Report to the

U.S. Consumer Product Safety Commission

by the

CHRONIC HAZARD ADVISORY PANEL ON PHTHALATES

AND PHTHALATE ALTERNATIVES

July 2014

APPENDIX A

DEVELOPMENTAL TOXICITY

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ABBREVIATIONS*

3β-HSD	3β-hydroxysteroid dehydrogenase
AA	antiandrogenicity; antiandrogenic
ADHD	attention deficit hyperactivity disorder
ADI	acceptable daily intake
AGD	anogenital distance
AGI	anogenital index
ASD	Autistic Spectrum Disorders
CRA	cumulative risk assessment
ASTDR	Agency for Toxic Substances and Disease Registry
ATBC	acetyl tributyl citrate
BASC-PRS	Behavior Assessment System for Children-Parent Rating Scales
BBP	butylbenzyl phthalate
BIBRA	British Industrial Biological Research Association
BMDL	benchmark dose (lower confidence limit)
BNBA	Brazelton Neonatal Behavioral Assessment
BRIEF	Behavior Rating Inventory of Executive Function
BSI	behavioral symptoms index
CBCL	Child Behavior Check List
CDC	Centers for Disease Control and Prevention, U.S.
CERHR	Center for the Evaluation of Risks to Human Reproduction
CHAP	Chronic Hazard Advisory Panel
CHO	Chinese hamster ovary
CNS	central nervous system
CPSC	Consumer Product Safety Commission, U.S.
CPSIA	Consumer Product Safety Improvement Act of 2008
CSL	cranial suspensory ligament
cx-MIDP	mono(carboxy-isononyl) phthalate (also, CNP, MCNP)
cx-MINP	mono(carboxy-isooctyl) phthalate (also COP, MCOP)
DBP	dibutyl phthalate
DCHP	dicyclohexyl phthalate
DEHA	di(2-ethylhexyl) adipate
DEHP	di(2-ethylhexyl) phthalate
DEHT	di(2-ethylhexyl) terephthalate
DEP	diethyl phthalate
DHEPP	di-*n*-heptyl phthalate
DHEXP	di-*n*-hexyl phthalate
DHT	dihydrotestosterone
DI	daily intake
DIBP	diisobutyl phthalate
DIDP	diisodecyl phthalate
DIHEPP	diisoheptyl phthalate
DIHEXP	diisohexyl phthalate

* List applies to main report and all appendices.

DINP	diisononyl phthalate
DINCH®	1,2-cyclohexanedicarboxylic acid, diisononyl ester
DINX	1,2-cyclohexanedicarboxylic acid, diisononyl ester
DIOP	diisooctyl phthalate
DMP	dimethyl phthalate
DNHEXP	di-*n*-hexyl phthalate
DNOP	di-*n*-octyl phthalate
DPENP	di-*n*-pentyl phthalate
DPHP	di(2-propylheptyl) phthalate
DPS	delayed preputial separation
DSP	decrease spermatocytes and spermatids
DVO	delayed vaginal opening
ECHA	European Chemicals Agency
ECMO	extracorporeal membrane oxygenation
ED_{50}	median effective dose
EPA	Environmental Protection Agency, U.S.
EPW	epididymal weight
FDA	Food and Drug Administration, U.S.
f_{ue}	urinary excretion factor
GD	gestational day
GGT	gamma-glutamyl transferase
GLP	good laboratory practices
grn	granulin
HBM	human biomonitoring
hCG	human chorionic gonadotrophin
HI	hazard index
HMW	high molecular weight
HQ	hazard quotient
IARC	International Agency for Research on Cancer
ICH	International Conference on Harmonisation
insl3	insulin-like factor 3
IP	intraperitoneally
LD	lactation day
LH	luteinizing hormone
LMW	low molecular weight
LOAEL	lowest observed adverse effect level
LOD	limit of detection
LOQ	limit of quantitation
MBP	monobutyl phthalate
MBZP	monobenzyl phthalate
MCPP	mono(3-carboxypropyl) phthalate
MDI	mental development index
MECPP	mono(2-ethyl-5-carboxypentyl) phthalate
MEHP	mono(2-ethylhexyl) phthalate
MEHHP	mono(2-ethyl-5-hydroxyhexyl) phthalate
MEOHP	mono(2-ethyl-5-oxohexyl) phthalate

MEP	monoethyl phthalate
MIBP	monoisobutyl phthalate
MINP	mono(isononyl) phthalate
MIS	Mullerian inhibiting substance
MMP	monomethyl phthalate
MNG	multinucleated gonocyte
MNOP	mono-*n*-octyl phthalate
MOE	margin of exposure
MSSM	Mount Sinai School of Medicine
MW	molecular weight
NA	not available
NAE	no antiandrogenic effects observed
NHANES	National Health and Nutritional Examination Survey
NNNS	NICU Network Neurobehavioral Scale
NOAEL	no observed adverse effect level
NOEL	no observed effect level
NR	nipple retention
NRC	National Research Council, U.S.
NTP	National Toxicology Program, U.S.
OECD	Organisation for Economic Cooperation and Development
OH-MIDP	mono(hydroxy-isodecyl) phthalate
OH-MINP	mono(hydroxy-isononyl) phthalate
OR	odds ratio
oxo-MIDP	mono(oxo-isodecyl) phthalate
oxo-MINP	mono(oxo-isononyl) phthalate
PBR	peripheral benzodiazepine receptor
PDI	psychomotor developmental index
PE	phthalate ester
PEAA	potency estimates for antiandrogenicity
PND	postnatal day
PNW	postnatal week
POD	point of departure
PODI	point of departure index
PPARα	peroxisome proliferator-activated receptor alpha
PPS	probability proportional to a measure of size
PSU	primary sampling unit
PVC	polyvinyl chloride
RfD	reference dose
RTM	reproductive tract malformation
SD	Sprague-Dawley
SDN-POA	sexually dimorphic nucleus of the preoptic area
SFF	Study for Future Families
SHBG	sex-hormone binding globulin
SR-B1	scavenger receptor class B1
SRS	social responsiveness scale
StAR	steroidogenic acute regulatory protein

SVW	seminal vesicle weight
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TDI	tolerable daily intake
TDS	testicular dysgenesis syndrome
TEF	toxicity equivalency factors
TOTM	tris(2-ethylhexyl) trimellitate
TPIB	2,2,4-trimethyl-1,3 pentanediol diisobutyrate
T PROD	testosterone production
TXIB®	2,2,4-trimethyl-1,3 pentanediol diisobutyrate
UF	uncertainty factor

1 Introduction

1.1 Male Sexual Differentiation in Mammals

Phthalates can induce a number of types of toxicities in animals, however, the most extensively studied is male developmental toxicity in the rat. As discussed in detail in the main report, phthalates have been shown to disrupt testicular development leading to reproductive tract dysgenesis. Because the developmental toxicity studies reviewed in this section relate to various aspects of male sexual differentiation, a brief introduction to this subject, taken directly from the 2008 National Research Council (NRC) publication: *Phthalates and Cumulative Risk Assessment: The Tasks Ahead* (2008), is herein provided.

> "Sexual differentiation in males follows complex interconnected pathways during embryo and fetal developments that have been reviewed extensively elsewhere (see, for example, Capel, 2000; Hughes, 2001; Tilmann and Capel, 2002; Brennan and Capel, 2004).
>
> Critical to the development of the male mammals is the development of the testis in embryonic life from a bipotential gonad (a tissue that could develop into a testis or an ovary). The 'selection' is genetically controlled in most mammals by a gene on the Y chromosome. The sex-determining gene (sry in mice and SRY in humans) acts as a switch to control multiple downstream pathways that lead to the male phenotype. Male differentiation after gonad determination is exclusively hormone-dependent and requires the presence at the correct time and tissue location of specific concentrations of fetal testis hormones-Mullerian inhibiting substance (MIS), insulin-like factors, and androgens. Although a female phenotype is produced independently of the presence of an ovary, the male phenotype depends greatly on development of the testis. Under the influence of hormones and cell products from the early testis, the Mullerian duct regresses and the mesonephric duct (or Wolffian duct) gives rise to the epididymis and vas deferens. In the absence of MIS and testosterone, the Mullerian ductal system develops further into the oviduct, uterus, and upper vagina, and the Wolffian duct system regresses. Those early events occur before establishment of a hypothalamic-pituitary-gonadal axis and depend on local control and production of hormones (that is, the process is gonadotropin-independent). Normal development and differentiation of the prostate from the urogenital sinus and of the external genitalia from the genital tubercle are also under androgen control. More recent studies of conditional knockout mice that have alterations of the luteinizing-hormone receptor have shown normal differentiation of the genitalia, although they are significantly smaller.
>
> Testis descent appears to require androgens and the hormone insulin-like factor 3 (insl3; Adham *et al.*, 2000) to proceed normally. The testis in early fetal life is near the kidney and attached to the abdominal wall by the cranial suspensory ligament (CSL) and gubernaculum. The gubernaculum contracts, thickens, and develops a bulbous outgrowth; this results in the location of the testes in the lower abdomen (transabdominal descent). The CSL regresses through an androgen-dependent process. In the female, the CSL is retained with a thin gubernaculum to maintain ovarian position. Descent of the testes

through the inguinal ring into the scrotum (inguinoscrotal descent) is under androgen control.

Because the majority of studies discussed below were conducted in rats, it is helpful to compare the rat and human developmental periods for male sexual differentiation. Production of fetal testosterone occurs over a broader window in humans (gestation weeks 8–37) than in rats (gestation days [GD] 15–21). The critical period for sexual differentiation in humans is late in the first trimester of pregnancy, and differentiation is essentially complete by 16 weeks (Hiort and Holterhus, 2000). The critical period in rats occurs in later gestation, as indicated by the production of testosterone in the latter part of the gestational period, and some sexual development occurs postnatally in rats. For example, descent of the testes into the scrotum occurs in gestation weeks 27–35 in humans and in the third postnatal week in rats. General, the early postnatal period in rats corresponds to the third trimester in humans."

As the authors of the 2008 NRC conclude "…it is clear that normal differentiation of the male phenotype has specific requirements for fetal testicular hormones, including androgens, and therefore can be particularly sensitive to the action of environmental agents that can alter the endocrine milieu of the fetal testis during the critical periods of development."

1.2 The Rat Phthalate Syndrome

Studies conducted over the past 20 plus years have shown that phthalates produce a syndrome of reproductive abnormalities when administered to pregnant rats during the later stages of pregnancy, *e.g.*, GD 15–20. This syndrome of reproductive abnormalities, known as the rat phthalate syndrome, is characterized by malformations of the epididymis, vas deferens, seminal vesicles, prostate, external genitalia (hypospadias), and by cryptorchidism (undescended testes) as well as by retention of nipples/areolae (sexually dimorphic structures in rodents) and demasculinization of the perineum, resulting in reduced anogenital distance (AGD). The highest incidence of reproductive tract malformations is observed at higher phthalate dose levels, whereas changes in AGD and nipple/areolae retention are frequently observed at lower phthalate dose levels (Mylchreest *et al.*, 2000).

Mechanistically, phthalate exposure can be linked to the observed phthalate syndrome abnormalities by an early phthalate-related disturbance of normal fetal testicular Leydig function and/or development (Foster, 2006). This disturbance is characterized by Leydig cell hyperplasia or the formation of large aggregates of Leydig cells at GD 21 in the developing testis. These morphological changes are preceded by a significant reduction in fetal testosterone production, which likely results in the failure of the Wolffian duct system to develop normally, thereby contributing to the abnormalities observed in the vas deferens, epididymis, and seminal vesicles. Reduced testosterone levels also disturb the dihydrotestosterone (DHT)-induced development of the prostate and external genitalia by reducing the amount of DHT that can be produced from testosterone by 5α-reductase. Because DHT is required for the normal apoptosis of nipple anlage in males and also for growth of the perineum to produce the normal male AGD, changes in AGD and nipple retention are consistent with phthalate-induced reduction in testosterone levels. Although testicular descent also requires normal testosterone levels, another Leydig cell product, insl3 (insulin-like factor 3), also plays a role. Phthalate exposure has been shown to decrease

insl3 gene expression, and mice in which the insl3 gene has been deleted show complete cryptorchidism.

1.3 The Phthalate Syndrome in Other Species (excluding humans)

Although the literature is replete with information about the phthalate syndrome in rats, there is, interestingly, a relative dearth of information about the phthalate syndrome in other species. In a study by Higuchi *et al.* (2003), **rabbits** were exposed orally to 0 or 400 mg dibutyl phthalate (DBP)/kg-day from GD 15–29 and male offspring were examined at 6, 12, and 25 weeks of age. The most pronounced effects observed were decreased testes weights at 12 weeks and accessory gland weights at 12 and 25 weeks as well as abnormal semen characteristics, *e.g.*, decreased sperm concentration/total sperm/normal sperm and an increase in acrosome-nuclear defects. In a study by Gaido *et al.* (2007), **mice** were exposed 0, 250, or 500 mg DBP/kg-day from GD 16 to 18, male fetuses were collected on day 19, and their testes were removed for histopathology. Similar to the rat, DBP significantly increased seminiferous cord diameter, the number of multinucleated gonocytes per cord, and the number of nuclei per multinucleated gonocyte. In a separate set of experiments, dosing with levels as high as 1500 mg DBP/kg-day from GD 14 to 16 did not significantly affect fetal testicular testosterone concentration even though the plasma concentrations of monobutyl phthalate (MBP) in mice were equal to or greater than the concentrations in maternal and fetal rats. In a third set of experiments, *in utero* exposure to DBP led to the rapid induction of immediate early genes, as in the rat; however, unlike in the rat, expression of genes involved in cholesterol homeostasis and steroidogenesis were not decreased. In another study, reported only in abstract form, Marsman (1995) exposed **mice** to 0, 1, 250, 2,500, 5,000, 7,500, 10, 000, or 20,000 ppm DBP in feed during gestation and lactation. No pups were delivered in the 20,000 ppm group, and only 1 pup survived past lactation day 1 in the 10,000 ppm group. Although the author states that "No treatment-related gross lesions were identified at necropsy, and no histopathological lesions definitively associated with treatment were observed in male or female mice in the 7,500 ppm group," he also states that "Developmental toxicity and fetal and pup mortality were suggested at concentrations as low as 7,500 ppm." Two studies have been published on the toxicity of phthalates (specifically DBP/MBP) in marmosets. In one study (Hallmark *et al.*, 2007), 4-day-old **marmosets** were administered 500 mg/kg-d MBP for 14 days after which blood was obtained for the measurement of testosterone levels and the testes were removed for histopathological examination. In a second acute study, nine males 2–7 days of age were administered a single oral dose of 500 mg/kg-d, and a blood sample was obtained 5 hours later for measurement of testosterone levels. Results showed that MBP did suppress testosterone production after an acute exposure; however, this suppression of testosterone production was not observed when measurements were taken 14 days after the beginning of exposure to MBP. The authors speculate that the initial MBP-induced inhibition of steroidogenesis in the neonatal marmoset leads to a "reduced negative feedback and hence a compensatory increase in luteinizing hormone (LH) secretion to restore steroid production to normal levels." In a follow-up study, McKinnell *et al.* (2009) exposed pregnant marmosets from ~7 to 15 weeks gestation with 500 mg/kg-d MBP, and male offspring were studied at birth (1–5 days; n= 6). Fetal exposure to 500 mg/kg-d MBP did not affect gross testicular morphology, reproductive tract development, testosterone levels, germ cell number and proliferation, Sertoli cell number or germ:Sertoli cell ratio.

1.4 Mechanism of Action

Initial mechanistic studies centered on phthalates acting as environmental estrogens or antiandrogens; however, data from various estrogenic and antiandrogenic screening assays clearly showed that while the parent phthalate could bind to steroid receptors, the developmentally toxic monoesters exhibited little or no affinity for the estrogen or androgen receptors (David, 2006). Another potential mechanism of phthalate developmental toxicity is through peroxisome proliferator-activated receptor alpha (PPARα). Support for this hypothesis comes from data showing that circulating testosterone levels in PPARα-null mice were increased following treatment with di(2-ethylhexyl) phthalate (DEHP) compared with a decrease in wild-type mice, suggesting that PPARα plays a role in postnatal testicular toxicity. PPARα activation may play some role in the developmental toxicity of nonreproductive organs (Lampen *et al.*, 2003); however, data linking PPARα activation to the developmental toxicity of reproductive organs are lacking.

Because other studies had shown that normal male rat sexual differentiation is dependent upon three hormones produced by the fetal testis, *i.e.*, an anti-Mullerian hormone produced by the Sertoli cells, testosterone produced by the fetal Leydig cells, and insulin-like hormone 3 (insl3), several laboratories conducted studies to determine whether the administration of specific phthalates to pregnant dams during fetal sexual differentiation that caused demasculinization of the male rat offspring would also affect testicular testosterone production and insl3 expression. Studies by Wilson *et al.* (2004), Howdeshell *et al.* (2007), and Borch *et al.* (2006b) reported significant decreases in testosterone production and insl3 expression after DEHP, DBP, and butylbenzyl phthalate (BBP), and by DEHP + DBP (each at one-half of its effective dose). The study by Wilson *et al.* (2004) also showed that exposure to DEHP (and similarly to DBP and BBP) altered Leydig cell maturation, resulting in reduced production of testosterone and insl3, from which they further proposed that the reduced testosterone levels result in malformations such as hypospadias, whereas reduced insl3 mRNA levels lead to lower levels of this peptide hormone and abnormalities of the gubernacular ligament (agenesis or elongated and filamentous) or freely moving testes (no cranial suspensory or gubernacular ligaments). Together, these studies identify a plausible link between inhibition of steroidogenesis in the fetal rat testes and alterations in male reproductive development. In addition, other phthalates that do not alter testicular testosterone synthesis (diethyl phthalate [DEP]; Gazouli *et al.*, 2002) and gene expression for steroidogenesis (DEP and dimethyl pthalate [DMP]; Liu *et al.*, 2005) also do not produce the phthalate syndrome malformations produced by phthalates that do alter testicular testosterone synthesis and gene expression for steroidogenesis (Gray *et al.*, 2000; Liu *et al.*, 2005).

Complementary studies have also shown that exposure to DBP *in utero* leads to a coordinated decrease in expression of genes involved in cholesterol transport (peripheral benzodiazepine receptor [PBR], steroidogenic acute regulatory protein [StAR], scavenger receptor class B1 [SR-B1]) and steroidogenesis (Cytochrome P450 side chain cleavage [P450scc], cytochrome P450c17 [P450c17], 3β-hydroxysteroid dehydrogenase [3β-HSD]), leading to a reduction in testosterone production in the fetal testis (Shultz *et al.*, 2001; Barlow and Foster, 2003; Lehmann *et al.*, 2004). Interestingly, Lehmann *et al.*, (2004) further showed that DBP induced significant reductions in SR-B1, 3β-HSD, and c-Kit (a stem cell factor produced by Sertoli cells that is essential for normal gonocyte proliferation and survival) mRNA levels at doses (0.1 or 1.0

mg/kg-d) that approach maximal human exposure levels. The biological significance of these data are not known, given that no statistically significant observable adverse effects on male reproductive tract development have been identified at DBP dose <100 mg/kg-d and given that fetal testicular testosterone is reduced only at dose levels equal to or greater than 50 mg/kg-d.

Thus, current evidence suggests that once the phthalate monoester crosses the placenta and reaches the fetus, it alters gene expression for cholesterol transport and steroidogenesis in Leydig cells. This, in turn, leads to decreased cholesterol transport and decreased testosterone synthesis. As a consequence, androgen-dependent tissue differentiation is adversely affected, culminating in hypospadias and other features of the phthalate syndrome. In addition, phthalates (DEHP and DBP) also alter the expression of insl3, leading to decreased expression. Decreased levels of insl3 result in malformations of the gubernacular ligament, which is necessary for testicular descent into the scrotal sac.

Summary of Mechanism of Action Studies									
Chemical	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
DBP	↓	↓		↓		↓	↓	↓	
BBP	↓	↓							
DEHP	↓	↓	↓	↓	↓	↓	↓	↓	↓
DEHP+DBP	↓	↓	↓	↓					
DNOP									
DINP	↓	↑	↓	↓	↑			↑	
DIDP									
DMP									
DEP									
DIBP	↓	↓		↓		↓		↓	↓
DPENP	↓	↓	↓	↓					
ATBC									
DEHA									
DINX									
DEHT									
TOTM									
TPIB									

1 = Testosterone
2 = insl3 (Insulin-like factor 3)
3 = CYP11A (Rate-limiting enzyme responsible for the conversion of cholesterol to pregnenolone)
4 = StAR = Steroidogenic Acute Regulated Protein, involved in mitochondrial cholesterol uptake
5 = LH = Lutenizing Hormone
6 = SR-B1 = Scavenger Receptor B-1, responsible for cholesterol uptake by Leydig cells
7 = PBR = Peripheral Benzodiazepene Receptor, involved in mitochondrial cholesterol uptake
8 = CYP450scc = Cytochrome P450 side chain cleavage enzyme, steroid converting enzyme
9 = SF-1 = Nuclear Receptor Steroidogenic Factor-1, regulates expression of genes involved in steroidogenesis

1.5 Cumulative Exposures to Phthalates

In a 2007 study, Howdeshell *et al.,* reported the results of the cumulative effects of DBP and DEHP on male rat reproductive tract development, steroid hormone production, and gene expression following exposure of Sprague-Dawley rats on GD 8–18. Pregnant rats were gavaged with vehicle control, 500 mg/kg DBP alone, 500 mg/kg DEHP alone, or a combination of DBP and DEHP (500 mg/kg for each phthalate). The mixture of DBP + DEHP elicited dose-additive effects, *i.e.,* increased incidence of epididymal agenesis and reduced androgen-dependent organ weights as well as decreased fetal testosterone, and expression of insl3 and CYP11a.

In a follow-up publication, Howdeshell *et al.,* (2008) reported studies in which they characterized the dose response effects of six individual phthalates (BBP, DBP, DEHP, DEP, diisobutyl phthalate [DIBP], and di-*n*-pentyl phthalate [DPENP]) on GD 18 testicular testosterone production following exposure of Sprague-Dawley rats on GD 8–18. Results showed that testosterone production was significantly reduced at doses of 300 mg/kg-d or higher of BBP, DBP, DEHP, and diisodecyl phthalate (DIDP) and at doses as low as 100 mg/kg-d of DPENP. In a follow-up study, dams were dosed via gavage from GD 8 to 18 with either vehicle or 7 dose levels of a mixture of BBP, DBP, DEHP, DIBP (each at 300 mg/kg-d) plus (DPENP) at 100 mg/kg-d. This mixture was administered at 100, 80, 60, 40, 20, 10, and 5% of the top dose (1300 mg/kg-d). Administration of the mixture of five antiandrogenic phthalates reduced fetal testicular testosterone production at doses of 26 mg/kg-d (20% of the top dose, which contains BBP, DBP, DEHP, and DIBP at 60 mg/kg-d per chemical and 20 mg DIPENP/kg/day) and higher. The authors conclude that their data demonstrate that "individual phthalates with a similar mechanism of action can elicit cumulative, dose additive effects on fetal testosterone production and pregnancy when administered as a mixture."

1.6 Developmental Toxicity of Phthalates in Rats

The goal of this appendix is to systematically review the published, peer-reviewed literature reporting the *in utero* exposure of phthalates in pregnant rats. After careful consideration by the committee, this review is limited to the three permanently banned phthalates (DBP, BBP, and DEHP), the three phthalates currently on an interim ban (di-*n*-octyl phthalate [DNOP], diisononyl phthalate [DINP], and DIDP), and eight other phthalates (DMP, DEP, DPENP/DPP, DIBP, dicyclohexyl phthalate [DCHP], di-*n*-hexyl phthalate [DHEXP], diisooctyl phthalate [DIOP], and di(2-propylheptyl) phthalate [DPHP]). Because the first six of these phthalates were extensively reviewed by a phthalates expert panel in a series of reports from the National Toxicology Program (NTP) Center for the Evaluation of Risks to Human Reproduction (CERHR) in 2002, our review of these phthalates begins with a brief summary of these NTP reports, which is then followed by a review of the literature since those reports. For the eight other phthalates that were not reviewed by the NTP panel, the following review covers all the relevant studies available to the committee. From the available literature for each of these 10 phthalates, we then identified the most sensitive developmentally toxic endpoint in a particular study as well as the lowest dose that did not elicitan adverse effect (no observed adverse effect level [NOAEL]). Finally, we evaluated the "adequacy" of particular studies to derive a NOAEL. Our criteria for an adequate study from which a NOAEL could be derived are: 1) at least three dose levels and a concurrent control should be used, 2) the highest dose should induce some developmental and/or maternal toxicity and the lowest dose level should not produce either

maternal or developmental toxicity, 3) each test and control group should have a sufficient number of females to result in approximately 20 female animals with implantation sites at necropsy, and 4) pregnant animals need to be exposed during the appropriate period of gestation. In addition, studies should follow the Organisation for Economic Cooperation and Development (OECD) Guideline for the Testing of Chemicals (OECD 414, adopted 22 January 2001).

As part of the charge to the committee, we were also asked to evaluate the potential developmental toxicity of phthalate substitutes. The phthalate substitutes include acetyl tributyl citrate (ATBC), di(2-ethylhexyl) adipate (DEHA), diisononyl 1,2-dicarboxycyclohexane (DINX), di(2-ethylhexyl) terephthalate (DEHT/DOTP), trioctyltrimellitate (TOTM), and 2,2,4-trimethyl-1,3-pentanediol-diisobutyrate (TPIB).

2 Permanently Banned Phthalates (DBP, BBP, DEHP)

2.1 Di-n-Butyl Phthalate (DBP) (84-74-2)

2.1.1 2002 Summary of the NTP-CERHR Report

The 2002 summary of the NTP-CERHR report on the reproductive and developmental toxicity of DBP (NTP, 2000) concludes that, as of their report, the expert panel could locate "no data on the developmental or reproductive toxicity of DBP in humans." However, on the basis of available animal data, the panel concluded that it "has high confidence in the available studies to characterize reproductive and developmental toxicity based upon a strong database containing studies in multiple species using conventional and investigative studies. When administered via the oral route, DBP elicits malformations of the male reproductive tract via a disturbance of the androgen status: a mode of action relevant for human development. This anti-androgenic mechanism occurs via effects on testosterone biosynthesis and not androgen receptor antagonism. DBP is developmentally toxic to both rats and mice by the oral routes; it induces structural malformations. A confident NOAEL of 50 mg/kg-d by the oral route has been established in the rat. Data from which to confidently establish a lowest observed adverse effect level (LOAEL)/NOAEL in the mouse are uncertain." These statements are made primarily on the basis of studies by Ema *et al.*, (1993; 1994; 1998) and Mylchreest *et al.*, (1998; 1999; 2000). Finally, studies by Saillenfait *et al.*, (1998) and Imajima *et al.*, (1997) indicated that the monoester metabolite of DBP is responsible for the developmental toxicity of DBP.

2.1.2 Relevant Studies Published Since the 2002 Summary of the NTP-CERHR Report

Zhang *et al.* (2004) reported a study in which rats were given DBP by gavage at levels of 0, 50, 250, and 500 mg/kg-d from GD 1 to postnatal day (PND) 21. "Severe damage to the reproductive system of mature F_1 male rats included testicular atrophy, underdeveloped or absent epididymis, undescended testes, obvious decline of epididymal sperm parameters, total sperm heads per g testis, decrease of organ/body weight ratio of epididymis and prostate and was observed in the group treated with 250 mg/kgBW/d and higher." A NOAEL for developmental toxicity of DBP was 50 mg/kg-d was established based upon pup body weight and male reproductive lesions.

Lee *et al.* (2004) reported a study in which Sprague-Dawley rats were given DBP at dietary concentrations of 0, 20, 200, 2000, and 10,000 ppm from GD 15 to PND 21. At PND 11 in males, a significant reduction of spermatocyte development was observed at 2000 ppm and above. At PND 21, a significant reduction of testicular spermatocyte development was observed at 20 ppm and above and decreased epididymal ductal cross-section at 2000 ppm and above. The authors also noted significant adverse effects on mammary gland development in females at 20 ppm and above on PND 21, but not on PND 11 or 20.

Howdeshell *et al.* (2007) reported a study in which pregnant Sprague-Dawley rats were gavaged on GD 14–18 with doses of DBP or DEHP at 500 mg/kg; or a combination of DBP and DEHP (500 mg/kg each chemical). DBP and DEHP significantly reduced anogenital distance on PND 3, increased the number of areolae per PND 14 males, and increased the number of nipples per adult male, whereas the DBP + DEHP dose increased the incidence of these reproductive malformations by more than 50%. The authors concluded that "individual phthalates with a similar mechanism of action, but with different active metabolites (monobutyl phthalate versus monoethylhexyl phthalate), can elicit dose-additive effects when administered as a mixture."

Jiang *et al.* (2007) reported a study in which timed-mated rats were given DBP by gastric intubation at doses of 0, 250, 500, 750, or 1000 mg/kg-d from GD 14 to 18. DBP significantly increased the incidence of cryptorchidism in male pups at doses of 250, 500, and 750 mg/kg-d and the incidence of hypospadias and a decrease in anogenital distance at doses of 500 and 750 mg/kg-d. They also reported significant decreases in serum testosterone concentration in PND 70 male offspring at DBP doses of 250, 500, and 750 mg/kg-d.

Mahood *et al.* (2007) reported a study in which timed-mated Wistar rats were given DBP by gavage at doses of 0, 4, 20, 100, or 500 mg/kg-d from GD 13.5 to either 20.5 or 21.5.

Struve *et al.* (2009) reported a study in which pregnant Sprague-Dawley CD rats were given DBP at doses of 0, 100, and 500 mg/kg-d via the diet from GD 12 to 19. DBP significantly decreased the anogenital distance in male offspring at 500 mg/kg-d, significantly reduced fetal testicular testosterone concentrations at 100 and 500 mg/kg-d when measured at 24 hours after removal of DBP from the diet, and at 500 mg/kg-d when measured 4 hours after removal of DBP from the diet. DBP also induced a significant dose-dependent reduction in testicular mRNA concentrations of scavenger receptor class B, member 1; steroidogenic acute regulatory protein; cytochrome P45011a1; and cytochrome P45017a1 at 100 and 500 mg/kg-d when evaluated 4 hours after the end of dietary exposure on GD 19.

Kim *et al,* (2010) reported a study in which pregnant Sprague-Dawley rats were given DBP at doses of 0, 250, 500, or 700 mg/kg-d on GD 10–19. DBP significantly increased the incidence of hypospadias and cryptorchidism in male offspring, decreased the weights of the testis and epididymis, decreased the anogenital distance, and decreased the levels of dihydrotestosterone and testosterone in rats treated with DBP at 700 mg/kg-d.

Studies cited above are summarized in Table A-1.

Table A-1 DBP developmental toxicity studies—antiandrogenic effects.

STUDY	AGENT	STRAIN/ SPECIES	# DOSE LEVELS	DOSING REGIMEN	# ANIMALS /DOSE	# LITTERS /DOSE	MATERNAL TOXICITY	ENDPOINT	NOAEL
Mylchreest *et al.* (2000)	DBP	S-D	0, 0.5, 5, 50, 100, 500 mg/kg-d	GD 12–21; gavage	19–20; 11@ 500 mg/kg-d	19–20; 11@ 500 mg/kg-d	no	↓ male AGD; ↑ hypospadias @ 500 mg/kg-d; ↑ nipple retention @ 100 mg/kg-d	50 mg/kg-d
Higuchi *et al.* (2003)	DBP	Rabbits	0, 400 mg/kg-d	GD 15–29; PNW 4–12	5–8	5–8	no	↑ hypospadia, cryptorchid testes; ↓ testes weight, sperm concentration	NA
Zhang *et al.* (2004)	DBP	S-D	0, 50, 250, 500 mg/kg-d	GD 1–PND21 gavage	20	14–16	no	↓ pup body weight; ↓ male AGD @ PND4; ↓ sperm @ 250 mg/kg-d	50 mg/kg-d
Lee *et al.* (2004)	DBP	S-D	0, 20, 200, 2000, 10,000 ppm	GD 15–PND 21 Diet	6–8	6–8	yes; maternal body weight @ 10,000 ppm	↓ male AGD;↑ nipple retention @ 10,000ppm; ↓ sperm development @ 20ppm	<20ppm based upon ↓ sperm development @ 20ppm
Carruthers & Foster (2005)	DBP	S-D	0, 500 mg/kg-d	GD 14–15, 15–16, 16–17, 17–18, 18–19, 19–20	9–16		no	↓ male AGD, ↓ epididymal weight, & epididymal agenesis @ 500 mg/kg-d after exposures on GD 16–18	NA
Howdeshell *et al.* (2007)	DBP; DBP+ DEHP	S-D	0, 500 mg/kg-d	GD 14–18 gavage	6	6	no	↓ male AGD @ 500 mg/kg-d	NA
Jiang *et al.* (2007)	DBP	S-D	0, 250, 500,750, 1000 mg/kg-d	GD 14–18 gavage	10	10	yes @ 750 & 1000 mg/kg-d	↓ male AGD and ↑ hypospadias @ 500 & 750 mg/kg-d: ↑ cryptorchidism and serum testosterone concentration @ 250 mg/kg-d	<250 mg/kg-d based upon ↑ cryptorchidism and serum testosterone concentration @ 250 mg/kg-d

STUDY	AGENT	STRAIN/ SPECIES	# DOSE LEVELS	DOSING REGIMEN	# ANIMALS /DOSE	# LITTERS /DOSE	MATERNAL TOXICITY	ENDPOINT	NOAEL
Mahood *et al.* (2007)	DBP	Wistar	0, 4, 20, 100, 500 mg/kg-d	GD 13.5–20.5/21.5	3–16	3–16	not reported	↑ cryptorchidism @ 500 mg/kg-d;↑ MNGs @ 100 mg/kg-d;↓ testosterone @ 100 mg/kg-d	20 mg/kg-d based upon ↓ testosterone @ 100 mg/kg-d
Howdeshell *et al.* (2008)	DBP	S-D	0, 33, 50, 100, 300, 600 mg/kg-d	GD 8–18	3–4	3–4	no	↓ testicular testosterone production @ 300 mg/kg-d and above	
Struve *et al.* (2009)	DBP	S-D	0, 100, 500 mg/kg-d	GD 12–19 diet	9	9	no	↓ male AGD @ 500 mg/kg-d; ↓ fetal testosterone @ 100 mg/kg-d @ 24 hrs	<100 mg/kg-d based upon ↓ fetal testosterone @ 100 mg/kg-d @ 24 hrs
Kim *et al.* (2010)	DBP	S-D	0, 250, 500, 700 mg/kg-d	GD 10–19	?	?	NA	↓ male AGD and ↑ nipple retention @ 500 mg/kg-d and above; ↑ cryptorchidism and hypospadias @ 700 mg/kg-d; ↓ serum DHT and testosterone @ 700 mg/kg-d	250 mg/kg-d based upon ↓ male AGD and ↑ nipple retention @ 500 mg/kg-d

S-D = Sprague-Dawley; GD = gestation day; AGD = anogenital distance; PNW = postnatal week; PND = postnatal day; MNG = multinucleated gonocyte; DHT = dihydrotestosterone; NOAEL = no observed adverse effect level

2.1.3 Consensus NOAEL for DBP

The studies listed in Table A-1 clearly indicate that DBP is developmentally toxic when exposure occurs later in gestation (during fetal development). Although several of these studies report a specific NOAEL, not all studies were amenable to the identification of a NOAEL. For example, the studies of Carruthers and Foster (2005) and Howdeshell *et al.* (2007) were designed to obtain mechanistic data and therefore did not include multiple doses. The study by Higuchi *et al.* (2003) is interesting because it demonstrates that DBP produces effects in rabbits similar to those seen in the rat, but again, only one dose was used, thus precluding the determination of a NOAEL. Other studies (Lee *et al.*, 2004; Jiang *et al.*, 2007; Struve *et al.*, 2009), which did use at least 3 doses, used fewer than the recommended number of animals/dose (20/dose). The study by Kim *et al.* (2010) used multiple doses; however, it was difficult to ascertain how many animals were used per dose. The studies of Mylchreest *et al.* (2000) and Zhang *et al.* (2004), on the other hand, used multiple doses and approximately 20 animals/dose. In the absence of maternal toxicity, Mylchreest reported an increase in nipple retention in male pups at 100 mg/kg-d, whereas Zhang *et al.* reported increased male AGD at 250 mg/kg-d. In both studies, these LOAELs correspond to a NOAEL of 50 mg/kg-d. A NOAEL of 50 mg/kg-d is supported by the study of Mahood *et al.* (2007), which reported a LOAEL of 100 mg/kg-d for decreased fetal testosterone production after exposure to DBP. Using the data of Mylchreest *et al.* (2000) and Zhang *et al.* (2004), the Chronic Hazard Advisory Panel (CHAP) committee assigns a NOAEL of 50 mg/kg-d for DBP.

2.2 Butylbenzyl Phthalate (BBP) (85-68-7)

2.2.1 2002 Summary of the NTP-CERHR Report

The 2002 summary of the NTP-CERHR report (NTP, 2003a) on the reproductive and developmental toxicity of BBP concludes that, as of their report, the expert panel could locate "no human data" on the developmental or reproductive toxicity of BBP. However, on the basis of available animal data, the panel concluded that (1) "the data in rats and mice are adequate for a prenatal assessment of fetal growth, lethality, and teratogenicity." (2) "None of the studies included a postnatal evaluation of androgen-regulated effects (*e.g.*, nipple retention, testicular descent, or preputial separation) that were the most sensitive indicators of developmental toxicity of DBP." (3)"Prenatal studies with BBP monoesters (MBP and monobenzyl phthalate [MBZP]) were sufficient to determine that both metabolites contribute to developmental toxicity." These statements are based primarily upon the studies by Field *et al.* (1989), Ema *et al.* (1990; 1992; 1995), and Price *et al.* (1990). The studies by Field *et al.* (1989) and Ema *et al.* (1992) reported that the developmental NOAELs in Sprague-Dawley and Wistar rats ranged from 420 to 500 mg/kg-d, respectively. The NTP-CERHR panel noted, however, that it was not confident in these NOAELs because the prenatal studies (GD 7–15) examined would not detect effects such as altered anogenital distance, retained nipples, delays in acquisition of puberty, and malformations of the post-pubertal male reproductive system.

2.2.2 Relevant Studies Published Since the 2002 Summary of the NTP-CERHR Report

Gray *et al.* (2000) reported a study in which Sprague-Dawley rats were given BBP (as well as DEHP, DINP, DEP, DMP, or DEHT/DOTP) by gavage at 0 or 750 mg/kg-d from GD 14 to PND 3. Males in the BBP-treated groups exhibited significantly shortened AGD, female-like areolae/nipples, decreased testes weights, and a significant incidence of reproductive malformations (cleft phallus, hypospadias). The authors note that of the phthalates tested, BBP, DEHP, and DINP altered sexual differentiation whereas DOTP, DEP, and DMP did not. They also noted that BBP and DEHP were of equivalent potency, whereas DINP was about an order of magnitude less active.

Nagao *et al.* (2000) reported a two-generation study in which Sprague-Dawley rats were exposed to oral doses of BBP at 0, 20, 100, or 500 mg/kg-d from 2 weeks before mating through cohabitation, gestation, and lactation until postpartum day 21. BBP produced a significant reduction in AGD in male pups and increased AGD in female pups at 500 mg/kg-d. In addition, preputial separation in male pups was delayed and serum concentrations of testosterone were decreased at 500 mg/kg-d.

Piersma *et al.* (2000) reported a study in which Harlan Cpb-WU rats were gavaged with BBP at doses of 0, 270, 350, 450, 580, 750, 970, 1250, 1600, or 2100 mg/kg-d for GD 6–15 or GD 6–20. BBP exposure was associated with skeletal anomalies (reduced rib size, fusion of two ribs, and incompletely ossified or fused sternebrae) at the middle or high doses (exact doses not specified). Anopthalmia was found in several pups after exposure to 750 and 970 mg/kg-d from day 6–15 and 6–20. Cleft palate was found in two cases at 750 mg/kg-d and one at 1250 mg/kg-d after exposure from GD 6–20. Two cases of exencephaly were observed in the 750 mg/kg-d group after exposure from GD 6–20. Finally, the incidence of retarded fetal testicular caudal migration increased in a dose-related fashion.

Saillenfait *et al.* (2003) reported studies in which OF1 outbred mice or Sprague-Dawley rats were given oral doses of BBP at 0, 280, 560, 1120, or 1690 mg/kg on GD 8 and 10. Similarly, mice and rats were given oral doses of MBP at doses of 0, 200, 400, 800, or 1200 mg/kg-d or MBZP at doses of 0, 230, 460, 920, or 1380 mg/kg-d. In mice, external malformations (exencephaly, facial cleft, meningocele, spina bifida, onphalocele, acephalostomia) were seen in animals dosed with 560 mg/kg-d BBP and above, 200 mg/kg-d MBP and above, and 920 mg/kg-d MBZP and above. In rats 5% of fetuses were exencephalic at the highest BBP dose; however, this effect did not appear to reach statistical significance.

Tyl *et al.* (2004) reported two-generation studies in which rats were exposed to dietary BBP at concentrations of 0, 750, 3750, and 11,250 ppm during a 10-week pre-breeding period and then during mating, gestation, and lactation. There were no effects on parents or offspring at BBP exposures of 750 ppm (50 mg/kg-d). At 3750 ppm (250 mg/kg-d), BBP induced a reduction in AGD in F1 and F2 male offspring. At 11,250 ppm (750 mg/kg-d), BBP induced a reduction in F1 and F2 male AGD and body weights/litter during lactation, delayed acquisition of puberty in F1 males and females, retention of nipples and areolae in F1 and F2 males, and male

reproductive system malformations (hypospadias, missing epididymides, testes, prostate, and abnormal reproductive organ size and/or shape). The authors concluded that the NOAEL for F1 parental systemic and reproductive toxicity was 3750 ppm (250 mg/kg-d), the offspring toxicity NOAEL was 3750 ppm (250 mg/kg-d), and the NOAEL for offspring toxicity was 750 ppm (50 mg/kg-d).

Studies cited above are summarized in Table A-2.

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Table A-2 BBP developmental toxicity studies—antiandrogenic effects.

STUDY	AGENT	STRAIN/ SPECIES	# DOSE LEVELS	DOSING REGIMEN	# ANIMALS /DOSE	# LITTERS /DOSE	MATERNAL TOXICITY	ENDPOINT	NOAEL
Gray *et al.* (2000)	BBP	S-D	0, 750 mg/kg-d	GD 14–PND 1	8	8	no	↓ male AGD; ↓ testes weight; ↑ nipple retention; ↓ epididymal weight	NA
Nagao *et al.* (2000)	BBP	S-D	0, 20, 100, 500 mg/kg-d	Two-generation study; GD 1–PND 21	25	25	yes; ↑ liver, kidney & thyroid gland weights @ 500 mg/kg-d	↓ male & female pup weight on PND 0 @ 100 mg/kg-d and above; ↓ male AGD & ↑ female AGD @ 500 mg/kg-d; ↓ serum testosterone @ 500 mg/kg-d	100 mg/kg-d based upon ↓ male AGD & ↑ female AGD @ 500 mg/kg-d; ↓ serum testosterone @ 500 mg/kg-d
Piersma *et al.* (2000)	BBP	Harlan Cpb-WU	0, 270, 350, 450, 580, 750, 970, 1250, 1600, 2100 mg/kg-d	GD 6–20 (also GD 6–15)	10		yes; death @ highest two doses; ↑ resorptions @ 750 mg/kg-d and above	dose-dependent ↓ in fetal testicular caudal migration & ↓ fetal testis weight	benchmark dose of 95 mg/kg-d for testicular dislocation
Ema and Myawaki (2002)	BBP	Wistar rat	0, 250, 500, 1000 mg/kg-d	GD 15–17	16	16	yes, ↓ maternal body weight @ 500 mg/kg-d and above	↑ incidence of undescended testes and ↓ male AGD @ 500 mg/kg-d and above	250 mg/kg-d
Saillenfait *et al.* (2003)	BBP	S-D; OF1 mice	0, 280, 560, 1120, 1690 mg/kg-d	GD 8 & 10	Rat 7–13; mice 15–23				NA
Saillenfait *et al.* (2003)	MBP	S-D: OF1 mice	0, 400, 800, 1200 mg/kg-d	GD 8 & 10	Rat 7–13; mice 15–23				NA
Saillenfait *et al.* (2003)	MBzP	S-D; OF1 mice	230, 460, 920, 1380 mg/kg-d	GD 8 & 10	Rat 7–13; mice 15–23				NA

STUDY	AGENT	STRAIN/ SPECIES	# DOSE LEVELS	DOSING REGIMEN	# ANIMALS /DOSE	# LITTERS /DOSE	MATERNAL TOXICITY	ENDPOINT	NOAEL
Ema *et al.* (2003)	MBP	Wistar rat	0, 167, 250, 375 mg/kg-d	GD 15–17	16	16	yes, ↓ maternal weight gain on days 18–21 @ 167 mg/kg-d and higher	↑ incidence of undescended testes and ↓ male AGD @ 250 mg/kg-d and above	167 mg/kg-d on the basis of ↑ incidence of undescended testes and ↓ male AGD @ 250 mg/kg-d and above
Tyl *et al.* (2004)	BBP	CD	0, 750, 3750, 11,250 ppm	two-generation study; GD 1–PND 21	20	20	yes; ↓ maternal body weight during gestation & lactation @ 11,250 ppm	F1 & F2 ↓ male AGD @ 3750 ppm and above; F1 ↓ testes weight @ 3750 ppm and above; F1 and F2 ↑ nipple retention @ 11,250 ppm; F1 ↑ male reproductive tract malformations, *e.g.*, hypospadias @ 11,250ppm	750 ppm (=50 mg/kg-d) on the basis of F1 & F2 ↓ male AGD @ 3750 ppm and above; F1 ↓ testes weight @ 3750 ppm and above
Howdeshell *et al.* (2008)	BBP	S-D	0, 100, 300, 600, 900	GD 8–18	2–9	2–9	yes	↓ testicular testosterone production @ 300 mg/kg-d and above	

S-D = Sprague-Dawley; GD = gestation day; PND = postnatal day; AGD = anogenital distance; NA = not available; NOAEL = no observed adverse effect level

2.2.3 Consensus NOAEL for BBP

The study by Gray *et al.* (2000) could not be used to generate a NOAEL because only one dose was used, whereas, the study by Saillenfait *et al.* (2003) could not be used because the sensitive period for the disruption of male fetal sexual development in the rat (GD 15–21) was not included in the study's exposure protocol (GD 7–13). The remaining studies were judged to be adequate for determining a NOAEL for BBP. In the Nagao *et al.* (2000) study, the CHAP determined a NOAEL of 100 mg/kg-d, Piersma *et al.* (2000) calculated a benchmark dose of 95 mg/kg-d, the CHAP determined a NOAEL of 250 mg/kg-d from the data of the Ema and Myawaki (2002) and a NOAEL of 167 mg/kg-d from the data of Ema *et al.* (2003). Finally, Tyl *et al.* (2004) determined a NOAEL of 50 mg/kg-d from data generated in their two-generation study. Thus, the NOAELs range from a low of 50 to a high of 250 mg/kg-d. The CHAP decided to take the conservative approach and recommend a NOAEL of 50 mg/kg-d for BBP.

2.3 Di(2-ethylhexyl) Phthalate (DEHP) (117-81-7)

2.3.1 2002 Summary of the NTP-CERHR Report

The 2002 summary of the NTP-CERHR report on the reproductive and developmental toxicity of DEHP concludes that, as of their report (Kavlock *et al.*, 2002), "There were no studies located on the developmental toxicity of DEHP or its metabolites in humans." In contrast, 41 prenatal developmental toxicity studies in animals in which assessments were made just prior to birth "were remarkably consistent." "DEHP was found to produce malformations, as well as intrauterine death and developmental delay. The pattern of malformations seen in fetuses is consistent across studies. It included morphological abnormalities of the axial skeleton (including tail), cardiovascular system (heart and aortic arch), appendicular skeleton (including limb bones, finger abnormalities), eye (including open eye), and neural tube (exencephaly). The NOAEL based upon malformations in rodents was ~40 mg/kg-d and a NOAEL of 3.7–14 mg/kg-d was identified for testicular development/effects in rodents." The panel noted that the examination of effects during late gestation and neonatal periods is "quite recent and incomplete." The panel also expressed concerns about *in utero* exposures in humans given that (1) "exposures may be on the order of 3–30 μg/kg bw/day," (2) "the most relevant rodent data suggest a NOAEL for testis/developmental effects of 3.7–14 mg/kg-d," (3) "even time-limited exposures are effective at producing irreversible effects," and (4) "the active toxicant mono(2-ethylhexyl) phthalate (MEHP) passes into breast milk and crosses the placenta."

In a 2006 NTP-CERHR Expert Panel update on the reproductive and developmental toxicity of DEHP (NTP, 2006), the panel reviewed several human studies and concluded that there is "insufficient evidence in humans that DEHP causes developmental toxicity when exposure is prenatal … or when exposure is during childhood." These conclusions were based upon the reports of Latini *et al.* (2003), Swan *et al.* (2005), Rais-Bahrami *et al.* (2004), and Colon *et al.* (2000). The panel also reviewed additional animal studies published since their first report, and on the basis of these reports, concluded that there is "sufficient evidence that DEHP exposure in rats causes developmental toxicity with dietary exposure during gestation and/or early postnatal life at 14–23 mg/kg bw/day as manifested by small or absent male reproductive organs." Multiple other studies showed effects on the developing male reproductive tract at higher dose levels. These conclusions are supported by studies of Shirota *et al.* (2005), Moore *et al.* (2001),

Borch *et al.* (Borch *et al.*, 2003; 2004; 2006b), Jarfelt *et al.* (2005), Li *et al.* (2000), Cammack *et al.* (2003), and Gray *et al.*, (2000).

2.3.2 Relevant Studies Published Since the 2006 Update Summary of the NTP-CERHR Report

Grande *et al.* (2006) reported studies in which Wistar rats were given DEHP by gavage from GD 6 to lactation day 22 at doses of 0, 0.015, 0.045, 0.135, 0.405, 1.215, 5, 15, 45, 135, or 405 mg/kg-d, and effects on female rat reproductive development were assessed. DEHP induced a significant delay in the age at vaginal opening at exposures of 15 mg/kg-d and above as well as a trend for a delay in the age at first estrus at 135 and 405 mg/kg-d. Anogenital distance and nipple development were unaffected. Based upon delayed pubertal development at 15 mg/kg-d, the authors set the NOAEL for female reproductive development at 5 mg DEHP/kg bw/day.

Andrade *et al.* (2006a) reported studies in which Wistar rats were given DEHP by gavage from GD 6 to lactation day 22 at doses of 0, 0.015, 0.045, 0.135, 0.405, 1.215, 5, 15, 45, 135, or 405 mg/kg-d, and effects on male rat reproductive development were assessed. DEHP induced delayed preputial separation at exposures of 15 mg/kg-d and above, increased testis weight on PND 22 at doses of 5, 15, 45, and 135 mg/kg-d, and nipple retention and reduced AGD at a dose of 405 mg/kg-d. On the basis of increased testis weight on PND 22, the authors set the NOAEL at 1.215 mg/kg-day.

Christiansen *et al.* (2010) reported studies in which Wistar rats were given DEHP by gavage from GD 7 to PND 16 at doses of 10, 30, 100, 600, or 900 mg DEHP/kg-day. DEHP induced decreased AGD, increased incidence of nipple retention, and mild dysgenesis of the external genitalia at 10 mg/kg-day. Higher doses of DEHP induced histopathological effects on the testes, reduced testis weight, and expression of androgen-related genes in the prostate. The authors note that the effects seen at 10 mg/kg-d are “consistent with the EU NOAEL of 5 mg/kg-day for DEHP.”

Studies cited above are summarized in Table A-3.

Table A-3 DEHP developmental toxicity studies.

STUDY	AGENT	STRAIN/ SPECIES	# DOSE LEVELS	DOSING REGIMEN	# ANIMALS /DOSE	# LITTERS /DOSE	MATERNAL TOXICITY	ENDPOINT	NOAEL
Gray *et al.* (2000),	DEHP	S-D	0, 750 mg/kg-d	GD 14–PND 1	16	16	yes, ↓ maternal weight gain @ 750 mg/kg-d	male AGD; testes weight; nipple retention; epididymal weight	NA
Moore *et al.* (2001)	DEHP	S-D	0, 375, 750, 1500 mg/kg-d	GD 3–PND 21	5–8		yes, ↓ maternal weight gain on GD 16–20 at @ 750 and 1500 mg/kg-d	↓ male AGD; ↑ nipple retention; ↑ incidence of permanent nipple retention @ 375 mg/kg-d; ↑ incidence of undescended testes; ↓ testes, epididymides, and glans penis weights; ↓ epididymal sperm number @ 750 and 1500 mg/kg-d	NA
NTP (2004)	DEHP	S-D	1.5, 10, 30, 100, 300, 1000, 7500, 10,000 ppm					↑ reproductive organ abnormalities @ 300 ppm (14–23 mg/kg-d) and above	100 ppm (3–5 mg/kg-d)
Borch *et al.* (2004)	DEHP	Wistar rat	0, 300, 750 mg/kg-d	GD 1–21	8	8	NA	↓ testicular testosterone production/content @ 300 & 750 mg/kg-d; ↓ male AGD @ 750 mg/kg-d	
Jarfelt *et al.* (2005)	DEHP	Wistar rat	0, 300, 750 mg/kg-d	GD 7–PND 17	20	11–15	↓ maternal weight gain @ 300 and 750 mg/kg-d, but not statistically significant	↓ male AGD, ↑ incidence of nipple retention & ↓ testes and epididymis weights @ 300 and 750 mg/kg-d	

STUDY	AGENT	STRAIN/ SPECIES	# DOSE LEVELS	DOSING REGIMEN	# ANIMALS /DOSE	# LITTERS /DOSE	MATERNAL TOXICITY	ENDPOINT	NOAEL
Shirota *et al.* (2005)	DEHP	S-D	0, 125, 250, 500 mg/kg-d	GD 7–18	11–12	11	no	↑ degeneration of germ cells and hyperplasia of interstitial cells in the fetal testis @ 250 mg/kg-d and above	125 mg/kg-d on basis of ↑ degeneration of germ cells and hyperplasia of interstitial cells in the fetal testes @ 250 mg/kg-d and above
Grande *et al.* (2006)	DEHP	Wistar rat	0, .015, .045, .135, 1.215, 5, 15, 45, 136, 405 mg/kg-d	GD 6–PND 22	11–16	11–16	no	delay in mean age at vaginal opening @ 15 mg/kg-d and above; no effect on female AGD or nipple retention at any dose	5 mg/kg-d based on delay in mean age at vaginal opening @ 15 mg/kg-d
Andrade *et al.* (2006a)	DEHP	Wistar rat	0, .015, .045, .135, 1.215, 5, 15, 45, 136, 405 mg/kg-d	GD 6–PND 22	11–16	11–16	no	delay in the age of preputial separation @ 15 mg/kg-d and above; ↓ male AGD and ↑ incidence of nipple retention @ 405 mg/kg-d	5 mg/kg-d based on delay in preputial separation
Howdeshell *et al.* (2008)	DEHP	S-D	0, 100, 300, 600, 900 mg/kg-d	GD 8–18	4	4	no	↓ testicular testosterone production @ 300 mg/kg-d and above	
Gray *et al.* (2009)	DEHP	SD rat	0, 11, 33, 100, 300 mg/kg-d	GD 8–17	13–14	13–14≤	no	↑ incidence of pups with phthalate syndrome at doses of 11 mg/kg-d and above	≤11 mg/kg-d based upon ↑ incidence of pups with phthalate syndrome at doses of 11 mg/kg-d and above
Christiansen *et al.* (2010)	DEHP	Wistar rat	0, 3, 10, 30, 100, 300, 600, 900 mg/kg-d	GD 7–21 and PND 1–16		13–15 @ 10-100 mg/kg-d; 6–7 @ 300–900 mg/kg-d	no	↓ male AGD and ↑ nipple retention @ 10 mg/kg-d	3 mg/kg-d based upon ↓ male AGD and ↑ nipple retention LOAEL @ 10 mg/kg-d

STUDY	AGENT	STRAIN/ SPECIES	# DOSE LEVELS	DOSING REGIMEN	# ANIMALS /DOSE	# LITTERS /DOSE	MATERNAL TOXICITY	ENDPOINT	NOAEL
Hannas *et al.* (2011)	DEHP	S-D and Wistar	0, 100, 300, 500, 625, 750, 875 mg/kg-d	GD 14–18	3–6			↓ testosterone production in both strains @ 300 mg/kg-d and higher; ↓expression of insl3 mRNA @ 625 mg/kg-d and higher; ↓ expression of StAR and Cyp11a mRNAs @ 500 mg/kg-d and above	100 mg/kg-d based on testosterone LOAEL of 300 mg/kg-d

S-D = Sprague-Dawley; GD = gestation day; PND = postnatal day; AGD = anogenital distance; NA = not available; insl3= insulin-like factor 3; StAR = steroidogenic acute regulatory protein; NOAEL = no observed adverse effect level; LOAEL = lowest observed adverse effect level

2.3.3 Consensus NOAEL for DEHP

The Gray *et al.* (2000) study could not be used to identify a NOAEL because only one dose was used. The studies of Moore *et al.* (2001), Borch *et al.* (2004), and Jarfelt *et al.* (2005) could not be used because in each case the lowest dose used produced a significant effect and therefore a NOAEL could not be determined. The studies of Grande *et al.* (2006), Andrade *et al.* (2006a), Gray *et al.* (2009), and Christiansen *et al.* (2010) are all-well designed studies employing multiple doses at the appropriate developmental window and using relatively large numbers of animals per dose group. Although different phthalate syndrome endpoints were used to set a NOAEL, the resulting NOAELs cluster tightly around a value of 3–11 mg/kg-d. It is noteworthy that this cluster is consistent with the NOAEL identified in the NTP study (4.8 mg/kg-d; Foster *et al.*, 2006). In contrast, using fetal testosterone production as an endpoint, Hannas *et al.* (2011) reported a LOAEL of 300 mg/kg-d and a NOAEL of 100 mg/kg-d, a NOAEL approximately 10 times the one derived using morphological endpoints. Using a weight-of-evidence approach, the CHAP has conservatively set the NOAEL for DEHP at 5 mg/kg-d.

3 Interim Banned Phthalates

3.1 Di-*n*-octyl Phthalate (DNOP) (117-84-0)

3.1.1 2002 Summary of the NTP-CERHR Report

The 2002 summary of the NTP-CERHR report on the reproductive and developmental toxicity of DNOP (NTP, 2003e) concludes that, as of their report, the expert panel could locate no data on the developmental or reproductive toxicity of DNOP in humans. The panel reviewed five animal studies involving prenatal exposure to DNOP in mice and rats (Singh *et al.*, 1972; Gulati *et al.*, 1985; Hardin *et al.*, 1987; Heindel *et al.*, 1989; Hellwig *et al.*, 1997). It should be noted that in all but one study, exposure to DNOP occurred before gestational day 15 in the rat and day 13 in the mouse. Although they concluded that "available studies do suggest a developmental toxicity response with gavage or i.p. administration with very high doses," the panel also noted that the limited study designs of the five studies reviewed "do not provide a basis for comparing consistency of response in the two species, nor do they allow meaningful assessment of dose-response relationships and determination of either LOAELs or NOAELs with any degree of confidence." The panel concluded by stating that the "experimental data are insufficient to permit a firm judgment about DnOP's potential to pose a developmental toxicity hazard to humans."

3.1.2 Relevant Studies Published Since the 2002 Summary of the NTP-CERHR Report

A PubMed literature search using the terms *di*-n-*octyl phthalate* and *developmental toxicity* or *DNOP* and *developmental toxicity* did not uncover any studies since the 2002 summary of the NTP-CERHR report.

3.1.3 Consensus NOAEL for DNOP

Only one study, Saillenfait *et al*. (2011), was of appropriate design to provide a meaningful NOAEL; however, no antiandrogenic effects were observed in this study. This study did, however, report a dose-related increase in supernumerary ribs at maternally nontoxic doses. Because of the lack of relevant data, a consensus NOAEL could not be determined.

3.2 Diisononyl Phthalate (DINP) (28553-12-0; 68515-48-0)

3.2.1 2002 Summary of the NTP-CERHR Report

The 2002 summary of the NTP-CERHR report on the reproductive and developmental toxicity of DINP (NTP, 2003c) concluded that, as of their report, there were "no human data located for Expert Panel review." The panel did review two rat studies evaluating prenatal developmental toxicity of DINP by gavage on GD 6–15 (Hellwig *et al.*, 1997; Waterman *et al.,* 1999), the developmental toxicity of DINP in a two-generation study in rats (Waterman *et al.,* 2000), and a prenatal developmental toxicity of isononyl alcohol, a primary metabolite of DINP (Hellwig and Jackh, 1997). The two rat prenatal studies showed effects on the developing skeletal system and kidney following oral exposures to DINP from GD 6–15, while in the two-generation study in rats, effects on pup growth were noted. The prenatal developmental toxicity study with isononyl alcohol provided evidence that this primary metabolite of DINP "is a developmental and maternal toxicant at high (~1000 mg/kg) oral doses in rats." From these studies, the panel concluded that the toxicology database "is sufficient to determine that oral maternal exposure to DINP can result in developmental toxicity to the conceptus." The panel also noted that "some endpoints of reproductive development that have been shown to be sensitive with other phthalates, were not assessed." Therefore, the panel recommended that "a perinatal developmental study in orally exposed rats that addresses landmarks of sexual maturation such as nipple retention, anogenital distance, age at testes descent, age at prepuce separation, and structure of the developing reproductive system in pubertal or adult animals exposed through development" should be considered.

3.2.2 Relevant Studies Published Since the 2002 Summary of the NTP-CERHR Report

Gray *et al.* (2000) reported a study in which Sprague-Dawley rats were given DINP (as well as BBP, DEHP, DEP, DMP, or DOTP) by gavage at 0 or 750 mg/kg-d from GD 14 to PND 3. DINP significantly induced increased the incidence of male offspring with areolae (with and without nipple buds) and increased incidence of male offspring with malformations of the androgen-dependent organs and testes The authors note that of the phthalates tested, DINP, BBP, and DEHP altered sexual differentiation whereas DOTP, DEP, and DMP did not. They also noted that DINP was about an order of magnitude less active than BBP and DEHP, which were of equivalent potency.

Masutomi *et al.* (2003) reported a study in which Sprague-Dawley rats were exposed to DINP in the diet at 0, 400, 4,000, or 20,000 ppm from gestational day 15 to PND 10. DINP significantly reduced maternal weight gain, postnatal weight gain and testis weights before puberty, but did not see any alterations in AGD.

Lee *et al.* (2006) reported a study in which Wistar-Imamichi rats were exposed to DINP in the diet at 0, 40, 400, 4000, or 20,000 ppm from gestational day 15 to PND 21. The authors reported that DINP induced a reduction in AGD at all levels tested; however, their statistical analyses apparently used the individual fetus rather than the litter as the unit of measurement, thus calling into question their conclusion.

Boberg *et al.* (2011) reported a study in which Wistar rats were exposed to DINP by gavage at 0, 300, 600, 750, or 900 mg/kg-d from gestation day 7 to PND 17. DINP significantly altered testis histology (*e.g.*, multinucleated gonocytes) at 600 mg/kg-d and above, increased nipple retention in males at 600 mg/kg-d and above, decreased sperm motility at 600 mg/kg-d and above, and decreased AGD in males at 900 mg/kg-d. The authors also reported a reduction in testicular testosterone levels at all doses tested; however, these reductions did not reach statistical significance, probably due to the small number of litters sampled for this endpoint. On the basis of these results, the authors conclude that the NOAEL for DINP-induced reproductive toxicity in the rat is 300 mg/kg-d.

Studies cited above are summarized in Table A-4

3.2.3 Consensus NOAEL for DINP

Several of the studies listed in Table A-4 were judged to be inadequate for ascertaining a NOAEL for DINP, *e.g.*, the Gray *et al.* (2000) study used only one dose and the Matsutomi *et al.* (2003), Borch *et al.* (2004), and Adamsson *et al.* (2009) studies used relatively small numbers of animals per dose group. In contrast, the Boberg *et al.*, (2011) study used multiple doses (4 plus control), exposure occurred during the developmentally sensitive period (GD 7–PND 17), and used a relatively high number of dams per dose (16). On the basis of increased nipple retention at 600 mg/kg-d, the authors report a NOAEL of 300 mg/kg-d. Furthermore, several of the other studies, although not "adequate" on their own for the determination of a NOAEL for DINP, do provide supporting data. For example, the Hass *et al.* (2003) study, reported only as an abstract, also reported a NOAEL of 300 mg/kg-d based on increased nipple retention. In addition, the Hannas *et al.* (2011) study found a LOAEL of 500 mg/kg-d based on decreased fetal testosterone production, suggesting that the NOAEL for this endpoint is somewhere below this level. Thus, on the basis of available studies, the CHAP committee sets the NOAEL for DINP at 300 mg/kg-d.

Table A-4 DINP developmental toxicity studies.

STUDY	AGENT	STRAIN/ SPECIES	DOSE LEVELS	DOSING REGIMEN	ANIMALS/ DOSE	LITTER S/DOSE	MATERNAL TOXICITY	ENDPOINT	NOAEL
Gray *et al.* (2000)	DINP	S-D	0, 750 mg/kg-d	GD 14–PND 3 gavage	14	14	yes, ↓ maternal weight gain @ 750 mg/kg-d	↑ nipple retention	NA
Waterman *et al.* (2000)	DINP	S-D	0, 0.5, 1.0, 1.5 % in one-generation study; 0, 0.2, 0.4, 0.8 % in two-generation study	One- & two-generation studies diet	30	?	yes, ↓ maternal weight gain @ 1.0% and above in one-generation and 0.8% in two-generation studies	CERHR panel concluded that the LOAEL for developmental effects (reduced pup weight) was 143 mg/kg-d for the gestational exposure; no effects on testicular development, testicular descent, & penile development (hypospadias)	CERHR could not establish a NOAEL
Hass *et al.* (2003)	DINP	Wistar	0, 300, 600, 750, 900 mg/kg-d	GD 7–17				↑ nipple retention on PND 13 @ 600 mg/kg-d and above; ↓ male AGD @ 750 mg/kg-d	300 mg/kg-d based on ↑ nipple retention on PND 13 @ 600 mg/kg-d
Masutomi *et al.* (2003)	DINP	S-D	0, 400, 4000, 20,000 ppm	GD 15–PND 10 diet	5–6	5–6	yes, ↓ maternal weight gain @ 20,000 ppm	↓ absolute & relative prepubertal testes weight @ 20,000 ppm	4000 ppm (?)
Borch *et al.* (2004),	DINP	Wistar rat	0, 750 mg/kg-d	GD 1–21 gavage	8	8	NA	↓ testicular testosterone production/content	NA
Lee *et al.* (2006)	DINP	Wistar rat	0, 40, 400, 4000, 20,000 ppm	GD 15–PND 21 diet	?	?		↓ male AGD @ 40 ppm and above; ↑ female AGD @ 20,000 ppm; ↑ in hypothalamic p130 mRNA @ 40 ppm and above	?

STUDY	AGENT	STRAIN/ SPECIES	DOSE LEVELS	DOSING REGIMEN	ANIMALS/ DOSE	LITTER S/DOSE	MATERNAL TOXICITY	ENDPOINT	NOAEL
Adamsson *et al.* (2009)	DINP	SD	0, 250, 750 mg/kg-d	ED 13.5–17.5 gavage	7–8	7–8	no	↑ P450scc, GATA-4 & *Insl-3* mRNAs @ 750mg/kg-d	250 mg/kg-d on the basis of ↑ P450scc, GATA-4 & insl-3 mRNAs @ 750 mg/kg-d
Boberg *et al.* (2011)	DINP	Wistar	0, 300, 600, 750, 900 mg/kg-d	GD 7–PND 17 gavage	16	10	no	↑ multinucleated gonocytes & nipple retention @ 600 mg/kg-d and above; ↓ testicular testosterone content @ 600 mg/kg-d and AGD @ 900 mg/kg-d	300 mg/kg-d reported by authors
Hannas *et al.* (2011)	DINP	SD	0, 500, 760, 1000, 1500 mg/kg-d	GD 14–18	3–6	3–6	no	↓ fetal testosterone production @ 500 mg/kg-d and above; ↓ StaR and Cyp11a mRNA levels @ 1000 mg/kg-d and above	? somewhere below 500 mg/kg-d based upon testosterone LOAEL

S-D = Sprague-Dawley; GD = gestation day; PND = postnatal day; NA = not available; CERHR = Center for the Evaluation of Risks to Human Reproduction; NOAEL = no observed adverse effect level; LOAEL = lowest observed adverse effect level; AGD = anogenital distance; insl3 = insulin-like factor 3; StAR = steroidogenic acute regulatory protein

3.3 Diisodecyl Phthalate (DIDP) (26761-40-0; 68515-49-1)

3.3.1 2002 Summary of the NTP-CERHR Report

The 2002 summary of the NTP-CERHR report (NTP, 2003b) on the reproductive and developmental toxicity of diisodecyl phthalate (DIDP) concludes that, as of their report, the expert panel concluded that there were "no human data located for Expert Panel review." The panel did review two developmental toxicity studies in rats (Hellwig *et al.,* 1997; Waterman *et al.,* 1999) and one in mice (Hardin *et al.,* 1987) in which exposure was by gavage from GD 6 to 15 or from 6 to 13, respectively. The panel also reviewed two two-generation reproductive toxicity studies (Exxon, 1997; ExxonMobil, 2000) in which developmental effects were observed. Although prenatal exposures of DIDP to mice did not result in any observable developmental or maternal toxicity, the prenatal rat studies and the two-generation studies did demonstrate developmental toxicity, *i.e.,* increased fetal cervical and lumbar ribs, and adverse effects on pup growth and survival, respectively. From these studies, the panel concluded that the "oral prenatal developmental toxicity studies and the oral two-generation reproductive toxicity studies have shown no effects on the reproductive system in rats." In addition, the panel "noted that the endpoints of reproductive development that have been shown to be sensitive with other phthalates were examined in one of the two-generation reproductive toxicity studies."

3.3.2 Recent Studies Not Cited in the 2002 Summary of the NTP-CERHR Report

Hushka *et al.* (2001) reported two-generation studies in which Sprague-Dawley rats were exposed to DIDP in the feed at approximate doses of 15, 150, 300, or 600 mg/kg-d for 10 weeks prior to mating and throughout mating, gestation, and lactation, until PND 0, 1, 4, 7, 14, or 21. The authors state that there were "no differences in anogenital distance, nipple retention, or vaginal patency in the F2 offspring (Table 7)." Preputial separation was slightly but statistically significantly delayed in the 300 mg/kg-d dose group; however, the authors concluded that this difference "was deemed not adverse because the magnitude was so small."

Studies cited above are summarized in Table A-5.

3.3.3 Consensus NOAEL for DIDP

Neither of the published studies reported significant antiandrogenic effects; however, one report did find that DIDP exposure was associated with a dose-related increase in percent of fetuses with supernumerary cervical and lumbar ribs (Waterman *et al.,* 1999). A 2003 NTP reevaluation of the Waterman *et al*. data led the Expert Panel for the Center for the Evaluation of Risks to Human Reproduction to set a NOAEL at 100 mg/kg-d, based upon the increased supernumerary ribs.

4 Other Phthalates

4.1 Dimethyl Phthalate (DMP) (131-11-3)

Although an early study by Singh *et al.* (1972) suggested that gestational exposure to DMP (0.4–1.3 g/kg intraperitoneally (IP) on gestational days 5, 10, and 15) increased the incidence of skeletal defects in rats, subsequent studies by Plasterer *et al.* (1985), Field *et al.* (1993), and Gray

et al. (2000) uniformly found that DMP was not a developmental toxicant in mice (Plasterer) or rats (Field and Gray). Plasterer *et al.* administered DMP to CD-1 mice by gavage at a single dose (at or just below the threshold of adult lethality) on GD 7–14 and reported that DMP had no effect on maternal or fetal survival and produced no congenital anomalies. Field *et al.* exposed rats to DMP from GD 6 to 15 at doses of 0, 0.25, 1, and 5% in feed (approximately 0.2–4.0 g/kg/day). Although high-dose DMP caused maternal toxicity (increased maternal liver weight and reduced weight gain), there was no effect of DMP "on any parameter of embryo/fetal development." Gray *et al.* administered DMP to rats at an oral dose of 0.75 g/kg from gestational day 14 to postnatal day 3 and reported that DMP was ineffective in altering sexual differentiation and inducing reproductive malformations observed after exposure to other phthalates (DEHP, BBP, and DINP).

Table A-5 DIDP developmental toxicity studies.

STUDY	AGENT	STRAIN/ SPECIES	# DOSE LEVELS	DOSING REGIMEN	# ANIMALS /DOSE	# LITTERS /DOSE	MATERNAL TOXICITY	ENDPOINT	NOAEL
Waterman *et al.* (1999)	DIDP	S-D	0, 100, 500, 1000 mg/kg-d by gavage in one-generation study	GD 6–GD 15	25	22–25	↓ weight gain, food consumption at 1000 mg/kg-d	↑ incidence of supernumerary cervical (7^{th}) ribs & rudimentary lumbar (14^{th}) ribs	100 mg/kg-d
Hushka *et al. (2001)*	DIDP	S-D	0, 0.02, 0.04, 0.2, 0.4 or 0, 0.2, 0.4, 0.8% in two-generation studies	GD 1–PND 21 diet	30	?	no	Slight, but significant ↑ in age of preputial separation @ 0.4% (~300 mg/kg-d) (Table 7; deemed "…not adverse because the magnitude was so small.") No observed effects on AGD or nipple retention @ any dose.	0.2% (~150 mg/kg-d)

S-D = Sprague-Dawley; GD = gestation day; PND = postnatal day; AGD = anogenital distance; NOAEL = no observed adverse effect level

4.1.1 Consensus NOAEL for DMP

The available data, particularly the studies of Field *et al.* (1993) (GD 6–15 exposure) and Gray *et al.* (2000) (GD 14–PND 3 exposure), support the conclusion that DMP is not a developmental toxicant.

4.2 Diethyl Phthalate (DEP) (84-66-2)

Although an early study by Singh *et al.* (1972) suggested that gestational exposure to DEP (600 to 1900 mg/kg IP on gestational days 5, 10, and 15) increased the incidence of skeletal defects in rats, subsequent studies by Field *et al*. (1993), and Gray *et al.* (2000) found that DEP was not a developmental toxicant in rats. Field *et al*. exposed rats to DEP from GD 6 to15 at doses of 0, 0.25, 2.5, or 5% in feed (approximately 200 to 4000 mg/kg-d). Although high-dose DMP caused maternal toxicity (reduced weight gain), there was no effect of DEP "on any parameter of embryo/fetal development." Gray *et al*. administered DEP to rats at an oral dose of 750 mg/kg-d from gestational day 14 to postnatal day 3 and reported that DEP was ineffective in altering sexual differentiation and inducing reproductive malformations observed after exposure to other phthalates (DEHP, BBP, and DINP).

4.2.1 Consensus NOAEL for DEP

The available data, particularly the studies of Field *et al*. (1993) (GD 6–15 exposure) and Gray *et al*. (2000) (GD 14–PND 3 exposure), support the conclusion that DEP is not a developmental toxicant.

4.3 Diisobutyl Phthalate (DIBP) (84-69-5)

Borch *et al.* (2006a) exposed pregnant Wistar rats to DIBP at 0 or 600 mg/kg-d from gestation day 7 to either 19 or 20/21. At this dose of DIBP, they observed significant reductions in anogenital distance, testicular testosterone production, testicular testosterone content, and expression of P450scc and StAR proteins in Leydig cells. In two different studies, Saillenfait *et al*. (2006; 2008) exposed pregnant Sprague-Dawley rats from gestation day 6 to 20 to DIBP at 0, 250, 500, 750, or 1000 mg/kg-d (Saillenfait *et al.*, 2006) or from gestation day 12–21 at 0, 125, 250, 500, or 625 mg/kg-d. In the 2006 study the authors found that the incidence of male fetuses with undescended testes was significantly elevated at 750 and 1000 mg/kg-d. In the later study, the authors found that DIBP caused reduced anogenital distance and increased nipple retention in males at 250 mg/kg-d and higher, and hypospadias and undescended testes at 500 mg/kg-d and higher. Boberg *et al*. (2008) exposed pregnant Wistar rats from gestation day 7 to 21 to DIBP at 600 mg/kg-d and observed reduced anogenital distance in males, testosterone production, and expression of testicular insl3 and genes related to steroidogenesis. Howdeshell *et al*. (2008) exposed pregnant Sprague-Dawley rats from gestation day 8–18 to DIBP at 0, 100, 300, 600, or 900 mg/kg-d and observed reduced fetal testicular testosterone production at 300 mg/kg-d and above. Finally, Hannas *et al.* (2011) exposed pregnant Sprague-Dawley rats from gestation day 14 to 18 to DIBP at 0, 100, 300, 600, or 900 mg/kg-d and observed reduced fetal testicular testosterone production at 300 mg/kg-d and above.

4.3.1 Consensus NOAEL for DIBP

The Boberg *et al.* (2008) study results could not be used to determine a NOAEL because only one dose was used. The Howdeshell *et al.* (2008) study, which used multiple doses but small numbers of animals per dose group, was designed, as the authors point out " to determine the slope and median effective dose (ED_{50}) values of the individual phthalates and a mixture of phthalates and not to detect NOAELs or low observable adverse effect levels." The same is true for the Hannas *et al.* (2011) study, which also used multiple doses but small numbers of animals per dose group. The two Saillenfait studies (2006; 2008) both included multiple doses and exposure during the appropriate stage of gestation, and employed relatively large numbers of animals per dose. Using the more conservative of the two NOAELs from the 2008 Saillenfait study, the CHAP assigns a NOAEL of 125 mg/kg-d for DIBP.

4.4 Di-*n*-pentyl Phthalate (DPENP/DPP) (131-18-0)

A PubMed search using the terms *dipentyl phthalate* and *developmental toxicity* or *DPENP* and *developmental toxicity* identified three articles, one by Heindel *et al.* (1989), one by Howdeshell *et al.* (2008), and the other by Hannas *et al.* (2011). Heindel *et al.* (1989) used a continuous breeding protocol to expose CD-1 mice to 0.5, 1.25, or 2.5% DPENP in the diet from 7 days prior to and during a 98-day cohabitation period. DPENP exposure adversely affected the reproductive system as evidenced by a complete inhibition of fertility at 1.25 and 2.5% DPENP, and reduced fertility at 0.5% DPENP. DPENP treatment was also associated with decreased body weight, increased liver weight, decreased testis and epididymis weights, decreased epididymal sperm concentration, and elevated seminiferous tubule atrophy. Howdeshell *et al.* (2008) exposed pregnant Sprague-Dawley rats from gestation day 8 to 18 to DPENP at doses of 0, 25, 50, 100, 200, 300, 600, or 900 mg/kg-d, and then measured fetal testicular testosterone production on gestational day 18. They found that testosterone production was significantly reduced at doses of DPENP at 100 mg/kg-d and above. Hannas *et al.* (2011) dosed pregnant rats with 0, 300, 600, 900, or 1200 mg/kg on GD 17, or 0, 11, 33, 100, or 300 mg/kg on GD 14–18, and then evaluated fetal testicular testosterone production on GD 17.5 or GD 18, respectively. They also dosed pregnant rats on GD 8–18 with 0, 11, 33, 100, or 300 mg/kg-d and evaluated early postnatal endpoints in male offspring. Results showed that DPENP significantly reduces fetal testicular testosterone production (at 300 mg/kg-d or higher after a 1-day exposure and 33 mg/kg-d after a 5-day exposure), StAR, Cyp11a, and *Ins13* gene expression levels (100 mg/kg-d after a 5-day exposure), and induced early postnatal reproductive alterations in male offspring (anogenital distance at 100 mg/kg-d and nipple retention at 300 mg/kg-d). The authors note that the reduction in fetal testicular testosterone production occurred as early as 5 hours following dosing and that a dose as low as 33 mg/kg-d makes fetal testicular testosterone production a more sensitive endpoint for the antiandrogenic action of phthalate compounds than genomic and early postnatal endpoints. The authors also note that DPENP is 8-fold more potent in decreasing fetal testicular testosterone production, 4.5-fold more potent in inducing nipple retention, and 2-fold more potent in reducing anogenital distance compared with DEHP. Finally, the authors conclude that the "consistency in DPENP potency from fetal endpoints to postnatal effects supports the hypothesis that fetal declines in androgen production are causally linked to postnatal malformations in androgen-sensitive tissues."

Table A-6 DIBP developmental toxicity studies.

STUDY	AGENT	STRAIN/ SPECIES	# DOSE LEVELS	DOSING REGIMEN	# ANIMALS /DOSE	# LITTERS /DOSE	MATERNAL TOXICITY	ENDPOINT	NOAEL
Borch *et al.* (2006a)	DIBP	Wistar rat	0, 600 mg/kg-d	GD 7–GD 19 or GD 20/21 gavage	6 or 8 (?)		NA	↓ testicular production & content; male AGD adjusted for body weight on GD 20/21 @ 600 mg/kg-d; ↑ female AGD adjusted for body weight @ 600 mg/kg-d on GD 20/21	NA
Saillenfait *et al.* (2006)	DIBP	S-D	0, 250, 500, 750, 1000 mg/kg-d	GD 6–20	23–24	20-21	yes, ↓ maternal body weight (GD 6–9) @ 500 mg/kg-d and above	↑ in visceral & skeletal malformation; ↑ in male fetuses with undescended testes @ 500 mg/kg-d, significant @750 mg/kg-d and above when evaluated on GD 21	Authors suggest 250 mg/kg-d based on the dose-dependent effects on testes migration.
Saillenfait *et al.* (2008)	DIBP	S-D	0, 125, 250, 500, 625 mg/kg-d	GD 12–21 gavage	11–14	7–14	no	↓ male AGD (on PND 1), ↑ nipple retention (PND 12–14) @ 250 mg/kg-d; delayed onset of puberty & ↑ hypospadias, cleft prepuce & undescended testis @ 500 mg/kg-d and above	125 mg/kg-d Based on ↓ male AGD (on PND 1), ↑ nipple retention (PND 12–14) @ 250 mg/kg-d
Boberg *et al.* (2008)	DIBP	Wistar rat	0, 600 mg/kg-d	GD 7–21 gavage	8	8		↓ expression of SR-B1, StAR, P450Scc, CYP17, SF1, insl3 on GD 19 & GD 20/21; PPARα on GD 19 @ 600 mg/kg-d	NA

STUDY	AGENT	STRAIN/ SPECIES	# DOSE LEVELS	DOSING REGIMEN	# ANIMALS /DOSE	# LITTERS /DOSE	MATERNAL TOXICITY	ENDPOINT	NOAEL
Howdeshell *et al.* (2008)	DIBP	S-D	0, 100, 300, 600, 900 mg/kg-d	GD 8–18	5–8	5–8		↓ fetal testicular testosterone production @ 300 mg/kg-d and above	100 mg/kg-d based upon ↓ fetal testicular testosterone production @ 300 mg/kg-d
Hannas *et al.* (2011)	DIBP	S-D	0,100, 300, 600, 900 mg/kg-d	GD 14–18	3–6	3–6		↓ fetal testosterone production @ 300 mg/kg-d and above; ↓ Cyp11a expression at 100 mg/kg-d and above and ↓ expression of StAR at 300 mg/kg-d and above	100 mg/kg-d based upon ↓ fetal testicular testosterone production @ 300 mg/kg-d

S-D = Sprague-Dawley; GD = gestation day; NA = not available; AGD = anogenital distance; PND = postnatal day; SR-B1 = scavenger receptor class B1; StAR = steroidogenic acute regulatory protein; PPARα = peroxisome proliferator-activated receptor alpha; ins3 = insulin-like factor 3; NOAEL = no observed adverse effect level

4.4.1 Consensus NOAEL for DPENP/DPP

There are only two studies available describing the effects of DPENP on reproductive development in rats after *in utero* exposure during late gestation. Although these studies were not designed to determine NOAELs, the data presented on the effects of DPENP on fetal testosterone production and gene expression of target genes involved in male reproductive development revealed that reduction in testosterone production was the most sensitive endpoint, with a LOAEL of 33 mg/kg-d (Hannas *et al.,* 2011). Thus, on the basis of this study, the CHAP assigns the NOAEL for DPENP/DPP at 11 mg/kg-d.

4.5 Dicyclohexyl phthalate (DCHP) (84-61-7)

Hoshino *et al.* (2005) conducted a two-generation reproductive toxicity study in which male and female Sprague-Dawley rats of parental (F0) and F1 generation were exposed to DCHP in the diet at concentrations of 0, 240, 1200, or 6000 ppm. DCHP caused a decrease in anogenital distance and an increase in nipple retention in F1 males at 6000 ppm and in F2 males at 1200 ppm and above. Based on the LOAEL in F2 males, the authors report a NOAEL of 240 ppm (16–21 mg/kg-d).

Yamasaki *et al.* (2009) exposed pregnant Sprague-Dawley rats on gestation day 6 to postnatal day 20 to DCHP at 0, 20, 100, or 500 mg/kg-d and observed prolonged preputial separation, reduced anogenital distance, increased nipple retention, and increased hypospadias in male offspring in the 500 mg/kg-d group. Using 500 mg/kg-d as the LOAEL, the NOAEL would be 100 mg/kg-d.

Saillenfait *et al.* (2009) reported a study in which they exposed pregnant Sprague-Dawley rats from gestational day 6–20 to DCHP at 0, 250, 500, or 750 mg/kg-d. Like DHEXP also studied by the same group, DCHP caused a significant and dose-related decrease in anogenital distance in male fetuses at all doses. Unlike DHEXP, DCHP did not cause a significant increase in the incidence of male fetuses with undescended testis or dose-related increases in cleft palate, eye defects, or axial skeleton abnormalities.

4.5.1 Consensus NOAEL for DCHP

Two of the three studies (Hoshino *et al.,* 2005; Yamasaki *et al.,* 2009) available report DCHP-induced effects on male reproductive development (decreased anogenital distance and nipple retention in males) and the third study (Saillenfait *et al.,* 2009) reported only the former. The Saillenfait (2009) study could not be used to determine a NOAEL because the lowest dose used in their study was a LOAEL. Of the two remaining studies, the two-generation study by Hoshino *et al.* (2005) reported adverse effects on male reproductive development at a calculated dose of 80–107; NOAEL of 16–21 mg/kg-d, whereas the Yamasaki *et al.* (2009) prenatal study reported adverse effects on male reproductive development at a dose of 500 mg/kg-d; NOAEL of 100 mg/kg-d. Using the more conservative of the two NOAELs, the CHAP assigns a NOAEL of 16 mg/kg-d for DCHP.

Table A-7 DCHP developmental toxicity studies.

Study	Agent	Strain/ Species	Dose levels	Dosing regimen	Animals/ dose	Maternal toxicity	Endpoint	NOAEL
Hoshino *et al.* (2005)	DCHP	S-D	0, 240, 1200, 6000 ppm	two-generation	20–24		↓ AGD and ↑ nipple retention @ 1200ppm and above in F2 males	240 ppm (16-21 mg/kg-d) based upon ↓ AGD and ↑ nipple retention @ 1200ppm and above in F2 males
Yamasaki *et al.* (2009)	DCHP	S-D	0, 20, 100, 500 mg/kg-d	GD 6–PND 20	10		↓ AGD, ↑ nipple retention and hypospadias @ 500 mg/kg-d	100 mg/kg-d based upon ↓ AGD, ↑ nipple retention and hypospadias @ 500 mg/kg-d
Saillenfait *et al.* (2009)	DCHP	S-D	0, 250, 500, 750 mg/kg-d	GD 6–20	24–25	yes	↓ male AGD @ 250 mg/kg-d and above	NA

S-D = Sprague-Dawley; AGD = anogenital distance; NA = not available; NOAEL = no observed adverse effect level

4.6 Di-*n*-hexyl Phthalate (DHEXP/DnHP) (84-75-3)

4.6.1 2002 Summary of the NTP-CERHR Report

The 2002 summary of the NTP-CERHR report (Kavlock *et al.*, 2002; NTP, 2003d) on the reproductive and developmental toxicity of DHEXP/DnHP indicates that no human developmental toxicity data were located by the expert panel. Animal data are limited to one screening assay in which a "massive oral dose (9,900 mg/kg-d) was administered to 48 mice on GD 6–13. None of the 34 pregnant dams gave birth to a live litter." Based on the available studies, the panel concludes that the "the database is insufficient to fully characterize the potential hazard. However, the limited oral developmental toxicity data available (screening level assessment in the mouse) are sufficient to indicate that DHEXP is a developmental toxicant at high doses (9900 mg/kg-d). These data were inadequate for determining a NOAEL or LOAEL because only one dose was tested."

4.6.2 Relevant Studies Published Since the 2002 Summary of the NTP-CERHR Report

Saillenfait *et al.* (2009) reported a study in which they exposed pregnant Sprague-Dawley rats from gestational day 6 to 20 to DHEXP at 0, 250, 500, or 750 mg/kg-d. DHEXP caused a significant and dose-related decrease in anogenital distance in male fetuses at all doses and a significant increase in the incidence of male fetuses with undescended testes at 500 mg/kg-d and above. In addition, DHEXP caused dose-related increases in cleft palate, eye defects, and axial skeleton abnormalities.

4.6.3 Consensus NOAEL for DHEXP

Although the study by Saillenfait *et al.* (2009) is fairly robust, *i.e.*, multiple doses, number of animals per dose group (20–25), and appropriate exposure time, no NOAEL for the most sensitive developmental reproductive endpoint (anogenital distance) could be ascertained because the lowest dose tested was the LOAEL.

4.7 Diisooctyl Phthalate (DIOP) (27554-26-3)

The only available data on developmental effects come from a parental study in which female rats were administered 0, 5, or 10 mL/kg DIOP (0, 4,930, or 9,860 mg/kg), using the reported density of 986 kg/m^3 (NICNAS, 2008) on days 5, 10, and 15 of gestation by intraperitoneal injection (as cited in Grasso, 1981; ECB, 2000). No increase in fetal mortality or skeletal abnormalities was observed. It was reported that there was a high incidence of soft tissue abnormalities in both treated groups, but quantitative data were not provided in the available summary.

4.7.1 Consensus NOAEL for DIOP

The lack of comprehensive developmental toxicity studies using DIOP as a test substance supported the conclusion that there was "inadequate evidence" for the designation of DIOP as a "developmental toxicant."

4.8 Di(2-propylheptyl) phthalate (DPHP) (53306-54-0)

A gestational exposure study of DPHP in rats is available as a brief report of preliminary results (BASF, 2003). Groups of presumed pregnant female Wistar rats (25/group) were administered 0, 40, 200, or 1,000 mg DPHP/kg-day by gavage (vehicle not specified) on GDs 6 through 19. At necropsy (not specified but presumably GD 20), 17–25 females per group had implantation sites. Maternal toxicity occurred in the high-dose group (1,000 mg/kg-day), as evidenced by insufficient care of fur, 32% reduced food consumption on GDs 6–10, and 30% reduced corrected body weight gain. Significant loss of body weight (magnitude not specified) occurred on GD 6–8. Gross necropsy showed that two high-dose females had hydrometra (accumulation of fluid in the uterus). Examination of the uterus showed that high-dose females had increased postimplantation loss compared with controls (21.3 vs. 6.2%). In addition, 17/20 high-dose females (it is unclear what happened with the remaining five females in this group) had viable fetuses, and in 3 dams, only resorptions were found in the uterus (2.2 vs. 0.5% in controls). Exposure to DPHP did not cause teratogenicity, but fetuses from high-dose females showed a statistically significant increased incidence of soft tissue variations (dilated renal pelvis), which according to the researchers, was just outside the historical control range. It should be noted that this study is also summarized in the review by Fabjan *et al.* (2006), which states that the rates of soft tissue, skeletal, and total variations were slightly but statistically significantly increased in high-dose fetuses. Fabjan *et al.* (2006) also reported a screening developmental toxicity study (citation not provided) in which pregnant rat dams were treated with DPHP on GD 6–15 by gavage with no maternal or fetal effects at the high dose of 1,000 mg/kg-day. No data were shown, and no further details were provided in the available reports of these studies.

4.8.1 Consensus NOAEL for DPHP

Overall, an insufficient amount of animal data and poorly described methodologies in studies using DPHP as a test substance supported the conclusion that there was "insufficient evidence" for the designation of DPHP as a "developmental toxicant."

Table A-8 Consensus reference doses for antiandrogenic endpoints.

PHTHALATE	NOAEL mg/kg-d	UNCERTAINTY FACTOR	RfD mg/kg-d
DBP	50	100	0.50
BBP	50	100	0.50
DEHP	5	100	0.05
DNOP	NA	NA	
DINP	300	100	3.0
DIDP	≥600	NA	
DMP	≥750	NA	
DEP	≥750	NA	
DIBP	125	100	1.25
DPENP (DPP)	11	100	0.11
DCHP	16	100	0.16
DNHEXP	≤ 250	NA	
DIOP	NA	NA	
DPHP	NA	NA	

NOAEL = no observed adverse effect level; NA = not available; RfD = reference dose

Table A-9 Summary of animal male developmental toxicology.

PE	Testis malform. /histopathology	Testis wt.	Seminal vesicle	Epididymal wt.	Cryptorchidism	Hypospadias	Gubernacu-lar malformations
DBP	↑	↓	↓	↓	↑	↑	↑
BBP	↑	↓	↓	↓	↑	↑	↑
DEHP	↑	↓	↓	↓	↑	-	
DNOP							
DINP	-	↓	-	-			
DIDP							
DMP	-	-	-	-			
DEP	-	-	-	-	-	-	-
DIBP	↑	↓	↓?	↓	↑	↑	↑?
DPP	↑	↓		↓	↑?	↑?	↑?
DHEXP					↑		
DCHP					↑	↑	
DIOP							
DPHP							
ATBC							
DEHA		-	-	-			
DINCX					-?	-?	-?
DEHT							
TOTM							
TPIB							

↑= increase; ↓= decrease; - = not affected; PE = phthalate esters

5 Prenatal Phthalate Exposures and Neurobehavioral Effects

Studies reviewed in the previous section have provided extensive documentation that phthalates induce the phthalate syndrome in rats and that one of the early manifestations of this syndrome is the reduction of testosterone production. Because gonadal steroids play an essential role in the process of brain sexual differentiation during embryonic development and early postnatal life, some developmental toxicology studies have also focused on the neurobehavioral effects of prenatal exposures to various phthalates.

Gray *et al.* (2000) treated pregnant Sprague-Dawley rats from gestation day 14 to postnatal day 3 with 0 or 750 mg DEHP, BBP, or DINP/kg-d and examined mounting behavior in a subset of control and treated males. The authors report that 4/6 treated males displayed mounts with pelvic thrusts versus 2/3 controls and conclude that "these data do not support the hypothesis that PEs alter sexual differentiation of central nervous system (CNS) with respect to male rat sexual behavior."

Moore *et al.* (2001) treated pregnant Sprague-Dawley rats from gestation day 3 through postnatal day 21 with 0, 375, 750, or 1,500 mg DEHP/kg/day, and males from litters so treated were examined for masculine sexual behaviors as adults. Nine of 16 DEHP-treated males failed to ejaculate during sexual behavior testing compared to 1 of 8 control males. Eight of these 9 had no intromissions and 5 failed to mount a single time. The authors could find no evidence that the abnormal sexual behaviors observed in the DEHP-exposed male rats was caused by effects on androgen concentrations in adulthood or by abnormal male reproductive organs. Instead, they suggest that the *in utero* and lactational DEHP exposure causes incomplete sexual differentiation of the CNS.

Masutomi *et al.* (2003) fed pregnant Sprague-Dawley rats 400, 4000, or 20,000 ppm DINP from gestation day 15 to postnatal day 10 and then did volume measurements on the sexually dimorphic nucleus of the preoptic area (SDN-POA), which is sensitive to exogenous androgens, at prepubertal necropsy. Although the SDN-POA in males was >10 larger than in females, there were no significant differences in SDN-POA values between controls and DINP-treated groups for either sex.

Takagi *et al.* (2005) fed pregnant CD (SD) IGS rats 4000 or 20,000 ppm DINP/kg/day from gestation 15 to postnatal day 10, at which time pups were killed, brains were fixed and sectioned, the SDN-POA localized and isolated, and total RNA extracted. Using this SDN-POA RNA and real-time RT-PCR, the authors determined the expression levels for ERα, ERβ, PR, and SRC-1 mRNAs. The only significant change observed was a decreased expression of PR in females after treatment with 20,000 ppm.

Lee *et al.* (2006) fed pregnant Wistar rats either DBP (20, 200, 2,000, or 10,000 ppm), DINP (40, 400, 4,000, or 20,000 ppm), or DEHA (480, 2,400 or 12,000 ppm) from gestation day 15 to the day of weaning (PND 21). On PND 7 a subset of rats was killed, their brains removed, and the entire hypothalamus removed and frozen for RNA isolation. The RNA was used to determine the expression levels of granulin (grn) and p130 mRNAs by RT-PCR. DBP induced increased expression of grn in females at 2000 ppm and above, and DINP induced increased grn expression

in females at all doses except 4000 ppm. In contrast, DBP induced increased expression of p130 in males at low doses (20 and 200 ppm), but not at high doses, whereas DINP induced increased expression of p130 in males at all doses tested. On PND 20–21, copulatory behavior was assessed for both males and females. Whereas the copulatory behavior of females was significantly inhibited at all doses of DBP and DINP, the effects of these phthalates on male copulatory behavior were complex, *e.g.*, 200 and 2,000 ppm DBP decreased the number of ejaculations while in the 10,000 ppm exposed rats, the number of ejaculations was increased.

Dalsenter *et al.* (2006) treated pregnant Wistar rats by gavage with 0, 20, 200, or 500 mg/kg-d DEHP from gestational day 14 through postnatal day 3, and adult males were then evaluated for sexual behavior (mount and intromission latencies, number of intromissions up to ejaculation, ejaculatory latency, and intromission frequency). Males exposed *in utero* to 500 mg/kg-d DEHP exhibited impaired sexual behavior as evidenced by increased intromission latency and increased number of intromissions up to ejaculation.

Andrade *et al.* (2006b) treated pregnant Wistar rats by gavage from gestation day 5 to lactation day 21 with 0, 0.015, 0.045, 0.135, 0.405, 1.215, 5, 15, 45, 135, or 405 mg DEHP/kg-d. Males from treated litters were tested as adults on postnatal day 130 for sexual behavior (mount and intromission latencies, number of intromissions up to ejaculation, ejaculatory latency, and intromission frequency). No effects on male sexual behavior were observed at any dose of DEHP tested.

Boberg *et al.* (2011) reported a study in which Wistar rats were exposed to DINP by gavage at 0, 300, 600, 750, or 900 mg/kg-d from gestation day 7 to PND 17. A subset of male and female animals from each dose group was weaned at PND 21 and used for behavioral testing (motor activity and habituation capability, and Morris maze learning and memory). Although DINP did not affect male behavior as tested, DINP-exposed females showed a dose-dependent improvement in spatial learning and memory abilities, which was statistically significant at the highest dose.

6 Developmental Toxicity of Phthalate Substitutes

6.1 Acetyl Tributyl Citrate (ATBC) (77-90-7)

A two-generation reproduction study in Sprague-Dawley rats was reported by Robins (1994). ATBC was mixed in the diet at concentrations to give 0, 100, 300, 1000 mg/kg-d. Males were exposed for 11 weeks, females were exposed for 3 weeks before mating, during mating, and through gestation and lactation. Male and female pups were given diets with ATBC for 10 weeks after weaning. There were no reproductive or developmental effects attributable to ATBC at any dose level (Table A-10).

Chase and Willoughby (2002) reported a one-generation reproduction study (summary only) in Wistar rats given ATBC in the diet at concentrations to provide 0, 100, 300, or 1000 mg/kg-d four weeks prior to and during mating plus during gestation and lactation. The F0 parents produced an F1 generation of litters. No systemic or reproductive effects were seen at any dose level.

6.1.1 Consensus NOAEL for ATBC

In both the Chase and Willoughby (2002) and the Robins (1994) studies, the highest dose tested, 1000 mg/kg-d, was also the NOAEL (Table A-11). Although these were not peer-reviewed studies and ATBC was administered in the diet rather than by gavage, the CHAP recommends a NOAEL of 1000 mg/kg-d but with an additional uncertainty factor of 10 being used in calculating the reference dose.

6.2 Di(2-ethylhexyl) Adipate (DEHA) (103-23-1)

Dalgaard (2002; 2003) reported on perinatal exposure of Wistar rats by gavage at dose levels of 0, 800, or 1200 mg/kg-d on gestation day 7 through postnatal day 17. This was a dose range finding study to examine pups for evidence of antiandrogenic effects—none were observed. Decreased pup weights were seen at both dose levels. In the main study, DEHA was given by gavage at dose levels of 0, 200, 400, or 800 mg/kg-d on gestation day 7 through postnatal day 17. No antiandrogenic effects were seen; a NOAEL of 200 mg/kg-d was based on postnatal deaths.

6.2.1 Consensus NOAEL for DEHA

The Dalgaard *et al.* (2003) study employed 3 dose groups (plus control), 20 dams/ dose, an appropriate exposure regimen (gestation day 7–17), and observed no antiandrogenic effects at any dose. Thus, the CHAP recommends a NOAEL of 800 mg/kg-d for DEHA but with an additional uncertainty factor of 10 being used to calculate the reference dose (RfD) given that this NOAEL is based upon one unreplicated study.

6.3 Diisononyl 1,2-dicarboxycyclohexane (DINX) (474919-59-0)

A PubMed search for the terms *diisononyl 1,2-dicarboxycyclohexane* and *developmental toxicity* or *DINCH*® and *developmental toxicity* failed to identify any peer-reviewed articles.

A two-generation reproduction study was reported by SCENIHR (2007) in summary form only. Because the study used OECD TG 416, it was likely conducted in rats. Dose levels by diet were 0, 100, 300, or 1000 mg/kg-d. There were no effects on fertility or reproductive performance in F0 or F1 parents and no developmental toxicity in F1 or F2 pups. A substudy designed to look for antiandrogenic effects showed no developmental toxicity at any dose level.

Prenatal developmental toxicity was also evaluated (BASF, 2005) in rats and rabbits that were orally administered DINX during gestation (at dose levels as high as 1200 mg/kg-d on gestational days 6–19 in the rat and 0, 100, 300 or 1000 mg/kg-d on gestation days 6–29 in the rabbit). No effects were observed in either species, suggesting apparent NOAELs of 1200 mg/kg-d in rats and 1000 mg/kg-d in rabbits.

6.3.1 Consensus NOAEL for DINX

Although the studies cited suggest a NOAEL in rats of 1000 mg/kg-d, these were not peer-reviewed studies; therefore CHAP members did not have access to protocol details or actual data. Given the limitation of non- peer-reviewed studies, the CHAP recommends a NOAEL for DINX of 1000 mg/kg-d but with an additional uncertainty factor of 10 being used to calculate the reference dose.

6.4 Di(2-ethylhexyl) Terephthalate (DEHT/DOTP) (6422-86-2)

Gray *et al.* (2000) reported a study to look for antiandrogenic effects of DEHT. Pregnant Sprague-Dawley rats were dosed by gavage with 0 or 750 mg/kg-d on gestation day 14 through postnatal day 3. No antiandrogenic effects were observed.

Faber *et al.* (2007b) reported the results of a two-generation reproduction study in Sprague-Dawley rats given DEHT in the diet. The dietary admix was given to males and females for 70 days prior to mating plus during pregnancy and lactation. Concentrations in the diet gave 0, 158, 316, or 530 mg/kg-d to males and 0, 273, 545, or 868 mg/kg-d to females. No adverse effects on reproduction were observed in either generation at any dose level. Weight gain was decreased in F0 high-dose males. Weight gain was decreased in F1 and F2 males at the top two dose levels. The NOAEL for reproductive effects was 530 mg/kg-d; the NOAEL for parental and pup systemic toxicity was 158 mg/kg-d.

This same group also reported the results of a developmental toxicity study in which rats or mice were fed DEHT at levels of 0, 226, 458, or 747 mg/kg-d (rats) or 197, 592, or 1382 mg/kg-d from GD 0 to 20 (rat) or 0 to18 (mice). Mean numbers of implantation sites, early resorptions, late resorptions, fetal sex ratios, preimplantation loss, malformations, or variations were unaffected at any concentration level in the rat or mouse. There was a slight reduction in maternal weight gain at the highest dose level rat group and the mid- and high-dose mouse groups. The NOAEL for maternal toxicity was 458 mg/kg-d in rats and 197 mg/kg-d in mice.

6.4.1 Consensus NOAEL for DEHT

The Gray *et al.* (2000) study, which used only one dose group and only eight animals per dose group, reported no antiandrogenic effects of DEHT (DOTP) at the only dose tested, 750 mg/kg-d. The Faber *et al.* (2007b) prenatal developmental toxicity study, which used multiple doses and 25 animals per dose group, also observed no antiandrogenic effects at the highest dose tested, *i.e.*, 747 mg/kg-d from GD 0 to 20 in Sprague-Dawley rats. On the basis of these two studies and the results of the two-generation study in rats, the CHAP recommends a NOAEL for DEHT of 750 mg/kg-d.

6.5 Trioctyl Trimellitate (TOTM) (3319-31-1)

A one-generation reproduction study was reported in Sprague-Dawley rats given TOTM by gavage at dose levels of 0, 100, 300, or 1000 mg/kg-d (JMHW, 1998). Males were dosed for 46 days, females for 14 days prior to mating and during mating through lactation day 3. Histologic examination showed a decrease in spermatocytes and spermatids at the top two dose levels. No other reproductive toxicity was seen. The NOAEL was 100 mg/kg-d.

Pre- and postnatal effects of TOTM in Sprague-Dawley rats were reported from Huntingdon Life Sciences (2002). Rats were given 0, 100, 500, or 1050 mg/kg-d by gavage on days 6–19 of pregnancy or day 3 through day 20 of lactation. There were no significant effects on developmental measures but there was a slight delay in the retention of areolar regions on postnatal day 13, but not day 18 (not considered to be toxicologically significant). The high dose of 1050 mg/kg-d was identified as a NOAEL in this study for developmental effects.

6.5.1 Consensus NOAEL for TOTM

As on ATBC and DINX, there is a lack of peer-reviewed studies on TOTM. Nevertheless, the data available from the Japanese toxicity testing report showing decreases in spermatocytes and spermatids in males exposed to TOTM and the "slight delay in the retention of areolar regions" (nipple retention) in the Huntingdon Life Sciences study suggests at the very least that additional studies are required. Lacking these, the CHAP recommends that the conservative NOAEL of 100 mg/kg-d derived in the Japanese study be assigned for TOTM.

6.6 2,2,4-Trimethyl-1,3-pentanediol-diisobutyrate (TPIB) (6846-50-0)

In the combined repeated dose and reproductive/developm**e**ntal toxicity screening test described in the repeat-dose section above, male and female Sprague-Dawley rats were administered gavage doses of 0, 30, 150, or 750 mg/kg-d TPIB from 14 days before mating until 30 days after (males) or day 3 of lactation (females) (JMHLW, 1993; OECD, 1995; Eastman, 2007). TPIB had no significant effect on mating, fertility, the estrus cycle, delivery, or lactation period. Parameters evaluating developmental toxicity were limited to body weights at postnatal days (PND) 0 and 4, and autopsy findings at PND 4; these examinations revealed no TPIB-related effects at any dose. The reproductive and developmental NOAEL, therefore, is 750 mg/kg-d.

A reproductive/developmental toxicity screening test was performed by Eastman Chemical Company under OECD test guideline 421 (Eastman, 2001). Sprague-Dawley rats (12/sex/dose) received dietary doses of 0, 120, 359, or 1135 mg/kg-d (females) or 0, 91, 276, or 905 mg/kg-d (males) for 14 days before mating, during mating (1–8 day), throughout gestation (21–23 days), and through PND 4–5. Significant reductions in mean body weight, body weight gain, and feed consumption/utilization were observed in both sexes of the parental generation at the high dose level, but were transient in nature. Reductions in mean number of implantation sites were observed in the high-dose group and correlated to the number of corpora lutea. However, there was no corresponding effect on pre- or post-implantation loss, or litter size on PND 0. Mean litter weights in the high-dose group were statistically lower than those of the control group on PND 0 and 4, an effect attributed to the smaller litter sizes rather than a difference in individual pup size. The mean number of live pups at PND 4 was lower in high-dose litters compared to control litters. Mean absolute epididymal sperm counts were statistically lower in all treated groups compared to the control group; however, when counts were normalized for organ weight, values were not statistically different. Males in the high- and low-dose groups had lower mean absolute and/or relative testicular sperm counts. The significance of this was unclear, as there was no effect on relative epididymal sperm counts, fertility, or microscopic lesions in the testes. Authors considered both sperm type changes to be nonadverse. Other reproductive parameters, including reproductive organ weights, gross or microscopic lesions, and mean sperm motility were not affected. Study authors concluded that the NOAEL for reproductive or developmental toxicity was 276 mg/kg-d for males and 359 mg/kg-d for females, based on decreased total litter weight and litter size on PND 4, decreased number of implants and number of corpora lutea (Eastman Chemical 2001).

6.6.1 Consensus NOAEL for TPIB

Although there are data in the Versar report (Versar/SRC, 2010, cited verbatim above), the two studies cited were conducted by Eastman Chemical (2001; 2007) and the data therein have not been published in the peer-reviewed literature. Nonetheless, in neither study is there any indication of any antiandrogenic effects of TXIB® when administered to females at doses as high as 1125 mg/kg-d for 14 days before mating, during mating (1–8 day), throughout gestation (21–23 days), and through PND 4–5. Thus, the developmental NOAEL for TXIB® is greater than 1125 mg/kg-d.

Table A-10 summarizes peer-reviewed developmental toxicity studies on phthalate substitutes.

Table A-10 Developmental toxicity of phthalate substitutes.

STUDY	AGENT	STRAIN/ SPECIES	# DOSE LEVELS	DOSING REGIMEN	# ANIMALS /DOSE	# LITTERS /DOSE	MATERNAL TOXICITY	ENDPOINT	NOAEL
No peer-reviewed studies located	ATBC								
Dalgaard *et al.* (2003)	DEHA	Wistar	0, 800, 1200 mg/kg-d in dose finding study; 0, 200, 400, 800 mg/kg-d in main study	GD 7–17 in dose finding study; GD 7–PND 17	8 in dose finding; 20 in main study	7 in dose finding study; 15–18 in main study	yes @ 1200 mg/kg-d; length of pregnancy ↑, male and female pup birth weights ↓ @ 800 mg/kg-d	no effects on male AGD, nipple retention or testosterone levels observed at any dose level	Authors give 200 mg/kg-d based on dose-dependent ↑ in postnatal death that almost reached significance @ 400 mg/kg-d
No peer-reviewed studies located	DINCH ®								
Gray *et al.* (2000)	DOTP/ DEHT	S-D	0, 750 mg/kg-d	GD 14–PND 3	8			No antiandrogenic effects	NA
Faber *et al.* (2007a)	DEHT	S-D	0, 0.3, 0.6, 1.0 % in diet= 0, 226, 458, 747 mg/kg-d	GD 0–20	25	23–24	yes, ↓ maternal body weight & liver weight @ 1.0% (747 mg/kg-d)	No developmental toxicity observed	747 mg/kg-d for developmental toxicity; 458 mg/kg-d for maternal toxicity
Faber *et al.* (2007a)	DEHT	CD1 mice	0, 0.1, 0.3, 0.7% in diet= 0, 197, 592, 1382 mg/kg-d	GD 0–18	25	21–24	yes, ↓ liver weight @ 0.3% (592 mg/kg-d) and above	No developmental toxicity observed	1382 mg/kg-d for developmental toxicity; 197 mg/kg-d for maternal toxicity
Faber *et al.* (2007b)	DEHT	S-D	0, 0.3, 0.6, 1.0% in diet	two-generation study	30	30?	yes, ↑ lethality in F0 and F1 dams @ 1.0%; ↑ female liver weights @ 0.6% and above	No developmental toxicity observed	1382 mg/kg-d for developmental toxicity; 226 mg/kg-d for maternal toxicity
No peer-reviewed studies located	TOTM								

GD = gestation day; PND = postnatal day; AGD = anogenital distance; S-D = Sprague-Dawley; NA = not available

Table A-11 NOAELs for phthalate substitutes.

Phthalate Substitute	NOAEL
ATBC	1000
DEHA	800
DINX	1000
DEHT	750
TOTM	100
TPIB	≥1125

NOAEL = no observed adverse effect level

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