200 mg/kg/day. Effects on serum testosterone levels and spermatogenesis were only observed at $100 mg/kg/day.

Based on the results from Gray et al. (1994), Ostby et al. (1997), and Hellwig et al. (2000), in this study, vinclozolin in corn oil will be administered by oral gavage once daily on gd 6 through pnd 21 at 0 mg/kg/day (vehicle control; the same control group as for DBP since same vehicle is used for both chemicals), 50 mg/kg/day (considered the LOAEL for male reproductive tract malformations), and 100 mg/kg/day (a clear effect level).

3.3 Allocation and Treatment of F0 Maternal Animals

All sperm-positive F0 female rats (presumed pregnant dams) will be assigned to treatment groups by a stratified randomization method designed to provide uniform mean body weights across dosage groups at the initiation of gestation (gd 0). Vinclozolin or DBP in vehicle, or the vehicle alone, will be administered by gavage once daily, from gd 6 through pnd 21 (day of birth designated pnd 0) at a 5.0 ml/kg dosing volume. The volume of dosing formulation given to presumed pregnant females will be adjusted, based on each animal's most recent body weight or the current weight on a scheduled weighing day. The dosing formulations will be administered using a 16-gauge, two-inch curved dosing needle (Perfektum®, Popper and Sons, New Hyde Park, NY), fitted to a syringe of appropriate volume. The route of administration (gavage) was specified by the Sponsor and was the route employed by the studies used for dose selection (see Section 3.2).

3.4 Observation of F0 Maternal Animals

3.4.1 Clinical Observations

Clinical observations of F0 maternal animals will be documented at least once daily on gd 0-5 (prior to dosing period) and at least twice daily, at dosing and one to two hours postdosing, throughout the dosing period (gd 6 through pnd 21). The examining technicians will be unaware of the test materials or of dosage levels. Observations will be made for (but not limited to):

a. Any response with respect to body position, activity, coordination, or gait
b. Any unusual behavior such as head flicking, compulsive biting or licking, circling, etc.
c. The presence of:
    1. Convulsions, tremors, or fasciculations
    2. Increased salivation
    3. Increased lacrimation or red-colored tears (chromodacryorrhea)

4. Increased or decreased urination or defecation (including diarrhea)
5. Piloerection
6. Mydriasis or miosis (enlarged or constricted pupils)
7. Unusual respirations (fast, slow, labored, audible, gasping, or retching)
8. Vocalization

3.4.2 F0 Maternal Body Weights

All F0 dams will be weighed in the morning on gd 0 and daily in the morning during the dosing period on gd 6 through pnd 21, for adjustment of dosing volume based on the most recent body weight. Maternal body weights will be reported for gd 0, 6, 9, 12, 15, 18, and 20 and for pnd 0, 4, 7, 14, and 21. F0 maternal weight gains will be calculated for gd 0-6 (pretreatment), 6-9, 9-12, 12-15, 15-18, 18-20, pnd 0-4, 4-7, 7-14, 14-21, gd 0-20 (gestation period), gd 6-20 (gestational treatment period), and for pnd 0-21 (lactational treatment period).

3.4.3 F0 Maternal Parturition and Lactation

Beginning on gd 20, each female will be examined twice daily (a.m. and p.m.) for evidence of littering. Dosing will continue through parturition through pnd 21. If the dam is in the process of littering at the usual time of dosing, she will not be dosed at that time but will be dosed after littering is completed but no later than 1530 hours, so that at lease 18 hours will elapse between doses. Females who are littering at morning and afternoon checks will have this information recorded on the gestational sheet and will be dosed on the next scheduled dosing day. Signs of dystocia or other signs of difficulty at parturition will be recorded. Dams that have not produced a litter by calculated gd 26 will be necropsied. Apparently nonpregnant uteri will be stained in 10% ammonium sulfide (Salewski, 1964) to confirm pregnancy status. Any dams whose whole litters are born dead or die prior to pnd 21 will be sacrificed, and the number of uterine implantation scars will be recorded.

3.5 F1 Progeny

3.5.1 Mortality, Body Weights, and Clinical Observations

All pups will be counted, sexed, weighed, and examined as soon as possible on the day of birth (designated as pnd 0) to determine the number of viable and stillborn members of each litter. Thereafter, litters will be evaluated for survival, sex, gross observations, and body weights on pnd 4, 7, and 14. Any pup which appears moribund or dies during lactation will be necropsied, when possible, to investigate the cause of death and internal status of the reproductive system. No organs will be weighed or saved.

3.5.2 Standardization of Litter Sizes

On pnd 4, the size of each litter will be adjusted to ten pups, maximizing the number of male pups retained. Natural litters with ten or fewer pups will not be culled. All culled pups will be sacrificed by decapitation. The culled pups will be examined viscerally to confirm the internal sex. The F0 dams will be allowed to rear their remaining F1 young to pnd 21. On pnd 21, each litter will be weaned.

3.5.3 Anogenital Distance and Nipple Retention

Anogenital distance will be recorded with the individual pup weight on pnd 0, using an ocular reticle calibrated to a stage micrometer (precision to 0.1 mm), and on pnd 21 weaning necropsy using a Vernier calipers (precision to 0.1 mm). The presence or absence of retained nipples and areolae on the ventrum will be recorded for all F1 males at approximately pnd 11-13. Any males with one or more nipples or areolae will be uniquely marked within the litter (dye on tail) until weaning.

3.5.4 Selection at Weaning

When each F1 litter has reached pnd 21, the F1 males will be weight ranked within litters and pair matched (e.g., one each of the two heaviest males from each litter will go into the pnd 21 necropsy group or the retention group, etc.). Three males per litter (weight matched to the retained males) will be sacrificed, and any females will be sacrificed. The remaining males in each litter (weight mated to the pnd 21 necropsy males) will be retained until scheduled sacrifice.

3.6 F1 Postwean Observations and Procedures

3.6.1 Body Weights and Clinical Observations

The F1 male pups which are selected at weaning will be retained until pnd 95±5 without dosing. The pups will be examined daily for adverse effects and will be weighed twice weekly during this period, based on their individual ages.

3.6.2 Cleavage of the Balanopreputial Gland (preputial separation)

Each retained male pup will be observed, beginning on pnd 35, for evidence of preputial separation (acquisition of puberty). The characteristic is present when the prepuce can be completely retracted to expose the glans penis. Male pups will be examined daily, and individual body weights at acquisition will be recorded until all male pups have this response.

3.7 Necropsy of F0 Females and F1 Offspring Males and Females

3.7.1 Necropsy of F0 Females

F0 females which are moribund, abort, or deliver early (with offspring not viable outside the uterus) will be sacrificed by CO_2 asphyxiation, necropsied, and discarded. Intact fetuses (*in utero*, aborted, or delivered early) will be examined externally and viscerally (with focus on the reproductive system) and discarded. Any F0 dams whose whole litters are born dead or die prior to pnd 21 will be sacrificed and examined as described below.

On pnd 21 of each F1 litter, each F0 dam will be euthanized by CO_2 asphyxiation. The thoracic and abdominal organs will be examined for grossly evident morphological changes, and uterine implantation scars will be counted and recorded. Organs or tissues showing macroscopic abnormalities will be retained in neutral buffered 10% formalin. Microscopic examination of the tissues may be undertaken only if considered necessary by the Study Director to interpret the findings of the study. Uteri from any F0 females who appear nonpregnant will be stained with 10% ammonium sulfide (Salewski, 1964) for confirmation of pregnancy status and count of implantation sites, if any. F0 maternal carcasses and nonretained tissues will be discarded.

3.7.2 Necropsy of F1 Males and Females on Pnd 21

At necropsy, the three weight-matched selected males per litter will be euthanized by CO_2 asphyxiation. Anogenital distance will be recorded for all pups selected for necropsy after euthanasia and body weights recorded (see Section 3.7.4 for a description of external and internal examinations of individual male pups). Any remaining F1 females on pnd 21 will be euthanized and necropsied to confirm sex by internal examination of the reproductive system.

3.7.3 Necropsy of F1 Males on Pnd 95±5

The F1 retained males will be weighed and then euthanized by CO_2 asphyxiation. See Section 3.7.4 for the description of the external and internal examination of each male pup. Anogenital distance will be measured by Vernier calipers (precision to 0.1 mm) at necropsy.

3.7.4 External and Internal Examination of F1 Males at Necropsy

Each male selected for pnd 21 necropsy or adult necropsy will be examined externally at necropsy as follows:

- Note any unusual malformations or anomalies
- Shave ventral surface from inguinal region to neck, count nipples and areolas, and record position of areolas and nipples
- Check animals for hypospadias, epispadias, and cleft phallus, and measure AGD
- Note if testes are obviously undescended (testis descent is usually completed in CD® (SD) rats by pnd 16-18)
- Note if inguinal regions are soiled with urine
- Note if prepuce is partially or entirely detached from glans penis, especially if a persistent thread of tissue is present along frenulum

The reproductive organs will be carefully observed for the following:

- Location of each testis (scrotal, abdominal, gubernaculum attached to abdominal wall)
- Gubernacular cords, present or absent, and length in mm
- Note, if present, cranial suspensory ligaments
- Note if testes are small, absent, fluid filled, enlarged, appear infected, or other
- Note if epididymides are small, absent, or infected (record region of effects)
- Note if ventral prostate is small, absent, or infected
- Note if dorsolateral prostate is small, absent, or infected
- Note if seminal vesicles are small, absent, infected, or one side larger than the other
- Note if coagulating glands are small, absent, infected, one side larger than the other, or detached from seminal vesicles
- Note if kidneys display hydronephrosis or calcium deposits
- Note presence of hydroureter(s)
- Note presence of urinary bladder stones or blood in urinary bladder

The following organs will be weighed:

- Each testis individually
- Each corpus plus caput epididymides
- Each cauda epididymides
- Entire seminal vesicle, plus coagulating glands with fluid as a unit, if possible
- Entire prostate, then ventral and dorsal lateral lobes separately
- Paired adrenals
- Liver
- Levator ani plus bulbocavernosus
- Cowper's (bulbourethral) glands as a pair
- Glans penis

The whole pituitary will be frozen and delivered to the U.S. EPA's National Health and Environmental Effects Research Laboratory (NHEERL) (Attn: Dr. Ralph Cooper). All other organs will be examined and weighed, as described above, and discarded.

4.0 STATISTICAL ANALYSES

The unit of comparison will be the pregnant female, the F1 pup, or the retained F1 male offspring, as appropriate.

4.1 Independent Continuous Data

These types of data are measured on animals that are statistically independent (i.e., not from the same litters) and includes such continuous endpoints as ***F0 maternal body weights, feed consumption, organ weights, gestational length,*** and ***litter size***. Treatment groups for each chemical will be compared to the concurrent control group using either parametric ANOVA under the standard assumptions or robust regression methods (Zeger and Liang, 1986; Royall, 1986; Huber, 1967) which do not assume homogeneity of variance or normality. The homogeneity of variance assumption will be examined via Levene's test (Levene, 1960).

If Levene's Test indicates lack of homogeneity of variance ($p<0.05$), robust regression methods will be used to test all treatment effects. The robust regression methods use variance estimators that make no assumptions regarding homogeneity of variance or normality of the data. They will be used to test for linear trends across dose within chemicals, as well as overall treatment group differences (via Wald chi-square tests). Significant overall treatment effects will be followed by single degree-of-freedom *t*-tests for exposed vs. control group comparisons, if the overall treatment effect is significant. If Levene's test does not reject the hypothesis of homogeneous variances, standard ANOVA techniques will be applied for comparing the treatment groups within chemicals. The GLM procedure in SAS® 8 (SAS Institute Inc., 1999a,b,c,d,e, 2000) will be used to test for linear trend, evaluate the overall effect of treatment and, when a significant treatment effect is present, to compare each exposed group within chemicals to the concurrent control group via Dunnett's Test (Dunnett, 1955, 1964). Standard ANOVA methods, as well as Levene's Test, are available in the GLM procedure of SAS® Version 8, and the robust regression methods are available in the REGRESS procedure of SUDAAN® Release 8.0 (RTI, 2001). Both packages are currently in use on GLP studies.

We will compare the ***F0 maternal post-implantation loss percentage*** across dose groups within chemicals using weighted ANOVA techniques. Since percentage data derived from litters tend to have unequal variances, we will apply the arcsine of the square root transformation to the litter percentages prior to analysis. We will then perform the ANOVA weighted by the number of implants

(denominator of the post-implantation loss percentage) in order to further stabilize the variances. In the presence of significant treatment effects, Dunnett's test will be used for pairwise comparisons to control. The average post-implantation loss percentage (prior to transformation) will be presented for each dose group within chemical, with sample size and standard error.

4.2 Independent Binary Data

These types of data are measured on animals that are statistically independent (i.e., not from the same litters) and includes binary endpoints such as the F0 maternal reproductive indices (e.g., ***mating, fertility,*** and ***live litter indices***). All indices will be analyzed by Fisher's Exact test for overall heterogeneity among treatment groups and by an exact version of the Cochran-Armitage test for linear trend on proportions (Cochran, 1954; Armitage, 1955; Agresti, 1990). When the overall Fisher's Exact test is significant ($p<0.05$), pairwise comparisons of individual exposed groups within chemicals vs. control are performed using pairwise Fisher's Exact tests. All of these tests can be obtained via the FREQ procedure in SAS 8. The SAS MULTTEST procedure can be used to obtain *p*-value adjustments for the multiple treatment comparisons resulting from repeated applications of Fisher's Exact test. The *p*-value adjustments are based on the bootstrap and permutation resampling techniques of Westfall and Young (1993).

4.3 Cluster-Correlated Data

Cluster-correlated data are those which are measured on more than one pup/sex/litter. Such endpoints include, for example, ***F1 periodic pup body weights during lactation, the periodic pup survival indices, the lactation index, the percentage of stillborns and live births, the sex ratio, the percentage of male pups with areolae and/or nipples on PND 11-13 and 21,*** and the ***anogenital distance*** (a developmental landmark). GEE regression methods (Zeger and Liang, 1986; Liang and Zeger, 1986) in SAS® 8 or SUDAAN® 8.0 will be used to evaluate overall significance, test for linear trend across dose groups within chemicals, and test pairwise comparisons to the control group values. For anogenital distance, a body weight covariate may be included in the regression model. Some of these outcomes are continuous (e.g., body weights, anogenital distance) and some are binary (e.g., periodic pup survival). Ordinal outcomes would include those measured on a severity scale, such as none, mild, moderate, and severe. The analyses will compare the results for adult offspring with those of weanlings.

No matter what type of endpoint is examined (e.g., continuous, binary, ordinal), when multiple offspring from the same litter are used in the analysis, care must be taken to adjust for the resulting correlation of responses within litters. Intracluster correlation, or the tendency for littermates to respond similarly, tends to inflate the true variance of parameter estimates, including percentages and

regression coefficients used in these analyses. In other words, offspring within litters are not statistically independent, and failure to account for this in the statistical analysis will result in underestimated standard errors and false-positive tests for treatment effects (Haseman and Kupper, 1979). Hence, the chance of finding false-positive results is increased when the clustering is ignored.

To incorporate the effects of intracluster correlation and reduce the chances of finding spurious results, a model-fitting method designed specifically for clustered data in which the outcomes may or may not be normally distributed is recommended. Recent advances in analyzing longitudinal and cluster-correlated data for generalized linear models (Zeger and Liang, 1986; Zeger, Liang, and Albert, 1988; Lipsitz, Kim, and Zhao, 1994) have led to new methods for handling binary, categorical, and continuous outcomes on offspring clustered within litters. The techniques (otherwise referred to as ***Generalized Estimating Equations***, or ***GEEs***) make no strict distributional assumptions about the endpoint of interest (e.g., normality) or the correlations among clustered observations, thereby providing flexibility for a variety of analytical settings.

The generalized linear model provides a unified approach for modelling continuous and non-continuous response variables. For continuous outcomes, the distribution of the responses within litters is assumed to be normal. The expected value of the response is related directly to a linear function of the covariates. For binary outcomes, the distribution of the responses within litters is assumed to be binomial. A logit transformation is used to relate the expected value of the response $prob(Y=1|x)$ to a linear function of the covariates (Morgan, 1992). For ordinal outcomes, a multinomial distributional assumption and a cumulative logit transformation is used, leading to the proportional odds model (McCullagh, 1980). The GEE approach requires only specification of the relationship between the mean and variance of the correlated outcomes within each litter. Only the mean, $prob(Y=1|x)$ or $E(Y|x)$, needs to be correctly specified for the estimated regression parameters to be approximately unbiased.

Zeger and Liang (1986) and Liang and Zeger (1986) showed how to use GEEs to solve for the regression parameters and their estimated variances in the generalized linear model when the data are cluster-correlated. In the linear and logistic regression setting, the model parameters can be estimated in the usual way, using standard methods such as maximum likelihood or ordinary least squares. These estimates are identical to those obtained if the data were independent, and they are known to have desirable statistical properties even under cluster sampling (e.g., they are asymptotically normal and unbiased). Robust variance estimates of model parameters fully account for the intracluster correlation of responses. This technique is valid for any underlying correlation structure within a litter, as long as the litters are statistically independent.

In order to gain efficiency (i.e., reduce the variance of estimated parameters), a pairwise correlation model for the offspring within a litter can be specified, and estimates of the correlation parameters are used in the estimation of regression coefficients. However, the correlation pattern is treated as a nuisance parameter, and not explicitly included in the regression model. The regression coefficient estimates and their robust variance estimates are approximately unbiased regardless of whether or not the correlation structure is correctly specified.

Many correlation structures have been developed for this purpose, with working independence implying no correlation within litters, an exchangeable structure denoting equal pairwise correlations for all pairs of offspring within a litter (popular for developmental toxicology studies), and a time-dependent structure (e.g., auto-regressive or *m*-dependent) used frequently for longitudinal designs. When the working correlation structure is consistent with empirical correlations, efficiency is maximized. However, a robust variance estimator for regression parameters provides valid and unbiased inferences even when the working correlation structure has been misspecified.

Wald chi-square test statistics are used to evaluate the significance of model parameters relating to treatment effects. For a single degree-of-freedom hypothesis (e.g., high dose vs. control, linear trend), the Wald chi-square test statistic reduces to a standard normal deviate, or the regression coefficient estimate divided by its standard error. For multiple degree-of-freedom hypotheses (e.g., the overall effect of treatment), the Wald chi-square test is analogous to the ANOVA F-test for linear models with continuous responses.

The application of the estimating equation approach to developmental toxicology studies has been demonstrated by many authors, among them Bieler and Williams (1995), Liang and Hanfelt (1994), Carr and Portier (1993), Lefkopoulou, Moore, and Ryan (1989), Lockhart, Piegorsch, and Bishop (1992), Rao and Colin (1991), and Williams (1982).

Software for implementing the GEE approach can be found in the GENMOD procedure of SAS® 8 (SAS Institute Inc., 1999a,b,c,d,e, 2000) as well as SUDAAN® 8.0 (RTI, 2001). SUDAAN was developed at the Research Triangle Institute, and fits linear, logistic, multinomial logistic, log-linear, and proportional hazards models to clustered and longitudinal data. Both independent and exchangeable working correlation structures are available in the REGRESS, LOGISTIC, LOGLINK, and MULTILOG procedures, which fit linear, logistic, log-linear (Poisson models) and multinomial logistic models to continuous, binary, count, ordinal, and nominal categorical data using the methods of Liang and Zeger (1986) and Lipsitz et al. (1994). SUDAAN allows for fixed-effect covariates that are either measured at the fetus (e.g., fetal body weight) or litter levels (e.g., dose group). Cluster sizes can vary, and missing data are assumed to be missing completely at random (meaning that the probability of a missing value is equal for all offspring within a litter).

4.4 Developmental Landmarks

Developmental landmarks include, for example, F1 litter ***time to vaginal opening and preputial separation,*** and the ***anogenital distance***. Since data for time to vaginal opening and preputial separation will be recorded on independent observations and should not be censored, these will be analyzed using either parametric ANOVA under the standard assumptions or robust linear regression methods, and possibly including body weight at acquisition as a covariate in the regression model. Details on the ANOVA and robust regression methods can be found in the sections above. Analysis of anogenital distance requires different methodology and is described in an earlier section entitled ***Cluster-Correlated Data.***

4.5 Statistical Outliers

A test for statistical outliers will be performed in the UNIVARIATE procedure of SAS 8® (SAS Institute Inc., 1999a,b,c,d,e, 2000) on F0 maternal body weights, feed consumption (in g/day), and retained F1 male body and organ weights. If examination of pertinent study data do not provide a plausible biologically sound reason for inclusion of the data flagged as "outlier," the data will be excluded from summarization and analysis and will be designated as outliers. If feed consumption data for a given animal for a given observational interval (e.g., pnd 0-7 or 7-14 during the lactational exposure period) are designated outliers or unrealistic, then summarized data for this animal encompassing this period (e.g., pnd 0-21 for the lactational exposure period) also will not include this value. For all statistical tests, $p \# 0.05$ (one- or two-tailed) will be used as the criterion for significance.

5.0 RETENTION OF SPECIMENS AND RECORDS

All specimens and records which remain the responsibility of RTI will be retained in the RTI archives for the length of time specified in the appropriate guidelines and regulations (see Section II; U.S. EPA, 1989; OECD, 1998). These materials will be stored for two years at the performing laboratory's expense. Beyond two years, continued retention will be at additional cost to the Sponsor.

6.0 GOOD LABORATORY PRACTICES (GLP)

RTI, through administration of a quality assurance program by the Quality Assurance Unit, assures compliance of all phases of toxicological studies with existing regulations and generally accepted good laboratory practices (see Section II, U.S. EPA, 1989; OECD, 1998).

7.0 REPORTS

7.1 Status Reports

Status reports will be provided to the Sponsor's Representative, with contents and frequency to be determined by the Sponsor's Representative and/or the Sponsor's Study Monitor.

7.2 Draft and Final Reports

A draft report will be submitted to the Sponsor's Representative within three months of the last necropsy date. The final report will include:

- Abstract
- Materials and Methods
- Results
- Discussion
- Conclusions
- References
- Summary in-life and necropsy data with statistical analyses
- Individual animal data: in-life and necropsy
- Protocol, any amendments, or any deviations from the protocol

Summary of F0 Maternal Data

a. Mean periodic maternal body weights and weight gains

b. Feed consumption (expressed as g/animal/day and g/kg body weight/day) during gestation and lactation

c. Survival indices

d. Gestational length

e. Mean litter size

f. Mean number of live and dead offspring

g. Prenatal (postimplantation) loss (%) $= \frac{\text{No. implantation scars} - \text{No. live pups at birth}}{\text{No. implantation scars}} \times 100$

h. Number and percent of mothers showing treatment-related behavioral abnormalities in nesting and nursing

i. Gestational index (%) =

j. Gross necropsy

k. Number of uterine nidation scars at necropsy

Individual F0 Maternal Data

a. Identification number

b. Age at beginning of study $\frac{\text{No. pregnant females with live litters}}{\text{No. pregnant females}} \times 100$

c. Age at death and manner of death

d. Gestational and lactational body weights

e. Gestational and lactational feed consumption

f. Male rat (by identification number) used for mating

g. Gestational length in days

h. Total number of offspring per litter

i. Number and percent of live and dead offspring

j. General condition of offspring and mother through weaning

k. Gross necropsy and organ weights

l. Number of uterine nidation scars (implantation sites) at necropsy

Summary of F1 Litter Lactational Data

a. Total litter size

b. Number and percent of stillborn

c. Number and percent of live births

d. Anogenital distance and body weight on pnd 0 and 21

e. Periodic viability counts

f. Periodic body weights by sex per litter from birth to weaning (taken on pnd 0, 4, 7, 14, and 21 by individual pup)

g. Sex ratio (% males per litter)

h. Indices:

$$\text{Live birth index: } = \frac{\text{No. live pups at birth}}{\text{Total no. pups at born}} \times 100$$

$$\text{4-day survival index } = \frac{\text{No. pups surviving 4 days (precull)}}{\text{Total no. live pups at birth}} \times 100$$

$$\text{7-day survival index } = \frac{\text{No. pups surviving 7 days}}{\text{Total no. live pups at 4 days (postcull)}} \times 100$$

$$\text{14-day survival index } = \frac{\text{No. pups surviving 14 days}}{\text{Total no. live pups at 7 days}} \times 100$$

$$\text{21-day survival index } = \frac{\text{No. pups surviving 21 days}}{\text{Total no. live pups at 14 days}} \times 100$$

$$\text{Lactation index } = \frac{\text{No. pups surviving 21 days}}{\text{Total no. live pups at 4 days (postcull)}} \times 100$$

i. Number of male pups per litter with (and number per male of) areolae and/or nipples on pnd 11-13 and 21

Individual Data From Retained F1 Male Offspring

a. Identification number

b. Age at death and manner of death

c. Twice weekly body weights during postwean holding period

d. Age and body weight at acquisition of preputial separation

e. Organ weights

f. Reproductive system external and/or gross abnormalities

g. Anogenital distance at necropsy

Summary of Data From Retained Male F1 Offspring

a. Mean periodic body weights and weight gains

b. Age and body weight at acquisition of preputial separation

c. Organ weights

d. Reproductive system external and/or gross abnormalities

e. Presence of areolae and/or nipples at adult necropsy

f. Anogenital distance at adult necropsy

8.0 PERSONNEL

Study Director:	Julia D. George, Ph.D.
Project Toxicologist:	Rochelle W. Tyl, Ph.D., DABT
ARF Veterinarian:	Donald B. Feldman, D.V.M., ACLAM

ARF Manager:	Frank N. Ali, M.B.A., LATG, ILAM
Laboratory Supervisor:	Melissa C. Marr, B.A., LATG
Data Specialist and Reproductive Toxicity Supervisor:	Christina B. Myers, M.S.
Research Data Entry Assistant:	Timothy W. Wiley, B.S.
Research Biologist:	William R. Ross, B.A.
Biologists:	Vickie I. Wilson Lawson B. Pelletier, RVMT, LAT
Biological Laboratory Assistants:	Charlene N. Beauman, B.S. Marian V. Rieth, RVMT Dee A. Wenzel, RVMT, LATG Melody P. Gower

Additional study team members to be determined.

9.0 STUDY RECORDS TO BE MAINTAINED

Protocol and any Amendments

List of any Protocol Deviations

List of Standard Operating Procedures

Animal Requisition and Receipt Records

Quarantine Records

Temperature and Humidity Records for the Animal Room(s)

Animal Research Facility Room Log(s)

Durham City Water Analysis (analyzed monthly, reported annually)

Feed Type, Source, Lot Number, Dates Used, Certification, Analytical Results

Dosage Code Records Containing Five-Digit Rx Code, Color Code, and Concentration

F0 Mating Records

F0 Randomization Records

F0 Assignment to Study Records

Dose Formulation Receipt and Use Records

Balance Check Sheets (Dosing and Necropsy)

Dosing Records Including Clinical Signs, Maternal Weights

F1 Offspring - Postnatal: F1 pup sex, body weight, external observations on pnd 0, 4, 7, 14, and 21; anogenital distance on pnd 0 and 21; culling data (confirmation of sex of culled females); clinical signs; nipple/areolar retention on pnd 11-13; randomization of F1 males for weanling necropsy or retention

F1 Weanling Necropsy: Body weights, organ weights, anogenital distance in males, nipple/areolar retention in males, confirmation of sex in female weanlings, gross observations

F0 Maternal Necropsy Records

F1 Male Postwean Holding Period: Body Weights

Clinical Signs

Acquisition of Preputial Separation

F1 Male Necropsy Records: Organ weights, gross observations, anogenital distance, retention of nipples/areolae

Statistical Analysis Records

Temperature and Relative Humidity Records

Room Log Sheets

Feed and Water Analyses

Correspondence

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ATTACHMENT

Material Safety Data Sheets (MSDSs)