



TOXICOLOGICAL REVIEW

OF

Dibutyl Phthalate (Di-n-Butyl Phthalate)

(CAS No. 84-74-2)

**In Support of Summary Information on the
Integrated Risk Information System (IRIS)**

June 2006

NOTICE

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U.S. Environmental Protection Agency
Washington DC

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FOREWORD

The purpose of this Toxicological Review is to provide scientific support and rationale for the hazard and dose-response assessment in IRIS pertaining to chronic exposure to dibutyl phthalate. It is not intended to be a comprehensive treatise on the chemical or toxicological nature of dibutyl phthalate.

In Section 6, *Major Conclusions in the Characterization of Hazard and Dose Response*, EPA has characterized its overall confidence in the quantitative and qualitative aspects of hazard and dose response by addressing knowledge gaps, uncertainties, quality of data, and scientific controversies. The discussion is intended to convey the limitations of the assessment and to aid and guide the risk assessor in the ensuing steps of the risk assessment process.

For other general information about this assessment or other questions relating to IRIS, the reader is referred to EPA's IRIS Hotline at 202-566-1676 (phone), (202) 566-1749 (fax), or hotline.iris@epa.gov (Email address)..

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This document and the accompanying IRIS Summary have been peer reviewed by EPA scientists and independent scientists external to EPA. Comments from all peer reviewers were evaluated carefully and considered by the Agency during the finalization of this assessment. During the finalization process, the IRIS Program Director achieved common understanding of the assessment among the Office of Research and Development; Office of Air and Radiation; Office of Prevention, Pesticides, and Toxic Substances; Office of Solid Waste and Emergency Response; Office of Water; Office of Policy, Economics, and Innovation; Office of Children's Health Protection; Office of Environmental Information, and EPA's regional offices.

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1. INTRODUCTION

This document presents background and justification for the hazard and dose-response assessment summaries in EPA's Integrated Risk Information System (IRIS). IRIS Summaries may include oral and inhalation reference values (RfDs and RfCs) for chronic and less-than-lifetime exposure durations, and a carcinogenicity assessment.

The RfDs and RfCs provide quantitative information for use in risk assessments for health effects known or assumed to be produced through a nonlinear (possibly threshold) mode of action. The RfD (expressed in units of mg/kg-day) is defined as an estimate (with uncertainty spanning perhaps an order of magnitude) of a daily exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious effects during a lifetime. The inhalation RfC (expressed in units of mg/m³) is analogous to the oral RfD, but provides a continuous inhalation exposure estimate. The inhalation RfC considers toxic effects for both the respiratory system (portal-of-entry) and for effects peripheral to the respiratory system (extra-respiratory or systemic effects). Reference values are generally derived for chronic exposures (up to a lifetime), but may also be derived for acute (≤ 24 hours), short-term (up to 30 days), and subchronic (up to 10% of average lifespan), all considered to be daily exposures, continuously or intermittently, throughout the duration specified.

The carcinogenicity assessment provides information on the carcinogenic hazard potential of the substance in question and quantitative estimates of risk from oral and inhalation exposure. The information includes a weight-of-evidence judgment of the likelihood that the agent is a human carcinogen and the conditions under which the carcinogenic effects may be expressed. Quantitative risk estimates are derived from the application of a low-dose extrapolation procedure, and are presented in two ways to better facilitate their use. First, route-specific risk values are presented. The "oral slope factor" is an upper bound on the estimate of risk per mg/kg-day of oral exposure. Similarly, a "unit risk" is an upper bound on the estimate of risk per unit of concentration, either per $\mu\text{g/L}$ drinking water or per $\mu\text{g/m}^3$ air breathed. Second, the estimated concentration of the chemical substance in drinking water or air when associated with cancer risks of 1 in 10,000, 1 in 100,000, or 1 in 1,000,000 is also provided.

Development of these hazard identification and dose-response assessments for dibutyl phthalate has followed the general guidelines for risk assessment as set forth by the National Research Council (1983). EPA guidelines that were used in the development of this assessment

include the following: *Guidelines for Developmental Toxicity Risk Assessment* (U.S. EPA, 1991), *Benchmark Dose Technical Guidance Document* (U.S. EPA, 2000c), and *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005).

The literature search strategy employed for this compound was based on the CASRN and at least one common name. Any pertinent scientific information submitted by the public to the IRIS Submission Desk was also considered in the development of this document. The relevant literature was reviewed through February 2006.

2. CHEMICAL AND PHYSICAL INFORMATION

Di-n-butyl phthalate (dibutyl phthalate) is an inert, colorless, oily liquid with a low vapor pressure, soluble in most organic solvents but only slightly soluble in water. Selected information on the physical and chemical properties of dibutyl phthalate (CASRN 84-74-2) is summarized below (ATSDR, 2001):

Chemical Formula:	C ₁₆ H ₂₂ O ₄
Molecular Structure:	See section 3
Molecular Weight:	278.34 g/mol
Melting Point:	-35 °C
Boiling Point:	340 °C
Density:	1.04 kg/L at 20 °C
Water Solubility:	11.2 mg/L
Log K _{ow} :	3.70–4.72
Log K _{oc} :	3.14–4.17
Vapor Pressure:	2.01–2.7 × 10 ⁻⁵ mm Hg at 25 °C
Henry's Law Constant:	8.83 × 10 ⁻⁷ –4.5 × 10 ⁻⁶ atm·m ³ /mol

Dibutyl phthalate is used mainly as a speciality plasticizer for nitrocellulose, polyvinyl acetate, and polyvinyl chloride; a lubricant for aerosol valves; an antifoaming agent; a skin emollient; and a plasticizer in nail polish, fingernail elongators, and hair spray (WHO, 1997; Koo et al., 2004).

Dibutyl phthalate is relatively nonpersistent in air, surface water and soil with a half-life in these compartments of only a few days (WHO, 1997). Washout via rainfall or dry deposition is believed to play a significant role in the removal of dibutyl phthalate from the atmosphere. The photooxidation half-life of dibutyl phthalate in air ranges from 7.4 hours to 3.1 days. The photolytic half-life of dibutyl phthalate in water is estimated to be 144 days. The biodegradation half-life of dibutyl phthalate in natural surface waters ranges from 1 to 14 days and in groundwater ranges from 2 to 23 days. The half-life for dibutyl phthalate in soil is estimated at 2 to 23 days.

Complete biodegradation is rapid under aerobic conditions, but much slower under anaerobic conditions. Dibutyl phthalate would be expected to bioaccumulate as a result of its

high octanol-water partition coefficient. However, dibutyl phthalate is readily metabolized to more water soluble compounds. Consequently bioconcentration factors tend to be lower than predicted from the octanol-water partition coefficient (WHO, 1997).

3. TOXICOKINETICS

3.1. ABSORPTION AND DISTRIBUTION

Dibutyl phthalate is rapidly hydrolyzed to monobutyl phthalate and n-butanol by non-specific lipases and esterases found in the contents of the gastrointestinal (GI) tract, homogenates of intestinal mucosal cells, homogenates of liver, isolated liver microsomes, isolated liver mitochondria, homogenates of pancreas, homogenates of kidney, and blood (NIEHS, 1995, 1994; Tanaka, 1989; Foster et al., 1982; White et al., 1980; Kaneshima et al., 1978a,b; Takahashi and Tanaka et al., 1978; Rowland et al., 1977). The hydrolysis of 1 mg of dibutyl phthalate will form 0.266 mg of n-butanol [$1 \times (74.12/278.34)$] and 0.799 mg of monobutyl phthalate [$1 \times (222.35/278.34)$]. Toxicological data on n-butanol are summarized in a separate IRIS entry. Hydrolysis of the second butyl group does not occur to any significant extent. Quantitatively, the most important location for the production of monobutyl phthalate is the lumen of the intestine by enzymes secreted from the pancreas (White et al., 1983).

The monobutyl phthalate formed in the GI tract of the rat is rapidly and completely absorbed into the systemic circulation, widely distributed in the body, and does not bioaccumulate in tissues (Fennell et al., 2004; Saillenfait et al., 1998; NIEHS, 1994, 1995; Tanaka et al, 1978; Williams and Blanchfield, 1975).

In vitro studies with intestinal preparations from rat, baboon, ferret, and humans show a species similarity in the amount and rate of hydrolysis of dibutyl phthalate (Lake et al., 1977). Therefore, complete hydrolysis of dibutyl phthalate to monobutyl phthalate is also expected in humans. Anderson et al. (2001) conducted a study to determine a quantitative biomarker method for correlating human urinary phthalate monoester elimination with exposure to the corresponding phthalate diester. As stated by the authors, the study was conducted with an “ethically approved” protocol, but details of the ethics review were not provided. ¹³C- Dibutyl phthalate (0, 255, or 510 µg) was administered in a single dose to human volunteers (8 people in each group, sex and body weight not reported). The amount of ¹³C- monobutyl phthalate in urine was measured by high performance liquid chromatography-mass spectrometry after hydrolysis of conjugates. 24-Hour urine samples were collected 1 day before the dose, 1 day after the dose, and 2 and 6 days after the dose. The amount of ¹³C-monobutyl phthalate in feces was not measured. For dibutyl phthalate, 64% and 73% (average 69%) on a mole basis of the low and high dose, respectively, was excreted as monobutyl phthalate in the first 24 hours. No

measurable ¹³C- monobutyl phthalate was present in the second or sixth day collections of urine. These data confirm that nearly complete hydrolysis and absorption of dibutyl phthalate occur in humans. In addition the conversion factor (69%) can be used to assess human exposure to dibutyl phthalate from data on urinary levels of monobutyl phthalate.

Data on the internal distribution of monobutyl phthalate are not available in humans. However, monobutyl phthalate has been detected in human urine, blood, amniotic fluid, breast milk, and saliva as the result of environmental exposure to dibutyl phthalate. Non-specific esterases in some biological matrices, including amniotic fluid, saliva, and breast milk, could hydrolyze dibutyl phthalate from laboratory ware to monobutyl phthalate and could be the source of some of the monobutyl phthalate found in these tissues. Data for urine are reported in Section 3.6. Silva et al. (2003) report a median concentration of total monobutyl phthalate in serum of 14.4 µg/L of which approximately 25-30% is free monobutyl phthalate and the remainder is monobutyl phthalate glucuronide. Silva et al. (2004b) report a median concentration of total monobutyl phthalate in human amniotic fluid of 5.8 µg/L, a 95th percentile of 15.9 µg/L, and a maximum value of 263.9 µg/L (n = 54 with detection in 50 of the samples). Calafat et al. (2004) report a mean concentration of total monobutyl phthalate in human breast milk of 1.3 ± 1.5 (n = 3 with detection in 2 of the samples) µg/L. Silva et al. (2005) report a median concentration of total monobutyl phthalate in human saliva of 5.0 µg/L and a 95th percentile of 57.9 µg/L (n = 39 with detection in 33 of the samples). The percentage of free monobutyl phthalate and monobutyl phthalate glucuronide was not determined in amniotic fluid, breast milk, or saliva. These data imply that monobutyl phthalate will be widely distributed in human tissues.

In rats, approximately 60% of a single dermal dose of 157 µmol/kg was excreted in urine during a 7-day period (Elsisi et al., 1989). Dibutyl phthalate was applied to the skin in absolute ethanol and the ethanol was allowed to evaporate. The skin was then covered by a circular plastic cap which had been perforated with needle holes to allow aeration. The dibutyl phthalate remained on the skin for 7 days.

Scott et al. (1987) measured the absorption of dibutyl phthalate in vitro through human and rat epidermal membranes. Dibutyl phthalate was applied to the epidermal membrane in a 50% v/v aqueous ethanol evenly distributed over the membrane. Studies using rat epidermis were concluded within 8 hours; studies using human epidermis were continued for 30 hours. Rat skin was more permeable than human skin. Absorption rates are presented in Table 3-1.

Table 3-1. Absorption rate through human and rat skin

Permeability constant x 10 ⁻⁵ cm/hr ^a		Steady state absorption rate (µg/cm ² /hr ^a)		Lag time (hr)	
Human	Rat	Human	Rat	Human	Rat
0.23±0.06	8.95±0.09	0.07±0.02	9.33±0.09	2.9	0.4
(n = 15)	(n = 8)	(n = 15)	(n = 9)		

^aMean values ± SE; n = number of determinations.

Source: Scott et al., 1987.

No quantitative information was found for absorption following inhalation exposure.

3.2. METABOLISM

A number of reports have presented data on the metabolites of dibutyl phthalate in rats (Fennell et al., 2004; Saillenfait et al., 1998; NIEHS, 1995, 1994; Foster et al., 1982; Tanaka et al., 1978; Williams and Blanchfield, 1975; Albro and Moore, 1974). The major metabolites were monobutyl phthalate and its glucuronide (90-95% of the total). Only small quantities of dibutyl phthalate (non-detect to 2% of the total), phthalic acid (trace to 3% of the total), and products of ω and ω -1 oxidation (trace to 10% of the total) were found. N-Butanol was not measured in these studies. Glucuronidation and ω and ω -1 oxidation are believed to occur in liver microsomes. Monobutyl phthalate and monobutyl phthalate glucuronide have been found in human blood and urine, but the products of ω and ω-1 oxidation have not been measured (Silva et al., 2003).

In rat serum, 80-90% of the total monobutyl phthalate is free monobutyl phthalate and the remainder is the glucuronide conjugate (Fennell et al., 2004; Saillenfait et al., 1998; Williams and Blanchfield, 1975; Albro and Moore, 1974). However, the situation is reversed in humans where 25 to 30% of the total monobutyl phthalate in the serum is free monobutyl phthalate and the remainder is the glucuronide conjugate (Silva et al., 2003).

The metabolism of dibutyl phthalate is shown in Figure 1. The pathways were deduced from studies conducted in rats, guinea pigs, and hamsters. N-Butanol, produced in the first step, is not shown in the figure.

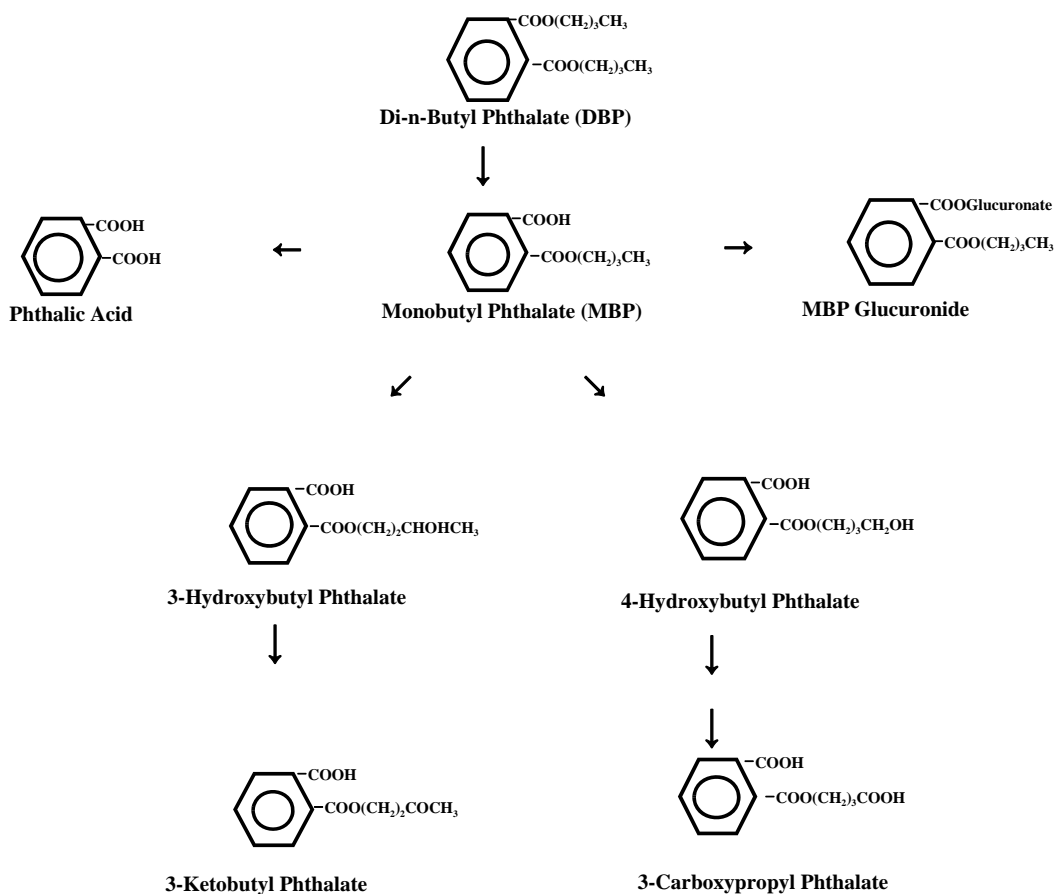


Figure 1. Metabolic Pathways

Source: ATSDR (2001)

3.3. ELIMINATION

More than 80 - 90% of an oral or intravenous dose of dibutyl phthalate is excreted within 24 to 48 hours in rats (Fennell et al., 2004; Saillenfait et al., 1998; Tanaka et al., 1978; Williams and Blanchfield, 1975). Similarly, complete elimination is observed following an intravenous dose of monobutyl phthalate (Kremer et al., 2005b). The major route of excretion is through the urine with a smaller amount excreted in feces (Fennell et al., 2004; Saillenfait et al., 1998; Foster et al., 1982; Tanaka et al., 1978; Williams and Blanchfield, 1975). There is some evidence for enterohepatic circulation from studies using animals with cannulated bile ducts (Tanaka et al.,

1978) and from a pharmacokinetic study (Fennell et al., 2004).

In the urine of rats following an exposure of 10 to 50 mg/kg, the distribution of monobutyl phthalate and monobutyl phthalate glucuronide was 1/3 and 2/3, respectively (Kremer et al., 2005b). In the urine of humans (n = 283) following environmental exposure to dibutyl phthalate, the median total concentration of monobutyl phthalate plus monobutyl phthalate glucuronide was 29.7 mg/L and the median free monobutyl phthalate concentration was 1.4 mg/L (Silva et al., 2003).

3.4. TOXICOKINETICS

Saillenfait et al. (1998) and Fennell et al. (2004) have conducted investigations of the pharmacokinetics of dibutyl phthalate in pregnant rats. Saillenfait et al. (1998) administered a single oral dose of radiolabelled dibutyl phthalate in mineral oil (500 or 1500 mg/kg body weight) to pregnant Sprague-Dawley rats (n = 4 to 6 per group per time point) on gestation day (GD) 14. The distribution and elimination of dibutyl phthalate metabolites were measured at 0.5, 1, 2, 4, 6, 8, 24, and 48 hours after exposure. Selected data for monobutyl phthalate (MBP) and its glucuronide conjugate (MBP-G) at the 500 mg/kg exposure are presented in Table 3-2.

Table 3-2. Distribution and toxicokinetic parameters of primary metabolites of Dibutyl Phthalate in pregnant Sprague-Dawley rats: 500 mg/kg exposure

Site/Parameter	Metabolite	
	MBP	MBP-G
<u>Maternal Plasma</u>		
C_{max} ($\mu\text{mol/g}$)	1.58 [#]	0.23
T_{max} (h)	1.4	1.8
$AUC_{0-\infty}$ ($\mu\text{mol}\cdot\text{h/g}$)	9.0	1.8
Elimination rate constant (h^{-1})	0.31	0.27
	[#] equal to 350 mg/L	
<u>Placenta</u>		
C_{max} ($\mu\text{mol/g}$)	0.39 [#]	0.04
T_{max} (h)	1.7	2.5

Site/Parameter	Metabolite	
	MBP	MBP-G
AUC _{0-∞} (μmol*h/g)	2.2	0.4
Elimination rate constant (h ⁻¹)	0.52	0.21
	#equal to 87 mg/L	
<u>Embryo</u>		
C _{max} (μmol/g)	0.38 [#]	Not detected
T _{max} (h)	1.4	
AUC _{0-∞} (μmol*h/g)	1.3	
Elimination rate constant (h ⁻¹)	0.71	
	#equal to 84 mg/L	
<u>Amniotic Fluid</u>		
C _{max} (μmol/g)	0.29 [#]	Not detected
T _{max} (h)	2.6	
AUC _{0-∞} (μmol*h/g)	1.7	
Elimination rate constant (h ⁻¹)	0.33	
	#equal to 64 mg/L	

Source: Saillenfait et al., 1998

Fennell et al. (2004) administered a single oral dose of radiolabelled dibutyl phthalate in corn oil (50, 100, or 250 mg/kg body weight) to pregnant Sprague-Dawley rats (n = 30 per group) on GD 20. The distribution and elimination of dibutyl phthalate metabolites were measured at 5, 15, and 30 minutes and 1, 2, 4, 8, and 24 hours after exposure. Selected data for monobutyl phthalate (MBP) and its glucuronide conjugate (MBP-G) at the 50 and 100 mg/kg body weight exposure are presented in Table 3-3.

Table 3-3. Distribution of toxicokinetic parameters of primary metabolites of dibutyl phthalate in pregnant Sprague-Dawley rats: 50 and 100 mg/kg exposures

Site/Parameter	Metabolites			
	50 mg/kg Exposure		100 mg/kg Exposure	
	MBP	MBP-G	MBP	MBP-G
<u>Maternal Plasma</u>				
C _{max} (μmol/mL)	0.18 [#]	0.059	0.34 [*]	0.079
T _{max} (h)	0.5	1	0.5	1
AUC _{0-∞} (μmol*h/mL)	0.65	0.28	1.72	0.64
Half life (h)	2.87	2.91	2.75	2.89
	[#] equal to 40 mg/L		[*] equal to 76 mg/L	
<u>Fetal Plasma</u>				
C _{max} (μmol/mL)	0.074 [#]	0.018	0.15 [*]	0.042
T _{max} (h)	1	4	1	4
AUC _{0-∞} (μmol*h/mL)	0.47	0.19	1.12	0.50
Half life (h)	4.2	6.5	4.58	4.92
	[#] equal to 16 mg/L		[*] equal to 33 mg/L	
<u>Amniotic fluid</u>				
C _{max} (μmol/mL)	0.015 [#]	0.013	0.048 [*]	0.028
T _{max} (h)	4	8	4	8
AUC _{0-∞} (μmol*h/mL)	0.27	0.6	0.73	1.93
Half life (h)	11.06	28.87	6.48	60.32
	[#] equal to 3.4 mg/L		[*] equal to 11 mg/L	

Source: Fennell et al. 2004.

Kremer et al. (2005a, abstract only) administered dibutyl phthalate daily by gavage in corn oil to pregnant Sprague-Dawley rats at 100 mg/kg on GDs 12–19 and measured the concentration of monobutyl phthalate and monobutyl glucuronide in maternal and fetal plasma on GD 19–21. Values estimated by EPA from the plots supplied by Dr. Kremer are presented in Table 3-4.

Table 3-4. Toxicokinetic parameters of primary metabolites of Dibutyl Phthalate in pregnant Sprague-Dawley rats: 100 mg/kg exposure

Site/Parameter	Metabolites	
	MBP	MBP-G
<u>Maternal Plasma</u>		
C_{max} (mg/L)	130	Not Reported
T_{max} (h)	1	
<u>Fetal Plasma</u>		
C_{max} (mg/L)	42	13
T_{max} (h)	1	4

Source: Kremer et al. 2005a.

The maximum concentration of monobutyl phthalate in the fetal plasma from the single exposure study of 33 mg/L (Fennell et al., 2004) is comparable to that from the multiple exposure study of 42 mg/L (Kremer et al., 2005a) at the 100 mg/kg exposure level.

Kremer et al. (2005b) conducted a study to evaluate the pharmacokinetics of monobutyl phthalate and monobutyl phthalate glucuronide in pregnant rats following intravenous (i.v.) dosing with monobutyl phthalate. Pregnant dams were dosed with aqueous monobutyl phthalate (0, 10, 30, or 50 mg/kg) on gestation day 19. The pharmacokinetics of monobutyl phthalate and its glucuronide conjugate were rapid. Monobutyl phthalate was rapidly distributed into maternal tissues. The authors stated that excessive tailing for monobutyl phthalate occurred after 8 hours at the 10 mg/kg exposure and yielded unreasonably large values for some of the calculated monobutyl phthalate pharmacokinetic parameters. Accordingly, these values are not reported here. The half-life for elimination of monobutyl phthalate at 30 and 50 mg/kg was 1.7 and 2.6 hours, respectively, and the volume of distribution at steady state was 0.51 L and 0.36 L, respectively. Monobutyl phthalate glucuronide appeared in maternal blood at 5 minutes following the i.v. bolus dose, was at a plateau for approximately 2 hours, and was near or below background within 24 hours. The half-life for depletion at 30 and 50 mg/kg was 2.1 and 1.7 hours, respectively. In the urine the distribution of monobutyl phthalate and monobutyl phthalate glucuronide was 1/3 and 2/3, respectively. The non-linear increases in monobutyl

phthalate Cmax and AUC suggest that metabolism of monobutyl phthalate and/or clearance become saturated at higher doses. Fennell et al (2004) found similar non-linear behavior and proposed that metabolism of monobutyl phthalate was saturated at higher doses. However, Kremer et al. (2005b) found no evidence that metabolism of monobutyl phthalate to monobutyl phthalate glucuronide was saturated at the exposures tested in this study. To explain the data, Kremer et al. (2005b) hypothesized that clearance of monobutyl phthalate is predominately through glomerular filtration at high monobutyl phthalate concentrations, while metabolism to monobutyl phthalate glucuronide is dominant at low monobutyl phthalate concentrations. The pharmacokinetic results of Kremer et al. (2005b) are consistent with two previous studies (Fennell et al., 2004; Saillenfait et al., 1998) that utilized oral doses of dibutyl phthalate. Kremer et al. (2005b) suggest that chemical (dibutyl phthalate versus monobutyl phthalate), vehicle (oil versus aqueous), dose level and route (oral versus i.v.) have minimal effects on the maternal pharmacokinetics of monobutyl phthalate and monobutyl phthalate glucuronide. The study provides a direct pharmacokinetic analysis for monobutyl phthalate and monobutyl phthalate glucuronide at biologically relevant exposures in pregnant rats during male reproductive development, and indicates that future pharmacokinetic or toxicology studies can reliably utilize oral dosing with dibutyl phthalate.

Calafat et al. (2006) examined the concentration of monobutyl phthalate in amniotic fluid on gestation day 18 and maternal urine on gestation day 17 after oral exposure to dibutyl phthalate during pregnancy. Dibutyl phthalate was administered by oral gavage to pregnant Sprague-Dawley rats at an exposure of 0, 100, or 250 mg/kg-day starting on gestational day 13. In urine most of the monobutyl phthalate was glucuronidated; in contrast in amniotic fluid free monobutyl phthalate predominated. Statistically significant differences were found in monobutyl phthalate concentration in amniotic fluid between animals within the same group treated with the same amount of dibutyl phthalate ($p < 0.0001$) and between animals in groups treated with different amount of dibutyl phthalate ($p < 0.0001$). In animals treated with 100 mg/kg-day, the mean concentration of total monobutyl phthalate in urine ($n=6$) was 2480 $\mu\text{g/mL}$. In animals treated with 250 mg/kg-day, the mean concentration of total monobutyl phthalate in urine ($n=6$) was 2022 $\mu\text{g/mL}$. In animals treated with 100 mg/kg-day, the mean concentration of total monobutyl phthalate in amniotic fluid was 1.4 $\mu\text{g/mL}$. In animals treated with 250 mg/kg-day, the mean concentration of total monobutyl phthalate in amniotic fluid was 13.4 $\mu\text{g/mL}$.

3.5. PHYSIOLOGICALLY-BASED TOXICOKINETIC MODELS

Keys et al. (2000) developed a physiologically based pharmacokinetic (PBPK) model for dibutyl phthalate in adult rats. The model was fit to data obtained by NIEHS (1995, 1994). The hydrolysis of dibutyl phthalate in the small intestine is simulated by a first order rate constant. Absorption of monobutyl phthalate from the small intestine is also simulated with a first order rate constant. Elimination of monobutyl phthalate is simulated with a single Michaelis-Menton function representing all combined pathways. A number of different model structures were fit to the data. The model providing the best fit to the data was a combined diffusion-limited and pH trapping model without enterohepatic circulation. Details of the model are not presented because the model lacks a fetal compartment.

Kremer et al. (2005a, 2004 abstracts only) are developing a PBPK model for dibutyl phthalate in pregnant rats that includes compartments for the placenta, the fetus, and amniotic fluid. The model was fit to data obtained by Fennell et al. (2004) and Kremer et al. (2005a). The model has a flow-limited structure. Hydrolysis of dibutyl phthalate to monobutyl phthalate in the GI tract is simulated as a Michaelis-Menton function. Uptake of monobutyl phthalate from the small intestine is simulated with a first order rate constant. Elimination of monobutyl phthalate is through metabolism to the glucuronide conjugate in the liver following Michaelis-Menton kinetics and excretion of the conjugate in the urine. A sensitivity analysis indicated that for the dam the uptake rate constant from the GI compartment and the diffusion constant into slowly perfused tissues are the variables that have the largest effect on model predictions; and that for the fetus the rate of diffusion from the placenta to the fetus and the placenta-to-blood partition coefficient are the variables that have the largest effect on model predictions. Quantitative data from the model are not reported in the abstract.

Clewell and Andersen (2004, abstract only) are developing a model describing the concentration of testosterone, dihydrotestosterone, and estradiol in the perinatal rat. This model is relevant to dibutyl phthalate as the mode of action for the malformations of the male reproductive tract are mediated by decreases in testosterone and dihydrotestosterone in the fetal testis. The long term objective is to link this model with the model for dibutyl phthalate in pregnant rats (Clewell et al., 2006; Kremer et al., 2005a, 2004).

Clewell et al. (2006, abstract only) are developing a model that combines a previous model for adult male rats (Keys et al., 2000) and pregnant female rats (Kremer et al. 2005a,

2004). The combined model uses a common description of in vivo kinetics and life-stage specific physiology. To enhance the utility for extrapolation to humans, the model has descriptions for potential biomarkers from each major metabolite route (hydrolysis, glucuronidation, and hydroxylation) and enterohepatic recirculation, urinary and fecal excretion, and placental transfer. The model successfully describes biliary and fecal excretion (50% and 9% of the dose, respectively), and dibutyl phthalate, monobutyl phthalate, and monobutyl phthalate glucuronide in serum and urine after iv dosing with dibutyl phthalate. The model also reproduces serum monobutyl phthalate after oral dosing with monobutyl phthalate and dibutyl phthalate. The model was tested in gestation after adding prenatal growth and placental transfer. From the fits to the data, glucuronidation appears to be increased in the pregnant female. Fetal UDP-glucuronidase activity leads to increased monobutyl phthalate in maternal serum. The kinetics in both adult and fetus at gestational day 19 are dominated by cycling of metabolites between free and conjugated forms.

Although a completed physiologically based pharmacokinetic model for both the rat and human is not yet available, it might be possible to use the available pharmacokinetic data to provide an estimate of the relative exposure of the rat and human fetus to the toxicologically active metabolite, monobutyl phthalate, during the critical window for development of the male reproductive tract. Information on the internal dose at the same external exposure for the rat and human could be used to calculate a data-derived interspecies uncertainty factor. In the absence of this information, EPA would apply the default value of 10 for the interspecies uncertainty factor.

As determined from the structure of the pharmacokinetic model (Kremer et al., 2005a, 2004), the important factors that determine exposure to the fetus include: hydrolysis of dibutyl phthalate to monobutyl phthalate in the intestinal lumen (H), absorption of the monobutyl phthalate across the lining of the GI tract and delivery to the liver (A), clearance of monobutyl phthalate (C), and diffusion of monobutyl phthalate from the maternal circulation across the placenta into the fetus (D). Each of these determinants of exposure is discussed below and a bounding estimate is provided for the relative exposure of the rat and human fetus. A formula expressing the relative exposure (E) of the rat and human fetus (with the trailing subscript designating the species) is:

$$E_R/E_H = [H_R/H_H] \times [A_R/A_H] \times [C_R/C_H] \times [D_R/D_H]$$

Hydrolysis of dibutyl phthalate and absorption of monobutyl phthalate

In rats dibutyl phthalate is completely hydrolyzed to monobutyl phthalate in the lumen of the intestine and is rapidly and completely absorbed into the systemic circulation (Fennell et al., 2004; Saillenfait et al., 1998; NIEHS, 1995, 1994; Takahashi and Tanaka, 1989; White et al., 1983, 1980; Kaneshima et al., 1978a,b; Tanaka et al., 1978; Rowland et al., 1977; Williams and Blanchfield, 1975). The time to achieve maximum concentration in the maternal circulation for the rat is 0.5 to 1.4 hours (Kremer et al., 2005a; Fennell et al., 2004; Saillenfait et al., 1998). An in vitro study shows a species similarity in amount and rate of hydrolysis of dibutyl phthalate between the rat, baboon, ferret, and human (Lake et al., 1977). In a human study, Anderson et al. (2001) showed that 69% of a known exposure to dibutyl phthalate appeared in urine within 24 hours. These data are comparable to what is observed in rats (52% from Foster et al., 1983 and 88% from Tanaka et al., 1978) and support the conclusion that complete hydrolysis of dibutyl phthalate and complete absorption of monobutyl phthalate also occur in the human. The resulting values for both hydrolysis (H) and absorption (A) for the rat and human are 1.

Clearance of monobutyl phthalate

Total clearance of monobutyl phthalate is due to any clearance of monobutyl phthalate by the kidney and metabolism to toxicologically inactive species. The free monobutyl phthalate concentration in blood (C_{free} in mg/L) at steady state will follow the equation:

$$C_{\text{free}} = D / (KC * v_{\text{free}})$$

where D is the exposure in mg/kg per day, KC is the clearance rate (1/day), v_{free} is the fractional volume of distribution (L/kg), and $(KC * v_{\text{free}})$ is a blood clearance rate. The rat-to-human ratio for the concentration at the same exposure is therefore

$$C_{\text{free}_R} / C_{\text{free}_H} = (KC * v_{\text{free}})_H / (KC * v_{\text{free}})_R.$$

Previously a difference in the amount of free monobutyl phthalate versus monobutyl phthalate glucuronide in urine was proposed as an explanation of the differential species sensitivity of rats and hamsters (Foster et al., 1983). The free monobutyl phthalate and monobutyl phthalate glucuronide have both been measured in rat and human serum. Rats have greater relative amounts of free monobutyl phthalate (Kremer et al., 2005a, 2005b; Fennell et al., 2004; Saillenfait et al., 1998) and humans typically have greater relative amounts of monobutyl phthalate glucuronide, albeit with significant variability (Silva et al. 2003). However, the ratio

of free monobutyl phthalate to monobutyl phthalate glucuronide depends on the clearance rate of both compounds from the blood, while as shown above, the clearance of free monobutyl phthalate from blood alone is the quantity of interest. Sufficient pharmacokinetic data is available to estimate clearance parameters from blood in the rat for both free monobutyl phthalate and monobutyl phthalate glucuronide (Kremer et al. 2005b; Fennell et al. 2004; Saillenfait et al., 1998). However, the only direct pharmacokinetic data available in humans is an upper bound on the half-life of total monobutyl phthalate (free and glucuronidated) reported in Anderson et al. (2001). In particular, Anderson et al. (2001) reported that no urinary monobutyl phthalate (either free or glucuronidated) was detected more than 24 hours after a bolus dose of dibutyl phthalate. This information is insufficient to specify the clearance rate of free monobutyl phthalate ($KC \cdot v_{\text{free}}$) from blood in the human because (1) the half-life reflects the clearance rate of the rate-limiting process, not necessarily just clearance of free monobutyl phthalate; and (2) the blood clearance rate also depends on the volume of distribution (v_{free}), which cannot be accurately estimated in humans given current data.

In addition, data from Silva et al. (2003) and Anderson et al. (2001) appear to imply a very small volume of distribution in humans for both free monobutyl phthalate and monobutyl phthalate glucuronide, in apparent conflict with the extensive distribution in human tissues suggested by reports of monobutyl phthalate being found in human saliva, breast milk, and amniotic fluid (Silva et al., 2005; Calafat et al., 2004, 2006; Silva et al., 2004). This can be illustrated by considering that the median serum level of total monobutyl phthalate was 14.4 $\mu\text{g/L}$ with 25 - 30% of that being free monobutyl phthalate (Silva et al., 2003). EPA calculated the concentration of free monobutyl phthalate to be 4.2 $\mu\text{g/L}$ from these data. Assuming that a 70 kg adult has about 3.5 L of serum, 50.4 μg of total monobutyl phthalate is in serum (14.4 $\mu\text{g/L} \times 3.5 \text{ L}$). The corresponding value for free monobutyl phthalate is 14.7 μg in serum (4.2 $\mu\text{g/L} \times 3.5 \text{ L}$). The daily exposure in humans estimated from the urinary excretion data is approximately 1.1 $\mu\text{g/kg-day}$ or 77 μg for a 70 kg adult. (See Section 3.6.) Thus, these data show that approximately 65% of the daily dose (50.4/77) remains in the serum as total monobutyl phthalate, and 19% of the daily dose (14.7/77) remains in the serum as free monobutyl phthalate, suggesting that diffusion of both monobutyl phthalate and its glucuronide are restricted in the human, perhaps due to binding to serum proteins that are not present in rats. As noted previously, it is possible that some of the monobutyl phthalate in human tissues results from esterase activity on dibutyl phthalate present as a contaminant from laboratory ware. It is possible that there is a systematic error in the data of Silva et al. (2003) or Anderson et al. (2001). Neither data sets have been confirmed by an independent investigation, and quantitative

estimates (e.g., based on pharmacokinetic data) of the effective volume of distribution in humans for monobutyl phthalate or its glucuronide have yet to be reported.

In summary, the data are insufficient data to estimate with confidence the relative clearance of monobutyl phthalate in rats and humans.

Diffusion of monobutyl phthalate across the placenta into the fetus

The determinants of the concentration of monobutyl phthalate in the fetus are the concentration on monobutyl phthalate in the maternal circulation and the partition coefficient for monobutyl phthalate across the placenta (Kremer et al., 2005a, 2004). These abstracts do not present a value for the placenta: blood partition coefficient. As the placenta is a richly perfused tissue, the rat liver: blood partition coefficient of 1.9 (Keys et al., 2000) is used. No data are available to estimate a placenta: blood partition coefficient in humans. For other chemicals with PBPK models with a fetal compartment, reported values for the human placenta: blood partition coefficient are nearly equal to or slightly exceed those for the rat (Gentry et al., 2002; Gargas et al., 2000a,b). In the boron assessment, EPA tacitly assumed the placenta: blood partition coefficients for the rat and human were equal (U.S. EPA, 2004). For monobutyl phthalate, the potential range for the placenta: blood partition coefficient in humans is taken as 1.9 to 2.8 (1 to 1.5 times the value for the rat).

Glucuronidation of monobutyl phthalate occurs readily in the adult liver but the activity in the fetal liver is limited. The development of UDP glucuronyl transferase activity for different substrates in the developing rat liver is known to occur during late gestation (late fetal group, [GD] 16-20) or around birth (neonatal group, day 2 following birth) (Wishart, 1978). The life stage that would contain the isoform that metabolizes monobutyl phthalate is not known. Calafat et al. (2006) found only a small amount of monobutyl phthalate glucuronide in rat amniotic fluid on GD 18 when pregnant rats were exposed on GD 17. However, Fennel et al. (2004) and Kremer et al. (2005b) did find monobutyl phthalate glucuronide in the plasma of the fetal rat following exposure on GD 20 or GD 19, indicating that late in gestation the UDP-glucuronide transferase is being expressed in the fetal rat liver. However, during the critical window for development of the male reproductive tract in the rat (GD 16 - 18, Carruthers and Foster, 2005), the rat fetus will have only limited ability to metabolize monobutyl phthalate to the glucuronide. Data indicate that the human fetus does not have the capability of glucuronidation (U.S. EPA, 2001). Therefore, it is logical to assume that the relative exposure to monobutyl phthalate in the rat and human fetus will be primarily determined by the

concentration of monobutyl phthalate in the maternal circulation and the placenta: blood partition coefficient.

In rats the critical window for the development of the male reproductive tract is GD 16–18 (Carruthers and Foster, 2005). In humans the critical window for the development of the male reproductive tract is the late first trimester. Gentry et al. (2003) conducted an evaluation using developed PBPK models of the potential changes in tissue dosimetry in offspring during pregnancy with a number of chemicals that are not metabolized in the fetus. The modeling took into account fetal growth. The results showed that the concentration of chemical in the fetal blood remained nearly constant across the full nine months of gestation.

Data show that the depletion of monobutyl phthalate is slower from the fetal compartment than from the maternal compartment. Following a single exposure on GD 20, Fennell et al. (2004) reported a half time for elimination of monobutyl phthalate from the plasma of the full term rat fetus of 4.20 hours; the comparable value for the maternal plasma was 2.87 hours. Kremer et al. (2005a) did not report quantitative data on elimination rate following exposure from GD 12–19, but inspection of the plots presented in the poster session shows a slower depletion from the fetus compared to the dam. There are no data to estimate the relative rates of depletion from the fetal and maternal compartments in the human. It is expected, however, that a similar slower rate of depletion of monobutyl phthalate from the fetal compartment will occur in the human.

Conclusion

Because of the limited quantitative understanding of the clearance of monobutyl phthalate in humans, EPA concludes that the current data are not sufficiently robust to support changing the default value of the pharmacokinetic portion of the interspecies uncertainty factor. Quantitative data on the effective whole body volume of distribution of monobutyl phthalate and its glucuronide, independent replication of the human Silva et al. (2003) and Anderson et al. (2001) human studies, and human pharmacokinetic data than included blood time-courses would all be of great utility in reducing these uncertainties.

3.6. ENVIRONMENTAL EXPOSURE TO HUMANS

Because dibutyl phthalate is used in a wide variety of consumer products, exposure to humans is ubiquitous. All of the studies reported below used techniques that measured total

monobutyl phthalate (free monobutyl phthalate plus its glucuronide conjugate) by first treating the sample with glucuronidase.

Blount et al. (2000) reported the concentration of total monobutyl phthalate in urine from 289 randomly selected individuals from the United States. The urine samples were collected from adults during 1988 - 1994 as part of the Third National Health and Nutrition Examination Survey (NHANES III). This sampling from the NHANES III population was not designed to be representative of the US population. When normalized to creatinine concentration in the urine, the 5th, 50th, and 95th percentiles were 9.3, 33.4, and 162 µg/gram creatinine, respectively, with a geometric mean of 36.9 µg/gram creatinine and a maximum of 2760 µg/gram creatinine. In this study 10 women of reproductive age had values >300 µg/gram creatinine. Hoppin et al. (2002) presented data on the concentration of total monobutyl phthalate in urine from 46 African-American women residing in Washington, DC. The mean value was 52.7 µg/gram creatinine, with a median value of 43.4 µg/gram creatinine and a maximum value of 157.3 µg/gram creatinine. Adibi et al. (2003) reported on total monobutyl phthalate values in urine among 25 residents of New York City. The median was 42.6 µg/gram creatinine; the mean was 54.4 ±24.5 µg/gram creatinine with a range of 21.3-105 µg/gram creatinine. Hauser et al. (2004) report a mean of 13.6 µg/L and a 95th percentile of 73.1 µg/L for 369 US men (not creatinine adjusted). In a German population of 85 people, Koch et al. (2003) report a mean of 195 µg/g creatinine and a 95th percentile of 531µg/g creatinine.

Additional data are available on the concentration of total monobutyl phthalate in urine from the NHANES samples collected in 1999-2000 and 2001-2002 (Silva et al., 2004a; DHHS, 2005). Selected data from these studies are reported in Table 3-5. For each age group, the data are reported as males and females combined. For survey years 1999-2000, the concentrations of mono-isobutyl phthalate and mono-n-butyl phthalate were measured together and expressed as a combined value. For survey years 2001-2002, mono-n-butyl phthalate levels were measured separately and only mono-butyl phthalate levels are given in the table. The concentrations of total monobutyl phthalate in urine in these studies are lower than those reported from NHANES III (Blount et al., 2000).

**Table 3-5. Total Monobutyl Phthalate in urine from NHANES data (DHHS, 2005)
concentration (µg/L)**

Group	Survey years	Geometric Mean	50 th Percentile	95 th Percentile	Sample Size
Total, age 6 and older	99-00	24.6	26.0	149	2541
	01-02	18.9	20.4	108	2782
6 - 11 years	99-00	41.4	40.0	163	328
	01-02	31.1	32.4	157	393
12 - 19 years	99-00	36.0	36.1	165	752
	01-02	25.1	26.4	147	742
> 20 years	99-00	21.6	23.0	142	1461
	01-02	17.0	19.1	95.4	1647
Males	99-00	22.0	23.1	115	1215
	01-02	17.7	19.3	95.2	1371
Females	99-00	27.3	30.0	167	1326
	01-02	20.2	21.6	120	1411

Creatinine adjusted (µg/gram)

Group	Survey years	Geometric mean	50 th Percentile	95 th Percentile	Sample Size
Total, age 6 and older	99-00	22.4	21.9	97.5	2541
	01-02	17.8	17.4	81.3	2772
6 - 11 years	99-00	41.9	38.9	159	328
	01-02	35.5	35.1	146	392
12 - 19 years	99-00	24.3	23.6	88.1	752
	01-02	19.4	20.3	88.6	742
> 20 years	99-00	20.4	19.5	91.0	1461
	01-02	16.1	15.4	71.6	1638
Males	99-00	17.3	17.0	63.6	1215
	01-02	14.4	13.7	60.0	1367
Females	99-00	28.6	28.6	131	1326
	01-02	21.7	21.5	91.5	1405

Several researchers have used data on the urinary excretion of total monobutyl phthalate and a pharmacokinetic calculation to estimate daily exposure to dibutyl phthalate (Koch et al., 2003; Koo et al., 2002; David, 2000; Kohn et al., 2000). Kohn et al. (2000) and Koo et al. (2002) used a linear two-compartment model and estimates of total elimination of monobutyl phthalate and fractional elimination of monobutyl phthalate in the urine in their calculation. Data on total elimination (94% from Tanaka et al., 1978) and fractional elimination in urine (52% from Foster et al., 1983) were derived from studies in rats. David (2000) and Koch et al. (2003) used a simplified formula but relied on the fractional elimination in urine in humans of 69% derived by Anderson et al. (2001) from a measured human exposure to dibutyl phthalate. David (2000), Kohn et al. (2000), and Koo et al. (2002) used the urinary excretion data for the US population (Blount et al., 2000) which included a total population of 289 individuals including 97 women of child bearing age (20-40 years). Koch et al. (2003) relied on urinary excretion data for a German population. David (2000) estimated a geometric mean, 95th percentile, and highest value of 1.56, 6.87, and 116.96 $\mu\text{g}/\text{kg}\text{-day}$, respectively. Kohn et al. (2000) estimated a median, 95th percentile, and maximum of 1.4, 6.5, and 50 $\mu\text{g}/\text{kg}\text{-day}$, respectively, for the general population; and 1.7, 32, and 113 $\mu\text{g}/\text{kg}\text{-day}$, respectively, for women 20–40 years of age. Koo et al. (2002) reported a mean, median, 5th percentile and 95th percentile of 1.66, 1.66, 0.31, and 8.78 $\mu\text{g}/\text{kg}\text{-day}$, respectively. Koch et al. (2003) reported a median and 95th percentile exposure of 5.2 and 16.2 $\mu\text{g}/\text{kg}\text{-day}$, respectively for the German population (n= 85).

Although a study (Kohn et al., 2000) using data from Blount et al. (2000) estimated a higher exposure to women of child-bearing age, an analysis by Silva et al. (2004a) using data from NHANES 1999-2000 did not show that women of child-bearing age have a higher concentrations of monobutyl phthalate in their urine.

4. HAZARD IDENTIFICATION

4.1. STUDIES IN HUMANS

Reports of declining sperm counts over the past 50 years have prompted several researchers to conduct studies on the correlation of decreased sperm count with exposure to phthalate esters. Murature et al. (1987) obtained data from 21 university student volunteers on mean sperm density and sperm concentration relative to the concentration of dibutyl phthalate in semen. When the concentration of dibutyl phthalate in the cellular fraction of ejaculates was plotted against either sperm density or total number of sperm for the same ejaculates, there appeared to be a decrease in mean sperm density with increasing concentration of dibutyl phthalate in the semen. The authors did not present a statistical analysis of these data. Exposure to other chemicals and demographic confounders were not evaluated. However, because monobutyl phthalate, rather than dibutyl phthalate, is the metabolite of concern, the significance of this finding is unclear.

Duty et al. (2003a) recruited 168 men who were part of subfertile couples and who presented to the Massachusetts General Hospital andrology laboratory for semen analysis between January 2000 and April 2001. This was a cross sectional study in which semen and urine samples were collected from each subject on the same day as part of an infertility work-up. Semen parameters were categorized based on 1999 World Health Organization (WHO) reference values for sperm concentration (<20 million/ml) and motility (<50% motile), as well as Tygerberg strict criteria for morphology (<4% normal). The comparison group was men (n = 77) for whom these semen parameters were all above the reference values. The concentration of eight phthalate monoesters was measured in a single spot urine sample with high-performance liquid chromatography and tandem mass spectrometry. Exposure to chemicals other than phthalate esters was not evaluated. Specific gravity-adjusted phthalate monoester levels were subdivided into tertiles (0–11.64, 12.24–20.13, and 20.16–433.93 ng/mL). There was a statistically significant relationship between tertiles of urinary monobutyl phthalate and decreased sperm motility (odds ratio per tertile = 1.0, 1.8 [95% CI 0.7–4.6], 3.0 [95% CI 1.2–7.6]; p-value for trend 0.02; n = 15, 21, or 27 in each tertile, respectively). There was a suggestive relationship between tertiles of urinary monobutyl phthalate and decreased sperm concentration (odds ratio per tertile = 1.0, 1.4 [95% CI 0.3–6.0], 3.3 [95% CI 0.9–12.6]; p-value for trend 0.07; n = 6, 6, or 10 for each tertile, respectively). There was also a statistically significant relationship for monobenzyl phthalate with sperm concentration. The authors pointed

out that a strength of the study included a reliable biomarker of exposure (phthalate monoesters in urine) rather than self-reported exposures, but a weakness in that the phthalate levels were based on a single spot urine sample from a limited number of subjects. The authors further cautioned that the results were also based on a single sperm analysis and that until the results are replicated in larger and more diverse populations, the consistency and wider relevance of the results remains unclear.

Duty et al. (2003b) further analyzed the integrity of the DNA in the sperm samples using the neutral single-cell microgel electrophoresis assay (comet assay). The concentration of monobutyl phthalate in the semen sample was not significantly associated with DNA fragmentation patterns as observed in the comet assay.

Duty et al. (2004) explored whether phthalates were associated with altered sperm movement characteristics. Two-hundred twenty subjects (male partners of a subfertile couple between 20 and 54 years of age and who presented to an andrology laboratory between January 2000 and October 2001) provided a semen sample for computer-aided sperm analysis (CASA) and a urine sample for measurement of phthalate monoesters (monoethyl phthalate, monobenzyl phthalate, monobutyl phthalate, mono-2-ethylhexyl phthalate, and monomethyl phthalate). Only data for monobutyl phthalate are reported here. Three CASA parameters, straight-line velocity (VSL), curvilinear velocity (VCL), and linearity (LIN), were used as measures of sperm progression, sperm vigor, and swimming pattern, respectively. As stated by the authors, there was a “suggestive negative” dose-response relationship (shown as the predicted change in mean sperm motion parameter for the second and third tertiles compared with the first tertile; p value for trend) for MBP and VSL ($-3.07 \mu\text{m/s}$, $-2.87 \mu\text{m/s}$; $p = 0.08$) and VCL ($-3.25 \mu\text{m/s}$, $-3.46 \mu\text{m/s}$; $p = 0.2$).

Swan et al. (2005) investigated anogenital distance (AGD) and other genital measurements in male infants and prenatal exposure to phthalates. A standardized measure of AGD was obtained from boys 2–30 months of age ($n = 134$). AGD was significantly correlated with penile volume ($r^2 = 0.24$, $p=0.005$) and the proportion of boys with incomplete testicular descent ($r^2 = 0.23$, $p=0.007$). The researchers defined the anogenital index (AGI) as the AGD divided by body weight (kg) at examination and calculated the age-adjusted AGI by regression analysis. Nine phthalate monoester metabolites were measured in a single sample of the mother’s prenatal urine ($n = 85$, taken at a mean gestation time of 28.3 weeks) and were examined as predictors of age-adjusted AGI in regression and categorical analyses. An

individual mother's urine sample was matched with the age-adjusted AGI for the individual boy. Exposure to other chemicals was not investigated. The urinary phthalate monoesters that were inversely related to age-adjusted AGI included monoethyl phthalate ($p=0.012$), monoisobutyl phthalate ($p=0.014$), monobutyl phthalate ($p=0.023$), and monobenzyl phthalate ($p=0.05$). Odds ratios were calculated for shorter than expected age-adjusted AGI with the concentration of phthalate monoester in the mother's urine. The exposure groups were low ($<25^{\text{th}}$ percentile), medium ($\geq 25^{\text{th}}$ to $<75^{\text{th}}$ percentile), and high ($\geq 75^{\text{th}}$ percentile). The reported odds ratios for monoethyl phthalate were 1.0, 2.6 [95% CI, 0.9–7.8], and 4.7 [95% CI, 1.2–7.4]; for monoisobutyl phthalate were 1.0, 2.5 [95% CI, 0.8–7.4], and 7.3 [95% CI, 1.9–27.9]; for monobutyl phthalate were 1.0, 3.8 [95% CI, 1.2–12.3], and 10.2 [95% CI, 2.5–42.2]; and for monobenzyl phthalate were 1.0, 3.1 [95% CI, 1.001–9.8], and 3.8 [95% CI, 1.03–13.9]. The concentration of monobutyl phthalate in prenatal urine associated with short AGI in this study (50th percentile = 24.5 $\mu\text{g/L}$ and 75th percentile = 44.8 $\mu\text{g/L}$) are comparable to the concentration of monobutyl phthalate reported for women in NHANES 1999-2000 (50th percentile = 30.0 $\mu\text{g/L}$ and 75th percentile = 59.5 $\mu\text{g/L}$) and NHANES 2001-2002 (50th percentile = 21.6 $\mu\text{g/L}$ and 75th percentile = 46.7 $\mu\text{g/L}$) (DHHS, 2005).

The associations between male genital development and phthalate exposure reported by Swan et al. (2005) are consistent with the effects found by others in prenatal rodents following oral exposure to dibutyl phthalate and monobutyl phthalate (see data reported in Section 4.3.1 and Section 4.3.2 following) and support the hypothesis that prenatal exposure to phthalates at environmental levels can affect male reproductive development in humans. However, as pointed out by the authors, Swan et al. (2005) has a number of limitations. The analysis is based on a single measurement of AGD in boys and a single measurement of phthalate esters in maternal urine. Although the AGD is a standard measure in the rodent developmental bioassay, the reliability of this measurement in humans has not been established. Use of the measurement of AGD in larger studies in a range of diverse human populations will be needed to obtain normative data. The measurement of AGD in this study was not conducted in infants of the same age as is done in the standard rodent developmental bioassay. The optimal timing for measurement of AGD in boys has not been established. In an attempt to provide a standard measure for comparison in boys of different ages, the measured AGD was normalized to body weight to give the AGI. The reliability of the AGI has not been established for humans. The maternal urine samples were collected late in pregnancy (mean = 28.3 weeks) and the measured phthalate metabolite levels may not reflect exposure during the most sensitive developmental window in the male fetus. Finally, the mothers were exposed to multiple phthalates at detectable

levels.

Additional limitations on Swan et al. (2005) were discussed in a commentary by Sharpe (2005). Sharpe points out that Swan et al. (2005) show an association between maternal exposure to phthalates and AGI in boys, but they do not show that one caused the other or that exposure to phthalates caused reduced production of testosterone. They also do not show that exposure to phthalates caused abnormalities as all the boys were “normal” at the time of examination. Sharp (2005) also points out that the findings need to be confirmed independently as the association with AGI with maternal exposure to phthalates could be fortuitous. There could be other lifestyle factors that cause a woman to be exposed to phthalates that might themselves cause the reduction in AGI or reduced testosterone in the fetus.

Main et al. (2006) investigated whether phthalate monoester contamination of human breast milk had any influence on the postnatal surge of reproductive hormones in newborn boys as a sign of testicular dysgenesis. Main et al. (2006) obtained biologic samples from 1997 to 2001 from a prospective Danish-Finnish cohort study on cryptorchidism. They analyzed individual breast milk samples collected as additive aliquots postnatally (n = 130; 62 cryptorchid and 68 healthy boys) for phthalate monoesters [mono-methyl phthalate, mono-ethyl phthalate, mono-n-butyl phthalate, mono-benzyl phthalate, mono-2-ethylhexyl phthalate, and mono-isononyl phthalate]. They analyzed serum samples (obtained in 74% of all boys) for gonadotropins, sex-hormone binding globulin, testosterone, and inhibin B. All phthalate monoesters were found in breast milk with large variations. The median, minimum, and maximum values for monobutyl phthalate were 9.6, 0.6, and 10,900 µg/L, respectively. Values for the other phthalate monoesters are not reported here. No association was found between phthalate monoester levels and cryptorchidism. However, mono-ethyl phthalate and monobutyl phthalate showed positive correlations with sex-hormone binding globulin ($r = 0.323$, $p = 0.002$ and $r = 0.272$, $p = 0.01$, respectively); mono-methyl phthalate, mono-ethyl phthalate, and monobutyl phthalate with the luteinizing hormone:free testosterone ratio ($r = 0.21-0.323$, $p = 0.002-0.044$); and mono-isononyl phthalate with luteinizing hormone ($r = 0.243$, $p = 0.019$). Monobutyl phthalate was negatively correlated with free testosterone ($r = -0.22$, $p = 0.033$). Other phthalate monoesters showed similar but non-significant tendencies. These data show concordance with rodent data showing a reduced concentration of testosterone with increasing perinatal exposure to some phthalates.

4.2. PRECHRONIC AND CHRONIC STUDIES IN ANIMALS

Single Exposure

Dibutyl phthalate is not very acutely toxic to rodents. Reported LD₅₀ values following oral administration to rats range from 8000 mg/kg to at least 20,000 mg/kg (WHO, 1997). In mice LD₅₀ values are 5000–16,000 mg/kg (WHO, 1997). Reported LD₅₀ values following intraperitoneal administration are 4000–7000 mg/kg in rats and 3000–6000 mg/kg in mice (WHO, 1997). The dermal LD₅₀ in rabbits is >4000 mg/kg (WHO, 1997). Signs of toxicity include general depression of activity, labored breathing, and lack of coordination.

Following intraperitoneal injection, monobutyl phthalate is more toxic than dibutyl phthalate. The LD₅₀ was 1000 mg/kg in the mouse (WHO, 1997).

Fukuoka et al. (1990) studied biochemical changes associated with dibutyl phthalate induced testicular atrophy. Male Wistar rats (n = 27 in each group) were given a single oral dose of 0 or 2400 mg/kg. Three rats/group were killed after 3, 6, or 12 hours, or 1, 2, 3, 4, 5, or 7 days later, and the testes were removed and weighed. The left testis was examined for histopathological changes. The right testis was analyzed for cholesterol, fructose, glucose, inositol, lactic acid, and sorbitol concentrations and the activities of aldose reductase, sorbitol dehydrogenase, succinate dehydrogenase, acid phosphatase, alkaline phosphatase, aspartate aminotransferase, γ -glutamyl transferase, lactate dehydrogenase, lactate dehydrogenase isozymes, and leucine aminopeptidase activity.

Absolute and relative testicular weights were decreased at 3 hours and 3, 4, 5, and 7 days. The maximum decrease of absolute weight of 30% and relative weight of 46% was observed after 7 days. Testicular atrophy developed by day three; however, sloughing of germ cells was seen as early as 6 hours after dosing. The sloughing was characterized by dissociation of mature germ cells, such as spermatozoa, spermatids, and spermatocytes, from the germinal epithelium of seminiferous tubules. Dibutyl phthalate induced transient increases in alkaline phosphatase and leucine aminopeptidase activity at 6 and 12 hours, respectively. γ -Glutamyl transferase and lactate dehydrogenase showed sustained increases starting at 3 and 12 hours, respectively. Aldose reductase activity was decreased starting at 6 hours, which was followed by decreases in sorbitol dehydrogenase and succinate dehydrogenase on day two. All lactate dehydrogenase isozyme activities except isozyme 2 were increased. Lactate dehydrogenase isozymes 4 and 5 showed the earliest increases, starting at 6 hours post exposure. Sorbitol and fructose

concentrations were decreased 3 and 12 hours after treatment, respectively. Inositol, lactic acid, and cholesterol concentrations were increased after 6, 12, and 24 hours, respectively. The authors suggest that dibutyl phthalate induced testicular atrophy is associated with disturbances in enzymes that are linked to Sertoli cell functions that are required for germ cell maturation. The lowest-observed-adverse-effect level (LOAEL) is 2400 mg/kg-day, the only exposure tested.

Zhou et al. (1990) investigated the mechanisms of dibutyl phthalate induced sloughing of germ cells in 18 animals, enzyme activities in the testicular cells of 30 animals, and changes in testicular levels of metal ions in 42 animals. Adult male Wistar rats were administered by gavage a single oral dose of 2400 mg/kg. Rats were killed up to 168 hours after dosing. Testes showed sloughing of the germ cells in treated rats at 6 hours with more severe sloughing at 24 and 48 hours after dosing. Monobutyl phthalate reached peak concentration in the testis 3 hours after oral dosing. However, the peak concentration of monobutyl phthalate in the Sertoli cells occurred earlier. The interstitial cells showed a decrease in the activity of succinate dehydrogenase at 3 hours ($p < 0.05$) and an increase in the activity of γ -glutamyl transferase at 6 hours ($p < 0.05$). The Sertoli cells showed decreases in the activity of succinate dehydrogenase at 3 hours ($p < 0.05$). The germ cells showed a decrease in the activity of sorbitol dehydrogenase at 3 and 6 hours ($p < 0.05$) and increases in the activities of lactate dehydrogenase at 3 and 6 hours ($p < 0.05$) and alkaline phosphatase at 6 hours ($p < 0.05$). There was also a decrease in the iron levels in testes. The LOAEL is 2400 mg/kg-day, the only exposure tested.

Short-term Exposure

Fukuoka et al. (1989) treated male Wistar rats ($n = 28$ in each group) with 0 or 2400 mg/kg-day by gavage for 7 days. Rats were killed at various times during and up to 96 hours after treatment ended. The testes were removed and weighed. The left testis was examined for histopathological changes. The right testis was homogenized and the homogenates were assayed for phospholipids, triacylglycerols, cholesterol, glucose, fructose, galactose, inositol, aldose reductase, zinc, iron, and sorbitol dehydrogenase. Blood samples were collected and assayed for fructose, glucose, and inositol. Dibutyl phthalate caused slight sloughing of germ cells in the seminiferous tubules after 24 hours. Severe sloughing was observed at 48 hours. The germ cells almost completely disappeared from the germinal epithelium after 5 and 7 days with only Sertoli cells left in the germinal epithelium. Testicular fructose and glucose concentrations were decreased after 24 hours ($p < 0.05$) and not detectable at 48 hours and longer. Testicular zinc and iron concentrations were decreased and inositol and cholesterol were increased after 48 hours. Testicular triacylglycerols, cholesterol, and phospholipids containing choline and ethanolamine

residues were decreased at later times. Sorbitol dehydrogenase activity was significantly elevated after 24 hours ($p < 0.05$) and significantly decreased after 5 and 7 days ($p < 0.05$). Blood fructose, glucose, and inositol concentrations were not significantly affected at 24 hours. The authors concluded that dibutyl phthalate causes sloughing of germ cells from seminiferous tubules leaving only Sertoli cells. The decreases in glucose and fructose concentration suggest that dibutyl phthalate may disturb an interaction between Sertoli cells and germ cells. The LOAEL is 2400 mg/kg-day, the only exposure tested.

NTP (1991) conducted a 14-day range finding study in CD Sprague-Dawley rats ($n = 8$ per sex per group, 10 weeks of age). Dibutyl phthalate was administered in the feed at 0, 1000, 5000, 10,000, 15,000, or 20,000 ppm (exposures in males were 0, 70, 340, 650, 910, or 1190 mg/kg-day and in females were 0, 70, 350, 700, 930, or 1150 mg/kg-day). The animals were monitored for mortality, clinical signs of toxicity, food consumption, and body weight. No animals died and there were no clinical signs of toxicity reported during the study. Food consumption over the 14-day study was decreased at the two highest exposures (19 and 21% in males and 13 and 18% in females, respectively). Body weights were reduced in males at the two highest exposures (10 and 8%, respectively) and in females at the three highest exposures (10, 7, and 12%, respectively). These results were used to select the exposures of 0, 1000, 5000, or 10,000 ppm in the continuous breeding study (NTP, 1995).

Srivastava et al. (1990) administered dibutyl phthalate to young male Wistar rats (5 weeks old, $n = 6$ in each group) by gavage in peanut oil at 0, 250, 500, or 1000 mg/kg-day for 15 days. A significant decrease in testis weight was observed at 500 and 1000 mg/kg-day (64 and 48%, respectively). Histopathological examination revealed degeneration of seminiferous tubules at all exposures (5, 20, and 70% at the low, mid, and high exposure, respectively). The activities of testicular enzymes associated with postmeiotic spermatogenic cells, such as sorbitol dehydrogenase and acid phosphatase, were decreased significantly at 500 and 1000 mg/kg-day (17 and 26%, respectively, for sorbitol dehydrogenase and 25 and 36%, respectively, for acid phosphatase), while that of lactate dehydrogenase was significantly increased at all exposures (16, 25, and 48%, respectively). The activities of enzymes associated with premeiotic spermatogenic cells, Sertoli cells or interstitial cells, β -glucuronidase, γ -glutamyl transpeptidase, and glucose-6-phosphate dehydrogenase were significantly increased at all exposures (β -glucuronidase 22, 39, and 74%, respectively; γ -glutamyl transpeptidase 26, 40, and 67%, respectively; glucose-6-phosphate dehydrogenase 32, 39, and 52%, respectively). The LOAEL is 250 mg/kg-day, the lowest exposure tested.

Walseth and Nilsen (1986) investigated the effect of dibutyl phthalate on cytochrome P450 metabolic activity in rat liver and lung. Male Sprague-Dawley rats (250 grams, n = 5 per group) were treated with dibutyl phthalate by gavage in corn oil for 5 days at 0, 2.8, 28, or 280 mg/kg-day. In the liver there was an increase of 48% in cytochrome P450 activity at the lowest exposure and a 28% increase in cytochrome c reductase at the two lower exposures. These changes persisted after a 4 week recovery period. No effect was observed in the lung.

Inhalation studies

Walseth and Nilsen (1984) investigated the effect of inhaled dibutyl phthalate on cytochrome P-450-mediated metabolism in rat liver and lung. Sprague-Dawley rats (250 grams, n = 15 per group) were exposed 6 hours per day for 5 days to dibutyl phthalate at 0, 5.7, 28.4, or 79.5 mg/m³. No changes were observed in liver microsomal P-450 levels at any exposure. However, at the highest exposure there was a 63% reduction in the microsomal cytochrome P-450 content of the lung.

McKee et al. (2005, abstract only) conducted an inhalation study in rats with dibutyl phthalate. Rats (strain and number exposed not reported) were exposed 6 hours/day, 5 days/week, for four weeks to 0, 1, 5, 50, or 500 mg/m³. Following the final exposure, the animals were sacrificed and the respiratory tract and other key tissues were examined for histological changes. All rats survived the exposure period. The only clinical observation of note was the formation of a red crust at the snouts of some animals from the 500 mg/m³ group. There were no effects on motor activity or differences between groups in the functional observation battery. There were no effects on hematological, clinical chemistry, or urinalysis parameters. There was a significant increase in absolute (but not relative) lung weight and a reduction in absolute (but not relative) testis weight in the animals exposed at 500 mg/m³. However, as these differences were not associated with histopathological changes, the authors considered these effects incidental and not adverse.

Histopathological evaluation of the nasal cavity revealed treatment-related hyperplasia and/or hypertrophy of the mucus cells in levels II, III, and IV. In level II the effects were located in the lateral sinus; in level III in the maxillary sinus; and in level IV in the nasopharyngeal duct. The epithelium in the respective areas was regular with no infoldings and no evidence of inflammation. The incidence of mucous cell hyperplasia and hypertrophy in nasal sections, graded as minimal to slight, was 0, 2, 6, 11, and 15 in male rats and 0, 7, 13, 14, and 15 in female rats. In the larynx, treatment-related squamous cell-like metaplasia, graded as minimal, was

observed at the base of the epiglottis with an incidence of 0, 1, 3, 4, and 5 in males and 0, 1, 3, 5, and 4 in females. There were no histological changes in trachea, lungs, or mediastinal lymph nodes, and no histological findings of note in any other organs examined. As these data are reported in an abstract only, a no-observed-adverse-effect level (NOAEL) and LOAEL are not assigned for this study.

Subchronic Exposure

BASF (1992, as summarized in NTP-CERHR, 2000) conducted a 3 month study in male and female Wistar rats. Rats (10 of each sex per dose, 6 weeks old) received dibutyl phthalate in the diet at 0, 400, 2000, or 10,000 ppm (equivalent to 0, 27, 142, or 688 mg/kg-day in males and 0, 33, 162, or 816 mg/kg-day in females). A battery of hematological, clinical chemical, and urinalysis tests were conducted after approximately 45 days of exposure and at the end of the study. Cyanide insensitive palmitoyl-CoA oxidation was also determined as a measure of peroxisome proliferation. Neurological function, using the EPA functional observation battery, was assessed prior to exposure and on days 34, 59, and 90. Testes were examined for histological changes after fixing in Bouin's solution. No effects were observed on body weight, neurological function, or the testes at any exposure. There was a statistically significant increase in relative liver and kidney weights in females and an increase in palmitoyl-CoA oxidation in both males and females at the highest exposure. However, quantitative data for these effects are not reported in NTP-CERHR (2000). The NOAEL for neurological and testicular effects is 688 mg/kg-day, the highest exposure tested. The NOAEL for effects in the liver is 142 mg/kg-day and the LOAEL is 688 mg/kg-day.

NTP (1995) conducted a 13-week evaluation of the toxicity of dibutyl phthalate in male and female F344 rats. Rats (n = 10 of each sex in each group) received dibutyl phthalate in the diet at 0, 2500, 5000, 10,000, 20,000, or 40,000 ppm (equivalent to 0, 176, 359, 720, 1540, or 2964 mg/kg-day in males and 0, 177, 356, 712, 1413, or 2943 in females). No deaths occurred. Markedly reduced final mean body weights were observed in males and females in the 40,000 ppm groups (a decrease of 45 and 73%, respectively). Increases in relative liver weight were observed in males that received 5000 ppm or greater (of 18, 32, 54, and 70%, respectively) and in females that received 10,000 ppm or greater (increases of 11, 25, and 78%, respectively). Testis and epididymal weights of males in the 20,000- and 40,000-ppm groups were lower than those of the controls. Hypocholesterolemia was observed in male and female rats receiving 20,000 or 40,000 ppm, and hypotriglyceridemia was detected in males in all exposed groups and in females receiving 10,000 ppm or greater. Elevations in alkaline phosphatase activity and bile

acid concentration in male and female rats were considered indicative of cholestasis.

Morphologic evaluation confirmed the toxicity of dibutyl phthalate to the liver and testis of rats. Microscopic examination of the liver revealed hepatocellular cytoplasmic alterations, consistent with glycogen depletion, in male and female rats receiving 10,000 ppm or greater. In the liver of rats in the 40,000-ppm groups, small, fine eosinophilic granules were also observed in the cytoplasm of hepatocytes. Ultrastructural examination suggested the presence of increased numbers of peroxisomes, and peroxisomal enzyme activity (palmitoyl-CoA oxidase activity) was elevated in the livers of rats administered 5000 ppm or greater. In males, increases of 1.9-, 5.7-, 9.7-, and 13.5-fold, respectively, were observed; in females increases of 1.7-, 2.6-, 11-, and 32.5-fold, respectively, were observed. Lipofuscin accumulation was detected in rats receiving 10,000 ppm or greater.

Histopathologic examination of the testes revealed degeneration of the germinal epithelium. There was a mild to marked focal lesion in the 10,000- and 20,000-ppm groups and a marked diffuse lesion in all males in the 40,000 ppm group resulting in almost complete loss of the germinal epithelium at 40,000 ppm. Testicular zinc concentrations were lower in the 20,000- and 40,000-ppm groups than in the controls. Serum testosterone values were also lower at these concentrations than in controls. Spermatogenesis was evaluated in males in the 0-, 2500-, 10,000-, and 20,000-ppm groups. At 20,000 ppm, spermatid heads per testis and per gram testis, epididymal spermatozoal motility, and the number of epididymal spermatozoa per gram epididymis were lower than in the controls. All of these findings are consistent with the marked loss of germinal epithelium at this exposure. The NOAEL for effects in the testis is 359 mg/kg-day (5000 ppm), and the LOAEL is 720 mg/kg-day (10,000 ppm). The NOAEL for effects in the liver is 176 mg/kg-day (2500 ppm), and the LOAEL is 359 mg/kg-day (5000 ppm).

NTP (1995) conducted a 13-week toxicity study in male and female B6C3F1 mice. Mice received 0, 1250, 2500, 5000, 10,000, or 20,000 ppm dibutyl phthalate in feed (equivalent to 0, 163, 353, 812, 1601, or 3689 mg/kg-day in males and 0, 238, 486, 971, 2137, or 4278 mg/kg-day in females). No deaths occurred during this study. Mean body weights were decreased by 13% in males and 13% in females at 20,000 ppm. An increase in relative liver weight was observed in males and females at 10,000 ppm or greater (an increase of 16% and 38% in males, respectively, and 19% and 52% in females, respectively). Although no gross lesions were observed at necropsy, microscopic examination revealed hepatocellular cytoplasmic alterations, consistent with glycogen depletion, in male mice receiving 10,000 or 20,000 ppm and female

mice receiving 20,000 ppm. Small, fine eosinophilic granules were also observed in the cytoplasm of hepatocytes in males and females in the 20,000 ppm groups. Lipofuscin accumulation in the liver was detected in males and females receiving 10,000 ppm or greater. The NOAEL is 5000 ppm (equivalent to 812 mg/kg-day in males and 971 mg/kg-day in females) and the LOAEL is 10,000 ppm (equivalent to 1601 mg/kg-day in males and 2137 mg/kg-day in females).

Smith (1953) fed diets containing 0, 0.01, 0.05, 0.25, or 1.25% dibutyl phthalate to groups of 10 male Sprague-Dawley rats for 1 year. Estimated exposures were 0, 5, 25, 125, or 600 mg/kg-day. One-half of the rats receiving the highest exposure died during the first week of the study. The remaining animals survived with no apparent ill effects. There was no effect of treatment on gross pathology or hematology. While it was stated that several organs were sectioned and stained, no histopathological examination was reported. The frank effect level is 600 mg/kg-day; the NOAEL is 125 mg/kg-day.

Chronic Exposure and Cancer Bioassays

There are no chronic studies and no chronic bioassays for cancer.

4.3. REPRODUCTIVE/DEVELOPMENTAL STUDIES

4.3.1. Studies with Dibutyl Phthalate

Cummings and Gray (1987) investigated the effects of dibutyl phthalate on maternal physiological parameters involved with reproductive performance. Pregnant Holtzman rats (n = 6-8 per group) were administered dibutyl phthalate by gavage in sesame oil on GDs 1-8 at 0, 500, 1000, or 2000 mg/kg-day. Animals were sacrificed on day 9 of gestation. Dibutyl phthalate had no effect on the decidual cell response, pregnant uterine weight, number of implantation sites, ovarian weight, or serum progesterone concentration during early pregnancy. The NOAEL is 2000 mg/kg-day, the highest exposure tested.

Ema et al. (1993) investigated the teratogenic effects of dibutyl phthalate in Wistar rats. Pregnant rats (n = 11-13 per group) were given dibutyl phthalate by gavage in olive oil at 0, 500, 630, 750, or 1000 mg/kg-day on GDs 7-15. A significant decrease in the maternal body weight gain was found at an exposure of 630 mg/kg-day and above (a decrease of 31, 65, and 85%, respectively). Maternal death and complete resorption of implanted embryos in all the surviving dams were observed in the 1000 mg/kg-day group. Significantly increased incidence

of postimplantation loss (52 and 93.6%, respectively, at 630 and 750 mg/kg-day) and decreased fetal weight (a decrease of 9 and 18% in males, respectively, and of 10 and 14% in females, respectively, at 630 and 750 mg/kg-day) were reported. The incidence of fetuses with malformations was higher in the 630 and 750 mg/kg-day groups (30 and 100% of litters affected, respectively). The difference was statistically significant only in the 750 mg/kg-day group. Cleft palate was observed predominantly. The NOAEL is 500 mg/kg-day, and the LOAEL is 630 mg/kg-day.

Ema et al. (1994) investigated the critical gestational period for testicular impairment resulting from exposure to dibutyl phthalate in pregnant Wistar rats. Rats (n = 10–12 per group) were given dibutyl phthalate by gavage in olive oil at 0, 750, 1000, or 1500 mg/kg-day on GDs 7–9, 10–12, or 13–15. Postimplantation loss was 100% for each period of treatment at 1500 mg/kg-day. A significant increase in postimplantation loss was found in dams at an exposure of 750 and 1000 mg/kg-day regardless of the days of treatment (59.6 and 45.8%, respectively, on GDs 7–9; 50.5 and 63.8%, respectively, on GDs 10–12; and 36.8 and 64.4%, respectively, on GDs 13–15). No evidence of teratogenicity was detected on GDs 10–12. Treatment on GDs 7–9 at exposures of 750 and 1000 mg/kg-day caused a significant increase in the number of skeletal malformations (more than 89% of litters affected), such as deformity of the vertebral column in the cervical and thoracic regions and of the ribs. Treatment on GDs 13–15 at exposures of 750 and 1000 mg/kg-day resulted in a significant increase in the incidence of fetuses with external and skeletal malformations (more than 78% of the litters affected), such as cleft palate and fusion of the sternbrae. The LOAEL is 750 mg/kg-day, the lowest exposure tested.

Ema et al. (1997) further investigated the susceptible gestational period for the developmental toxicity of dibutyl phthalate. Wistar rats (n = 10–12 per group) were given a single exposure to dibutyl phthalate by gavage in olive oil at 0 or 1500 mg/kg on one of GDs 6–16. A significant increase in the incidence of postimplantation loss (greater than 25.6% versus 11.7% in controls) was found on GDs 6, 8–10, and 12–16. Significant increases were found in the incidences of fetuses with skeletal malformations on GD 8, of fetuses with skeletal and internal malformations on GD 9, and of fetuses with external and skeletal malformations on GD 15. Deformity of the cervical vertebrae was frequently observed on GD 8. Deformity of the cervical and thoracic vertebrae and ribs and dilatation of the renal pelvis were predominantly found in fetuses of dams treated on GD 9. Cleft palate and fusion of the sternbrae were exclusively detected after treatment on day 15. The LOAEL is 1500 mg/kg-day, the only

exposure tested.

Ema et al. (1998) also evaluated the developmental toxicity of dibutyl phthalate during the second half of pregnancy. Wistar rats (n = 11 per group) were fed a diet containing dibutyl phthalate at 0, 0.5, 1.0, or 2.0% ad libitum on GDs 11–21 of pregnancy. Average daily intakes of dibutyl phthalate were 0, 331, 555, or 661 mg/kg-day. No significant changes were detected in the incidence of postimplantation loss, the numbers of live fetuses, resorptions, and dead fetuses. The weights of male and female fetuses at the highest exposure were significantly decreased (12% in males and 13% in females). The incidences of fetuses with cleft palate (36%) and fetuses with fusion of the sternbrae (100%) were increased at the highest exposure. The incidence of fetuses with cryptorchidism (undescended testis) was significantly increased at the two higher exposures (64 and 100%, respectively). There were significant decreases in the AGD of male fetuses in the two higher exposures (a decrease of 24 and 42%, respectively). The AGD of female fetuses was not affected at any exposure. The NOAEL for developmental toxicity in this study is 331 mg/kg-day, and the LOAEL is 555 mg/kg-day.

Ema et al. (2000a) investigated the effects of dibutyl phthalate on reproductive function in pregnant and pseudopregnant (females mated with vasectomized male) rats. Wistar rats (n = 13 in each group) were given dibutyl phthalate by gavage in olive oil at 0, 250, 500, 750, 1000, 1250, or 1500 mg/kg-day on GDs 0–8. Pregnancy outcomes were evaluated on day 20. The same amounts of dibutyl phthalate were given on days 0 to 8 to pseudopregnant rats with an induced decidual cell response. The decidual cells are responsible for formation of the uterine endometrium. Uterine weight on day 9 served as an index of the uterine decidualization. Dibutyl phthalate caused significant increases in the incidences of preimplantation loss in females successfully mated at 1250 and 1500 mg/kg-day (43.7 and 62.1%, respectively, versus 4.6% in controls) and the incidence of postimplantation loss in females having implantations at 750 mg/kg-day and above (31.6, 61.1, 54.2, and 62.7%, respectively, versus 9.4% in controls). The uterine decidualization in pseudopregnant rats was significantly decreased at 750 mg/kg-day and above. The NOAEL in this study is 500 mg/kg-day, and the LOAEL is 750 mg/kg-day.

Ema et al. (2000b) investigated the critical time for exposure to dibutyl phthalate on the development of the reproductive system in male offspring. Pregnant Wistar rats (n = 13 per exposure group) were given dibutyl phthalate by gavage in olive oil at 0, 1000, or 1500 mg/kg-day on GDs 12–14 or 18–20 or at 0, 500, 1000, or 1500 mg/kg-day on GDs 15–17. A significant decrease in the maternal body weight gain was found in the groups treated with

dibutyl phthalate regardless of the days on which 1000 and 1500 mg/kg-day was given (a decrease of 70 and 38%, respectively, on GDs 12–14; a decrease of 40 and 63%, respectively, on GDs 15–17; and a decrease of 23 and 30%, respectively, on GDs 18–20). A significant increase in the number of resorptions per litter was found in the groups given dibutyl phthalate at 1500 mg/kg-day on GDs 12–14 (10.5% versus 0.8% in controls) and GDs 15–17 (4.2% versus 1.2% in controls). The weights of male and female fetuses were significantly decreased (greater than 10%) in the groups given dibutyl phthalate at 1000 and 1500 mg/kg-day on GDs 12–14 and GDs 18–20 and at 1500 mg/kg-day on GDs 15–17. A significant increase in the incidence of fetuses with undescended testes was found at 1500 mg/kg-day on GDs 12–14 (50% of litters affected) and at all exposures on GDs 15–17 (more than 80% of litters affected). A significant decrease in the AGD of male fetuses was observed in the groups treated with dibutyl phthalate regardless of the days of treatment. The greatest effect occurred after treatment on GDs 15–17 (decreases of 17, 36, and 44%, respectively). The AGD of female fetuses in the dibutyl phthalate-treated groups was comparable to that in the control group. The authors concluded that days 15–17 of pregnancy was the most susceptible period for dibutyl phthalate-induced undescended testis and decreased AGD in male offspring. The LOAEL is 500 mg/kg-day, the lowest exposure tested.

Saillenfait et al. (1998) evaluated the developmental toxicity of dibutyl phthalate in Sprague-Dawley rats exposed to dibutyl phthalate on a single day of gestation. Pregnant females (n = 27 in each group) were given a single dose of 0, 500, 1000, 1500, or 2000 mg/kg of dibutyl phthalate by gavage in mineral oil on GD 14 and were necropsied on GD 21. At 1500 and 2000 mg/kg, there was an increased incidence of resorptions (31 and 41%, respectively, versus 8% in control) and reduced mean fetal body weight (5.18 g and 5.14 g, respectively, versus 5.49 g in control). Higher incidences of skeletal variations were found at doses 1500 and 2000 mg/kg (49.6% and 66.7% of fetuses affected, respectively, versus 27.1% in control). The NOAEL is 1000 mg/kg, and the LOAEL is 1500 mg/kg-day.

Gray et al. (1999) studied the reproductive and developmental effects of dibutyl phthalate. In the multi-generational protocol, the P0 generation is exposed by daily gavage from weaning, through puberty, young adulthood, mating, and lactation. F1 pups are evaluated through mating, but they are exposed only via the dam during gestation and lactation. In the trans-generational protocol, dams are dosed only during gestation (starting after implantation) and lactation. The offspring and the sire are not exposed to dibutyl phthalate directly. In a multi-generational assessment using Long Evans hooded rats treated with 0, 250, 500, or 1000 mg/kg-day (10 per sex per group) by gavage in mineral oil, preputial separation was significantly

delayed in P0 males at each exposure (control 39.50 ± 0.90 days; low dose 42.60 ± 1.10 days; mid dose 43.40 ± 1.05 days; high dose 44.40 ± 0.53 days). In P0 females there was no change in vaginal opening or evidence of induced persistent vaginal cornification. In this study in the P0 generation at exposures of 500 and 1000 mg/kg-day, there was a significant reduction in fertility in both males and females that were mated with untreated animals. In males infertility was related to testicular atrophy and reduced sperm production, while treated females cycled and mated successfully but many treated females at 500 mg/kg-day aborted their litters around mid-pregnancy. In the F1 offspring at 250 and 500 mg/kg-day, there were malformations (50 and 100 % of litters affected, respectively) and reduced fecundity when animals were mated under continuous breeding conditions over 11 breeding cycles (the number of pups/litters were 179/24; 76/10, and 20/4, respectively). Malformations included a low incidence of hypospadias, testicular non-descent, anophthalmia, uterus unicornus, and renal agenesis. In a trans-generational protocol, four Long Evans hooded female rats were exposed on GD 16 - 19 to 500 mg/kg-day dibutyl phthalate or eight Sprague-Dawley females were exposed from GD 14 to PND 3 to 500 mg/kg-day dibutyl phthalate. The male offspring from both studies showed reduced anogenital distance, retained nipples, and reduced tissue weights measured in adult animals in androgen-dependent tissues (prostate, seminal vesicles, testis, and epididymis). The LOAEL in this study is 250 mg/kg-day, the lowest exposure tested.

Mylchreest et al. (2002, 2000, 1999, 1998) conducted a series of studies on the effect of dibutyl phthalate on the development and function of the male reproductive tract. In the first study (Mylchreest et al., 1998), pregnant Sprague-Dawley CD rats (n = 10 in each group) were given dibutyl phthalate by gavage in corn oil at 0, 250, 500, or 750 mg/kg-day throughout pregnancy and lactation until postnatal day (PND) 20. Maternal body weights throughout the dosing period were comparable in all groups. At 750 mg/kg-day, the number of live pups per litter at birth was decreased (9.0 vs. 12.3 in control). AGD was decreased at birth in the male offspring at 500 and 750 mg/kg-day (a decrease of 13 and 24%, respectively). In male offspring evaluated at 100 days old, a number of reproductive tract malformations were reported. The epididymis was absent or underdeveloped in 9, 50, and 71% of offspring at 250, 500, and 750 mg/kg-day, respectively, and was associated with testicular atrophy and widespread germ cell loss. Hypospadias (an abnormal location of the urethral opening in the penis) occurred in 3, 21, and 43% of males. Ectopic or absent testes occurred in 3, 6, and 29% of males at 250, 500, and 750 mg/kg-day, respectively. None of these effects occurred in controls. Absence of prostate gland and seminal vesicles as well as small testes and seminal vesicles were noted at 500 and 750 mg/kg-day. Vaginal opening and estrous cyclicity, both estrogen-dependent events, were

not affected in the female offspring, although low incidences of reproductive tract malformations were observed at 500 and 750 mg/kg-day. No skeletal or external malformations were reported. The LOAEL is 250 mg/kg-day, the lowest exposure tested.

In the second study (Mylchreest et al., 1999), pregnant Sprague-Dawley CD rats (n = 10 per group) received dibutyl phthalate by gavage in corn oil at 0, 100, 250, or 500 mg/kg-day on GDs 12–21. Dibutyl phthalate at 500 mg/kg-day caused hypospadias; cryptorchidism; agenesis of the prostate, epididymis, and vas deferens; degeneration of the seminiferous epithelium; and interstitial cell hyperplasia of the testis. Dibutyl phthalate at 250 and 500 mg/kg-day also produced an increase in retained areolas or thoracic nipples and a decrease in AGD. Interstitial cell adenoma occurred at 500 mg/kg-day in two males. The only effect seen at 100 mg/kg-day was delayed preputial separation. However, at this exposure the effect was attributed at least in part to one markedly affected litter. The delay in preputial separation was not observed in a subsequent more detailed study (Mylchreest et al., 2000). Accordingly, 100 mg/kg-day is considered a NOAEL in this study and the LOAEL is 250 mg/kg-day.

Mylchreest et al. (2000) conducted another study to establish a NOAEL for developmental toxicity. Pregnant Sprague-Dawley CD rats were given dibutyl phthalate by gavage in corn oil at 0, 0.5, 5, 50, or 100 mg/kg-day (n = 19–20 per group) or 500 mg/kg-day (n = 11) on GD 12–21. In male offspring AGD was decreased at 500 mg/kg-day. A statistically significant increase ($p < 0.05$, using a nested analysis) in retained areolas or nipples on PND 14 were present in 31 and 90% of male pups at 100 and 500 mg/kg-day, respectively (80 and 100% of litters affected, respectively). The individual litter data are presented in Appendix C. At 500 mg/kg-day, male pups had a nipple bud similar to those of females. The areolas and nipples in the thoracic area were the most prominent. Nipple development was more rudimentary at 100 mg/kg-day but was still clearly different from control males. The nipple bud was rarely visible, but a dark spot on the skin was apparent at the position of the nipple, also mainly in the thoracic area. In the control group, only one male pup had nipple buds; others had a discoloration of the skin in the nipple region, but it was faint and did not show a predilection for the thoracic area. In contrast to Mylchreest et al. (1999), preputial separation was not delayed at any exposure in males with normal external genitalia. Hypospadias was observed in 5/58 rats (4/11 litters) at 500 mg/kg-day. Absent or partially developed epididymis (23/58 rats in 9/11 litters), vas deferens (16/58 animals in 9/11 litters), seminal vesicles (4/58 rats in 4/11 litters), and ventral prostate (1/58 animals) occurred at 500 mg/kg-day. In 110-day-old males at 500 mg/kg-day, the weights of the testes, epididymis, dorsolateral and ventral prostates, seminal vesicles, and levator

ani-bulbocavernosus (LABC) muscle were decreased. At 500 mg/kg-day, widespread seminiferous tubule degeneration was seen in 25/58 rats (in 9/11 litters), focal Leydig cell hyperplasia in 14/58 rats (in 5/11 litters), and Leydig cell adenoma in 1/58 rats (in 1/11 litters). The NOAEL is 50 mg/kg-day, and the LOAEL is 100 mg/kg-day.

Mylchreest et al. (2002) conducted another study to determine whether dibutyl phthalate causes pathologic changes and alterations in androgen status in the testis during the prenatal period of male reproductive tract differentiation. Pregnant Sprague-Dawley CD rats were given 0 or 500 mg/kg-day dibutyl phthalate by gavage in corn oil on GD12–21. Dams were killed on GD 14, 16, 18, or 21 and pups examined. At GDs 16–21, dibutyl phthalate caused hyperplasia of Leydig cells, many of which were 3 β -hydroxysteroid dehydrogenase (3 β -HSD)- and/or androgen receptor (AR)-positive. Focal areas of hyperplasia had increased numbers of Leydig cells positive for proliferating cell nuclear antigen (PCNA). At GD 21, testis atrophy was apparent, and seminiferous cords were enlarged and contained multinucleated gonocytes that, unlike controls, were PCNA-positive. Dibutyl phthalate markedly decreased testicular testosterone levels at GDs 18 and 21. Fewer epididymal ducts and reduced AR staining in some ducts were evident with dibutyl phthalate treatment. The authors concluded that the observed Leydig cell proliferation is a compensatory mechanism to increase testicular steroidogenesis triggered by testosterone insufficiency. The failure of Leydig cell proliferation to correct the androgen concentration results in reproductive tract malformations. The multinuclearity and proliferation of gonocytes suggests an underlying Sertoli cell dysfunction. The LOAEL is 500 mg/kg-day, the only exposure tested.

Barlow and Foster (2003) described the male reproductive tract lesions in fetal, early postnatal, and young adult male rats following exposure to dibutyl phthalate in utero. Pregnant Sprague-Dawley rats were exposed to 500 mg/kg-day dibutyl phthalate by gavage in corn oil on GDs 12–21. Male reproductive tracts were examined on GDs 16–21 and on PNDs 3, 7, 16, 21, 45, and 70. In the fetal testis, large aggregates of Leydig cells, multinucleated gonocytes, and increased numbers of gonocytes were first detected on GD 17 and increased in incidence to 100% by GDs 20 and 21. These lesions resolved during the early postnatal period, while decreased numbers of spermatocytes were noted on PNDs 16 and 21. Epididymal lesions were present at all times examined. In the fetus the epididymal lesion was observed as decreased coiling of the epididymal duct. The decreased coiling progressed on PND 45 to mild degeneration of the seminiferous epithelium. On PND 70, the lesion progressed to severe seminiferous epithelial degeneration. The degeneration was concurrent with malformed

epididymides, which caused obstruction of testicular fluid flow and secondary pressure atrophy in the seminiferous tubules. As the animals were only exposed in utero, the authors concluded that these findings indicated that dibutyl phthalate may initiate testicular and epididymal changes in the fetus that progress to clear malformations in adulthood. The LOAEL is 500 mg/kg-day, the only exposure tested.

Barlow et al. (2004) investigated the incidence and persistence of decreased AGD, increased areolae retention, and Leydig cell adenomas in adult rats following in utero exposure to dibutyl phthalate. Pregnant Sprague-Dawley rats [CRL:CD9SD)BR] were treated with dibutyl phthalate by gavage in corn oil at 0, 100, or 500 mg/kg-day on GDs 12–21. Ten dams were assigned to each group, with a total of 30 dams per replicate. Two replicates were used in the total study. Male offspring were allowed to mature to 6, 12, or 18 months. Gross malformations in the male reproductive tract and histological lesions in the testes were similar to those previously described. In this study testicular dysgenesis, a lesion of proliferating Leydig cells and aberrant tubules, was also observed. The incidence of this lesion was approximately 20% unilateral and 7–18% bilateral at 500 mg/kg-day and was similar among all ages, indicating a developmental alteration rather than an age-related change. Decreased AGD and areolae retention were found to be permanent changes following in utero exposure to 500 mg/kg-day. Decreased AGD was found to be a sensitive predictor of lesions in the male reproductive tract. Relatively small changes in AGD were associated with a significant incidence of male reproductive tract malformations. Exposure to dibutyl phthalate induced proliferative developmental lesions, some of which would have been diagnosed as Leydig cell adenomas by the morphological criteria of the Society of Toxicologic Pathology. These lesions were called Leydig cell adenomas in previous studies from this laboratory (Mylchreest et al., 2002, 2000, 1999). However, more detailed examination of the lesions in this study revealed that these lesions were dissimilar to traditional Leydig cell adenomas as the Leydig cells were poorly differentiated and the lesions contained aberrant seminiferous tubules. Only small numbers of classically defined hyperplasia and adenomas were observed in this study with no differences in incidence among the control and groups exposed to dibutyl phthalate.

Fisher et al. (2003) investigated the effects of dibutyl phthalate in Wistar rats following in utero exposure by gavage in corn oil at 500 mg/kg-day on GDs 13–21. Dibutyl phthalate induced a high rate (>60%) of cryptorchidism (mainly unilateral), hypospadias, infertility, and testis abnormalities similar to those in human testicular dysgenesis syndrome. Immunohistochemistry for cell specific protein markers and confocal microscopy were used to

track development of specific cell types from fetal life to adulthood. Sertoli cells were tracked with anti-Müllerian hormone (AMH), Wilm's tumor (WT-1) protein, and the p27^{kip} protein. Leydig cells were tracked with 3 β -HSD. Germ cells were tracked with DAZL protein. Peritubular myoid cells were tracked with smooth muscle actin. In scrotal and cryptorchid testes of males exposed to dibutyl phthalate, areas of focal dysgenesis were found that contained Sertoli and Leydig cells and gonocytes and partially formed testicular cords; these dysgenetic areas were associated with Leydig cell hyperplasia at all ages. Suppression (~90%) of testicular testosterone levels on GD 19, coincident with delayed peritubular myoid cell differentiation, may have contributed to the dysgenesis. Double immunohistochemistry using WT-1 (expressed in all Sertoli cells) and p27^{kip} (expressed only in mature Sertoli cells) revealed immature Sertoli cells in dysgenetic areas. Animals exposed to dibutyl phthalate also exhibited Sertoli cell-only (SCO) tubules, sporadically in scrotal and predominantly in cryptorchid testes, or foci of SCO within normal tubules in scrotal testes. In all SCO areas the Sertoli cells were immature. Intratubular Leydig cells were evident, and, where these occurred, Sertoli cells were immature and spermatogenesis was absent. Abnormal Sertoli cell-gonocyte interaction was evident at GD 19 coincident with appearance of multinucleated gonocytes, although these disappeared by PND 10 during widespread loss of germ cells. The authors concluded that these results suggest that dibutyl phthalate caused abnormal development of Sertoli cells or failure of maturation of Sertoli cells, which leads to abnormalities in Leydig cells and germ cells (gonocytes). The LOAEL is 500 mg/kg-day, the only exposure tested.

Higuchi et al. (2003) evaluated sequelae in male Dutch-belted rabbits following exposure to dibutyl phthalate at a level known to affect adversely testicular function in rodents without causing systemic toxicity. Rabbits were exposed to 0 (n = 5) or 400 mg/kg-day (n = 8) in utero (GDs 15–29) or during adolescence (postnatal weeks 4–12). Dibutyl phthalate was administered by gavage from a water/Karo light syrup mixture (60:40). Male offspring were examined at 6, 12, and 25 weeks of age. Another group was exposed after puberty (for 12 weeks) and examined at the conclusion of exposure. The most pronounced reproductive effects were in male rabbits exposed in utero. Male offspring in this group exhibited reduction in numbers of ejaculated sperm (reduced by 43%, p<0.01), in weights of testes (at 12 weeks, reduced by 23%, p<0.05) and in weights of accessory sex glands (at 12 and 25 weeks, reduced by 36%, p<0.01 and 27%, p<0.05, respectively). Serum testosterone levels were also reduced (at 6 weeks, by 32%, p<0.05) with a slight increase in histological alterations of the testis (p<0.05) and a doubling in the percentage (16–30%, p<0.01) of abnormal sperm and 1/17 males manifesting hypospadias, hypoplastic prostate, and cryptorchid testes. Cellular atypia in the cryptorchid testes included

irregular nuclear contours, chromatin clumps, and swollen mitochondria. These morphological features are similar to carcinoma in situ reported in humans with cryptorchid testes.

In the group exposed during adolescence, basal serum testosterone levels were reduced at 6 weeks ($p < 0.01$), while at 12 weeks testosterone production in vivo failed to respond normally to a gonadotropin releasing hormone challenge ($p < 0.01$). This assay assesses the ability of the pituitary-gonadal axis to respond to gonadotropin releasing hormone and stimulate the production of testosterone. In addition, weight of accessory sex glands was reduced at 12 weeks by 33% but not at 25 weeks. At 25 weeks there was a slight increase in the percentage of abnormal sperm in the ejaculate and 1/11 males was unilaterally cryptorchid. In both of these groups, daily sperm production, epididymal sperm counts, mating ability, and weights of body and nonreproductive organs were unaffected. In the group exposed after puberty, the only statistically significant change noted was a slight decrease (80–75%) in morphologically normal sperm. This change is not biologically significant. The LOAEL is 400 mg/kg-day, the only exposure tested, for in utero exposure. The NOAEL for exposure to the adult is 400 mg/kg-day.

Kim et al. (2004c) investigated the effect of dibutyl phthalate on the expression of several proteins in lung and liver of B6C3F1 mice. The focus of this study was on combined effects between exposure to ozone, 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone, and dibutyl phthalate. Only the results following exposure to dibutyl phthalate alone are reported here. B6C3F1 mice (5–6 weeks old) were fed a diet containing 0 or 5000 ppm dibutyl phthalate for 52 weeks (equivalent to an exposure of approximately 800–900 mg/kg-day from comparison with the study in mice reported by NTP, 1995). The number of animals used is not reported. Only qualitative results are reported in the paper. Dibutyl phthalate increased the DNA-binding activity of transcriptional factor NF- κ B in liver and lung. Expression of p105, p65, and p50 proteins was also increased, but I κ B binding activity was inhibited by dibutyl phthalate. The binding activity of AP-1 was enhanced in liver and lung. The expression of proteins c-jun, jun B, and jun D were enhanced, but expression of c-fos was depressed in both liver and lung.

Kim et al. (2004b, abstract only) studied the effects of in utero exposure to dibutyl phthalate on the development of reproductive organs and investigated the specific mechanisms causing these abnormalities in the male reproductive system. Pregnant Sprague-Dawley rats received dibutyl phthalate by gavage in corn oil at 0, 250, 500, or 700 mg/kg-day on GDs 10–19. Pups were killed at 31 or 42 days of age and blood was collected for serum testosterone analysis. Testis and accessory organs (epididymides, seminal vesicles, ventral prostate, LABC, Cowpers’

glands) were weighed and examined histologically. Steroid hormone receptor levels (androgen, estrogen, and orphan nuclear receptor) were also examined in the testes. At 31 days of age, effects of dibutyl phthalate on the male reproductive system were exposure-related. Dibutyl phthalate treatment significantly decreased the weights of testes and accessory sex organs and significantly delayed testis descent (no numerical data were provided in the abstract). Dibutyl phthalate also significantly increased the expression of estrogen receptor (ER) in the testes (data not shown). At 42 days of age, the animals treated with dibutyl phthalate did not show any change in weight of testis or accessory sex organs or changes in expression of androgen or estrogen steroid receptors in the testes. The LOAEL is most likely 250 mg/kg-day but this value cannot be confirmed because the data are not presented in the abstract.

Kleymenova et al. (2005) used rats exposed to dibutyl phthalate in utero to study fetal abnormalities underlying impaired spermatogenesis. Cell proliferation in the fetal testes was assessed on tissue slides by histological methods and bromodeoxyuridine (BrdU) incorporation. Apoptosis was measured by transferase dUTP nick end labeling (TUNEL) assay. Cadherins were analyzed by immunostaining. Rats were exposed to 500 mg/kg-day beginning at GD 12. Groups of animals were killed on successive days and analyzed for changes related to exposure to dibutyl phthalate. The total number of cells and the number of tubular cells in the testis were significantly decreased (two-way ANOVA), starting on GD 17. BrdU incorporation and TUNEL assays suggest that this decrease is caused by altered proliferation of Sertoli and peritubular cells, but not apoptosis. As early as GD 17, treated testes had fewer cords that were enlarged and less convoluted. Beginning on GD 20, Sertoli cells in treated testes exhibited hypertrophy. Multinucleated gonocytes, first detected on GD 19, appeared to be arrested in telophase/cytokinesis 1 phase of meiosis. Immunostaining for P- and N-cadherins suggested changes in Sertoli cell-gonocyte interactions. These data indicated that in utero exposure to dibutyl phthalate affects cord formation and development of Sertoli cells that may alter meiosis in gonocytes. These data support the hypothesis linking impaired spermatogenesis in the adults with failure of fetal Sertoli cells to proliferate and mature. The LOAEL is 500 mg/kg-day, the only exposure tested.

NTP (1995) conducted a continuous breeding study in Sprague-Dawley rats. These data are also reported in NTP (1991) and Wine et al. (1997). Animals (n = 40 in the control group and 20 in each exposure group) received 0, 1000, 5000, or 10,000 ppm dibutyl phthalate in feed (equivalent to 0, 52, 256, or 509 mg/kg-day in males and 0, 80, 385, or 794 mg/kg-day in females) during a 16-week mating period. Selected data are presented in Appendix D. Mean

body weights of exposed dams at delivery and during lactation generally decreased with increasing exposure concentration. Mating, pregnancy, and fertility indices of F₁ rats were lower in the 10,000 ppm group than in the controls. Germinal epithelial degeneration of the testes and absence or under development of the epididymides were noted in F₁ males in the 10,000-ppm group. Interstitial cell hyperplasia was noted in 7 of 10 males in the 10,000 ppm-group. There was a decrease in mean live pups per litter in F₁ and a decrease in adjusted mean live pup weight in F₂ at the lowest exposure (see Appendix D). Based on the results of the series of studies by Ema et al. (2000b, 1998, 1994, 1993) showing postimplantation loss and a decrease in fetal body weight and on the crossover trial in NTP (1995) showing a decrease in fetal body weight when only pregnant females were exposed to dibutyl phthalate, these effects are most likely due to effects in the female, rather the male. The statistically significant decrease in mean live pups per litter was not observed in the crossover trial. In part this was attributed to the decreased power of the crossover trial where only 1 litter was produced versus 5 litters in the main study (Wine et al., 1997). The LOAEL is 80 mg/kg-day (1000 ppm) in female rats.

Additional data on developmental effects in males from a separate multi-generation study were reported in an abstract (NTP, 2002). Sprague-Dawley rats (n = 17 of each sex) were administered dibutyl phthalate in the diet at 1, 4, 10, 30, 100, 1000, or 10000 ppm. NTP did not provide detailed exposure information in the abstract. Effects on sexual development were found only in males from dams in the 10,000 ppm group. On PND 1 there was a statistically significant decrease of 15% in AGD in F_{1a} pups. In F_{1b} pups the change in AGD was 10% (not statistically significant). In F_{2a}, F_{2b}, and F_{2c} pups there was a statistically significant decrease of 13–15% in AGD. In males born to dams treated at 10,000 ppm and mated with untreated males, there was a statistically significant decrease of 14% in AGD. In F_{1b} male pups there was a statistically significant delay of 2.0 days in preputial separation (45.58 days versus 43.60 days in controls) and a statistically significant delay of 2.8 days in testicular decent (26.54 days versus 23.77 days in controls).

NTP (1995) studies the effects of dibutyl phthalate in F344 rats following perinatal exposure. Dibutyl phthalate was administered in the diet to dams (n = 20 in each group) during gestation and lactation and to the pups postweaning for four additional weeks at concentrations of 0, 1250, 2500, 5000, 7500, 10,000, or 20,000 ppm. Based on measured food consumption and average body weight during the final four-week exposure period, exposure to males was 0, 143, 284, 579, 879, or 1165 mg/kg-day and to females was 0, 133, 275, 500, 836, or 1104 mg/kg-day. NTP did not provide average exposure for the 20,000 ppm group. Decreased

weight gains were noted in dams exposed to 20,000 ppm during gestation and to dams exposed to 10,000 ppm during lactation. The gestation index (number of live pups per breeding female) was significantly lower in the 20,000 ppm-group than in the controls, and pup mortality in this group was marked (100% by day 1 of lactation); however, survival was 89% or greater in all other treatment groups. The mean body weight of pups in the 10,000 ppm-group at day 28 of lactation was approximately 90% of the mean weight of control pups. Pups were weaned onto diets containing dibutyl phthalate at the same concentrations fed to dams. After an additional 4 weeks of dietary administration, final mean body weights of pups in the 10,000-ppm groups were 92% of the control value for males and 95% of the control value for females. An increase in relative liver weight was observed in males at 2500 ppm and above (increase of 10% and 29%, respectively) and in females receiving 5000 ppm (increase of 14%). No gross lesions were observed at necropsy. Moderate hypospermia of the epididymis was diagnosed in all male rats in the 7500- and 10,000-ppm groups; mild hypospermia of the epididymis was diagnosed in 2 of 10 males in the 5000-ppm group. No degeneration of the germinal epithelium was detected in the testes of these rats. The NOAEL in this study is 143 mg/kg-day (1250 ppm), and the LOAEL is 284 mg/kg-day (2500 ppm).

NTP (1995) conducted another study in F344 rats following perinatal exposure to dibutyl phthalate. Dams (n = 20 in each group) were administered diets containing 0 or 10,000 ppm during gestation and lactation, and weaned pups were administered the same diets as their dams received for an additional 4 weeks until the beginning of the 13-week exposure phase. Male and female rats then received diets containing dibutyl phthalate at concentrations of 0, 2500, 5000, 10,000, 20,000, or 40,000 ppm for 13 weeks. Based on measured food consumption and average body weight in the adult phase, exposure to males was 0, 138, 279, 571, 1262, or 2495 mg/kg-day and to females was 0, 147, 294, 593, 1182, or 2445 mg/kg-day. No mortality or toxicity was observed in dams during the perinatal phase of the study; however, before pups were culled at 4 days postpartum, the percentage of live pups per litter was 86% to 93% that of the controls. Through weaning, litter weights of exposed pups ranged from 89% to 92% of the control values. Ten control and ten exposed pups per sex were examined at the time of weaning; an increase in liver weight and markedly increased peroxisomal enzyme activities (approximately nine-fold greater than the control values) were observed in exposed pups. Body weights of the perinatally exposed pups remained lower than those of the controls throughout the 4-week period before the 13-week adult exposures began.

During the 13-week adult exposure phase, the final mean body weight of males in the

10,000 ppm:0 ppm control group (rats treated at 10,000 ppm were returned to the control for 13 weeks) was 95% that of the controls. The body weight gain of females in the 10,000 ppm:0 ppm group was greater than that of the unexposed controls, and the final body weights of these two groups were similar. Body weight gains of rats treated with dibutyl phthalate as adults decreased with increasing exposure concentration; for rats that received 10,000 ppm followed by 40,000 ppm for 13 weeks, final body weights were 51% of the control value for males and 74% of the control value for females.

The increase in relative liver weight regressed in rats in the 10,000 ppm:0 ppm groups but was observed in male rats receiving 5000 ppm or greater (an increase of 15%, 29%, 59%, and 77%, respectively) and in females receiving 10,000 ppm and above (an increase of 18%, 33%, and 75%, respectively). In males that received 20,000 ppm as adults, testes and epididymal weights were less than in the controls; males in the 40,000 ppm group also had a lower testes weight than the controls. Hypotriglyceridemia was detected in females receiving 20,000 or 40,000 ppm and in males receiving 10,000 ppm or greater. Elevations in alkaline phosphatase activities and bile acid concentrations in male and female rats receiving 20,000 or 40,000 ppm as adults were indicative of cholestasis.

Microscopic examination revealed hepatocellular cytoplasmic alteration, consistent with glycogen depletion, in male and female rats receiving a concentration of 10,000 ppm or greater. In the liver of rats receiving 40,000 ppm, small, fine eosinophilic granules were also observed in the cytoplasm of hepatocytes. Ultrastructural examination suggested the presence of increased numbers of peroxisomes. Lipofuscin accumulation was detected in rats that received 10,000 ppm or greater. Consistent with the regression of the increased liver weight in rats in the 10,000 ppm:0 ppm and 10,000 ppm:2500 ppm groups, peroxisomal enzyme activity was not elevated in these groups. Marked elevations of peroxisomal enzyme activity (palmitoyl-CoA oxidase activity) were detected, however, in males receiving 5000 ppm or greater (an increase of 1.7-, 4.1-, 11.3-, and 20.5-fold, respectively) and in females receiving 10,000 ppm or greater (an increase of 1.8-, 5-, and 19.5-fold, respectively).

Histopathologic examination of the testes revealed degeneration of the germinal epithelium, a mild to moderate focal lesion in rats in the 10,000- and 20,000-ppm groups and a marked diffuse lesion in all males receiving 40,000 ppm; at 40,000 ppm an almost complete loss of the germinal epithelium resulted. Testicular zinc concentrations were lower in the 40,000-ppm group than in the controls, a finding consistent with the marked loss of germinal epithelium

at this exposure. Spermatogenesis was evaluated in rats in the 0, 2500, 10,000, and 20,000 ppm groups. Rats administered 20,000 ppm had fewer spermatid heads per testis than the unexposed controls, and epididymal spermatozoal concentration was less than that in the 10,000 ppm:0 ppm group. The NOAEL for effects in the testis is 279 mg/kg-day (5000 ppm), and the LOAEL is 571 mg/kg-day (10,000 ppm). The NOAEL for effects in the liver is 138 mg/kg-day (2500 ppm), and the LOAEL is 279 mg/kg-day (5000 ppm).

Lamb et al. (1987) investigated the reproductive effects of dibutyl phthalate in Swiss (CD-1) mice using a continuous breeding protocol. These data are also reported in NTP (1984) and NTP (1995). Mice (n = 40 in control and 20 in each exposure group) were fed diets containing dibutyl phthalate at 0, 0.03, 0.3, or 1.0 % (equivalent to exposures of 0, 53, 525, or 1750 mg/kg-day). Both male and female mice were dosed for 7 days prior to and during a 98-day cohabitation period. Reproductive function was evaluated during the cohabitation period by measuring the number of litters per pair, the number of live pups per litter, pup weight, and offspring survival. At the highest dose only, dibutyl phthalate caused a reduction in the numbers of litters per pair (1.8 versus 4.85), in the numbers of live pups per litter (1.72 versus 12.08), and in the proportion of pups born alive (0.5 versus 1.0). A crossover mating trial using 1% dibutyl phthalate in the diet demonstrated that female mice, but not males, were affected. In the crossover trial, there was a significant decrease in the percentage of fertile pairs (21% versus 74%), in the number of live pups per litter (0.75 versus 7.71), in the proportion of pups born alive (0.63 versus 0.95), and in live pup weight (1.41 g versus 1.83 g). The NOAEL is 525 mg/kg-day, and the LOAEL is 1750 mg/kg-day.

NTP (1995) studies the effects of dibutyl phthalate in B6C3F1 mice following perinatal exposure. Dams (n = 20 in each group) received 0, 1250, 2500, 5000, 7500, 10,000, or 20,000 ppm dibutyl phthalate in feed during gestation and lactation. Pups were weaned onto the same diets as the dams received and were exposed for an additional 4 weeks. Based on measured food consumption and average body weight during the final four-week exposure period, exposure to males was 0, 199, 437, 750, 1286, or 3804 mg/kg-day and to females was 0, 170, 399, 714, or 1060 mg/kg-day. NTP did not provide average exposure for females in the 10,000 ppm group or for males or females in the 20,00 ppm group. The gestation period was longer in dams that received 2500 ppm or greater. A depression in body weight gain was noted in dams receiving 7500 ppm or greater. Only 5 of 20 females in the 10,000-ppm group delivered live pups. None of the 20 females receiving 20,000 ppm delivered live pups. Only one pup in the 10,000-ppm group survived past lactation day 1. No deaths of either male or female pups occurred after

weaning. The mean body weights of exposed male pups at weaning were decreased at 5000 and 7500 ppm (11 and 12%, respectively). The mean body weights of exposed female pups were similar to the control body weight at weaning and remained similar throughout the 4 weeks postweaning. An increase in relative liver weight was observed in male mice at 5000 ppm and above (an increase of 17 and 23%, respectively). No treatment-related gross lesions were identified at necropsy. No histopathologic lesions definitively associated with treatment were observed in male or female mice in the 7500-ppm groups. The one surviving male pup in the 10,000-ppm group had cytoplasmic alteration in the liver consistent with peroxisome proliferation. Developmental toxicity and fetal and pup mortality were suggested at concentrations as low as 7500 ppm. The NOAEL in this study is 437 mg/kg-day (2500 ppm), and the LOAEL is 750 mg/kg-day (5000 ppm).

Tsutsumi et al. (2004) investigated whether the testicular toxicity of dibutyl phthalate in adults is influenced by diminished renal function. Adult male F344 rats (six weeks old, eight groups of 5 animals each) were given weekly subcutaneous injections of folic acid at 0 or 300 mg/kg for five consecutive weeks to induce renal disease. In a pre-experiment, this treatment was shown by histological examination to induce interstitial nephritis characterized by focal atrophy of the tubules and an increase in interstitial connective tissue. Starting at the sixth week, the animals were given diets containing dibutyl phthalate at 0, 1200, 5000, or 20,000 ppm for 4 weeks (equivalent to 0, 61, 255, or 1535 mg/kg-day; the authors mentioned that the highest exposure is overestimated because of food spillage). In animals with renal disease at the highest exposure, there was a decrease in relative testes weight, degeneration of seminiferous tubules, decreased sperm number per epididymis, and an increase in the number of morphologically abnormal sperm. These data suggest that individuals with renal disease could be at greater risk from the effects of high exposure to dibutyl phthalate due to diminished capacity to excrete the metabolites of dibutyl phthalate through the kidney. However, this study cannot exclude the possibility that the apparent enhanced effects are due to impaired absorption of zinc by the intestinal tract caused by folic acid (Tavares et al., 2000; Hansen et al., 1995). Zinc is known to be an important factor in dibutyl phthalate induced reproductive toxicity (NTP, 1995; Fukuoka et al., 1989).

Salazar et al. (2004) investigated the effect of dibutyl phthalate administered in feed on reproductive performance and pre-pubertal development of offspring in Long Evans rats. Female rats (2 months old, n = 15 per group) were fed diets containing 0, 610, or 2500 ppm dibutyl phthalate for 2.5 months. The females were then mated with untreated males. Dams

continued to receive dibutyl phthalate throughout pregnancy and lactation. Pups were weaned on PND 22 and fed the same diet as their dams. The authors reported the exposure to dibutyl phthalate as 0, 12, or 50 mg/kg-day. The weight gain reported in control dams fed for three months was 69 grams and the weight gain was decreased 26% in both groups fed dibutyl phthalate. Pregnancy rate was significantly decreased at the highest exposure (58.3% versus 81.8% in control). There was no effect on litter size, pup survival, or pup sex ratio at any exposure. Pup weight on PND 2 was significantly decreased at both exposures (10% and 23%, respectively). Pups weight on PND 6 was decreased only at the low exposure (12%). Testis weight was decreased on PND 14 at both exposures (21%). Preputial separation was significantly delayed at both exposures (control at PND 36, low exposure PND 38, and high exposure PND 41). Vaginal opening was delayed at both exposures (at PND 37 versus PND 35 in control) and the time to first estrus was delayed at the high exposure (at PND 40 versus PND 38 in control).

There appear to be a number of problems with this study. First, the exposure reported by the authors appears incorrect. Details of the calculation are not provided in the paper. However, a pregnant rat normally consumes 8–12% of her body weight in food per day. Accordingly, the exposures should be closer to 0, 50–70, or 200–300 mg/kg-day. The author was contacted to try to resolve this discrepancy. However, no response was received. The decreased maternal weight gain at both exposures (decreases of 26%) indicates significant maternal toxicity that compromises the reliability of the results in the offspring (U.S. EPA, 1991). Significant maternal toxicity has not been observed in previous studies until the exposure was 500 mg/kg-day or higher (Ema et al., 1993). Fetal testis weight was not affected in previous studies until the exposure was 250 mg/kg-day or higher (Kim et al., 2004b). Preputial separation was not affected at an exposure of 500 mg/kg-day (Mylchreest et al., 2000) or at 712 mg/kg-day (Lee et al., 2004). Vaginal opening, day to first estrus, and estrous cyclicity were not affected at an exposure of 712 mg/kg-day (Lee et al., 2004). Because of the uncertainty in exposure, the significant maternal toxicity as shown by the decreased maternal weight gain, and the lack of concordance with other results at higher exposures, a NOAEL and LOAEL are not assigned for this study and it is not considered in the exposure-response determination.

Zhang et al. (2004) investigated the effect of dibutyl phthalate in Sprague-Dawley rats in utero and during lactation. Pregnant rats (n = 20 per exposure group) were treated with dibutyl phthalate by gavage in corn oil at 0, 50, 250, or 500 mg/kg-day from GD 1 to PND 21. The developmental condition of F₁ rats and the reproductive system of mature F₁ male rats were

monitored. Exposure to dibutyl phthalate had no effect on body weight gain in dams, on length of gestation, on sex ratio of live fetuses, or on pup survival to weaning. At 500 mg/kg-day there was a statistically significant decrease in live pups per litter (14% decrease). At 250 and 500 mg/kg-day there was a statistically significant decrease in live pup weight at birth (12% and 13% in males; 10% and 18% in females, respectively). At 250 and 500 mg/kg-day there was a statistically significant decrease in AGD in male pups at postnatal day 4 (11% and 25%, respectively) but no change in female pups. Necropsy revealed reproductive tract malformations in treated males. Undescended testes located in the abdominal cavity and underdeveloped epididymides were observed in two pups from the high dose group on PND 21. At PND 70, testicular atrophy occurred in 1 of 20 male pups in the 250 mg/kg-day group and in 6 of 20 male pups in the 500 mg/kg-day group. Underdeveloped epididymis occurred in 1 of 20 male pups in the 250 mg/kg-day groups and in 5 of 20 male pups in the 500 mg/kg-day group. Absence of epididymis occurred in 1 of 20 males in the 500 mg/kg-day group. There were no differences in absolute or relative testis and epididymis weight at PNDs 14 and 21. However, at PND 70, there was a statistically significant decrease in right epididymis weight at 250 and 500 mg/kg-day (16% and 29%, respectively) and a statistically significant decrease in prostate weight at 250 mg/kg-day (31%) but not at 500 mg/kg-day. There was a statistically significant decrease in percent motile sperm and in total sperm heads per testis at 250 and 500 mg/kg-day and a statistically significant decrease in total number of sperm at 500 mg/kg-day. Histological examination of the testis revealed mild degeneration of the seminiferous epithelium at 250 mg/kg-day and more severe degeneration at 500 mg/kg-day on PND 70. Based on the decrease in birth weight of pups, the decrease in AGD in male pups, the decrease in epididymis weight, and degeneration of the seminiferous epithelium, the LOAEL in this study is 250 mg/kg-day and the NOAEL is 50 mg/kg-day.

Kim et al. (2004a) investigated the effect of dibutyl phthalate administered subcutaneously on the development of male reproductive organs in early postnatal male rats. Male rats were injected with dibutyl phthalate in corn oil at 0, 5, 10, or 20 mg/animal on PNDs 5–14. Based on the estimated average body weight of the pups, EPA estimated the exposures to be 250, 500, or 1000 mg/kg-day. Animals were killed on PNDs 31 or 42 and the testes, epididymis, seminal vesicles, ventral prostate, LABC muscles, and Cowper's glands were weighed. The expression of AR, ERs, and steroidogenic factor-1 (SF-1) were examined in the testes using Western blots. Total body weights were significantly reduced on PNDs 29–31 (approximately 19% and 5%, respectively) but recovered by PND 42 in animals treated with 20 mg dibutyl phthalate. There was no effect on testicular descent at any exposure. At PND 42,

dibutyl phthalate at 20 mg/animal significantly reduced the weights of the testes (decrease of 15%) and accessory sex organs (seminal vesicles, decrease of 32%; LABC, decrease of 22% and Cowper's glands, decrease of 28%), but not the epididymis or ventral prostate. Serum testosterone levels did not show any significant changes at any exposure at PND 31 or 42. Histological examination showed mild diffuse Leydig cell hyperplasia in the interstitium of seminiferous tubules at 20 mg/animal on PND 31. Only a few multi-nuclear germ cells were observed. On PND 31, dibutyl phthalate at 20 mg/animal significantly decreased the expression of the AR, but not ER-alpha, whereas expression of ER-β and SF-1 were increased. On PND 42, dibutyl phthalate at 20 mg/animals inhibited the expression of ER-β but not AR, ER-alpha, or SF-1. In this study, adverse effects were observed only at the highest exposure of 20 mg/animal (approximately 1000 mg/kg-day). Because the exposure was by the subcutaneous route, a LOAEL and NOAEL are not assigned for this study.

Lee et al. (2004) evaluated the developmental toxicity of dibutyl phthalate during the period from late gestation through lactation. Pregnant Sprague Dawley rats [CD(SD)IGS, n = 6–8 at each exposure] were administered dibutyl phthalate at dietary concentrations of 0, 20, 200, 2000, or 10,000 ppm from GD 15 to PND 21. Based on measured dietary consumption and body weight, the dams were exposed to 0, 1.5–3.0, 14.4–28.5, 148–291, or 712–1372 mg/kg-day. The lower exposure was during gestation and the higher exposure was during late lactation (PNDs 10–21). Offspring were examined at various times after birth and into adulthood.

There was no significant change in body weight or food consumption in dams and no change in duration of pregnancy at any exposure. At 10,000 ppm at birth, there was a decrease in the percentage of male pups (24.7% versus 65.6% in control), a decrease in AGD (3.0 mm versus 3.7 mm in controls), and an increase in retained nipples by PND 14 (100% versus 0% in controls). At 10,000 ppm on PND 21, there was an increase in relative liver weight (29% in males and 27% in females) and a decrease in relative testes weight (19%). At this exposure there was also histopathological evidence of liver cell hypertrophy associated with increased eosinophilia in the cytoplasm. As measured by day of preputial separation, age of first estrus, and estrus cyclicity, there was no significant change in the onset of puberty in offspring at any exposure.

Adult offspring were examined at postnatal week 11 and 20. There was no consistent biologically significant change in body weight or most organ weights at either time. However, at postnatal week 11, relative pituitary weight was increased in males at 20, 200, and 2000 ppm

(increases of 16, 19, and 22%, respectively) but no change at 10,000 ppm but was decreased 36% in females at 10,000 ppm and accompanied by histopathological evidence of reduced pituitary size. At postnatal week 20, there was no change in relative pituitary weight in males but a decrease in females at 200, 2000, and 10,000 ppm (16, 16, and 23%, respectively). However, there was no histopathological evidence of reduced pituitary size in females at any exposure. Accordingly, the changes in relative pituitary weight in females are not considered a critical effect. There were changes noted in the percentage of follicle-stimulating hormone- (FSH), luteinizing hormone- (LH), or prolactin (PRL)-producing cells in the anterior pituitary of males and females at PND 21 and postnatal week 11. In males at 10,000 ppm there was a decrease in FSH-producing cells at PND 21 and an increase at postnatal week 11; an increase in LH-producing cells only at PND 21; and a decrease in PRL-producing cells only at postnatal day 21. In females there was a decrease in FSH-producing cells at 200, 2000, and 10,000 ppm at PND 21, but the decrease did not progress in magnitude with increasing exposure, and there was an increase only at 10,000 ppm at postnatal week 11. Also in females there was an increase in LH-producing cells at 2000 and 10,000 ppm at PND 21, but no change at any exposure at postnatal week 11. There was also a decrease in PRL-producing cells at 10,000 ppm only at PND 21. Because these changes do not show a consistent pattern, they are not considered adverse effects in this study.

There were changes noted in the mammary gland of females at PND 21 (hypoplasia of the alveolar bud, judged minimal to slight) at all exposures. However, as no changes were observed at postnatal week 11 and 20, these changes are not considered adverse effects.

There were statistically significant histological changes in the mammary gland of males at postnatal week 11 and 20. At postnatal week 11, statistically significant vacuolar degeneration occurred at all exposures and statistically significant alveolar atrophy occurred at 20, 2000, and 10,000 ppm. In addition there was a decrease in average size of mammary alveolar buds at postnatal week 11 at all exposures. The decreases ranged from 12 to 25%; however, there was no increase in effect with increasing exposure. Animals were not examined at postnatal week 20 for these effects. At postnatal week 20, statistically significant vacuolar degeneration occurred only at 200 ppm (graded minimal to slight) and statistically significant alveolar atrophy occurred at 200 (graded slight) and 2000 ppm (graded slight to moderate). Males exposed to 10,000 ppm were not examined for these changes at postnatal week 20. The biological significance of the changes observed in the mammary gland of males is unclear and they are not regarded as adverse effects in this study.

In males on PND 21, there was a reduction in spermatocyte development (statistically significant at all exposures; minimal to slight changes at 20, minimal changes at 200 ppm, slight to moderate changes at 2000 ppm, and moderate changes in all animals at 10,000 ppm), an increase in foci of aggregated Leydig cells (statistically significant at 2000 and 10,000 ppm), and decreased ductular cross section in the epididymis (statistically significant at 2000 and 10,000 ppm). In males at postnatal week 11, there was statistically significant loss of germ cell development in the testis at 2000 ppm (graded minimal in 4 of 8 animals) and 10,000 ppm (graded minimal in 7 and severe in 2 of 10 animals), and a statistically significant flattening of the surface epithelia of the ventral lobe of the prostate at 10,000 ppm. These changes were not statistically significant at any exposure at postnatal week 20, but the animals exposed to 10,000 ppm were not evaluated. As the loss of germ cell development observed at 2000 ppm at postnatal week 11 is not statistically significant at postnatal week 20, this effect is not considered an adverse effect at 2000 ppm.

Based on the increase in liver weight and histopathological changes in males and females at PND 21, the decrease in testes weight at PND 21, the loss of germ cell development in males observed at postnatal week 11, the reduction in AGD and nipple retention in males on PND 14, the LOAEL in this study is 10,000 ppm (equivalent to 712-1372 mg/kg-day) and the NOAEL is 2000 ppm (equivalent to 148-291 mg/kg-day).

Mahood et al. (2005) investigated the effect of dibutyl phthalate on Leydig cells in the fetal testis. Pregnant Wistar rats (n = 32 in each group) were given dibutyl phthalate at 0 or 500 mg/kg by gavage in corn oil on GDs 13.5–21.5. Fetuses were removed for examination on GDs 15.5, 17.5, 19.5, or 21.5. Male pups were examined on PND 4, 25, or 90. Exposure of dams to dibutyl phthalate caused abnormal aggregation of Leydig cells centrally in the fetal testis. This effect was apparent as early as GD 17.5 and was most pronounced on GD 21.5. The aggregation was not due to an increase in Leydig cell number. However, Leydig cell size was significantly reduced. In addition, testosterone levels measured by radioimmunoassay on GD 19.5 and 21.5 and expression of P450 side-chain cleavage enzyme measured by immunohistochemistry on GD 17.5 were both reduced. The Leydig cell aggregates did not exhibit evidence of focal proliferation at GDs 17.5 or 19.5. Using confocal microscopy and cell-specific markers for Leydig cells (3 β -HSD) and Sertoli cells (anti-Müllerian hormone), the researchers showed that the aggregates of fetal Leydig cells trap isolated Sertoli cells within them at GD 21.5. The areas of intermingled cells were still apparent on PND 4 when they formed misshaped seminiferous cords that appeared to trap Leydig cells within them. These centrally located dysgenic tubules

contained germ cells in early puberty, but by adulthood they contained only Sertoli cells. This observation implies that the presence of Leydig cells within the tubule interferes with spermatogenesis. The authors concluded that the aggregation of Leydig cells may be a key event in the toxicity of dibutyl phthalate and may give new insight into the fetal origins of testicular dysgenesis syndrome disorders in the human.

Kleyменова et al. (2005, abstract only) investigated the exposure response relationship for a number of cellular responses known to be affected by exposure to dibutyl phthalate. The responses investigated included the presence of multinucleated gonocytes, inhibition of cell proliferation, altered formation of seminiferous tubules, and disruption of cellular contacts between Sertoli and germ cells. Pregnant Sprague-Dawley rats were treated on GDs 12–20 with dibutyl phthalate by gavage in corn oil at 0, 0.1, 1, 10, 30, 50, 100, or 500 mg/kg-day. Two hours before killing on GD 21, the dams were injected with BrdU. Fetal testes were fixed in situ. Cellular responses were assessed using histochemical staining and immunostaining with antibodies to P-cadherins. Quantitative data were not reported in the abstract. The effect of dibutyl phthalate on cell size, total cell number, and the number for seminiferous tubules seen in cross section was significant at 50 mg/kg-day. There was an increased incidence of multinucleated gonocytes at 100 mg/kg-day. At exposures of 30 and 50 mg/kg-day, there was a disruption of Sertoli-germ cell contacts as indicated by changes in P-cadherin immunostaining.

Carruthers and Foster (2005) conducted a study to identify the critical days for the abnormal development of the male reproductive tract, specifically the testis and epididymis. Pregnant Sprague-Dawley rats ($n = 20$ in each group) were exposed to dibutyl phthalate by gavage in corn oil at 0 or 500 mg/kg-day on either GDs 14 and 15, 15 and 16, 16 and 17, 17 and 18, 18 and 19, or 19 and 20. AGD was measured on PNDs 1 and 13. Areola number was recorded only on PND 13. After weaning males were allowed to mature to PND 90 at which time they were necropsied. Areola number and AGD were recorded and testes, epididymides, seminal vesicles, prostate gland, and kidney, and liver were weighed. Blood serum was collected and assayed for total testosterone concentration.

There were no observable effects on litter size, sex ratio, or mortality of pups. Serum testosterone concentrations were not biologically affected at PND 90. Statistically significant permanent reductions in AGD were present in males exposed on GDs 15 and 16, or 18 and 19. On PND 13, areolae were present in males exposed to dibutyl phthalate on GDs 15 and 16, 16 and 17, 17 and 18, and 19 and 20; however significant permanent retention of areolae occurred

only in males exposed to dibutyl phthalate on GDs 16 and 17. Exposure to dibutyl phthalate on only GDs 17 and 18 elicited a significant reduction in epididymal weights; while exposure on only GDs 16 and 17 caused a significant increase in weights of testes due to edema. There was a statistically significant increase in the number of litters with malformations. Malformations of the seminal vesicles were significantly increased in males exposed only on GDs 15 and 16. Malformations of the epididymis, characterized by agenesis of various regions, and small or flaccid testes were significantly increased in males exposed only on GDs 17 and 18. Malformations of the coagulating gland were significantly increased in males exposed only on GDs 19 and 20. These findings suggest that the two-day exposure to dibutyl phthalate is highly detrimental to the developing reproductive tract of the male fetus and that the critical window for abnormal development is GDs 16–18. The LOAEL in this study is 500 mg/kg-day, the only exposure tested.

Studies on steroidogenesis and genomics

Based on data collected in its laboratory (Mylchreest et al., 2000, 1999, and 1998), a research group developed the hypothesis that many of the malformation observed in the developing male reproductive tract observed following exposure of the dam to dibutyl phthalate are due to a decrease in testosterone concentration in the Leydig cell of the fetal testis. Accordingly, the group conducted several studies to investigate the relationship between exposure of the dam to dibutyl phthalate and gene expression (mRNA and protein synthesis) for the genes involved in the synthesis of testosterone from cholesterol in the fetal testis. One study examined in detail the exposure response relationship for exposure to dibutyl phthalate and the decrease in testosterone concentration in the fetal testis.

Shultz et al. (2001) examined gene expression in the fetal rat testis following in utero exposure to dibutyl phthalate. Pregnant Sprague-Dawley rats received 0 or 500 mg/kg-day dibutyl phthalate by gavage in corn oil on GDs 12–21. Testes were isolated on GDs 16, 19, and 21. Global changes in gene expression were determined by microarray analysis. Selected genes were further examined by RT-PCR. Dibutyl phthalate reduced expression of the steroidogenic enzymes cytochrome P450 side chain cleavage (P450scc), cytochrome P450c17, and steroidogenic acute regulatory protein. Testicular testosterone and androstenedione were decreased on GDs 19 and 21, while progesterone was increased on GD 19. Testosterone-repressed prostate message-2 (TRPM-2) was upregulated, while c-kit (stem cell factor receptor) mRNA was downregulated following exposure to dibutyl phthalate. TRPM-2 and bcl-2 protein staining was elevated in GD 21 Leydig and Sertoli cells. The authors concluded that these

results suggest that the antiandrogenic effects of dibutyl phthalate are due to decreased testosterone synthesis in the fetal testes. In addition, enhanced expression of cell survival proteins such as TRPM-2 and bcl-2 may be involved in dibutyl phthalate induced Leydig cell hyperplasia, whereas downregulation of c-kit may play a role in gonocyte degeneration.

Barlow et al. (2003) investigated altered gene expression for enzymes in the testicular testosterone biosynthetic pathway following exposure to dibutyl phthalate. The objectives of this study were to develop a more detailed understanding of the effect of dibutyl phthalate on steroidogenesis using a robust study design with increased numbers of dams and fetuses compared with previous studies, and to explore messenger RNA expression for other critical genes involved in androgen biosynthesis and signaling. Additionally, immunohistochemical localization of protein expression for several key genes was performed to further confirm mRNA changes. Fetal Leydig cell lipid levels were also examined histochemically, using oil red O. Six to seven pregnant Crl:CD(SD)BR rats per group were gavaged with dibutyl phthalate in corn oil at 500 mg/kg-day on GDs 12–19. Testicular RNA isolated from three randomly selected GD 19 fetuses per litter was used for RT-PCR for the following genes: scavenger receptor class B-1 (SR-B1), steroidogenic acute regulatory protein (StAR), P450_{scc}, 3 β -HSD, P450c17, 17 β -hydroxysteroid dehydrogenase (17 β -HSD), AR, luteinizing hormone receptor (LHR), follicle-stimulating hormone receptor (FSHR), stem cell factor tyrosine kinase receptor (c-kit), stem cell factor (SCF), PCNA, and TRPM-2. mRNA expression was downregulated for SRB1, StAR, P450_{scc}, 3 β -HSD, P450c17, and c-kit; TRPM-2 was upregulated; and 17 β -HSD, AR, LHR, FSHR, and PCNA were not significantly changed. Immunohistochemical staining for c-kit was seen in fetal Leydig cells, which has not been previously reported. Downregulation of most of the genes in the testosterone biosynthetic pathway confirms and extends previous findings. Diminished Leydig cell lipid content and alteration of cholesterol transport genes also support altered cholesterol metabolism and transport as a potential mechanism for decreased testosterone synthesis following exposure.

Thompson et al. (2004) investigated the time course of onset and reversibility of the effects of dibutyl phthalate on the fetal testis and on cholesterol transport and steroidogenesis. Pregnant Sprague-Dawley rats (n = 4–5 per group) received dibutyl phthalate by gavage in corn oil at 0 or 500 mg/kg-day on GDs 12–17 with sacrifice on GDs 17–19; on GDs 12–18 with sacrifice on GD 18–19; or on GDs 12–19 with sacrifice on GD 19. Testes were removed for testosterone, mRNA, and protein analysis. Significant decreases in testosterone production and mRNA expression of SR-B1, P450_{scc}, StAR, and P450c17 were observed as early as GD 17.

Testosterone, mRNA, and protein levels remained low 24 hours after exposure but increased 48 hours after exposure. The concentration of testosterone relative to age-matched control was 46.6% on GD 17 and 17.8% on GD 18. The mean expression of the four genes relative to age-matched control was 46.4% on GD 17 and 15.4% on GD 18.

In another experiment reported in the same publication as described above, Thompson et al. (2004) treated pregnant dams with dibutyl phthalate by gavage in corn oil at 0 or 500 mg/kg-day beginning at GD 12 with sacrifice on GD 19. The start of exposure was shifted from GD 12 to one day later in gestation for each treatment group. The final group was treated only on GD 19. Testicular testosterone was measured in four fetuses, each from a separate litter. Significant decreases in fetal testicular testosterone, mRNA expression, and protein expression were evident in each exposure group. In groups treated on GD12 - 19, GD 13 - 19, GD 14 - 19, GD 15 - 19, GD 16 - 19, GD 17 - 19, or GD 18 - 19, the concentration of fetal testicular testosterone was decreased an average of 87%. In groups treated only on GD 19 and sacrificed 3 hours after exposure, the concentration of fetal testicular testosterone was decreased 56%. In a testis explant system, dibutyl phthalate caused diminished transport of cholesterol across the mitochondrial membrane and diminished function at each point in the testosterone biosynthetic pathway, except for the step catalyzed by 17 β -HSD. The transcriptional repression caused by dibutyl phthalate was not mediated by interference with SF-1.

Thompson et al. (2005) studied the precise timing of dibutyl phthalate-associated changes in testosterone concentration and gene expression in the fetal testis and corticosterone and gene expression in the fetal adrenal gland. For the study in the fetal testis, pregnant Sprague-Dawley rats were treated with 0 or 500 mg/kg dibutyl phthalate by gavage in corn oil at 0.5, 1, 2, 3, 6, 12, 18, or 24 hrs before sacrifice on GD 19. Testosterone concentration was measured by radioimmunoassay in fetal testes (4 litters, 3 fetuses per litter). Gene and protein expression was also measured (4-5 fetuses per group with each fetus taken from a separate litter). Testicular testosterone was decreased within one hour of exposure to dibutyl phthalate and preceded the repressed transcription of StAR (steroid acute regulatory protein), Scarb1 (scavenger receptor class B, member 1; also known as Sr-b1), Cyp11a1 (cytochrome P450 family 11, subfamily a, polypeptide 1; also known as P450_{scc}), and Cyp17a1 (cytochrome P450 family 17, subfamily a, polypeptide 1; also known as CYP17). StAR mRNA was significantly diminished 2 hours after exposure to DBP, but Cyp11a1, Cyp17a1, and Scarb1 did not show a significant decrease in expression until 6 hours after exposure to dibutyl phthalate. The decrease in testicular testosterone averaged 43% in animals sacrificed 1 to 6 hours after exposure. The

decrease in testicular testosterone averaged 77% in animals sacrificed 12, 18, or 24 after exposure. For the study in the fetal adrenal gland, pregnant Sprague-Dawley rats were treated with 0 or 500 mg/kg dibutyl phthalate by gavage in corn oil daily from GD 12 to 19 and sacrificed on GD 19. Total corticosterone was measured by radioimmunoassay in fetal adrenal glands (4 litters, 2 fetuses per litter). Protein expression for StAR, Scarb1, and Cyp11a1 was also measured in the fetal adrenal gland (4-5 fetuses per group with each fetus taken from a separate litter). The decrease in corticosterone production in the fetal adrenal (approximately 45% decrease) was not statistically significant. In addition the expression of Star, Scarb1, and Cyp11a1 in the adrenal was unaffected by dibutyl phthalate. Together these studies demonstrate that dibutyl phthalate initiates a rapid and dynamic change in gene expression in the fetal testis that likely plays a role in the reduction in steroidogenesis that is unique to the fetal testis relative to the steroidogenically active fetal adrenal.

Lehmann et al. (2004) investigated the exposure-response relationships for the effect of dibutyl phthalate on steroidogenesis in fetal rat testes. Pregnant Sprague-Dawley rats (n = 7 in control and 5 in each exposed group) were treated with dibutyl phthalate by gavage in corn oil at 0, 0.1, 1.0, 10, 50, 100, or 500 mg/kg-day on GDs 12-19. This phase of the study measured gene expression and protein synthesis. Separate groups of animals (n = 7) were treated with dibutyl phthalate by gavage in corn oil at 0, 0.1, 1.0, 10, 30, 50, 100, or 500 mg/kg-day on GDs 12-19 to determine fetal testosterone concentration. Testes were isolated on GD 19 and changes in gene and protein expression were quantified by RT-PCR and Western blot analysis. For determination of mRNA gene expression, total RNA was isolated from the testes of five individual fetuses representing four to five litters per treatment group. Rat specific primers and probes were used for the genes of interest. For Western blot analysis, whole testes from four individual fetuses per treatment group were solubilized. The samples were heated at 95 °C and equal concentrations of protein were added to each lane of a sodium dodecyl sulfate-polyacrylamide minigel. After electrophoresis, the proteins were transferred onto polyvinylidene difluoride membranes and quantified using specific primary antibodies. Fetal testicular testosterone concentration was determined from three to four individual fetuses from one to four litters per exposure group. Testes were homogenized and extracted with ethyl acetate and chloroform (4:1). After drying, the extract was dissolved in methanol and testosterone was quantified with a radioimmunoassay.

Exposure to dibutyl phthalate at 50 mg/kg-day and above resulted in significant reductions in mRNA and protein concentration for steps involved in cholesterol transport and

synthesis of testosterone including SR-B1, StAR, P450_{SCC}, and 3 β -HSD. mRNA expression and protein synthesis of insulin-like growth factor 3 (Insl3) was reduced at 500 mg/kg-day. Reductions in Insl3 result in cryptorchidism in rats. There was a statistically significant decrease ($p < 0.05$) in mean testosterone concentration per testis at 50 mg/kg-day (a decrease of 61%), but not at 30 mg/kg-day (a decrease of 26%). These data are reported in Appendix C. Lehmann et al. (2004) characterized the decrease in testosterone concentration at 50 mg/kg-day as a “lowest observable effect level (LOEL)” and stated that the effect was not “overtly adverse.” EPA, however, concludes that the effect at 50 mg/kg-day is an adverse effect, that is a Lowest Observed Adverse Effect Level (LOAEL). In this study, the NOAEL for the decrease in testosterone concentration is 30 mg/kg-day and the LOAEL is 50 mg/kg-day.

Wilson et al. (2004) investigated the effects of dibutyl phthalate on testosterone production and expression of the gene for insl3 during fetal development. Previous studies have shown that low levels or inactivation of the gene for insl3 results in altered gubernacular development, disrupted testis descent, and cryptorchidism in male mice. The gubernacular ligaments are involved in the descent of the testis into the scrotum. Pregnant Sprague-Dawley rats were given dibutyl phthalate by gavage in corn oil at 0 or 750 mg/kg-day on GDs 14–18. Ten animals were used in each group. On GD 18, testes were removed from the fetus and examined for effects on steroid hormone production and insl3 gene expression. Testosterone production was reduced by 90%, but progesterone production was not affected. The expression of insl3 mRNA was reduced by 80%. The LOAEL in this study is 750 mg/kg-day, the only exposure tested.

McKinnell et al. (2005) investigated the expression of Insl3 in rat Leydig cells from fetal life until adulthood and its relationship to cryptorchidism induced by exposure to dibutyl phthalate. In the absence of exposure to dibutyl phthalate, expression of Insl3 was strong on gestation day 17.5 to 19.5 and from postnatal day 35 onward. Expression of Insl3 was weak from gestation day 21.5 until puberty. Pregnant Wistar rats were exposed to 0 or 500 mg/kg-day dibutyl phthalate by gavage in corn oil on gestation day 13.5 to 21.5, an exposure known to increase the incidence of cryptorchidism. Expression of Insl3 and P450 side-chain cleavage enzyme in fetuses on gestation day 17.5 and 19.5 decreased dramatically after exposure of their dams to dibutyl phthalate, but there was no consistent correlation between this suppression and abnormal testis position in the fetus. A parallel study in which dams were exposed to flutamide at 50 mg/kg-day did not effect the expression of either protein. Flutamide at this exposure is known to induce a high rate of cryptorchidism in male pups because it is an androgen receptor

antagonist. In adult males exposed in utero to dibutyl phthalate from this study, Insl3 expression was markedly and consistently suppressed in the Leydig cells in 80% of the animals with cryptorchidism. Unexpectedly Insl3 expression was also suppressed in the Leydig cells in 50% of the animals with scrotal testes. The findings of this study indicate that cryptorchidism from exposure to dibutyl phthalate results from the combined suppression of both Insl3 and testosterone, rather than just suppression of the expression of Insl3, and does not involve antagonism of the androgen receptor. The LOAEL in this study is 500 mg/kg-day, the only exposure tested.

Bowman et al. (2005) conducted a study to identify changes in gene expression associated with altered morphology of the proximal Wolffian duct (the anatomical precursor to the epididymis, vas deferens, and seminal vesicles) following in utero exposure to dibutyl phthalate. Pregnant Crl:CD Sprague-Dawley rats were treated with 0 or 500 mg/kg-day dibutyl phthalate by gavage in corn oil on GDs 12–19 or 21. There were only small morphological differences between control and exposed fetuses on GD 19. On GD 21, 89% of the male fetuses (41/46) and 100% of the litters (7/7) showed marked underdevelopment of the Wolffian ducts, characterized by decreased coiling. No abnormalities were observed in controls. Messenger RNA was isolated from Wolffian ducts on GDs 19 and 21. DNA microarrays were used to identify candidate genes that could be associated with the morphological changes observed on GD 21. These candidate genes were analyzed by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Changes in mRNA expression were observed in genes within the insulin-like growth factor (IGF) pathway, the matrix metalloproteinase (MMP) family, the extracellular matrix, and in other developmentally conserved signaling pathways. On GD 19 immunolocalization of IGF-1 receptor protein demonstrated an increase in cytoplasmic expression in the mesenchymal and epithelial cells. There was also a variable decrease in the AR protein in ductal epithelial cells. This study provides insights into the effects of antiandrogens on the molecular mechanisms involved in Wolffian duct development. The altered morphology and changes in gene expression are suggestive of altered paracrine interactions between ductal epithelial cells and the surrounding mesenchyme during Wolffian duct differentiation due to lowered testosterone. The LOAEL in this study is 500 mg/kg-day, the only exposure tested.

Plummer et al. (2005, abstract only) conducted a study to investigate disruption of signaling pathways in fetal testis associated with testicular dysgenesis following exposure to dibutyl phthalate using transcriptional profiling and immunohistochemical analysis. Pregnant

Wistar rats (number not reported) were exposed to dibutyl phthalate at 0 or 500 mg/kg-day by gavage during gestation (exposure days not reported). High density microarray analysis (22K array) on GDs 15, 17, and 19 indicated that dibutyl phthalate altered the expression of three functionally distinct gene clusters. Quantitative data are not reported in the abstract. A battery of genes involved in the synthesis of testosterone [including SF-1, StAR, P450_{scC}, and 17-alpha-hydroxylase-17,20-lyase] were down-regulated. These changes correlated with a 70% reduction in fetal testosterone levels. Insl-3 and genes associated with cellular protection against oxidative stress were also down-regulated. SF-1 mRNA levels in individual litters correlated with changes in expression of the SF-1-regulated genes Insl3, StAR, and P450_{scC}. Immunohistochemical analysis of fetal testes indicated down-regulation of the SF-1 regulated gene, inhibin alpha. This occurred in Leydig cells but not Sertoli cells, suggesting a cell specific effect. In adulthood, 65% of rats exposed in utero to dibutyl phthalate had either bilateral or unilateral cryptorchidism. These results suggest that dibutyl phthalate-induced testicular dysgenesis involves effects on several different signaling pathways and is mediated in part through down-regulation of SF-1 together with a battery of genes regulated by this orphan nuclear receptor in a cell specific manner.

Liu et al. (2005) examined global gene expression in the fetal testis of the rat following in utero exposure to phthalate esters. Pregnant Sprague-Dawley rats were treated from GD 12 through 19 with 0 or 500 mg/kg-day phthalate ester by gavage in corn oil. The phthalate esters used were diethyl phthalate (DEP), dimethyl phthalate (DMP), dioctyl tere-phthalate (DOTP), dibutyl phthalate (DBP), diethylhexyl phthalate (DEHP), dipentyl phthalate (DPP), or butyl benzyl phthalate (BBP). Testes were isolated on GD 19, and global changes in gene expression were determined. Of the approximately 30,000 genes queried, expression of 391 genes was significantly altered following exposure to the developmentally toxic phthalates (DBP, BBP, DPP, and DEHP) relative to the control. The developmentally toxic phthalates were indistinguishable in their effects on global gene expression. No significant changes in gene expression were detected following exposure to the non-developmentally toxic phthalates (DMP, DEP, and DOTP). Gene pathways disrupted included those previously identified as targets for DBP, including cholesterol transport and steroidogenesis, as well as newly identified pathways involved in intracellular lipid and cholesterol homeostasis, insulin signaling, transcriptional regulation, and oxidative stress. Additional gene targets included alpha inhibin, which is essential for normal Sertoli cell development, and genes involved with communication between Sertoli cells and gonocytes. The common targeting of these genes by a select group of phthalates indicates a role for their associated molecular pathways in testicular development.

4.3.2. Studies with Monobutyl Phthalate

Ema et al. (1995) evaluated monobutyl phthalate for sensitive developmental exposure periods in Wistar rats. Pregnant rats (n = 11 to 15 per group) were given monobutyl phthalate by gavage in water at 0, 250, 500, or 625 mg/kg-day on GDs 7–15. Significant decreases in the maternal body weight gains were found at 500 and 625 mg/kg-day (a decrease of 45 and 73%, respectively). Significant increase in the incidence of postimplantation loss per litter (61.5 and 98.4% versus 10.5% in control) and decreases in the number of live fetuses per litter (5.3 and 0.3 versus 13.0 in control) and fetal weight (a decrease of 11 and 18%, respectively) were also detected at 500 mg/kg-day and above. The incidence of fetuses with malformations in the 500 and 625 mg/kg-day groups (more than 44% of litters affected) was higher than in the control group. Cleft palate, deformity of the vertebral column and dilatation of the renal pelvis were frequently observed. The LOAEL is 500 mg/kg-day, and the NOAEL is 250 mg/kg-day.

Ema et al. (1996) evaluated monobutyl phthalate for developmental toxicity in Wistar rats. Pregnant rats (n = 10 to 15 per group) were given monobutyl phthalate by gavage in water containing at 0, 500, 625, or 750 mg/kg-day on GDs 7–9, 10–12, or 13–15. A significantly increased incidence of postimplantation loss was found on GDs 7–9 (27.8 and 57.7% versus 13.3% in controls), on GDs 10–12 (46.4 and 86.9% versus 13.3% in controls) at exposures of 625 mg/kg-day and above, and on GDs 13–15 (34.7, 66.8, and 95.5% versus 13.3% in controls) at exposures of 500 mg/kg-day and above. No evidence of teratogenicity was found on GDs 10–12. A significantly increased incidence of fetuses with external malformations was found after treatment on GDs 7–9 and 13–15 at exposures of 625 and 750 mg/kg-day (more than 44% of litters affected). A significantly increased incidence of fetuses with skeletal malformations was observed after treatment on GDs 7–9 at exposures of 500 mg/kg-day and above (more than 60% of litters affected) and on GDs 13–15 at exposures of 625 mg/kg-day and above (55% of litters affected). Deformity of the cervical vertebrae was predominantly observed on GDs 7–9. Cleft palate and fusion of the sternbrae were exclusively found on GDs 13–15. The LOAEL is 500 mg/kg-day, the lowest exposure tested.

Ema and Miyawaki (2001a) investigated monobutyl phthalate for adverse effects on development of the reproductive system and the role of monobutyl phthalate in the antiandrogenic effects of dibutyl phthalate. Pregnant rats (n = 16 per group) were given monobutyl phthalate by gavage in water at 0, 250, 500, or 750 mg/kg-day on GDs 15–17 of pregnancy. Maternal body weight gain during the administration period was significantly

decreased at 500 mg/kg-day and higher (a decrease of 50 and 70%, respectively). A significant increase in the incidence of postimplantation embryonic loss (30.6 and 52.7% versus 6.5% in controls) was found at 500 mg/kg-day and higher. The body weights of male and female fetuses were significantly lower at 750 mg/kg-day (a decrease greater than 10%). A significant increase in the incidence of fetuses with undescended testes was found at 250 mg/kg-day and higher (greater than 38% versus 0% in controls). A significant decrease in the AGD of male fetuses was observed at 250 mg/kg-day and higher (a decrease of 10, 30, and 35%, respectively). The AGD in female fetuses was not affected. The LOAEL is 250 mg/kg-day, the lowest exposure tested.

Ema and Miyawaki (2001b) investigated the effects of monobutyl phthalate on reproductive function in pregnant and pseudopregnant rats. Wistar rats (n = 16 per group) were given monobutyl phthalate by gavage in water at 0, 250, 500, 750, or 1000 mg/kg-day on GDs 0–8. Pregnancy outcome was determined on GD 20. The effects of monobutyl phthalate on uterine function as a cause of early embryonic loss were also determined in pseudopregnant rats with an induced decidual cell response. The same doses of monobutyl phthalate were given to pseudopregnant rats on days 0 to 8 of pseudopregnancy and the uterine weight on day 9 served as an index of uterine decidualization. Monobutyl phthalate at 1000 mg/kg-day caused significant increases in the incidences of preimplantation loss (20.2% versus 5.9% in controls) in females successfully mated and of postimplantation loss (26.3% versus 9.1% in controls) in females having implantations. Uterine decidualization in pseudopregnant rats was significantly decreased at 1000 mg/kg-day. The authors concluded that these findings suggest that early embryonic loss due to monobutyl phthalate is mediated, at least in part, via suppression of uterine decidualization, an impairment of uterine function. The LOAEL is 1000 mg/kg-day, and the NOAEL is 750 mg/kg-day.

Saillenfait et al. (2001) conducted a study to characterize the embryotoxic effects of monobutyl phthalate and to evaluate its role in the developmental toxicity of dibutyl phthalate. Sprague-Dawley rats (n = 11 to 15 per group) were given a single administration of 0, 1.8, 3.6, 5.4, or 7.2 mmol/kg-day of dibutyl phthalate by gavage in mineral oil (equivalent to 0, 500, 1000, 1500, or 2000 mg/kg-day) or monobutyl phthalate by gavage in water on GD 10. Embryos were evaluated for growth and development on GD 12. The embryotoxic effects of monobutyl phthalate were compared to those of the parent compound. Both chemicals induced concentration-dependent developmental toxicity (i.e., decreased growth and malformations) which became apparent at 3.6 mmol/kg-day. Dibutyl phthalate and monobutyl phthalate were

approximately equally potent and produced qualitatively similar dysmorphogenic effects. The most common malformations involved the proencephalon, the optic system, and the mandibular and maxillary processes.

Saillenfait et al. (2001) also evaluated the embryotoxic potential of monobutyl phthalate *in vitro* using the rat whole embryo culture system. Day 10 embryos were cultured for 48 hr in the presence of 0.5 to 5.0 mM monobutyl phthalate. Monobutyl phthalate was a potent direct-acting embryotoxicant, causing concentration-related growth retardation and dysmorphogenesis. The spectrum of morphological defects observed in monobutyl phthalate exposed embryos *in vitro* was comparable to those seen *in vivo* in the embryos at the same developmental stage after maternal administration of dibutyl phthalate or monobutyl phthalate. The authors concluded that these data provide additional evidence in support of the hypothesis that monobutyl phthalate is the active species for the developmental toxicity of ingested dibutyl phthalate in rats. The LOAEL is 1000 mg/kg-day, and the NOAEL is 500 mg/kg-day.

Collectively, these studies with monobutyl phthalate, together with the information on toxicokinetics in section 3, clearly show that monobutyl phthalate is responsible for the toxic effect of dibutyl phthalate. The studies in section 3 show that monobutyl phthalate and its glucuronide conjugate are the major metabolites formed in rats and humans (Fennell et al., 2004; Saillenfait et al., 1998; NIEHS, 1995, 1994; Tanaka, 1989; Foster et al., 1982; White et al., 1980, 1983; Kaneshima et al., 1978a,b; Takahashi and Tanaka et al., 1978; Lake et al., 1977; Rowland et al., 1977; Williams and Blanchfield, 1975; Albro and Moore, 1974). Only insignificant amounts of other metabolites are formed. Studies further show that monobutyl phthalate readily crosses the placenta and is found in fetal plasma (Kremer et al., 2005a; Fennel et al., 2004; Saillenfait et al., 1998). The toxicological studies reported above show that monobutyl phthalate and dibutyl phthalate cause a similar spectrum of adverse effects in laboratory animals and both chemicals have similar exposure-response relationships.

4.4. OTHER STUDIES

Genetic Toxicity

Dibutyl phthalate did not induce reverse mutations in *Saccharomyces cerevisiae* in the presence or absence of a metabolic activation system (Shahin and von Borstel, 1977). Dibutyl phthalate produced a marginal response for sister chromatid exchange in a pseudodiploid Chinese hamster cell line (Don) in the absence of a metabolic activation system (Abe and Sasaki,

1977). Dibutyl phthalate was not mutagenic in Salmonella strains TA98, TA100, TA1535, TA1537 in the presence or absence of a liver S-9 metabolic activation system (Zeiger et al., 1985). Dibutyl phthalate produced significant increases in the frequency of mutations in the mouse lymphoma assay using L5178Y cells in the presence but not in the absence of an S-9 Arochlor-induced rat liver activation system (Barber et al., 2000). Dibutyl phthalate did not increase the frequency of transformations in Balb/3T3 cells (Barber et al., 2000). In peripheral blood samples obtained from male and female mice at the end of a 13-week study, frequencies of micronucleated normochromatic erythrocytes were similar between exposed and control mice (NTP, 1995). The highest exposure used in this study was 20,000 ppm dibutyl phthalate in feed (equivalent to 3689 mg/kg-day in males and 4278 mg/kg-day in females). Kleinsasser et al. (2001, 2000) demonstrated that dibutyl phthalate exhibited genotoxicity on human mucosa cells of the upper aerodigestive tract using the alkaline version of the microgel electrophoresis assay to detect single-strand breaks in DNA (Comet Assay).

Gene Expression

Kobayashi et al. (2003) used RT-PCR to examine changes in gene expression regulated by peroxisome proliferator-activated receptor γ (PPAR γ) and in gene expression related to the inhibin/activin-follistatin system in the rat testis induced by a single oral administration of dibutyl phthalate (2400 mg/kg, n = 32). An increase in cytochrome P450 4A1 mRNA, which is regulated by PPAR γ , was observed in both the testis and liver. There was also an increase in the mRNA level of plasminogen activator inhibitor-1 (PAI-1) in the testis, also suggesting activation of PPAR γ . This latter change may be related to the disruption of spermatogenesis. There was also significant suppression of the mRNA level of inhibin β_B and elevation in the mRNA level of follistatin, an activin-binding protein. Activin B, a homodimer of inhibin β_B , is known to stimulate spermatogonial proliferation. The time course of these changes in gene expression is consistent with the testicular atrophy observed at this exposure in previous studies from this group. This study suggests that the suppression of spermatogenesis resulting from the changes in the expression of genes involved in the inhibin/activin-follistatin system is one of the mechanisms in testicular atrophy induced by dibutyl phthalate.

You et al. (2004, abstract only) studied the effects of dibutyl phthalate exposure during gestation on the expression of enzymes in the maternal and fetal liver that are involved in the metabolism of steroid hormones. Pregnant Sprague-Dawley rats received dibutyl phthalate by gavage at 10 to 500 mg/kg-day on GDS 12-19. Protein and mRNA levels of CYP 2B (testosterone 16 β -hydroxylase), CYP 3A (testosterone 6 β -hydroxylase), and CYP 4A were

increased in both maternal and fetal liver in the 500 mg/kg-day group but not in the groups exposed to 50 mg/kg-day or lower. The 500 mg/kg-day exposure also increased the mRNA of hepatic estrogen sulfotransferase and UDP-glucuronosyltransferase 2B1 in the dams but not in the fetuses.

Fan et al. (2004a) investigated the effect of dibutyl phthalate on the expression of cytochrome P450 2C7 (CYP2C7), a retinoic acid and testosterone hydroxylase. In the first study, male and female F-344 rats were fed 0 or 20,000 ppm dibutyl phthalate for 13 weeks. In a follow-up study, male and female F-344 rats were fed 0 or 20,000 ppm for 1, 5, or 13 weeks. In a third study, male Sprague-Dawley rats were fed 0, 1,500, 2,500, 5,000, 10,000, or 20,000 ppm dibutyl phthalate for 13 weeks. Food consumption, body weight, or number of animals used were not reported for any study. In the first study dibutyl phthalate decreased the CYP2C7 mRNA levels in the liver but the decrease was not statistically significant. In contrast the expression of CYP2C7 protein from the same livers was significantly decreased in both males and females. In the follow-up study, expression of CYP2C7 protein was decrease at 1, 5, and 13 weeks but the decrease was only statistically significant at 5 weeks due to the variability in expression of the controls at 1 and 13 weeks. In the third study, exposure to 5000 ppm and above caused a statistically significant decrease in CYP2C7 expression.

Fan et al. (2004b) investigated the effect of dibutyl phthalate on cytochrome P450 enzymes that hydroxylate testosterone. In the first study male and female F-344 rats were fed diets containing 0 or 20,000 ppm for 13 weeks. In the second study male Sprague-Dawley rats were fed diets containing 0, 1,500, 2,500, 10,000, or 20,000 ppm dibutyl phthalate for 1, 5, or 13 weeks. There were five to six animals per group in each study. Food consumption and body weight were not reported for either study. In the first study there were increases in 6 β - and 16 β -testosterone and androstenedione hydroxylase activities and decreases in 16 α - and 2 α -testosterone hydroxylase activities. The authors attributed the latter decreases to cytochrome P450 2C11 (CYP2C11). The authors investigated other cytochrome P450 enzymes known to have 6 β - and 16 β -testosterone hydroxylase activity. Exposure to dibutyl phthalate at 20,000 ppm in both males and females increased the protein expression of cytochrome P450 3A1 (CYP3A1). Only females showed an increase in cytochrome P450 3A2 (CYP3A2). Both of these cytochromes have 6 β -testosterone hydroxylase activity. Exposure to dibutyl phthalate at 20,000 ppm in both males and females increased the protein expression of cytochrome P450 2B1 (CYP2B1), a cytochrome that has 16 β -testosterone hydroxylase activity. In this study there was also an increase in the protein expression of cytochrome P450 4A (CYP4A) in males but not in

females. This cytochrome has fatty acid ω -hydroxylase activity. In the second study, there was no significant increase in protein expression for CYP3A2 as a function of exposure concentration or duration.

Wyde et al. (2005) investigated the potential of dibutyl phthalate to activate nuclear receptors that regulate the expression of liver enzymes. Pregnant Sprague-Dawley rats were treated with dibutyl phthalate by gavage in corn oil at 0, 10, 50, or 500 mg/kg-day from gestation days 12 to 19. Maternal and fetal livers were collected on day 19 for analyses. Increased protein level and mRNA levels of cytochrome P450 2B1 (CYP2B1), cytochrome P450 3A1 (CYP3A1), and cytochrome P450 4A1 (CYP4A1) were found in both maternal and fetal livers in the 500 mg/kg-day group. Dibutyl phthalate at 500 mg/kg-day also caused an increase in the mRNA of hepatic estrogen sulfotransferase and UDP-glucuronosyltransferase 2B1 in the dams but not in the fetuses. Xenobiotic induction of CYP3A1 and 2B1 is known to be mediated by the nuclear receptors pregnane X receptor (PXR) and constitutive androstane receptor (CAR). In vitro transcriptional activation assays showed that dibutyl phthalate activates both PXR and CAR. However, monobutyl phthalate did not interact strongly with either CAR or PXR. These data indicate that hepatic steroid- and xenobiotic-metabolizing enzymes are susceptible to induction by dibutyl phthalate at the fetal stage. The effects on enzyme expression are likely mediated by xenobiotic-response transcriptional factors, including CAR and PXR.

Activation of Peroxisome Proliferators in Cell Culture

Hurst and Waxman (2003) reported that mono(n)butyl phthalate did not activate mouse or human PPAR α or PPAR γ in COS-1 transfected cells lines or PPAR α -responsive liver cells or PPAR γ -responsive adipocyte cells at a concentration of 300 μ M. In contrast using similar techniques, Bility et al. (2004) reported activation of mouse and human PPAR α in transfected 3T3-L1 cells. Mouse PPAR α was activated 3.7-fold at 100 μ M and human PPAR α was activated 2.5-fold at 200 μ M. Monobutyl phthalate did activate mouse PPAR β (4.7-fold at 3 μ M), but not human PPAR β . Monobutyl phthalate did not activate either mouse or human PPAR γ . These authors also reported that monobutyl phthalate stimulated the production of the PPAR α target gene mRNA for acyl CoA oxidase 2.8-fold at 10 and 100 μ M in rodent FaO but not human HepG2 liver cell lines.

Lapinskas et al. (2005) investigated the ability of dibutyl phthalate and monobutyl phthalate to activate peroxisome proliferator (PPAR) α , β , or γ using a transient transfection assay with human hepatoblastoma cell line HepG2. In this assay dibutyl phthalate, but not

monobutyl phthalate, weakly activated all three PPAR subtypes. Using a scintillation proximity assay, dibutyl phthalate, but not monobutyl phthalate, interacted directly with human PPAR α and PPAR γ . Neither dibutyl phthalate nor monobutyl phthalate induced protein expression of acyl-CoA oxidase in vitro using rat hepatoma cell line FGC4. Finally the authors examined the responses in the livers of wild-type and PPAR α -null mice treated with 2000 mg/kg-day dibutyl phthalate for 7 days. In this study wild-type, but not PPAR α -null mice exhibited significantly greater liver weights (data not shown). Further in wild-type mice, but not PPAR α -null mice, dibutyl phthalate increased protein expression of acyl-CoA oxidase and cytochrome P450 4A (CYP4A).

Estrogenic Activity

Zacharewski et al. (1998) tested dibutyl phthalate for estrogenic activity in vitro using ER competitive ligand-binding and mammalian- and yeast-based gene expression assays. In vivo assays included uterine wet weight and vaginal cell cornification using ovariectomized Sprague-Dawley rats. Dibutyl phthalate weakly competed with 17 β -estradiol for binding to the ER. In gene expression assays, dibutyl phthalate at 10 μ M exhibited 36% activity as compared to a 100% response observed with 10 nM estradiol. Dibutyl phthalate tested in vivo did not induce significant increases in uterine wet weight or affect the degree of vaginal epithelial cell cornification when tested at oral doses of 20, 200, or 2000 mg/kg.

Seidlová-Wuttke et al. (2004) investigated the ability of dibutyl phthalate to bind to ER α and ER β in vitro and its effect on the uterus, vagina, and bone density of ovariectomized rats. Dibutyl phthalate showed no binding to ER α at a concentration of 100 μ M and only very weak binding to ER β (4500-fold less than estradiol). Monobutyl phthalate was not tested. In an in vivo experiment, 12 ovariectomized Sprague-Dawley rats were administered dibutyl phthalate in feed at 0, 92.5, or 462.5 mg/kg-day for 3 months. Dibutyl phthalate had no effect on the weight of the uterus or the thickness of the uterine epithelium. There was an effect on the thickness of the endometrium (an increase of 40%) at the highest exposure and a decrease in the thickness of the myometrium at both exposures (a decrease of 29 and 31%, respectively). Dibutyl phthalate did not significantly effect gene expression of ER α or ER β in the uterus or the vagina. Dibutyl phthalate did significantly effect gene expression of complement C3 (a uterine protein) in uterus at the low exposure (decrease of 85%). Dibutyl phthalate decreased the gene expression of Cornifin in the vagina at the low exposure (decrease of 75%) but stimulated its expression at the high exposure (10-fold increase). Dibutyl phthalate had no effect on bone density, on serum osteocalcin, or serum cross (rat) laps.

Hong et al. (2005) investigated the estrogenic activity of dibutyl phthalate using in vitro and in vivo assays. Compared to 17beta-estradiol and 17alpha-estradiol, dibutyl phthalate induced only weak proliferation of MCF-7 cells (a human breast cancer cell line). The authors also investigated the alteration of the expression of Calbindin-D9k (CaBP-9k) in immature rats after oral treatment with a number of phthalates, including dibutyl phthalate at 600 mg/g-day. None of the phthalates induced the expression of CaBP-9k mRNA and its protein in the uterus as analyzed by Northern and Western blot analyses, respectively.

Seidlová-Wuttke et al. (2005) investigated the effects of dibutyl phthalate on estrogen-regulated fat depot and on serum TSH, T3, T4, LH, and serum lipid concentrations. Dibutyl phthalate was administered in feed for 3 months to previously ovariectomized Sprague-Dawley rats (n = 12 in each group) at 0, 82, or 393 mg/kg-day. Dibutyl phthalate had negligible effects on the endpoints examined.

In Vitro Developmental

Rhee et al. (2002) evaluated the potential of dibutyl phthalate to cause embryotoxicity in a short-term in vitro battery system using whole embryo, midbrain, and limb bud culture systems. Dibutyl phthalate at 10, 100, and 1000 µg/ml inhibited growth and development of the whole embryo and inhibited differentiation and showed cytotoxicity in mid-brain and limb bud cultures with a dose-response effect.

4.5. SYNTHESIS AND EVALUATION OF MAJOR NONCANCER EFFECTS

4.5.1. Oral

Dibutyl phthalate is rapidly hydrolyzed to monobutyl phthalate and n-butanol by non-specific lipases and esterases found in the contents of the GI tract, homogenates of intestinal mucosal cells, homogenates of liver, isolated liver microsomes, and isolated liver mitochondria, homogenates of pancreas, homogenates of kidney, and blood (NIEHS, 1995, 1994; Takahashi and Tanaka, 1989; White et al., 1980; Kaneshima et al., 1978a,b; Tanaka et al., 1978; Rowland et al., 1977). Hydrolysis of the second butyl group does not occur to any significant extent. Quantitatively, the most important location for the production of monobutyl phthalate is the lumen of the intestine by enzymes secreted from the pancreas (White et al., 1983). In vitro studies with intestinal preparations from rat, baboon, ferret, and humans show a species similarity in the hydrolysis of dibutyl phthalate (Lake et al., 1977).

The monobutyl phthalate formed in the intestinal tract is rapidly and completely absorbed into the systemic circulation and is widely distributed in the body (Fennell et al., 2004; Saillenfait et al., 1998; NIEHS, 1995, 1994; Tanaka et al., 1978; Williams and Blanchfield, 1975).

The major metabolites of dibutyl phthalate are monobutyl phthalate and its glucuronide. Only small quantities of dibutyl phthalate, phthalic acid, and the products of ω and ω -1 oxidation are found in blood and tissues of the rat (Fennell et al., 2004; Saillenfait et al., 1998; NIEHS, 1995, 1994; Foster et al., 1982; Tanaka et al., 1978; Williams and Blanchfield, 1975; Albro and Moore, 1974). In rat serum 80-90% of the total monobutyl phthalate is free monobutyl phthalate and the remainder (10-20%) is the glucuronide conjugate (Fennell et al., 2004; Saillenfait et al., 1998; Williams and Blanchfield, 1975; Albro and Moore, 1974). However, in humans approximately 25 - 30% of the total monobutyl phthalate in the serum is free monobutyl phthalate and the remainder (70-75%) is the glucuronide conjugate (Silva et al., 2003). The metabolites of dibutyl phthalate are rapidly excreted primarily in the urine and do not bioaccumulate in tissue (Fennell et al., 2004; Saillenfait et al., 1998; Foster et al., 1982; Tanaka et al., 1978; Williams and Blanchfield, 1975).

Keys et al. (2000) developed a PBPK model for dibutyl phthalate in adult rats. The model providing the best fit to the data was a combined diffusion-limited and pH trapping model without enterohepatic circulation. This model has only very limited usefulness as it lacks the fetal compartment. Clewell et al. (2006, abstract only) and Kremer et al. (2005a, 2004, abstracts only) are developing a PBPK model for dibutyl phthalate in pregnant rats. The model includes compartments for the placenta, the fetus, and amniotic fluid. The model has a flow-limited structure. A sensitivity analysis indicated that maternal monobutyl phthalate levels are primarily affected by the diffusion rate into slowly perfused tissues and the rate of uptake from the gastrointestinal tract; fetal monobutyl phthalate levels are primarily affected by the rate of diffusion from the placenta to the fetus and the placenta-to-blood partition coefficient.

The information on the hydrolysis of dibutyl phthalate in the GI tract, the lack of significant metabolism of the monobutyl phthalate that is absorbed to compounds other than the glucuronide conjugate, and the similar exposure response relationships in toxicity studies for dibutyl phthalate and monobutyl phthalate establish that monobutyl phthalate is responsible for the toxic effects of dibutyl phthalate.

Two studies have documented an association between some adult human semen measures with exposure to dibutyl phthalate (Murature et al., 1987) and phthalate monoesters (Duty et al., 2003a). One study documented an association between a decrease in AGI in male human infants and increasing maternal exposure to phthalate monoesters during pregnancy (Swan et al., 2005). Another study documented an association between decreased testosterone in male infants and increased monobutyl phthalate in breast milk of their mothers (Main et al., 2006). These studies support the hypothesis that exposure to phthalates may have effects on the male reproductive tract during development and in the adult. However, it is impossible to draw a causal relationship from these studies. None of the studies examined exposure to other chemicals or other lifestyle factors that could have contributed to the associations. In addition it is impossible to derive an exposure response relationship for dibutyl phthalate from these studies because people are exposed to multiple phthalate diesters from environmental sources.

Dibutyl phthalate is not very acutely toxic to rodents as the LD₅₀ values are in the grams per kilogram range.

There are extensive studies documenting developmental toxicity of dibutyl and monobutyl phthalate in rodents. The most complete description of effects on developmental toxicity in rats are the series of studies of Ema et al. (2000a, b, 1998, 1997, 1996, 1995, 1994, 1993), Ema and Miyawaki, 2001a,b); Barlow and Foster (2003), Barlow et al. (2004), and Mylchreest et al. (2002, 2000, 1999, 1998). The effects on fetal development consistently observed by Ema et al. include cleft palate, skeletal malformations, reduced AGD in males, cryptorchidism, and other male reproductive tract malformations. Barlow and Foster (2003) and Barlow et al. (2004) described male reproductive tract lesions in fetal, early postnatal, and young adult male rats following exposure in utero to dibutyl phthalate. They observed a variety of fetal testicular and epididymal lesions that persisted into adulthood. The studies of Mylchreest et al. (2002, 2000, 1999, 1998) found hypospadias, agenesis of epididymides or seminal vesicles, cryptorchidism, decreased AGD in males, delayed preputial separation, and retained areolas or nipples in males. The study of Mylchreest et al. (2000) showed a biologically significant effect on retained areolas or nipples at an exposure of 100 mg/kg-day with a no effect level at 50 mg/kg-day.

A number of studies have examined gene expression for the enzymes involved in steroid biosynthesis in rodents (Plummer et al., 2005; Lehmann et al., 2004; Thompson et al., 2005, 2004; Barlow et al., 2003; Fisher et al., 2003; Shultz et al., 2001). Collectively these studies

document that exposure to dibutyl phthalate disrupts steroid synthesis in the fetal testis. The most significant biological effect is the decrease in testicular testosterone in the fetus during the critical developmental window. The available data on the mode of action of dibutyl phthalate toxicity indicate that this biochemical change during the critical developmental window initiates the cascade of external and internal malformations of the male reproductive tract. Lehmann et al. (2004) established that a statistically significant reduction in testosterone concentration resulted from in utero exposure to 50 mg/kg-day but not from an exposure to 30 mg/kg-day. Thompson et al. (2004) established that following in utero exposure to 500 mg/kg-day the testosterone levels in the testes return to normal after the metabolites of dibutyl phthalate are cleared from the circulation. However, the malformations induced by exposure to 500 mg/kg-day persist into adulthood (Barlow et al, 2004; Barlow and Foster, 2003). Thus, although the inhibition of testosterone synthesis is reversible, the biological effects resulting from the decrease in testosterone during the critical developmental window are irreversible.

Dibutyl phthalate also causes reproductive toxicity in rats and mice. The most significant effects include increased prenatal mortality and decreased fetal body weight. At the lowest exposure tested in a continuous breeding study in rats, 80 mg/kg-day in females, there was a decrease in mean live pups per litter in F₁ and a decrease in mean live pup weight in F₂ (NTP, 1995).

There are several 90-day or longer studies (Higuchi et al., 2003; NTP, 1995; BASF, 1992). These studies have documented effects on the liver and testes. In the rat liver, there are effects on organ weight and histological and enzyme changes consistent with peroxisome proliferation. The lowest NOAEL for effects in the liver is 138 mg/kg-day with a LOAEL of 279 mg/kg-day (NTP, 1995). Effects in the testis include degeneration of the germinal epithelium with a NOAEL of 279 mg/kg-day and a LOAEL of 571 mg/kg-day (NTP, 1995). One study found no effects on neurological function in a standard battery of tests at 688 mg/kg-day (BASF, 1992).

There are no chronic bioassays. The data base on dibutyl phthalate is fairly complete and more than adequately documents the important toxicological endpoints. It is not likely that additional study will reveal an endpoint that occurs at an exposure below that which causes a decrease in testosterone in the fetal testes.

4.5.2. Mode-of-Action Information

The key steps in the mode of action for the developmental effects are shown in Figure 2. The key steps determining exposure include hydrolysis of dibutyl phthalate to monobutyl phthalate in the GI tract, absorption into the systemic circulation, and transfer of monobutyl phthalate to target tissues (the developing fetus, the liver, and the adult testes) by simple diffusion. The key steps in detoxification include metabolism of monobutyl phthalate to its glucuronide conjugate in the liver and excretion in the urine. See Kremer et al. (2005a, 2005b, 2004); Fennell et al. (2004); Silva et al. (2003); Keys et al. (2000); Saillenfait et al. (1998); NIEHS (1995, 1994); Takahashi and Tanaka (1989); White et al. (1983); Foster et al. (1982); White et al. (1980); Kaneshima et al. (1978a,b); Tanaka et al. (1978); Lake et al. (1977); Rowland et al. (1977); Williams and Blanchfield (1975); and Albro and Moore (1974) for more detailed information.

In the developing fetus the toxicological effects that occur at the lowest exposure are a variety of malformations of the male reproductive tract in structures that are dependent on the presence of testosterone and dihydrotestosterone. These include hypospadias; decrease in AGD; delayed preputial separation; agenesis of the prostate, epididymis, and vas deferens; degeneration of the seminiferous epithelium; interstitial cell hyperplasia of the testis; and the retention of thoracic areolas or nipples (Bowman et al., 2005; Barlow et al., 2004; Kim et al., 2004b; Kleymenova et al., 2005; Barlow and Foster, 2003; Fisher et al., 2003; Higuchi et al., 2003; Mylchreest et al., 2002, 2000, 1999, 1998; Ema et al., 2000b, 1998, 1997, 1994; Saillenfait et al., 1998). There is no evidence for comparable malformations in the female reproductive tract. There is no evidence that monobutyl phthalate has significant estrogenic activity or that it interacts with the androgen receptor.

Several studies have documented that the underlying key event for the toxicological effects listed above in the male fetus is a decrease in fetal testicular testosterone in Leydig cells (Thompson et al., 2005; Lehmann et al., 2004; Thompson et al., 2004). Additional studies have established that there is a decrease in cholesterol metabolism and in cholesterol transport genes as well as downregulation of most of the genes in the testosterone biosynthetic pathway in the fetus (Liu et al., 2005; Thompson et al., 2005; Plummer et al., 2005; Lehmann et al., 2004; Thompson et al., 2004; Barlow et al., 2003; Fisher et al., 2003; Shultz et al., 2001). The steps in testosterone synthesis disrupted in the Leydig cell of the fetal testes include transport of cholesterol esters from high density lipoproteins into the cell by SR-B1, delivery of cholesterol to the inner mitochondrial membrane by StAR, conversion of cholesterol to pregnenolone catalyzed by P450_{SCC}, and oxidation of progesterone to androstenedione catalyzed by CYP17.

Gubernacular malformations and the resulting cryptorchidism appear to be due to a separate mode of action involving reduced insulin-like growth factor 3 (Insl3) and reduced testosterone (McKinnell et al., 2005; Lehmann et al., 2004; Wilson et al., 2004). This mode of action in the rat appears to require a higher exposure to dibutyl phthalate than the mode of action discussed above. Dibutyl phthalate represses the expression of Insl3 (Lehmann et al., 2004; Wilson et al., 2004). This protein participates in the development of the gubernacular ligament and is essential for the transabdominal descent of the testis. The testosterone surge late in the developmental window is then responsible for the inguinoscrotal descent of the testis.

Although the mode of action was developed using data collected in rats, the mode of action is plausible for humans (Foster, 2006, 2005). Hydrolysis of dibutyl phthalate to monobutyl phthalate in the gastrointestinal tract, absorption of monobutyl phthalate, and transport of monobutyl phthalate to the fetal compartment occur in humans. The principal metabolites, monobutyl phthalate and its glucuronide conjugate, are found in human urine and tissues, including amniotic fluid (Silva et al., 2005, 2004a, 2004 b, 2003; Anderson et al., 2001). The biosynthetic pathway for the production of testosterone and dihydrotestosterone from cholesterol is identical in rats and humans. It is plausible that the regulation of expression of mRNA and synthesis of the necessary enzymes for this pathway are comparable in rats and humans. Testosterone and dihydrotestosterone are critical for normal development of the male reproductive tract in all species. Cryptorchidism and hypospadias, prominent effects of exposure to dibutyl phthalate in rats, are among the most common male birth defects in humans. Swan et al. (2005) observed an association between reduced anogenital index in human male infants with increasing maternal concentration of phthalate monoesters in urine (Swan et al., 2005). However, the Swan et al. (2005) study has several limitations that were discussed previously in Section 4.1. A decrease in anogenital distance is a prominent effect in rats following in utero exposure to dibutyl phthalate. Finally, Main et al. (2006) observed an association between reduced serum testosterone levels in newborn males with increasing phthalate monoesters in the breast milk of their mothers.

Additional molecular and cellular targets for monobutyl phthalate in the fetal testes are unknown. Some evidence suggests that failure of maturation of Sertoli cells and disruption of cell signaling to the Leydig cell is involved (Fisher et al., 2003). Steroidogenesis in the Leydig cell requires both LH signaling and arachidonic acid release from the cell membrane by cytosolic phospholipase A₂ (CPLA₂). Some evidence is available suggesting that monobutyl phthalate may inhibit CPLA₂ (Clewell et al., 2005, abstract only).

The general toxic effects on reproductive performance and on the developing fetus include a decrease in live pups per litter (Ema et al., 2000a, 1994, 1993; NTP, 1995; Lamb et al., 1987), a decrease in mean live pup weight (NTP, 1995), and skeletal and palate malformations (Ema et al., 2000b, 1998, 1997, 1994). These effects are observed in both males and females and in the absence of significant maternal toxicity. The decrease in mean litter size is probably the result of postimplantation loss. However, no evidence is available that points to a specific mode of toxicity for these effects.

The adult testis is also a target for dibutyl phthalate. The histopathological effects observed include degeneration of seminiferous tubules, degeneration of germinal epithelium, and sloughing of germ cells (NTP, 1995 ; Srivastava et al., 1990; Fukuoka et al., 1989). The mode of action for these effects is not known but is most likely due to alterations in Sertoli cell structure and function (Fisher et al., 2003, 1990; NTP, 1995; Srivastava et al., 1990; Zhou et al., 1990; Fukuoka et al., 1989). These alterations in the Sertoli cells lead to sloughing of the germinal epithelium.

The toxicological effects of dibutyl phthalate in rat liver (increase in liver weight and histopathological changes) are correlated with an increase in the marker enzyme (palmitoyl CoA oxidase) for peroxisome proliferation (NTP, 1995). However, data examining activation of PPAR α in cell cultures are somewhat contradictory (Lapinskas et al., 2005; Bility et al., 2004; Hurst and Waxman, 2003). However, the hepatic effects seen in vivo in rats are most likely due to PPAR α agonism. Other structurally similar phthalate esters (diethylhexyl phthalate and diisononyl phthalate) cause hepatic effects, including tumors, mediated by PPAR α agonism (Klaunig et al., 2003).

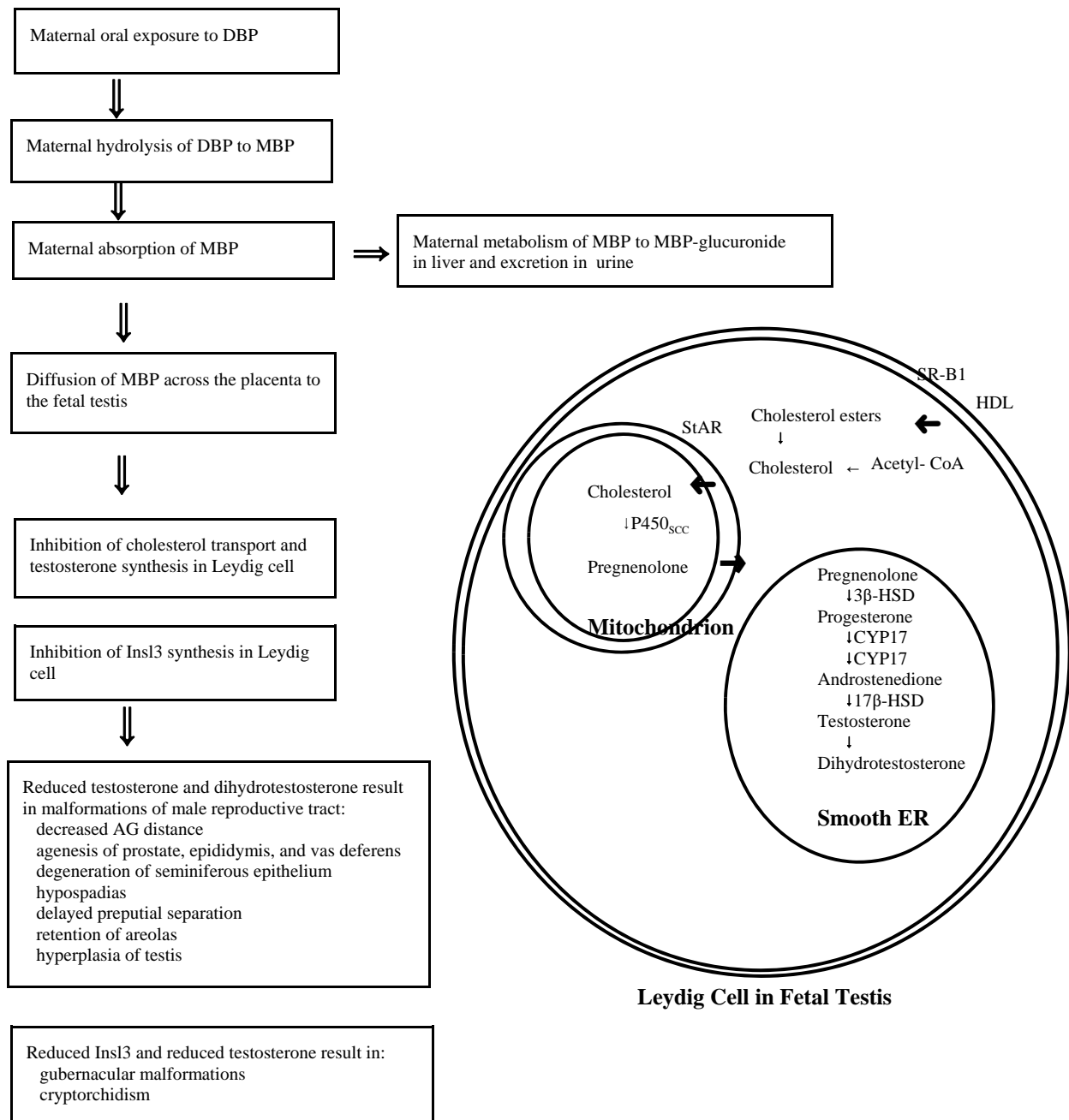


Figure 2. Proposed Mode of Action for Developmental Effects

Source: Adapted from Thompson et al., 2004

4.6. WEIGHT-OF-EVIDENCE AND CANCER CHARACTERIZATION

There are no chronic studies evaluating cancer effects in any species. Therefore, under the *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005), there is inadequate information to assess carcinogenic potential.

4.7. SUSCEPTIBLE POPULATIONS AND LIFE STAGES

The most sensitive life stage is the developing fetus. Oral exposure of pregnant rats to dibutyl phthalate causes effects on the developing male reproductive tract due to disruption of testosterone synthesis in the testis. There are no data suggesting that comparable effects occur in the developing female fetus. Although one study suggests that in utero exposure to dibutyl phthalate might have an effect on vaginal opening and the time to first estrus (Salazar et al., 2004), a more detailed study found no evidence for effects on age of first estrus or estrus cyclicity (Lee et al., 2004). Dibutyl phthalate also causes preimplantation and postimplantation fetal loss, decreased body weight, and malformations of the skeleton and palate in both male and female fetuses. Effects on the fetal and adult liver affect both males and females. Dibutyl phthalate also causes testicular lesions in adult males. As all the in utero effects occur at a much lower exposure than effects in the adult and are mediated by exposure to the female, the target population for primary consideration in a risk assessment is women of childbearing age.

5. DOSE-RESPONSE ASSESSMENTS

5.1. ORAL REFERENCE DOSE (RfD)

5.1.1. Choice of Principal Study and Critical Effect

The human epidemiological studies showing associations between some semen parameters with increasing environmental exposure to phthalate monoesters in adult males (Duty et al., 2003), a decrease in AGI in boys with increasing environmental exposure to phthalate monoesters in the mothers (Swan et al., 2005), and a decrease in testosterone in 3 month old male infants and an increase in monobutyl phthalate in breast milk in their mothers (Main et al., 2006) cannot be used to derive RfDs for dibutyl phthalate. Although these effects are consistent with the effects observed in studies in laboratory animals, the human epidemiological studies do not clearly establish a cause and effect relationship. The individuals in these studies were exposed to multiple phthalate esters and these exposures are highly correlated. In addition the individuals in these studies may have been exposed to other chemicals or have lifestyle factors that might contribute to the effects observed. Accordingly, the human epidemiological studies are only used qualitatively in this assessment.

The studies relevant for the development of acute, short-term, subchronic, and chronic RfDs are summarized in Figures 3, 4, and 5 and Tables 5-1, 5-2, and 5-3. Studies in Figures 3 and 4 and Tables 5-2, 5-3, and 5-3 are listed by increasing LOAEL; studies in Figure 5 are grouped by toxicological endpoint.

The studies showing developmental effects at the lowest exposure following exposure only in utero include the decrease in testosterone concentration in the fetal testes with a NOAEL and LOAEL of 30 mg/kg-day and 50 mg/kg-day, respectively (Lehmann et al., 2004); the increase in retained areolas or nipples in the male fetus with a NOAEL and LOAEL of 50 mg/kg-day and 100 mg/kg-day, respectively (Mylchreest et al., 2000); and the decrease in live pups per litter with a LOAEL of 80 mg/kg-day (NTP 1995). Studies showing comparable developmental effects following exposure in utero and during lactation include Zhang et al. (2004) with a NOAEL and LOAEL of 50 and 250 mg/kg-day, respectively, and Lee et al. (2004) with a NOAEL and LOAEL of 148-291 and 712-1372 mg/kg-day, respectively.

The studies showing effects on the testis include NTP (1995) and Fukuoka et al. (1990). In a study with exposure in utero, during lactation, and during adulthood (NTP, 1995), the

NOAEL and LOAEL were 279 mg/kg-day and 571 mg/kg-day, respectively. Studies showing effects following adult only exposure include Fukuoka et al. (1990) with a LOAEL of 250 mg/kg-day, and NTP (1995) with a NOAEL and LOAEL of 359 and 720 mg/kg-day, respectively.

A number of studies show effects in the liver (Lee et al., 2004; NTP, 1995; BASF, 1992). The study showing an effect at the lowest exposure is NTP (1995) with a NOAEL and LOAEL of 138 mg/kg-day and 279 mg/kg-day, respectively.

One study (BASF, 1992) shows a NOAEL for neurological effects (688 mg/kg-day).

As discussed above and shown in Figure 5, the developmental studies (Lehmann et al., 2004; Mylchreest et al., 2000; NTP, 1995) show effects at a lower exposure than effects in other target tissue (testis, liver, and nervous system)

The effects on testosterone concentration in the fetal testes (Lehmann et al., 2004) and the increase in retained areolas or nipples in male pups (Mylchreest et al., 2000) occur at the lowest exposure. The increase in retained areolas or nipples is considered an adverse effect and is irreversible. The NOAEL and LOAEL in this study are 50 and 100 mg/kg-day, respectively. In Lehmann et al. (2004) there was a statistically significant decrease in fetal testosterone concentration at 50 mg/kg-day, but not at 30 mg/kg-day. Although the decrease in testosterone in the fetal testis is reversible and returns to normal levels after the metabolites of dibutyl phthalate are cleared from the circulation, this biochemical change during the critical developmental window may initiate the cascade of irreversible malformations in the male reproductive tract as described in Section 4.5.2. Therefore, EPA considers the decrease in testosterone concentration an adverse effect in this study. Accordingly, the critical effect for chronic exposure to dibutyl phthalate is developmental toxicity (decreased testosterone in the fetal testes) and the principal study is Lehmann et al. (2004) with a NOAEL of 30 mg/kg-day and a LOAEL of 50 mg/kg-day. In this study exposure was on GDs 12 - 19 and there was a 61% decrease in testosterone in the fetal testis at the LOAEL. Other studies (Thompson et al., 2005, 2004a) showed a comparable decrease in fetal testosterone (43 - 77%) following a single exposure to dibutyl phthalate at 500 mg/kg-day on GD 19. It is not known whether the effects seen following repeated (8 days) administration of 50 mg/kg-day dibutyl phthalate would be evident after a single exposure. However, it is a plausible assumption for developmental toxic effects that “a single exposure at a critical time in development may produce an adverse

developmental effect, i.e., repeated exposure is not a necessary prerequisite for developmental toxicity to be manifested” as discussed in U.S. EPA’s 1991 Guidelines for Development Toxicity Risk Assessment. Therefore, EPA concluded that a single exposure to 50 mg/kg-day dibutyl phthalate during the critical developmental window may initiate the cascade of malformations in the male reproductive tract. Therefore, the Lehmann et al. (2004) study is applicable to the derivation of both the acute and short-term reference values. The effects on testosterone levels observed by Lehmann et al. (2004) also occur at doses that are lower than those observed in the available subchronic studies. Thus, Lehmann et al. (2004) was selected for the derivation of the subchronic and chronic reference values. Using the decrease in testosterone concentration in the fetal testes (Lehmann et al., 2004) as the critical effect to derive the reference value for all durations of exposure will likely protect children and adults from the other effects of dibutyl phthalate that require a higher exposure.

Figure 3. Exposure Response Array for Acute and Short Term Exposure to Dibutyl Phthalate

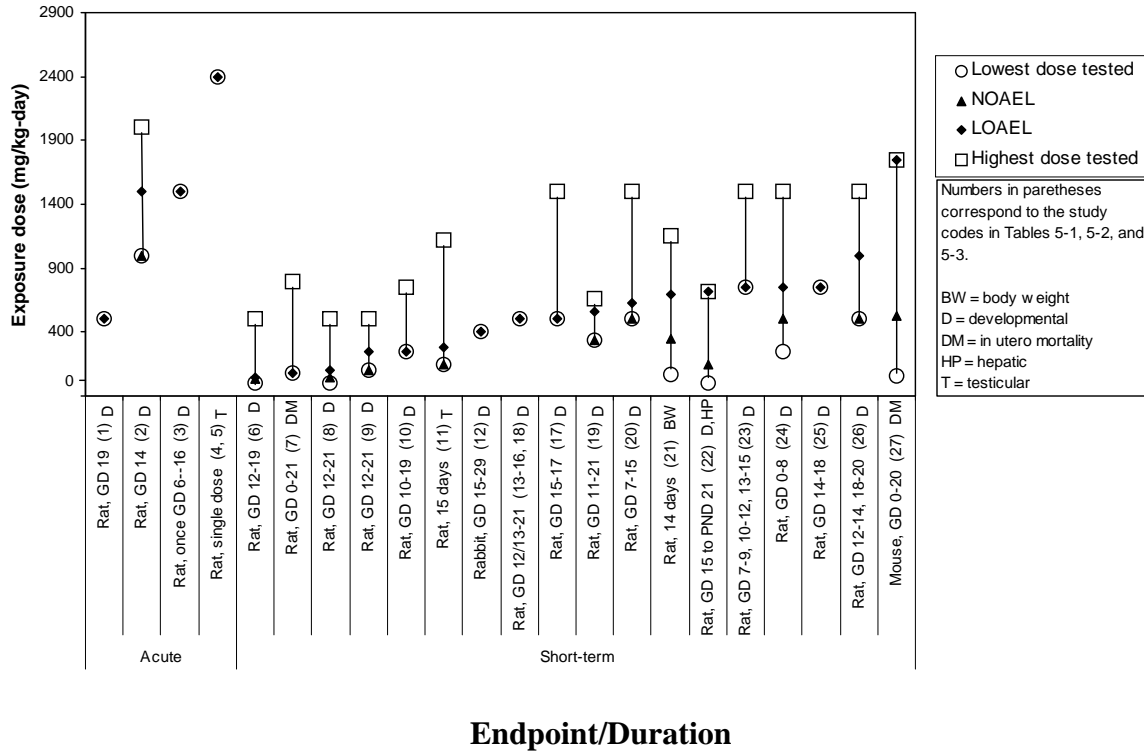


Figure 4. Exposure Response Array for Subchronic Exposure to Dibutyl Phthalate

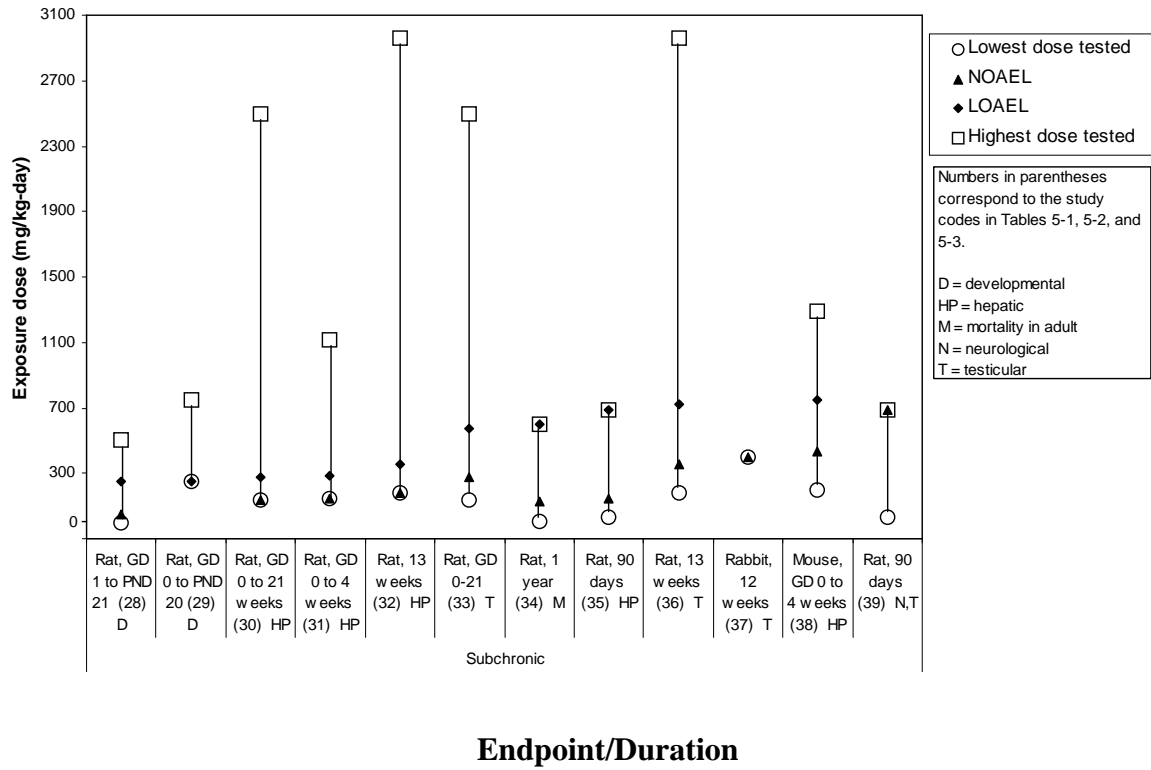


Figure 5. Exposure Response Array for Candidate Endpoints for RfD for Dibutyl Phthalate

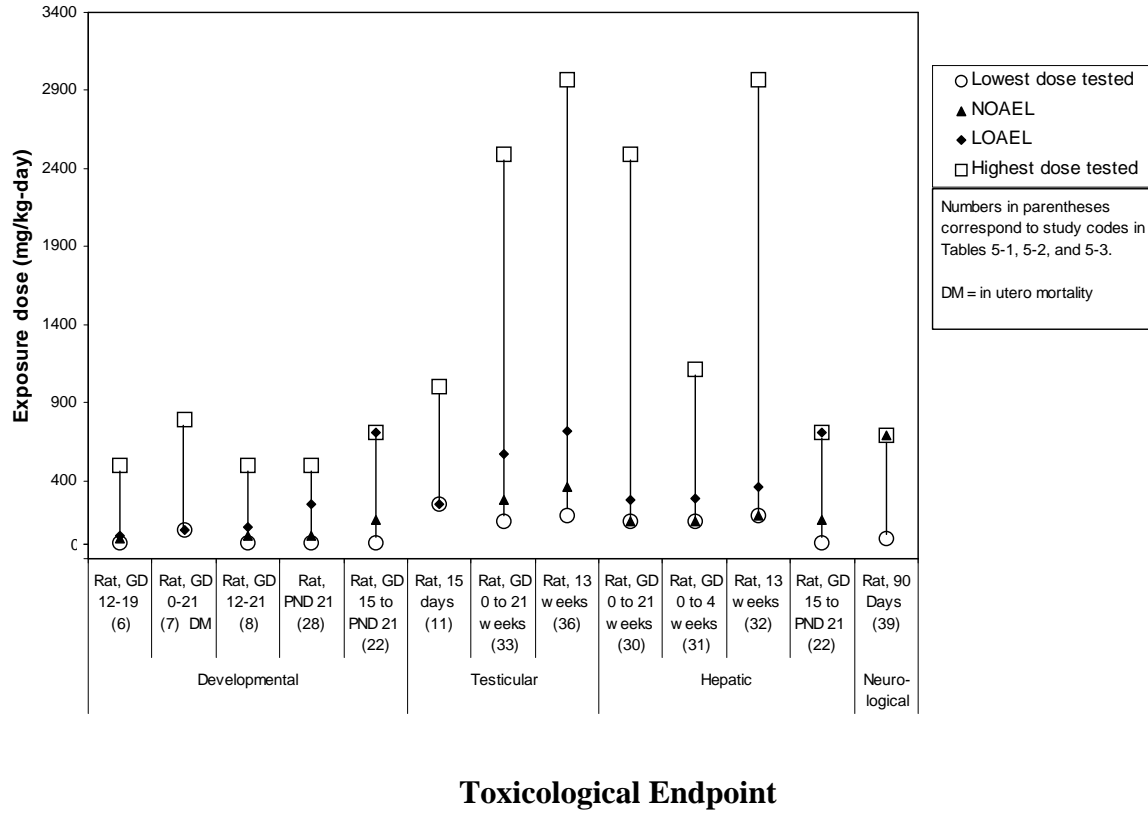


Table 5-1. Studies applicable to derivation of the acute RfD (exposure 1 day or less)

Study	Species, Duration, and Exposure	Effect	NOAEL, mg/kg-day	LOAEL, mg/kg-day
Lehmann et al., 2004 [#] -(6)	Rat; GD 12 - 19; 0, 0.1, 1, 10, 30, 50, 100, or 500 mg/kg-day	Decreased testosterone	30	50
Mylchreest et al., 2000 [#] -(8)	Rat; GD 12 - 21; 0, 0.5, 5, 50, or 100 mg/kg-day	Retained thoracic areolas or nipples in male pups	50	100
Thompson et al., 2004 and 2005 (1)	Rat; once GD 12 to 19; 0 or 500 mg/kg	Decreased testosterone		500
Carruthers and Foster, 2005 [#] (1)	Rat; GD 14-15, GD 15-16, GD 16-17, GD 17-18, GD 18-19, or GD 19-20; 0 or 500 mg/kg-day	Reduced AG distance from exposure on GD 15-16 and GD 18-19; Retained nipples from exposure on GD 16-17; Reduced epididymal weight from exposure on GD 17-18; Increased male reproductive tract malformations from exposure on GD 15-16, GD 17-18, and GD 19-20		500
Saillenfait et al., 1998 (2)	Rat; once GD 14; 0, 500, 1000, or 2000 mg/kg-day	Postimplantation loss, reduced fetal body weight, increased incidence of skeletal variations	1000	1500
Ema et al., 1997 (3)	Rat; once, GD 6 to GD 16; 0 or 1500 mg/kg	Postimplantation loss (GD 6, 8 through 10, 12 through 16), malformations (GD 8, 9, 15)		1500
Zhou et al., 1990 (4)	Rat, adult, 1 day, 0 or 2400 mg/kg	Sloughing of germ cells in testes		2400
Fukuoka et al., 1990 (5)	Rat, adult, 1 day, 0 or 2400 mg/kg	Sloughing of germ cells in testes		2400

[#] It is not known whether the effects seen following repeated administration of dibutyl phthalate would be evident after a single exposure. It is, however, a plausible assumption for developmental toxic effects that a single exposure at a critical time in development may produce an adverse developmental effect. Therefore, EPA considers these studies to be applicable to the derivation of the acute RfD.

Table 5-2. Studies applicable to derivation of the short-term RfD (exposure up to 30 days)

Study	Species, Duration, and Exposure	Effect	NOAEL, mg/kg-day	LOAEL, mg/kg-day
Lehmann et al., 2004 (6)	Rat; GD 12 - 19; 0, 0.1, 1, 10, 30, 50, 100, or 500 mg/kg-day	Decreased testosterone	30	50
NTP, 1995 (7)	Rat; continuous breeding, dams 16 weeks, each litter GD 0-21; 0, 80, 385, or 794 mg/kg-day	Decreased live pups/litter in F ₁ ; Adjusted mean live pup weight in F ₂		80
Mylchreest et al., 2000 (8)	Rat; GD 12 - 21; 0, 0.5, 5, 50, or 100 mg/kg-day	Retained thoracic areolas or nipples in male pups	50	100
Mylchreest et al., 1999 (9)	Rat; GD 12 - 21; 0, 100, 250, or 500 mg/kg-day	Male reproductive tract malformations	100	250
Kim et al., 2004b (10)	Rat; GD 10 - 19; 0, 250, 500, or 700 mg/kg-day	Decreased testes and accessory sex organ weight		250
Srivastava et al., 1990 (11)	Rat; adult, 15 days; 0, 250, 500, or 1000 mg/kg-day	Degeneration of seminiferous tubules		250
Higuchi et al., 2003 (12)	Rabbit; GD 15 - 29; 0 or 400 mg/kg-day	Decreased sperm production, decreased testes and accessory sex organ weight		400
Carruthers and Foster, 2005 (13)	Rat; GD 14-15, GD 15-16, GD 16-17, GD 17-18, GD 18-19, or GD 19-20; 0 or 500 mg/kg-day	Reduced AG distance from exposure on GD 15-16 and GD 18-19; Retained nipples from exposure on GD 16-17; Reduced epididymal weight from exposure on GD 17-18; Increased male reproductive tract malformations from exposure on GD 15-16, GD 17-18, and GD 19-20		500
Fisher et al., 2003 (13)	Rat; GD 13 - 21; 0 or 500 mg/kg-day	Male reproductive tract malformations		500
Mylchreest et al., 2002 (14)	Rat; GD 12 - 21; 0 or 500 mg/kg-day	Testicular atrophy, decreased testicular testosterone, Leydig cell hyperplasia		500

Study	Species, Duration, and Exposure	Effect	NOAEL, mg/kg-day	LOAEL, mg/kg-day
Barlow and Foster, 2003; Barlow et al., 2004 (15);	Rat; GD 12 - 21; 0 or 500 mg/kg-day	Male reproductive tract malformations		500
Kleymenova et al., 2004 (16)	Rat; GD 12 - 21; 0 or 500 mg/kg-day	Decrease in cells in testes		500
Ema et al., 2000b (17)	Rat; GD 15 - 17; 0, 500, 1000, or 1500 mg/kg-day	Male reproductive tract malformations		500
Mahood et al., 2005 (18)	Rat, GD 13.5 - 21.5; 0 or 500 mg/kg	Aggregation of Leydig cells		500
Ema et al., 1998 (19)	Rat; GD 11 - 21; 0, 331, 555, or 661 mg/kg-day	Male reproductive tract malformations	331	555
Ema et al., 1993 (20)	Rat; GD 7 - 15; 0, 500, 630, 750, or 1000 mg/kg-day	Postimplantation loss, decreased fetal body weight	500	630
NTP, 1991 (21)	Rat; adult, 14 days; 0, 70, 350, 700, 930, or 1150 mg/kg-day	Decreased body weight in females	350	700
Lee et al., 2004 (22)	Rat; GD15 to PND 21; 0, 1.5, 14.4, 148, or 712 mg/kg-day	Increased liver weight and histopathological changes; decreased testis weight; loss of germ cell development; reduced anogenital distance in males; and nipple retention in males	148	712
Ema et al., 1994 (23)	Rat; GD 7 - 9, GD 10 - 12, or GD 13 - 15; 0, 750, 1000, or 1500 mg/kg-day	Postimplantation loss (GD 7 - 9, 10 - 12, and 13 - 15), malformations (GD 7 - 9, 13 - 15)		750
Ema et al., 2000a (24)	Rat; GD 0 - 8; 0, 250, 500, 750, 1000, 1250, or 1500 mg/kg-day	Postimplantation loss, uterine decidualization	500	750

Study	Species, Duration, and Exposure	Effect	NOAEL, mg/kg-day	LOAEL, mg/kg-day
Wilson et al., 2004 (25)	Rat; GD 14 - 18; 0 or 750 mg/kg-day	Decreased testosterone and expression of insl3		750
Ema et al., 2000b (26)	Rat; GD 12 - 14, or GD 18 - 20; 0, 500, 1000, or 1500 mg/kg-day	Male reproductive malformations		1000
Lamb et al., 1987 (27)	Mice; continuous breeding, dams 105 days, each litter GD 0 - 20; 0, 53, 525, or 1750 mg/kg-day	Decreased mean live pups per litter, decreased litters per breeding pair	525	1750
Cummings and Gray, 1987	Rat; GD 1 - 8; 0, 500, 1000, or 2000 mg/kg-day	None	2000	
Fukuoka et al., 1989	Rat, adult, 7 days, 0 or 2400 mg/kg-day	Sloughing of germ cells in testes		2400

Table 5-3. Studies applicable to derivation of the subchronic RfD (exposure up to 10% of lifespan) and chronic RfD

Study	Species, Duration, and Exposure	Effect	NOAEL, mg/kg-day	LOAEL, mg/kg-day
Lehmann et al., 2004 (6)	Rat; GD 12 - 19; 0, 0.1, 1, 10, 30, 50, 100, or 500 mg/kg-day	Decreased testosterone	30	50
NTP, 1995 (7)	Rat; continuous breeding, dams 16 weeks, each litter GD 0 - 21; 0, 80, 385, or 794 mg/kg-day	Decreased live pups/litter in F ₁ , Adjusted mean live pup weight in F ₂		80
Mylchreest et al., 2000 (8)	Rat; GD 12 - 21; 0, 0.5, 5, 50, or 100 mg/kg-day	Retained thoracic areolas or nipples in male pups	50	100
Zhang et al., 2004 (28)	Rat; GD 1 to PND 21; 0, 50, 250, or 500 mg/kg-day	Decreased birth weight; male reproductive tract lesions	50	250
Mylchreest et al., 1998 (29)	Rat; GD 0 to PND 20; 0, 250, 500, or 750 mg/kg-day	Male reproductive tract malformations		250
NTP, 1995 (30)	Rat; perinatal and lactation plus 17 weeks; 0, 138, 279, 571, 1262, or 2495 mg/kg-day	Increased liver weight, peroxisome proliferation	138	279
NTP, 1995 (31)	Rat; perinatal and lactation plus 4 weeks; 0, 143, 284, 579, 879, or 1115 mg/kg-day	Increased liver weight	143	284
NTP, 1995 (32)	Rat; adult, 13 weeks; 0, 176, 359, 720, 1540, or 2964 mg/kg-day	Increased liver weight, peroxisome proliferation	176	359

Study	Species, Duration, and Exposure	Effect	NOAEL, mg/kg-day	LOAEL, mg/kg-day
NTP, 1995 (33)	Rat; perinatal and lactation plus 17 weeks; 0, 138, 279, 571, 1262, or 2495 mg/kg-day	Testicular lesions	279	571
Smith, 1953 (34)	Rat; adult, 1 year; 0, 5, 25, 125, or 600 mg/kg-day	Mortality during first week of study	125	600
BASF, 1992 (35)	Rat; adult, 90 days; 0, 27, 142, or 688 mg/kg-day	Increased palmitoyl-CoA oxidation in liver, increased relative liver and kidney weight in females	142	688
NTP, 1995 (36)	Rat; adult, 13 weeks; 0, 176, 359, 720, 1540, or 2964 mg/kg-day	Testicular lesions	359	720
Higuchi et al., 2003 (37)	Rabbit; adult, 12 weeks; 0 or 400 mg/kg-day	Slight decrease in % of morphologically normal sperm (not biologically significant)	400	
NTP, 1995 (38)	Mice; perinatal and lactation plus 4 weeks; 0, 199, 437, 750, or 1286 mg/kg-day	Decreased body weight in male offspring	437	750
BASF, 1992 (39)	Rat; adult, 90 days; 0, 27, 142, or 688 mg/kg-day	Lack of neurological and testicular effects	688	
NTP, 1995 (40)	Mice; adult, 13 weeks; 0, 163, 353, 812, 1601, or 3689 mg/kg-day	Increased liver weight	812	1601

5.1.2. Methods of Analysis

The NOAEL/LOAEL and benchmark dose methods are used to analyze the exposure response relationships. From each developmental study that is a candidate for determining the point of departure, the NOAEL and/or LOAEL and the BMDL_{1SD} and/or the BMDL₁₀ are presented to facilitate comparison of the studies at a comparable level of response. All exposures are in mg/kg-day. The NOAEL/ LOAEL is based on the statistical analysis presented in the original publications. EPA conducted the benchmark dose analysis. Details of the benchmark dose calculations are in Appendix B. In the absence of knowledge about the amount of change required to cause an adverse effect, the Benchmark Dose Technical Guidance Document (U.S EPA, 2000) recommends using a decrease of one standard deviation for a continuous endpoint and a 10% response level for a quantal endpoint as the benchmark response. As can be seen, the studies by Lehmann et al. (2004) and Mylchreest et al. (2000) show an exposure response relationship at the lowest exposure.

Table 5-4. Candidate points-of-departure for RfD development from studies of the systemic and reproductive/developmental toxicity of dibutyl phthalate

Study/Endpoint	NOAEL	LOAEL	BMDL _{1SD}	BMDL ₁₀
Lehmann et al. (2004) Decrease in testosterone	30	50	24.4	-
Mylchreest et al. (2000) Retained areolas/nipples	50	100	-	39.6
NTP (1995) Live pups/litter F ₁ , L3	-	80	246	121
Live pups/litter F ₁ , L1-5	-	80	154	165
NTP (1995) Adjusted mean live pup weight in F ₂	-	80	224	374

5.1.3. RfD Derivation

5.1.3.1. Point of Departure

The point of departure for all exposure durations (acute, short-term, subchronic and chronic) is the NOAEL of 30 mg/kg-day (Lehmann et al., 2004). The biochemical effect that occurs at 50 mg/kg-day in this study is a statistically significant decrease in testosterone concentration in the fetal testis. EPA considers this decrease in testosterone concentration an adverse effect, a LOAEL. This biochemical change during the critical developmental window

may initiate the cascade of malformations in the developing male reproductive tract.

There are a number of reasons why the benchmark dose approach was not used to establish the point of departure. The study was conducted in only a limited number of male fetuses (3-4 per exposure group). In addition, the fetuses were taken from different litters without considering the position in the litter. It is known that there are interactions among fetuses in the litter with effects on steroid synthesis depending on whether the fetus is surrounded by fetuses of the same or different gender (Clark et al., 1992; Even and vom Saal, 1992; Even et al., 1992; Nonneman et al., 1992; vom Saal and Dhar, 1992). There were an unequal number of dams represented across the exposure groups. In other studies from this laboratory (Barlow et al., 2003), there was a greater variability among fetuses from the same litter than from fetuses from different litters. These combined factors result in an inadequate ability to characterize variability in the data. Finally, it is not known how much of a decrease in testosterone concentration in the fetal testis is biologically significant, i.e., how much of a decrease is required to initiate the cascade of malformations of the male reproductive tract.

5.1.3.2. Uncertainty Factors

Total Uncertainty Factor. The total factor of 100 is used to develop reference values for all durations of exposure. Each individual factor is discussed below.

Interspecies. A ten-fold uncertainty factor is used for interspecies extrapolation. As discussed in Section 3.5, the existing pharmacokinetic data are not sufficiently robust to depart from the default value of 3 for the pharmacokinetic portion. The pharmacodynamic portion is retained at 3 as there are no data to justify a departure from the default value.

Intraspecies. A ten-fold uncertainty factor is used for intraspecies extrapolation as there are no data to justify a departure from the default value. Future results based on physiologically based toxicokinetic modeling might be used to lower this value.

LOAEL to NOAEL. A factor for extrapolation from a LOAEL to NOAEL was not used because the principal study established a NOAEL.

Subchronic to Chronic Exposure. Consistent with EPA practice (U.S EPA, 1991), an uncertainty factor was not used to account for the extrapolation from less than chronic exposure because developmental toxicity resulting from a narrow period of exposure was used as the

critical effect. The developmental period is recognized as a susceptible life stage when exposure during a time window of development is more relevant to the induction of developmental effects than lifetime exposure. In addition, the developmental effect occurs at an exposure lower than the exposure required for effects in other target organ (liver, testes, nervous system) following subchronic exposure.

Data Base. Although the data base lacks focused studies on developmental neurotoxicity, immunotoxicity studies, and chronic studies, an uncertainty factor for data base deficiencies is not considered necessary. Neither developmental neurotoxicity nor immunotoxicity are likely to be critical effects for dibutyl phthalate. There is considerable evidence that chemicals that cause peroxisome proliferation in rodent are likely to cause liver tumors in rodents at an exposure similar to that causing peroxisome proliferation (Klaunig et al., 2003). Dibutyl phthalate causes effects in the rodent liver when exposure is 279 mg/day. These liver effects are correlated with markers for peroxisome proliferation and they occur at an exposure much greater than the exposure causing effects in the male fetus. Accordingly, the lack of chronic studies for dibutyl phthalate is not considered a data base deficiency.

5.1.3.3. Acute RfD (exposure 1 day or less)

The Acute RfD is 0.3 mg/kg-day (30 mg/kg-day divided by 100). See 5.1.3.1 and 5.1.3.2.

5.1.3.4. Short-term RfD (exposure up to 30 days)

The Short-term RfD is 0.3 mg/kg-day (30 mg/kg-day divided by 100). See 5.1.3.1 and 5.1.3.2.

5.1.3.5. Subchronic RfD (exposure up to 10% of lifetime)

The Subchronic RfD is 0.3 mg/kg-day (30 mg/kg-day divided by 100). See 5.1.3.1 and 5.1.3.2.

An alternative is to base the subchronic RfD on the liver pathology from subchronic studies (NTP 1995). The NOAEL of 138 mg/kg-day would be the point of departure. Using a total uncertainty factor of 100 (10 for interspecies and 10 for intraspecies extrapolation), the subchronic RfD would be 1 mg/kg-day. The developmental effects described in Lehmann et al. (2004) are observed at doses lower than those found in the NTP (1995) study and are selected for the derivation of the RfD.

5.1.3.6. Chronic RfD (exposure up to a lifetime)

The Chronic RfD is 0.3 mg/kg-day (30 mg/kg-day divided by 100). See 5.1.3.1 and 5.1.3.2.

An alternative is to base the chronic RfD on the liver pathology from subchronic studies (NTP 1995). The NOAEL of 138 mg/kg-day would be the point of departure. The interspecies uncertainty factor would remain at 10; the intraspecies uncertainty factor would remain at 10; and an uncertainty factor for duration of exposure (subchronic to chronic exposure) would normally be applied. The value of the uncertainty for subchronic to chronic exposure is typically 1, 3, or 10. Only if the full value of 10 is used would the chronic RfD be lower than that based on Lehmann et al. (2004), 0.1 versus 0.3 mg/kg-day. As the liver pathology is most likely related to peroxisome proliferation, it is not likely that liver pathology will appear at considerably lower exposure if a lifetime study were conducted. The developmental effects described in Lehmann et al. (2004) are observed at doses lower than those found in the NTP (1995) study and are selected for the derivation of the RfD.

5.1.4. Previous Oral Assessment

The previous RfD (verified 08/26/1987) of 1E-1 mg/kg-day was based on the 1 year study of Smith (1953) with a NOAEL of 125 mg/kg-day, a LOAEL for increased mortality of 600 mg/kg-day, and a total uncertainty factor of 1000 (10 each for interspecies and intraspecies extrapolation and 10 for duration of exposure and deficiencies in the study).

5.2. INHALATION REFERENCE CONCENTRATION (RfC)

No data are available to derive the RfC for any duration of exposure. One study following inhalation exposure (Walseth and Nilsen, 1984) did not examine the respiratory tract for toxicity or sufficiently examine other relevant endpoints for exposure to dibutyl phthalate. Another four week inhalation study is available only as an abstract (McKee et al., 2005).

5.3. CANCER ASSESSMENT

As there is inadequate information to assess carcinogenic potential, a quantitative exposure response assessment for cancer has not been conducted.

6. MAJOR CONCLUSIONS IN THE CHARACTERIZATION OF HAZARD AND DOSE RESPONSE

Dibutyl phthalate has been reviewed by the National Toxicology Program Center for the Evaluation of Risks to Human Reproduction (NTP-CERHR, 2003, 2000; Kavlock et al., 2002). Although there are no data on the effects of dibutyl phthalate in humans, there is high confidence in the conclusion that dibutyl phthalate is a reproductive and developmental toxicant in a number of laboratory animal species and would present a reproductive and developmental hazard to humans. The exposure-response relationships for these effects are adequately documented in laboratory animals and were used to calculate benchmark doses for the effects. The principal study for the derivation for all duration RfDs is Lehmann et al. (2004). The critical effect is a decrease in testosterone in the fetal testis (developmental toxicity) with a NOAEL of 30 mg/kg-day. The total uncertainty factor is 100. The resulting RfD for all durations of exposure is 0.3 mg/kg-day. As the toxic effects of dibutyl phthalate occur in utero and are mediated by exposure to the female, the target population to consider in a risk assessment is women of childbearing age. Because dibutyl phthalate is used in a wide variety of consumer products, exposure to humans is ubiquitous. Several studies have documented exposure of the U.S. population by measuring monobutyl phthalate in urine (Blount et al., 2000; DHHS, 2005). One exposure reconstruction study (Kohn et al., 2000) using the data from Blount et al. (2000) calculated the exposure for the 95th percentile for the general population to be approximately 7 µg/kg-day and for the 95th percentile for women of childbearing age to be approximately 32 µg/kg-day. A comparable calculation has not been conducted from the NHANES 1999-2000 or NHANES 2001-2002 data sets.

The RfD merits a high confidence rating. There are extensive studies that document the developmental and reproductive effects of dibutyl phthalate. These effects occur at an exposure lower than the exposure necessary to cause effects in other organ system. Although there are no studies focusing on developmental neurotoxicity or immunotoxicity, there are no indications that developmental neurotoxicity or immunotoxicity would be critical effects. There are no chronic bioassays. However as discussed in Section 5.1.3.2, the lack of a chronic bioassay is not considered a data base deficiency.

There are no data to derive the RfC for any duration of exposure.

There is inadequate information to assess carcinogenic potential. Consequently a Cancer

Slope Factor and an Inhalation Unit Risk are not derived.

7. REFERENCES

- Abe, S and Sasaki, M. (1977) Chromosome aberrations and sister chromatid exchanges in Chinese hamster cells exposed to various chemicals. *J Natl Cancer Inst* 58:1635-1641.
- Adibi, JJ; Perera, FP; Jedrychowski, W; et al. (2003) Prenatal exposures to phthalate among women in New York City and Krakow, Poland. *Environ Health Perspect* 111:1719-1722.
- Albro, PW; Moore, B. (1974) Identification of the metabolites of simple phthalate diesters in rat urine. *J Chromatogr* 94:209-218.
- Anderson, WAC; Castle, L; Scotter, MJ; et al. (2001) A biomarker approach to measuring human dietary exposure to certain phthalate diesters. *Food Additives and Contaminants* 18:1068-1074.
- ATSDR (Agency for Toxic Substances and Disease Registry). (2001) Toxicological profile for di-n-butyl phthalate. Atlanta GA. Available at <http://www.atsdr.cdc.gov/toxprofiles/tp135.html>.
- Barber, ED; Cifone, M; Rundell, J; et al. (2000) Results of the L5178Y mouse lymphoma assay and the Balb/3T3 cell in vitro transformation assay for eight phthalate esters. *J Appl Toxicol* 20:69-80.
- Barlow, NJ; Foster, PMD. (2003) Pathogenesis of male reproductive tract lesions from gestation through adulthood following in utero exposure to di(n-butyl) phthalate. *Toxicol Pathol* 31:397-410.
- Barlow, NJ; McIntyre, BS; Foster, PMD. (2004) Male reproductive tract lesions at 6, 12, and 18 months of age following in utero exposure to di(n-butyl) phthalate. *Toxicol Pathol* 32:79-90.
- Barlow, NJ; Phillips, SL; Wallace, DJ; et al. (2003) Quantitative changes in gene expression in fetal rat testes following exposure to di(n-butyl) phthalate. *Toxicol Sci* 73:431-441.
- BASF. (1992) Study on the oral toxicity of dibutyl phthalate in Wistar rats. Administration via the diet over 3 months. 31S0449/89020: Eastman Kodak Company. As cited in NTP-CERHR expert panel report on di-n-butyl phthalate. NTP-CERHR-DBP-00.
- Bility, MT; Thompson, JT; McKee, RH; et al. (2004) Activation of mouse and human peroxisome proliferator-activated receptors (PPARs) by phthalate monoesters. *Toxicol Sci* 82:170-182.
- Blount, BC; Silva, MJ; Caudill, SP; et al. (2000) Levels of seven urinary phthalate metabolites in a human reference population. *Environ Health Perspect* 108:979-982.
- Bowman, CJ; Turner, KF; Sar, M; et al. (2005) Altered gene expression during rat Wolffian duct development following di(n-butyl) phthalate exposure. *Toxicol Sci* 86:161-174.
- Calafat, AM; Brock JW; Silva MJ; et al. (2006) Urinary and amniotic fluid levels of phthalate monoesters in rats after oral administration of di(2-ethylhexyl) phthalate and di-n-butyl phthalate. *Toxicol* 217:22-30.
- Calafat, AM; Slakman, AR; Silva, MJ; et al. (2004) Automated solid phase extraction and quantitative analysis of human milk for 13 phthalate metabolites. *J Chromatog B* 805:49-56.
- Carruthers, CM; Foster, P. (2005) Critical window of male reproductive tract development in rats following gestational exposure to di-n-butyl phthalate. *Birth Defects Research (Part B)* 74:277-285.
- Clark, MM; vom Saal, FS; Balef, BG Jr. (1992) Intrauterine positions and testosterone levels of adult male gerbils are correlated. *Physiol Behav* 51:957-960.

Clewell, R; Andersen, M. (2004) Pharmacokinetic model for testosterone and its metabolites, dihydrotestosterone and estradiol, in the perinatal rat. *Toxicologist* 78: abstract #2027.

Clewell, RA; Kremer, J; Andersen, M et al. (2006) Using a PBPK model to explore mechanisms of observed pharmacokinetic differences of phthalates across life-stages in rats. *Toxicologist* 80, abstract #1270.

Clewell, RA; Ross, SM; Gaido, KW; et al. (2005) Assessing the role of cytosolic phospholipase A (CPLA₂) inhibition in phthalate ester toxicity. *Toxicologist* 79: abstract #276.

Cummings, AM; Gray, LE jr. (1987) Dibutyl phthalate: maternal effects versus fetotoxicity. *Toxicol Lett* 39:43-50.

DHHS (Department of Health and Human Services). (2005) Third national report on human exposure to environmental chemicals. National Center for Environmental Health. NCEH Pub. No. 02-0716. Available at: <http://www.cdc.gov/exposurereport> .

David, RM. (2000) Exposure to phthalate esters. *Environ Health Perspect* 108:A440.

Duty, S; Calafat, AM; Silva, MJ; et al. (2004) The relationship between environmental exposure to phthalates and computer-aided sperm analysis motion parameters. *J Androl* 25:293-301.

Duty, SM; Silva, MJ; Barr, DB; et al. (2003a) Phthalate exposure and human semen parameters. *Epidemiol* 14:269-277.

Duty, SM; Singh, NP; Silva, MJ; et al. (2003b) The relationship between environmental exposures to phthalates and DNA damage in human sperm using the neutral comet assay. *Environ Health Perspect* 111:1164-1169.

Elsisi, AE; Carter, DE; Sipes IG. (1989) Dermal absorption of phthalate diesters in rats. *Fund Appl Toxicol* 12:70-77.

Ema, M; Amano, H; Itami, T; et al. (1993) Teratogenic evaluation of di-n-butyl phthalate. *Toxicol Lett* 69:197-203.

Ema, M; Amano, H; Ogawa, Y. (1994) Characterization of the developmental toxicity of di-n-butyl phthalate. *Toxicol* 86:163-174.

Ema, M; Harazono, A; Miyawaki, E; et al. (1997) Developmental effects of di-n-butyl phthalate after a single administration in rats. *J Appl Toxicol* 17:223-229.

Ema, M; Kurosaka, R; Amano, H; et al. (1995) Developmental toxicity evaluation of mono-n-butyl phthalate in rats. *Toxicol Lett* 78:101-106.

Ema, M; Kurosaka, A; Harazono, A; et al. (1996) Phase specificity of developmental toxicity after oral administration of mono-n-butyl phthalate in rats. *Arch Environ Contam Toxicol* 31:170-176.

Ema, M; Miyawaki, E. (2001a) Adverse effects on development of the reproductive system in male offspring of rats given monobutyl phthalate, a metabolite of dibutyl phthalate, during late pregnancy. *Repro Toxicol* 15:189-194.

Ema, M; Miyawaki, E. (2001b) Effects of monobutyl phthalate on reproductive function in pregnant and pseudopregnant rats. *Repro Toxicol* 15:261-267.

Ema, M; Miyawaki, E; Kawashima, K. (1998) Further evaluation of developmental toxicity of di-n-butyl phthalate following administration during later pregnancy in rats. *Toxicol Lett* 98:87-93.

Ema, M; Miyawaki, E; Kawashima, K. (2000a) Effects of dibutyl phthalate on reproductive function in pregnant and pseudopregnant rats. *Repro Toxicol* 14:13-19.

Ema, M; Miyawaki, E; Kawashima, K. (2000b) Critical period for adverse effects on development of reproductive system in male offspring of rats given di-n-butyl phthalate during later pregnancy. *Toxicol Lett* 111:271-278.

Even, MD; Dhar, MG; vom Saal, FS. (1992) Transport of steroids between fetuses via amniotic fluid in relation to the intrauterine position phenomenon in rats. *J Reprod Fertil* 96:709-716.

Even, MD; vom Saal, FS. (1992) Seminal vesicle and preputial gland response to steroids in adult male mice is influenced by prior intrauterine position. *Physiol Behav* 51:11-16.

Fan, L-Q; Brown-Borg, H; Brown, S; et al. (2004a) PPAR α activators down-regulate CYP2C7, a retinoic acid and testosterone hydroxylase. *Toxicol* 203:41-48.

Fan, L-Q; You, L; Brown-Borg, H; Brown, S; et al. (2004b) Regulation of phase I and phase II steroid metabolism enzymes by PPAR α activators. *Toxicol* 204:109-121.

Fennell, TR; Krol, WL; Sumner, SCJ; et al. (2004) Pharmacokinetics of dibutylphthalate in pregnant rats. *Toxicol Sci* 82:407-418.

Fisher, JS; Macpherson, S; Marchetti, N; et al. (2003) Human 'testicular dysgenesis syndrome': a possible model using in-utero exposure of the rat to dibutyl phthalate. *Human Repro* 18:1-13.

Foster, PM (2005) Mode of action: impaired fetal Leydig cell function – effects on male reproductive development produced by certain phthalate esters. *Crit Rev in Toxicol* 35:713-719.

Foster, PM (2006) Disruption of reproductive development in male rat offspring following in utero exposure to phthalate esters. *Int J androl* 29:140-147.

Foster, PM, Cook, MW, Thomas, LV, et al. (1982) Differences in urinary metabolic profile from di-n-butyl phthalate-treated rats and hamsters: a possible explanation for species differences in susceptibility to testicular atrophy. *Drug Metal Dispos* 11:59-61.

Fukuoka, M; Tanimota, T; Zhou, Y; et al. (1989) Mechanism of testicular atrophy induced by di-n-butyl phthalate in rats. Part 1. *J Appl Toxicol* 9:277-283.

Fukuoka, M; Zhou, Y; Tanake, A; et al. (1990) Mechanism of testicular atrophy induced by di-n-butyl phthalate in rats. Part 2. The effects on some testicular enzymes. *J Appl Toxicol* 10:285-293.

Gargas, ML; Tyler, TR; Sweeney LM; et al. (2000a) A toxicokinetic study of inhaled ethylene glycol monomethyl ether (2-ME) and validation of a physiologically based pharmacokinetic model for the pregnant rat and human. *Toxicol Appl Pharmacol* 165:53-62.

Gargas, ML; Tyler, TR; Sweeney, LM; et al. (2000b) A toxicokinetic study of inhaled ethylene glycol ethyl ether acetate and validation of a physiologically based pharmacokinetic model for rat and human. *Toxicol Appl Pharmacol* 165:63-73.

Gentry, PR; Covington, TR; Andersen, MA; et al. (2002) Application of a physiologically based pharmacokinetic model for isopropanol in the derivation of a reference dose and reference concentration. *Regul Toxicol Pharmacol* 36:51-68.

Gentry, PR; Covington, TR; and Clewell, HJ III. (2003) Evaluation of the potential impact of pharmacokinetic differences on tissue dosimetry in offspring during pregnancy and lactation. *Regul Toxicol Pharmacol* 38:1-16.

Gray, LE jr; Wolf, C; Lambright, C; et al. (1999) Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, p,p'-DDE, and ketoconazole) and toxic substances (dibutyl- and

diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. *Toxicol and Ind Health* 15:94-118.

Hansen, DK; Grafton, DF; Dial, SL; et al. (1995) Effect of supplemental folic acid on valproic acid-induced embryotoxicity and tissue zinc levels in vivo. *Teratol* 52:277-285.

Hauser, R; Meeker, JD; Park, S; et al. (2004) Temporal variability of urinary phthalate metabolite levels in men of reproductive age. *Environ Health Perspect* 112:1734-1740.

Higuchi, TT; Palmer, JS; Gray, LE jr.; et al. (2003) Effects of dibutyl phthalate in male rabbits following in utero adolescent, or postpubertal exposure. *Toxicol Sci* 72:301-313.

Hong, EJ; Ji, YK; Choi, KC; et al. (2005) Conflict of estrogenic activity by various phthalates between in vitro and in vivo models related to the expression of Calbindin-D9k. *J Reprod Dev* 51:253-263.

Hoppin, JA; Brock, JW; Davis, BJ; et al. (2002) Reproducibility of urinary phthalate metabolites in first morning urine samples. *Environ Health Perspect* 110:515-518.

Hurst, CH; Waxman, DJ. (2003) Activation of PPAR α and PPAR γ by environmental phthalate monoesters. *Toxicol Sci* 74:297-308.

Kaneshima, H; Yamaguchi, T; Okui, T; et al. (1978a) Studies on the effects of phthalate esters on the biological system (part 2) - In vitro metabolism and biliary excretion of phthalate esters in rats. *Bull Environ Contam Toxicol* 19:502-509.

Kaneshima, H; Yamaguchi, T; Itoh, K. (1978b) Studies on the effects of phthalate esters on the biological system (part 3). The in vitro metabolism of dibutyl phthalate in the small intestines of rats. *Bull Environ Contam Toxicol* 20:725-728.

Kavlock, R; Boekelheide, K; Chapin, R; et al. (2002) NTP Center for the evaluation of risks to human reproduction: phthalate expert panel report on the reproductive and developmental toxicity of di-n-butyl phthalate. *Repro Toxicol* 16:489-527.

Keys, DA; Wallace, DG; Kepler, TB; et al. (2000) Quantitative evaluation of alternative mechanisms of blood disposition of di(n-butyl) phthalate and mono(n-butyl) phthalate in rats. *Toxicol Sci* 53:173-184.

Kim, HS; Kim, TS; Shin, JH; et al. (2004a) Neonatal exposure to di(n-butyl) phthalate (DBP) alters male reproductive-tract development. *J Toxicol Environ Health Part A* 67:2045-2060.

Kim, T; Shin, J; Lee, S; et al. (2004b) Effects of in utero exposure of diethylstilbestrol and dibutyl phthalate on the testis descent in rat offspring. *Toxicologist* 78: abstract #574.

Kim, MY; Song, KS; Park, GH; et al. (2004c) B6C3F1 mice exposed to ozone with 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone and/or dibutyl phthalate showed toxicities through alternations of NF- κ B, AP-1, Nrf2, and osteopontin. *J Vet Sci* 5:131-137.

Klaunig, JE; Babich, MA; Baetcke, KP; et al. (2003) PPAR α agonist-induced rodent tumors: modes of action and human relevance. *Crit Revs Toxicol* 33:655-780.

Kleinsasser, NH; Kastenbauer, RR; Weissacher, H; et al. (2000) Phthalates demonstrate genotoxicity on human mucosa of the upper aerodigestive tract. *Environ Mol Mutagen* 35:9-12.

Kleinsasser, NH; Wallner, BC; Kastenbauer, ER; et al. (2001) Genotoxicity of di-butyl-phthalate and di-iso-butyl-phthalate in human lymphocytes and mucosal cells. *Teratog Carcinog Mutagen* 21:189-196.

- Kleyменова, E; Swanson, C; Boekelheide, K; et al. (2005) Exposure in utero to di(n-butyl) phthalate alters the vimentin cytoskeleton of fetal rat Sertoli cells and disrupts Sertoli Cell-gonocyte contact. *Biol Reprod* 73:482-490.
- Kleyменова, E; Swanson, C; Gaido, KW. (2005) Low dose cellular responses in the fetal rat testis in utero exposed to di-n-butyl phthalate. *Toxicologist* 79: abstract #1372.
- Kobayashi, T; Niimi, S; Kawasishi, T; et al. (2003) Changes in peroxisome proliferator-activated receptor γ -regulated gene expression and inhibin/activin-follistatin system gene expression in rat testis after administration of di-n-butyl phthalate. *Toxicol Lett* 138:215-225.
- Koch, HM; Drexler, H; and Angerer, J. (2003) An estimation of the daily intake of di(2-ethylhexyl)phthalate (DEHP) and other phthalates in the general population. *Inter J Hyg Environ Health* 206:77-83.
- Kohn, MC; Parham, F; Masten, SC; et al. (2000) Human exposure estimates for phthalates. *Environ Health Perspect* 108:A440-442.
- Koo, HJ; Lee, BM. (2004) Estimated exposure to phthalates in cosmetics and risk assessment. *J Toxicol Environ Health Part A* 67:1901-1914.
- Koo, J-W; Parham, F; Kohn, MC; et al. (2002) The association between biomarker-based exposure estimates for phthalates and demographic factors in a human reference population. *Environ Health Perspect* 110:405-410.
- Kremer, JJ; Clewell, RA; Borghoff SJ. (2004) Physiologically based pharmacokinetic (PBPK) modeling of di-n-butyl phthalate (DBP) in pregnant rats. *Toxicologist* 78: abstract #2028. Dr. Kremer also supplied the plots of data presented in the poster session.
- Kremer, JJ; Clewell, RA; Borghoff, SJ. (2005a) Prediction of di-n-butylphthalate (DBP) levels in pregnant rats using a physiologically based pharmacokinetic (PBPK) model. *Toxicologist* 79: abstract #862.
- Kremer, JJ; Williams, CC; Parkinson, HD; et al. (2005b) Pharmacokinetics of monobutylphthalate, the active metabolite of di-n-butylphthalate, in pregnant rats. *Toxicol Lett* 159:144-153.
- Lake, BG; Phillips, JC; Linnell, JC; et al. (1977) The in vitro hydrolysis of some phthalate diesters by hepatic and intestinal preparations from various species. *Toxicol Appl Pharmacol* 39:239-248.
- Lamb, JC IV; Chapin, RE; Teague, J; et al. (1987) Reproductive effects of four phthalic acid esters in the mouse. *Toxicol Appl Pharmacol* 88:255-269.
- Lapinskas, PJ; Brown, S; Leesnitzer, LM; et al. (2005) Role of PPARalpha in mediating the effects of phthalates and metabolites in the liver. *Toxicol* 207:149-163.
- Lee, K-Y; Shibutani, M; Takagi, H; et al. (2004) Diverse developmental toxicity of di-n-butyl phthalate in both sexes of rat offspring after maternal exposure during the period from late gestation through lactation. *Toxicology* 203:221-238.
- Lehmann, KP; Phillips, S; Sar, M; et al. (2004) Dose-dependent alterations in gene expression and testosterone synthesis in the fetal testes of male rats exposed to di (n-butyl) phthalate. *Toxicol Sci* 81:60-68.
- Liu, K; Lehmann, KP; Sar, M; et al. (2005) Gene expression profiling following in utero exposure to phthalate esters reveals new gene targets in the etiology of testicular dysgenesis. *Biol Reprod* 73:180-192.
- Mahood, IK; Hallmark, N; McKinnell, C; et al. (2005) Abnormal Leydig cell aggregation in the fetal testis of rats exposed to di-n-butyl) phthalate and its possible role in testicular dysgenesis. *Endocrinology* 146:613-623.

Main, KM; Mortensen, GK; Kaleva, MM; et al. (2006) Human breast milk contamination with phthalates and alterations of endogenous reproductive hormones in infants three months of age. *Env Health Perspect* 114:270-276.

McKee, R; David, R; Butala, JH; et al. (2005) Assessment of hazard potential and risk for asthmatic responses following residential exposure to phthalates. *Toxicologist* 79: abstract #264.

McKinnell, C, Sharpe, RM; Mahood, K; et al. (2005) Expression of insulin-like factor 3 protein in the rat testis during fetal and postnatal development and in relation to cryptorchidism induced by *in utero* exposure to di (n-butyl) phthalate. *Endocrin* 146:4536-4544.

Murature, DA; Tang, SY; Steinhardt, G ; et al. (1987) Phthalate esters and semen quality parameters. *Biomed and Environ Mass Spect.* 14: 473-477.

Mylchreest, E; Cattley, RC; Foster, PMD. (1998) Male reproductive tract malformations in rats following gestational and lactational exposure to di(n-butyl) phthalate: an antiandrogenic mechanism? *Toxicol Sci* 43:47-60.

Mylchreest, E; Sar, M; Cattley, RD; et al. (1999) Disruption of androgen-regulated male reproductive development by di(n-butyl) phthalate during late gestation in rats is different from flutamide. *Toxicol Appl Pharmacol* 156:81-95.

Mylchreest, E; Sar, M; Wallace, DG; et al. (2002) Fetal testosterone insufficiency and abnormal proliferation of Leydig cells and gonocytes in rats exposed to di(n-butyl) phthalate. *Repro Toxicol* 16:19-28.

Mylchreest, E; Wallace, DG; Cattley, RC; et al. (2000) Dose-dependent alterations in androgen-regulated male reproductive development in rats exposed to di(n-butyl) phthalate during late gestation. *Toxicol Sci* 55:143-151.

NIEHS (National Institute of Environmental Health Sciences). (1994) Di-n-butyl phthalate (DBP) in rodent plasma. Prestart toxicokinetic report.

NIEHS (National Institute of Environmental Health Sciences). (1995) Di-n-butyl phthalate. The toxicokinetics and metabolism of di-n-butyl phthalate.

NRC (National Research Council). (1983) Risk assessment in the federal government: managing the process. Washington DC. National Academy Press.

Nonneman, DL; Ganjam, VK; Welsons, WV; et al. (1992) Intrauterine position effects on steroid metabolism and steroid receptors of reproductive organs in male mice. *Biol Reprod* 47:723-729.

NTP (National Toxicology Program). (1984) Reproduction and fertility assessment in CD-1 mice when administered in the feed. NTP-84-411.

NTP (National Toxicology Program). (1991) Final report on the reproductive toxicity of di-n-butyl phthalate in Sprague-Dawley rats. NTIS No. PB92111996.

NTP (National Toxicology Program). (1995) NTP technical report on toxicity studies of dibutyl phthalate administered in feed to F344/N rats and B6C3F1 mice. Toxicity Report Series Number 30. NIH publication 95-3353.

NTP (National Toxicology Program). (2002) Dibutyl phthalate (CAS No. 84-74-2): Multigenerational reproductive assessment by continuous breeding when administered to Sprague-Dawley rats in the diet. NTP Report # RACB97003.

NTP-CERHR (National Toxicology Program Center for the Evaluation of Risks to Human Reproduction). (2000) NTP-CERHR expert panel report on di-n-butyl phthalate. NTP-CERHR-DBP-00. Available at: <http://cerhr.niehs.nih.gov> .

NTP-CERHR (National Toxicology Program Center for the Evaluation of Risks to Human Reproduction). (2003) NTP-CERHR monograph on the potential human reproductive and developmental effects of di-n-butyl phthalate (DBP). Available at: <http://cerhr.niehs.nih.gov> .

Plummer, S; Hallmark, N; Sharpe, R; et al. (2005) Identification of gene clusters and signaling pathways affected by dibutyl phthalate - nuclear receptor interactions in foetal rat testes. *Toxicologist* 79: abstract #2258.

Rhee, GS; Kim, SH; Kim, SS; et al. (2002) Comparison of embryotoxicity of ESBO and phthalate esters using an in vitro battery system. *Toxicol in Vitro* 16:443-448.

Rowland, IR; Cottrell, RC; Phillips, JC. (1977) Hydrolysis of phthalate esters by the gastro-intestinal contents of the rat. *Fd Cosmet Toxicol* 15:17-21.

Saillenfait, AM; Langonné, I; Leheup, B. (2001) Effects of mono-n-butyl phthalate on the development of rat embryos: in vivo and in vitro observations. *Pharmacol Toxicol* 89:104-112.

Saillenfait, AM; Payan, JP; Fabry, JP; et al. (1998) Assessment of the developmental toxicity, metabolism, and placental transfer of di-n-butyl phthalate administered to pregnant rats. *Toxicol Sci* 45:212-224.

Salazar, V; Castillo, C; Ariznavarreta, C; et al. (2004) Effect of oral intake of dibutyl phthalate on reproductive parameters of Long Evans rats and pre-pubertal development of their offspring. *Toxicol* 205:131-137.

Scott, RC; Dugard, PH; Ramsey, JD; et al. (1987) In vitro absorption of some o-phthalate diesters through human and rat skin. *Environ Health Perspect* 74:223-227.

Seidlová-Wuttke, D; Jarry, H; Christofel, J; et al. (2005) Effects of bisphenol-A (BPA), dibutylphthalate (DBP), benzophenone-2 (BP2), procymidone (Proc), and linurone (Lin) on fat tissue, a variety of hormones and metabolic parameters: A 3 months comparison with effects of estradiol (E2) in ovariectomized (ovx) rats. *Toxicol* 213:13-24.

Seidlová-Wuttke, D; Jarry, H; Wuttke, W. (2004) Pure estrogenic effect of benzophenone-2 (BP2) but not of bisphenol A (BPA) and dibutylphthalate (DBP) in uterus, vagina, and bone. *Toxicol* 205:103-112.

Shahin, M and Von Borstel, RC. (1977) Mutagenic and lethal effects of α -benzene hexachloride, dibutyl phthalate and trichloroethylene in *Saccharomyces cerevisiae*. *Mutat Res* 48:173-180.

Sharpe, RM. (2005) Phthalate exposure during pregnancy and lower anogenital index in boys: wider implications for the general population? *Environ Health Perspect* 113:A504-505.

Shultz, VD; Phillips, S; Sar, M; et al. (2001) Altered gene profiles in fetal rat testes after in utero exposure to di(n-butyl) phthalate. *Toxicol Sci* 64:233-242.

Silva, MJ; Barr, DB; Reidy, JA; et al. (2003) Glucuronidation patterns of common urinary and serum monoester phthalate metabolites. *Arch Toxicol* 77:561-567. Erratum in *Arch Toxicol* 79:302 (2005).

Silva, MJ; Barr, DB; Reidy, JA; et al. (2004a) Urinary levels of seven phthalate metabolites in the U. S. population from the National Health and Nutrition Examination Survey (NHANES) 1999-2000. *Environ Health Perspect* 112:331-338.

Silva, MJ; Reidy, JA; Herbert, AR; et al. (2004b) Detection of phthalate metabolites in human amniotic fluid. *Bull Environ Contam Toxicol* 72:1226-1231.

Silva, MJ; Reidy, JA; Samander, E; et al. (2005) Detection of phthalate metabolites in human saliva. *Arch Toxicol* 11:647-652.

- Smith, CC. (1953) Toxicity of butyl stearate, dibutyl sebacate, dibutyl phthalate and methoxyethyl oleate. *Arch Ind Hyg Occupat Med* 7:310-318.
- Srivastava, SP; Srivastava, S; Saxena, DK; et al. (1990) Testicular effects of di-n-butyl phthalate (DBP): biochemical and histopathological alterations. *Arch Toxicol* 64:148-152.
- Swan, SH; Main, KM; Liu, F; et al. (2005) Decrease in anogenital distance among male infants with prenatal phthalate exposure. *Environ Health Perspect* 113:1056-1061.
- Takahasi, T; Tanaka, A. (1989) Biochemical studies on in vitro hydrolysis of di-n-butyl phthalate isomers in rats. *Arch Toxicol* 63:72-74.
- Tanaka, A; Matsumoto, A; Yamaha, T. (1978) Biochemical studies on phthalic esters. III. Metabolism of dibutyl phthalate (DBP) in animals. *Toxicology* 9:109-123.
- Tavares, E; Carreras, O; Gomez-Tubio, A; et al. (2000) Effects of folic acid and amino acid supplements on zinc intestinal absorption in the progeny of ethanol-treated rats. *J Physiol Biochem* 56:247-256.
- Thompson, CJ; Ross, SM; Gaido, K. (2004) Di(n-butyl) phthalate impairs cholesterol transport and steroidogenesis in the fetal rat testis through a rapid and reversible mechanism. *Endocrinology* 145:1227-1237.
- Thompson, CJ; Ross, SM; Hensley, J; et al. (2005) Differential steroidogenic gene expression in the fetal adrenal versus the testis and rapid and dynamic response of the fetal testis to di(n-butyl) phthalate. *Biol Reprod* 73:908-917.
- Tsutsimi, T; Ichihara, T; Kawabe, M; et al. (2004) Renal toxicity induced by folic acid is associated with the enhancement of male reproductive toxicity of di(n-butyl)phthalate in rats. *Reprod Toxicol* 18:35-42.
- U.S. EPA. (1991) Guidelines for developmental toxicity risk assessment. Federal Register 56(234):63798-63826. Available from: <<http://www.epa.gov/iris/backgr-d.htm>>.
- U.S. EPA. (2000) Benchmark dose technical guidance document [external review draft]. EPA/630/R-00/001. Available from: <<http://www.epa.gov/iris/backgr-d.htm>>.
- U.S. EPA. (2001) Exploration of perinatal pharmacokinetic issues. EPA/630/R-01/004.
- U.S. EPA. (2004) Toxicological review of boron and compounds. Integrated Risk Information System (IRIS), National Center for Environmental Assessment, Office of Research and Development, Washington DC; EPA 635/04/052. Available from: <<http://www.epa.gov/iris>>.
- U.S. EPA. (2005) Guidelines for carcinogen risk assessment. Risk Assessment Forum, Washington, DC; EPA/630/P-03/001B. Available from: <http://www.epa.gov/iris/backgr-d.htm>.
- Vom Saal, FS; Dhar, MG. (1992) Blood flow in the uterine loop artery and loop vein is bidirectional in the mouse: implications for transport of steroids between fetuses. *Physiol Behav* 52:163-171.
- Walseth, F; Nilsen, OG. (1984) Phthalate esters. II. Effects of inhaled dibutylphthalate on cytochrome P-450 mediated metabolism in rat liver and lung. *Arch Toxicol* 55:132-136.
- Walseth, F; Nilsen, OG. (1986) Phthalate esters: effects of orally administered dibutylphthalate on cytochrome P-450 mediated metabolism in rat liver and lung. *Acta Pharmacol et Toxicol* 59:263-269.
- Walton, K; Dorne, JLCM; Renwick, AG. (2004) Species-specific uncertainty factors for compounds eliminated principally by renal excretion in humans. *Fd Chem Toxicol* 42:261-274.

White, RD; Carter, DE; Earnest; et al. (1980) Absorption and metabolism of three phthalate diesters by the rat small intestine. *Fd Cosmet Toxicol* 18:383-386.

White, RD; Ernest, DL; Carter, DE. (1983) The effect of intestinal esterase inhibition on the in vivo absorption and toxicity of di-n-butyl phthalate. *Fd Cosmet Toxicol* 21:99-101.

WHO (World Health Organization). (1997) International Programme on Chemical Safety Environmental Health Criteria 189 di-n-butyl Phthalate. Geneva. Available from: <http://www.who.int/ipcs/publications/ehc/>.

Williams, DT; Blanchfield, BJ. (1975) The retention, distribution, excretion and metabolism of dibutyl phthalate -7-¹⁴C in the rat. *J Agric Food Chem* 23:854-858.

Wilson VS; Lambright, C; Furr, J; et al. (2004) Phthalate ester-induced gubernacular lesions are associated with reduced insl3 gene expression in fetal rat testis. *Toxicol Lett* 146:207-215.

Wine, RN; Li, L-H; Barnes, LH; et al. (1997) Reproductive toxicity of di-n-butyl phthalate in a continuous breeding protocol in Sprague-Dawley rats. *Environ Health Perspect* 105:102-107.

Wishart, GJ. (1978) Functional heterogeneity of UDP-glucuronosyl-transferase as indicated by its differential development and inducibility by glucocorticoids. Demonstration of two groups within the enzyme's activity twelve substrates. *Biochem J* 174:485-489.

Wyde, MF; Kirwan, SE; Zhang, F; et al. (2005) Di-n-butyl phthalate activates constitutive androstane receptor and pregnane X receptor and enhances the expression of steroid-metabolizing enzymes in the liver of rat fetuses. *Toxicol Sci* 86:281-290.

You, L; Wyde, M; Kirwan, S; et al. (2004) Di-butyl phthalate activates the nuclear receptors CAR and PXR and enhances the expression of CYP 2B1 and 3A1 in maternal and fetal liver in the rat. *Toxicologist* 78: abstract #1374.

Zacharewski, TR; Meek, MD; Clemons, JH; et al. (1998) Examination of the in vitro and in vivo estrogenic activities of eight commercial phthalate esters. *Tox Sci* 46:282-293.

Zeiger, E; Haworth, S; Mortelmans, K; et al. (1985) Mutagenicity testing of di(2-ethylhexyl)phthalate and related chemicals in *Salmonella*. *Environ Mutagen* 7:213-232.

Zhang, Y; Jiang, X, Chen B. (2004) Reproductive and developmental toxicity in F1 Sprague-Dawley male rats exposed to di-n-butyl phthalate in utero and during lactation and determination of its NOAEL. *Repro Toxicol* 18:669-676.

Zhou, Y; Fukuoka, M; Tanaka, A. (1990) Mechanism of testicular atrophy induced by di-n-butyl phthalate in rats. Part 3. Changes in the activity of some enzymes in the Sertoli and germ cells, and in the levels of metal ions. *J Appl Toxicol* 10:447-453.

**APPENDIX A. SUMMARY OF EXTERNAL PEER REVIEW AND PUBLIC COMMENT
AND DISPOSITION**

to be added

APPENDIX B. BENCHMARK DOSE CALCULATIONS

1. Increase in retained areolas or nipples (Mylchreest et al., 2000)

The data for this calculation are in Appendix C. In developmental studies the dam and the litter, rather than the individual pups, are considered the experimental unit. The nested models account for this as well as intra-litter correlations of observed effects. The NCTR Nested model gave the best fit to the data considering P value, visual fit, and AIC.

```
=====
NCTR Model. $Revision: 1.9 $ $Date: 2000/03/01 15:36:12 $
Input Data File: C:\BMDS\MYLCHREEST_NESTED.(d)
Gnuplot Plotting File: C:\BMDS\MYLCHREEST_NESTED.plt
Wed Jan 12 23:12:14 2005
=====
```

BMDS MODEL RUN

~~~~~  
The probability function is:

$$\text{Prob.} = 1 - \exp[-(\alpha + \text{th1} * \text{Rij}) - (\beta + \text{th2} * \text{Rij}) * \text{Dose}^{\rho}],$$

where Rij is the centralized litter specific covariate.

Restrict Power  $\rho \geq 1$ .

Total number of observations = 109

Total number of records with missing values = 0

Total number of parameters in model = 11

Total number of specified parameters = 2

Maximum number of iterations = 250

Relative Function Convergence has been set to: 1e-008

Parameter Convergence has been set to: 1e-008

User specifies the following paramters:

theta1 = 0

theta2 = 0

Default Initial Parameter Values

alpha = 0.0813752

beta = 0.00021424

rho = 1.49035

phi1 = 0.206671

phi2 = 0.147391

phi3 = 0.111136

phi4 = 0

phi5 = 0.215374

phi6 = 0.0718363

Parameter Estimates

| Variable | Estimate   | Std. Err.   |
|----------|------------|-------------|
| alpha    | 0.0813752  | 0.0213036   |
| beta     | 0.00021424 | 0.000309926 |



|      |           |           |
|------|-----------|-----------|
| rho  | 1.49035   | 0.249328  |
| phi1 | 0.206671  | 0.175593  |
| phi2 | 0.147391  | 0.126114  |
| phi3 | 0.111136  | 0.0924766 |
| phi4 | 0         | 0.0931192 |
| phi5 | 0.215374  | 0.0671025 |
| phi6 | 0.0718363 | 0.185152  |

#### Analysis of Deviance Table

| Model         | Log(likelihood) | Deviance | Test DF | P-value   |
|---------------|-----------------|----------|---------|-----------|
| Full model    | -159.025        |          |         |           |
| Fitted model  | -236.658        | 155.266  | 102     | 0.0003395 |
| Reduced model | -341.889        | 365.727  | 108     | <.0001    |
| AIC:          | 487.317         |          |         |           |

#### Goodness of Fit

| <u>Dose</u> | <u>Litter Size</u> | <u>Est. Prob.</u> | <u>Expected</u> | <u>Observed</u> |
|-------------|--------------------|-------------------|-----------------|-----------------|
| 0           | 4                  | 0.078             | 0.313           | 0               |
| 0           | 5                  | 0.078             | 1.954           | 3               |
| 0           | 6                  | 0.078             | 1.407           | 0               |
| 0           | 7                  | 0.078             | 1.094           | 0               |
| 0           | 8                  | 0.078             | 3.126           | 2               |
| 0           | 9                  | 0.078             | 0.703           | 4               |
| 0           | 10                 | 0.078             | 0.782           | 0               |
| 0           | 14                 | 0.078             | 1.094           | 0               |
| 0.5         | 2                  | 0.078             | 0.156           | 0               |
| 0.5         | 3                  | 0.078             | 0.469           | 0               |
| 0.5         | 4                  | 0.078             | 0.313           | 0               |
| 0.5         | 5                  | 0.078             | 1.173           | 1               |
| 0.5         | 6                  | 0.078             | 1.408           | 0               |
| 0.5         | 7                  | 0.078             | 3.285           | 2               |
| 0.5         | 8                  | 0.078             | 2.503           | 5               |
| 5           | 1                  | 0.080             | 0.080           | 0               |
| 5           | 3                  | 0.080             | 0.241           | 0               |
| 5           | 4                  | 0.080             | 1.606           | 5               |
| 5           | 5                  | 0.080             | 1.205           | 4               |
| 5           | 6                  | 0.080             | 1.446           | 0               |
| 5           | 7                  | 0.080             | 2.249           | 1               |
| 5           | 8                  | 0.080             | 0.643           | 1               |
| 5           | 10                 | 0.080             | 0.803           | 2               |
| 50          | 3                  | 0.143             | 0.858           | 1               |
| 50          | 4                  | 0.143             | 1.716           | 2               |
| 50          | 5                  | 0.143             | 2.145           | 1               |
| 50          | 6                  | 0.143             | 3.432           | 0               |

|     |    |       |        |    |
|-----|----|-------|--------|----|
| 50  | 7  | 0.143 | 3.003  | 2  |
| 50  | 8  | 0.143 | 3.432  | 3  |
| 50  | 9  | 0.143 | 2.574  | 3  |
| 100 | 4  | 0.249 | 0.996  | 1  |
| 100 | 5  | 0.249 | 4.979  | 7  |
| 100 | 6  | 0.249 | 2.988  | 3  |
| 100 | 7  | 0.249 | 8.714  | 6  |
| 100 | 8  | 0.249 | 5.975  | 16 |
| 100 | 9  | 0.249 | 8.963  | 10 |
| 100 | 10 | 0.249 | 2.490  | 1  |
| 500 | 1  | 0.903 | 0.903  | 1  |
| 500 | 3  | 0.903 | 2.710  | 3  |
| 500 | 4  | 0.903 | 10.841 | 9  |
| 500 | 5  | 0.903 | 9.034  | 10 |
| 500 | 7  | 0.903 | 12.648 | 13 |
| 500 | 8  | 0.903 | 7.227  | 6  |
| 500 | 10 | 0.903 | 9.034  | 10 |

Chi-square = 47.66    DF = 36    P value = 0.0926

To calculate the BMD and BMDL, the litter specific covariate is fixed at the overall mean of the litter specific covariates: 6.192661

#### Benchmark Dose Computation

Specified effect = 0.05

Risk Type = Extra risk

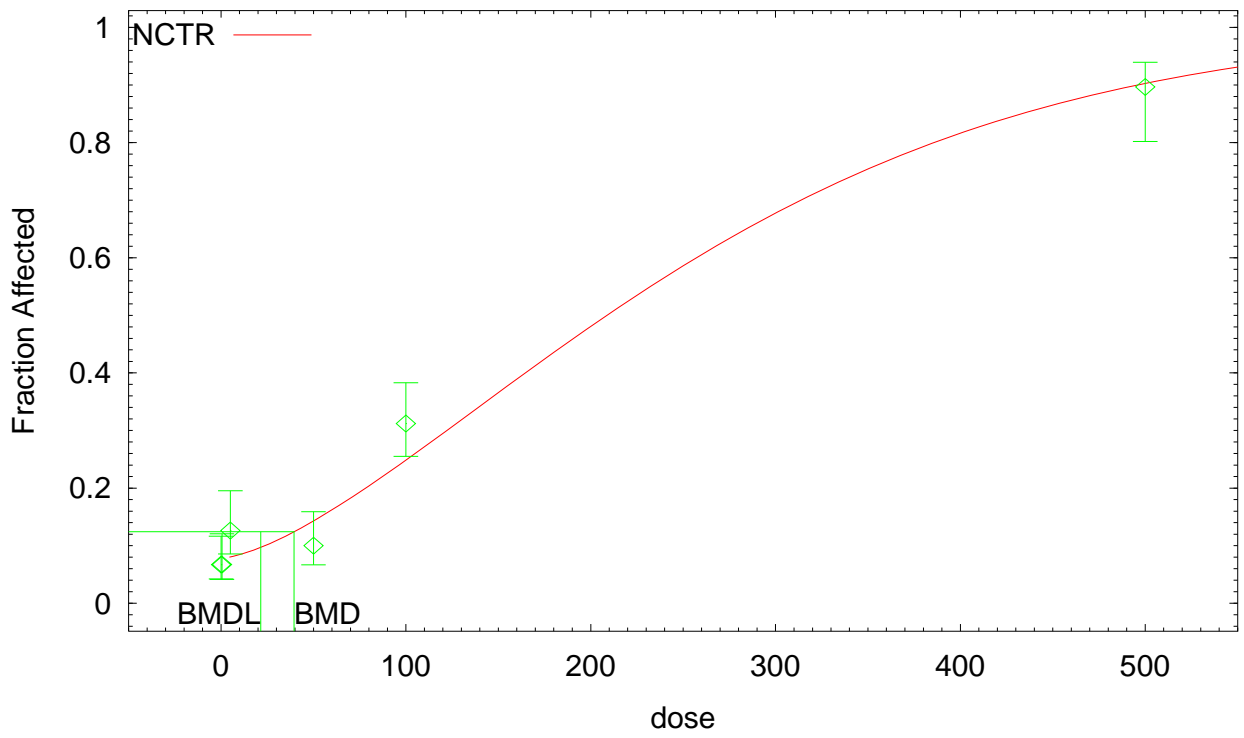
Confidence level = 0.950000

BMD = 39.4795

BMDL = 21.4693

The BMD<sub>01</sub> and the BMDL<sub>01</sub> are 13.2 and 5.3 mg/kg-day, respectively. The BMD<sub>10</sub> and the BMDL<sub>10</sub> are 64.0 and 39.6, respectively.

NCTR Model with 0.95 Confidence Level



23:12 01/12 2005

## 2. Mean live pups per litter in F1 (NTP, 1995)

The data for this calculation are in Appendix D. In this continuous breeding study, the experimental unit is the dam. Therefore, N is the number of dams delivering pups at each exposure. In F1 litter 3 showed a statistically significant response at the lowest exposure and is analyzed separately. The data are also analyzed using the combined data from all five litters in F1. There was a slight change in the number of dams delivering pups across the five litters. For simplicity in this analysis, N is set equal to the number of dams delivering pups in the first litter.

None of the models gave an adequate fit when data from all the exposure groups were used. When data from the highest exposure group was eliminated, only the linear model gave a satisfactory fit based on P value.

### Litter 3 Separately

```
=====
Polynomial Model. Revision: 2.2 Date: 9/12/2002
Input Data File: C:\BMDS\LIVE_PUPS_L3.(d)
Gnuplot Plotting File: C:\BMDS\LIVE_PUPS_L3.plt
Thu Sep 16 22:22:02 2004
=====
```

### BMDS MODEL RUN

```
~~~~~
The form of the response function is:
Y[dose] = beta_0 + beta_1*dose + beta_2*dose^2 + ...
Dependent variable = MEAN
Independent variable = COLUMN1
rho is set to 0
The polynomial coefficients are restricted to be negative
A constant variance model is fit
Total number of dose groups = 3
Total number of records with missing values = 0
Maximum number of iterations = 250
Relative Function Convergence has been set to: 1e-008
Parameter Convergence has been set to: 1e-008
Default Initial Parameter Values
alpha = 7.93105
rho = 0 Specified
beta_0 = 13.0618
beta_1 = -0.00728044
```

### Parameter Estimates

| Variable | Estimate | Std. Err. | 95.0% Wald Confidence Interval |                   |
|----------|----------|-----------|--------------------------------|-------------------|
|          |          |           | Lower Conf. Limit              | Upper Conf. Limit |
| alpha    | 7.86984  | 1.25218   | 5.41561                        | 10.3241           |

|        |             |            |            |             |
|--------|-------------|------------|------------|-------------|
| beta_0 | 13.2692     | 0.389005   | 2.5068     | 14.0317     |
| beta_1 | -0.00792696 | 0.00201502 | -0.0118763 | -0.00397759 |

Asymptotic Correlation Matrix of Parameter Estimates

|        |          |          |          |
|--------|----------|----------|----------|
|        | alpha    | beta_0   | beta_1   |
| alpha  | 1        | 9.9e-009 | 1.7e-008 |
| beta_0 | 9.9e-009 | 1        | -0.58    |
| beta_1 | 1.7e-008 | -0.58    | 1        |

Table of Data and Estimated Values of Interest

| Dose | N  | Obs Mean | Obs Std Dev | Est Mean | Est Std Dev | Chi^2 Res. |
|------|----|----------|-------------|----------|-------------|------------|
| 0    | 40 | 13.6     | 2.5         | 13.3     | 2.81        | 0.746      |
| 80   | 20 | 11.8     | 2.7         | 12.6     | 2.81        | -1.33      |
| 385  | 19 | 10.4     | 3.5         | 10.2     | 2.81        | 0.284      |

Model Descriptions for likelihoods calculated

Model A1:  $Y_{ij} = \mu(i) + e(ij)$   
 $\text{Var}\{e(ij)\} = \sigma^2$

Model A2:  $Y_{ij} = \mu(i) + e(ij)$   
 $\text{Var}\{e(ij)\} = \sigma(i)^2$

Model R:  $Y_i = \mu + e(i)$   
 $\text{Var}\{e(i)\} = \sigma^2$

Likelihoods of Interest

| Model  | Log(likelihood) | DF | AIC        |
|--------|-----------------|----|------------|
| A1     | -119.766815     | 4  | 247.533629 |
| A2     | -118.286233     | 6  | 248.572467 |
| fitted | -120.989977     | 2  | 245.979955 |
| R      | -128.059565     | 2  | 260.119130 |

Test 1: Does response and/or variances differ among dose levels (A2 vs. R)

Test 2: Are Variances Homogeneous (A1 vs A2)

Test 3: Does the Model for the Mean Fit (A1 vs. fitted)

Tests of Interest

| Test   | -2*log(Likelihood Ratio) | Test df | p-value |
|--------|--------------------------|---------|---------|
| Test 1 | 19.5467                  | 4       | <0.0001 |
| Test 2 | 2.96116                  | 2       | 0.2275  |
| Test 3 | 2.44633                  | 1       | 0.1178  |

The p-value for Test 1 is less than .05. There appears to be a difference between response and/or variances among the dose levels. It seems appropriate to model the data.

The p-value for Test 2 is greater than .05. A homogeneous variance model appears to be appropriate here.

The p-value for Test 3 is greater than .05. The model chosen appears to adequately describe the data.

#### Benchmark Dose Computation

Specified effect = 0.05

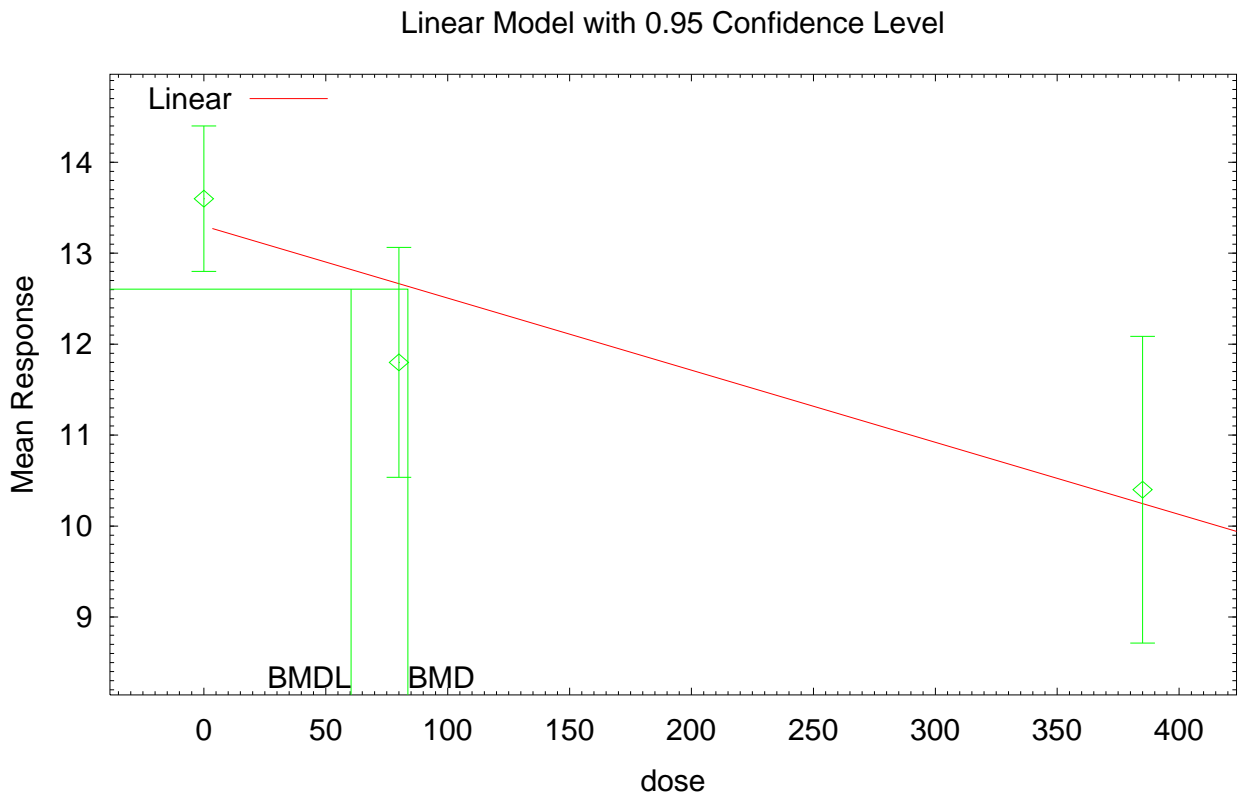
Risk Type = Relative risk

Confidence level = 0.95

BMD = 83.6968

BMDL = 60.3783

The  $BMD_{01}$  is 16.7 and the  $BMDL_{01}$  is 12.1. The  $BMD_{10}$  is 167 and the  $BMDL_{10}$  is 121. The  $BMD_{1SD}$  is 354 and the  $BMDL_{1SD}$  is 246.



22:22 09/16 2004

**Litters 1 through 5 combined**

=====  
Polynomial Model. Revision: 2.2 Date: 9/12/2002  
Input Data File: C:\BMDS\NTP\_PUPS\_LITTERL1\_5.(d)  
Gnuplot Plotting File: C:\BMDS\NTP\_PUPS\_LITTERL1\_5.plt  
Thu Sep 16 22:47:35 2004  
=====

**BMDS MODEL RUN**

~~~~~  
The form of the response function is:  
 $Y[\text{dose}] = \text{beta}_0 + \text{beta}_1 * \text{dose} + \text{beta}_2 * \text{dose}^2 + \dots$   
Dependent variable = MEAN  
Independent variable = COLUMN1  
The polynomial coefficients are restricted to be negative  
The variance is to be modeled as  $\text{Var}(i) = \text{alpha} * \text{mean}(i)^\rho$   
Total number of dose groups = 3  
Total number of records with missing values = 0  
Maximum number of iterations = 250  
Relative Function Convergence has been set to: 1e-008  
Parameter Convergence has been set to: 1e-008

Default Initial Parameter Values

alpha = 2.43605  
rho = 0  
beta\_0 = 12.613  
beta\_1 = -0.00438522

Parameter Estimates

| Variable | Estimate    | Std. Err.  | 95.0% Wald Confidence Interval |                   |
|----------|-------------|------------|--------------------------------|-------------------|
|          |             |            | Lower Conf. Limit              | Upper Conf. Limit |
| alpha    | 107239      | 253810     | -390219                        | 604696            |
| rho      | -4.32844    | 0.952748   | -6.19579                       | -2.46109          |
| beta_0   | 12.7783     | 0.186904   | 12.412                         | 13.1446           |
| beta_1   | -0.00550899 | 0.00129154 | -0.00804037                    | -0.00297762       |

Asymptotic Correlation Matrix of Parameter Estimates

|        | alpha  | rho    | beta_0 | beta_1 |
|--------|--------|--------|--------|--------|
| alpha  | 1      | -1     | 0.027  | -0.095 |
| rho    | -1     | 1      | -0.026 | 0.091  |
| beta_0 | 0.027  | -0.026 | 1      | -0.49  |
| beta_1 | -0.095 | 0.091  | -0.49  | 1      |

Table of Data and Estimated Values of Interest

| Dose | N  | Obs Mean | Obs Std Dev | Est Mean | Est Std Dev | Chi^2 Res. |
|------|----|----------|-------------|----------|-------------|------------|
| 0    | 40 | 12.9     | 1.3         | 12.8     | 1.32        | 0.583      |
| 80   | 20 | 11.9     | 1.3         | 12.3     | 1.42        | -1.37      |
| 385  | 19 | 11       | 2.2         | 10.7     | 1.95        | 0.764      |

Model Descriptions for likelihoods calculated

- Model A1:  $Y_{ij} = \mu(i) + e(ij)$   
 $\text{Var}\{e(ij)\} = \sigma^2$
- Model A2:  $Y_{ij} = \mu(i) + e(ij)$   
 $\text{Var}\{e(ij)\} = \sigma(i)^2$
- Model A3:  $Y_{ij} = \mu(i) + e(ij)$   
 $\text{Var}\{e(ij)\} = \alpha * (\mu(i))^{\rho}$
- Model R:  $Y_i = \mu + e(i)$   
 $\text{Var}\{e(i)\} = \sigma^2$

Likelihoods of Interest

| Model  | Log(likelihood) | DF | AIC        |
|--------|-----------------|----|------------|
| A1     | -73.140746      | 4  | 154.281491 |
| A2     | -68.689618      | 6  | 149.379236 |
| A3     | -69.491944      | 5  | 148.983888 |
| fitted | -70.546520      | 4  | 149.093039 |
| R      | -82.376933      | 2  | 168.753867 |

Explanation of Tests

- Test 1: Does response and/or variances differ among Dose levels? (A2 vs. R)
- Test 2: Are Variances Homogeneous? (A1 vs A2)
- Test 3: Are variances adequately modeled? (A2 vs. A3)
- Test 4: Does the Model for the Mean Fit? (A3 vs. fitted)

Tests of Interest

| Test   | -2*log(Likelihood Ratio) | Test df | p-value |
|--------|--------------------------|---------|---------|
| Test 1 | 27.3746                  | 4       | <0.0001 |
| Test 2 | 8.90225                  | 2       | 0.01167 |
| Test 3 | 1.60465                  | 1       | 0.2052  |
| Test 4 | 2.10915                  | 1       | 0.1464  |

The p-value for Test 1 is less than .05. There appears to be a difference between response and/or variances among the dose levels It seems appropriate to model the data.

The p-value for Test 2 is less than .05. A non-homogeneous variance model appears to be appropriate.

The p-value for Test 3 is greater than .05. The modeled variance appears to be appropriate here.



The p-value for Test 4 is greater than .05. The model chosen seems to adequately describe the data.

### Benchmark Dose Computation

Specified effect = 0.05

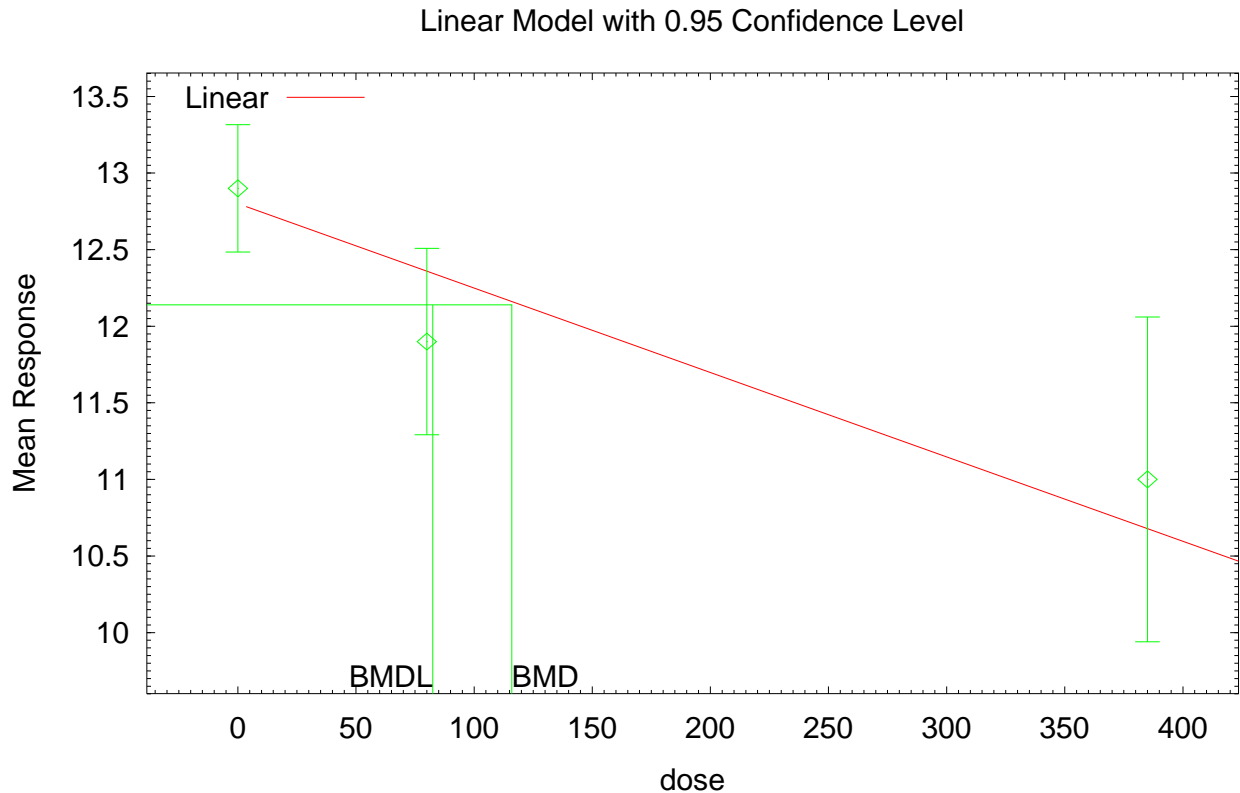
Risk Type = Relative risk

Confidence level = 0.95

BMD = 115.977

BMDL = 82.4497

The  $BMD_{01}$  is 23.2 and the  $BMDL_{01}$  is 16.5. The  $BMD_{10}$  is 232 and the  $BMDL_{10}$  is 165. The  $BMD_{1SD}$  is 240 and the  $BMDL_{1SD}$  is 154.



### 3. Adjusted mean live pup weight in F2 (NTP, 1995)

Data for the analysis are in Appendix C. There was a statistically significant reduction in adjusted live pup weight in F2. The linear model gave the best fit to the data considering P value, visual fit, and AIC.

=====

Polynomial Model. Revision: 2.2 Date: 9/12/2002  
Input Data File: C:\BMDS\PUP\_WT\_F2.(d)  
Gnuplot Plotting File: C:\BMDS\PUP\_WT\_F2.plt  
Tue Aug 03 22:15:11 2004

=====

#### BMDS MODEL RUN

~~~~~

The form of the response function is:

$$Y[\text{dose}] = \text{beta}_0 + \text{beta}_1 * \text{dose} + \text{beta}_2 * \text{dose}^2 + \dots$$

Dependent variable = MEAN

Independent variable = COLUMN1

rho is set to 0

Signs of the polynomial coefficients are not restricted

A constant variance model is fit

Total number of dose groups = 3

Total number of records with missing values = 0

Maximum number of iterations = 250

Relative Function Convergence has been set to: 1e-008

Parameter Convergence has been set to: 1e-008

Default Initial Parameter Values

alpha = 0.131716

rho = 0 Specified

beta\_0 = 5.88979

beta\_1 = -0.00107389

#### Parameter Estimates

| Variable | Estimate    | Std. Err.   | 95.0% Wald Confidence Interval |                   |
|----------|-------------|-------------|--------------------------------|-------------------|
|          |             |             | Lower Conf. Limit              | Upper Conf. Limit |
| alpha    | 0.131496    | 0.025544    | 0.0814305                      | 0.181561          |
| beta_0   | 5.89596     | 0.067075    | 5.7645                         | 6.02742           |
| beta_1   | -0.00109246 | 0.000301185 | -0.00168277                    | -0.000502146      |

#### Asymptotic Correlation Matrix of Parameter Estimates

|        | alpha    | beta_0   | beta_1  |
|--------|----------|----------|---------|
| alpha  | 1        | 7.9e-010 | -8e-010 |
| beta_0 | 7.9e-010 | 1        | -0.67   |
| beta_1 | -8e-010  | -0.67    | 1       |

Table of Data and Estimated Values of Interest

| Dose | N  | Obs Mean | Obs Std Dev | Est Mean | Est Std Dev | Chi^2 Res. |
|------|----|----------|-------------|----------|-------------|------------|
| 0    | 19 | 5.98     | 0.35        | 5.9      | 0.363       | 1.01       |
| 80   | 17 | 5.69     | 0.37        | 5.81     | 0.363       | -1.35      |
| 385  | 17 | 5.5      | 0.37        | 5.48     | 0.363       | 0.28       |

Model Descriptions for likelihoods calculated

- Model A1:  $Y_{ij} = \mu(i) + e(ij)$   
 $\text{Var}\{e(ij)\} = \sigma^2$
- Model A2:  $Y_{ij} = \mu(i) + e(ij)$   
 $\text{Var}\{e(ij)\} = \sigma(i)^2$
- Model R:  $Y_i = \mu + e(i)$   
 $\text{Var}\{e(i)\} = \sigma^2$

Likelihoods of Interest

| Model  | Log(likelihood) | DF | AIC        |
|--------|-----------------|----|------------|
| A1     | 28.762467       | 4  | -49.524933 |
| A2     | 28.795455       | 6  | -45.590910 |
| fitted | 27.262657       | 2  | -50.525314 |
| R      | 21.381979       | 2  | -38.763958 |

Test 1: Does response and/or variances differ among dose levels (A2 vs. R)

Test 2: Are Variances Homogeneous (A1 vs A2)

Test 3: Does the Model for the Mean Fit (A1 vs. fitted)

Tests of Interest

| Test   | $-2 \cdot \log(\text{Likelihood Ratio})$ | Test df | p-value   |
|--------|------------------------------------------|---------|-----------|
| Test 1 | 14.827                                   | 4       | 0.0006031 |
| Test 2 | 0.0659765                                | 2       | 0.9675    |
| Test 3 | 2.99962                                  | 1       | 0.08328   |

The p-value for Test 1 is less than 0.05. There appears to be a difference between response and/or variances among the dose levels. It seems appropriate to model the data.

The p-value for Test 2 is greater than 0.05. A homogeneous variance model appears to be appropriate here.

The p-value for Test 3 is greater than 0.05. The model chosen appears to adequately describe the data.

Benchmark Dose Computation

Specified effect = 0.05

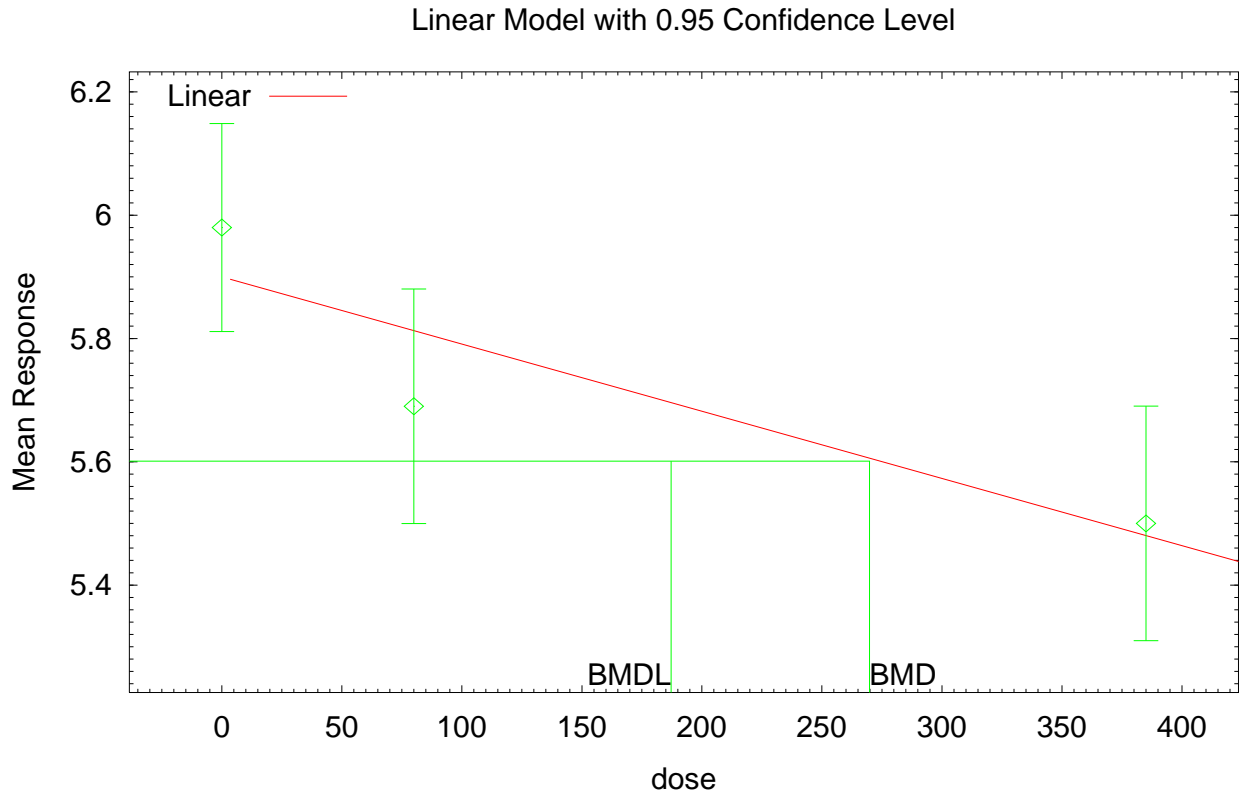
Risk Type = Relative risk

Confidence level = 0.95

BMD = 269.848

BMDL = 187.2

The  $BMD_{01}$  is 54.0 and the  $BMDL_{01}$  is 37.4. The  $BMD_{10}$  is 540 and the  $BMDL_{10}$  is 374. The  $BMD_{1SD}$  is 332 and the  $BMDL_{1SD}$  is 224.



22:15 08/03 2004

#### 4. Decrease in testosterone (Lehmann et al., 2004)

Each of the continuous models gave an adequate fit based on P value. The Hill model gave the best visual fit to the data and the lowest BMD and BMDL.

```
=====
Hill Model. $Revision: 2.1 $ $Date: 2000/10/11 21:21:23 $
Input Data File: C:\BMDS\LEHMANN-TESTOSTERONE.(d)
Gnuplot Plotting File: C:\BMDS\LEHMANN-TESTOSTERONE.plt
Wed Dec 08 10:14:17 2004
=====
```

#### BMDS MODEL RUN

The form of the response function is:

$$Y[\text{dose}] = \text{intercept} + v \cdot \text{dose}^n / (k^n + \text{dose}^n)$$

Dependent variable = MEAN

Independent variable = COLUMN1

rho is set to 0

Power parameter restricted to be greater than 1

A constant variance model is fit

Total number of dose groups = 8

Total number of records with missing values = 0

Maximum number of iterations = 250

Relative Function Convergence has been set to: 1e-008

Parameter Convergence has been set to: 1e-008

Default Initial Parameter Values

alpha = 0.15359

rho = 0 Specified

intercept = 1.2

v = -1.14

n = 1.0915

k = 138.095

#### Asymptotic Correlation Matrix of Parameter Estimates

|           | alpha | rho | intercept | v | n | k |
|-----------|-------|-----|-----------|---|---|---|
| alpha     | 1     | 0   | 0         | 0 | 0 | 0 |
| rho       | 0     | 1   | 0         | 0 | 0 | 0 |
| intercept | 0     | 0   | 1         | 0 | 0 | 0 |
| v         | 0     | 0   | 0         | 1 | 0 | 0 |
| n         | 0     | 0   | 0         | 0 | 1 | 0 |
| k         | 0     | 0   | 0         | 0 | 0 | 1 |

#### Parameter Estimates

| Variable | Estimate | Std. Err. |
|----------|----------|-----------|
| alpha    | 0.133761 | 1         |
| rho      | 0        | 1         |

|           |          |   |
|-----------|----------|---|
| intercept | 1.22551  | 1 |
| v         | -1.43767 | 1 |
| n         | 1.13564  | 1 |
| k         | 141.922  | 1 |

Table of Data and Estimated Values of Interest

| Dose | N | Obs Mean | Obs Std Dev | Est Mean | Est Std Dev | Chi^2 Res. |
|------|---|----------|-------------|----------|-------------|------------|
| 0    | 4 | 1.2      | 0.38        | 1.23     | 0.366       | -0.0698    |
| 0.1  | 4 | 1.24     | 0.3         | 1.23     | 0.366       | 0.0407     |
| 1    | 3 | 1.2      | 0.24        | 1.22     | 0.366       | -0.0557    |
| 10   | 4 | 1.19     | 0.38        | 1.16     | 0.366       | 0.0871     |
| 30   | 4 | 1.07     | 0.42        | 1.02     | 0.366       | 0.149      |
| 50   | 4 | 0.79     | 0.6         | 0.889    | 0.366       | -0.27      |
| 100  | 4 | 0.69     | 0.36        | 0.648    | 0.366       | 0.116      |
| 500  | 3 | 0.06     | 0.59        | 0.0654   | 0.366       | -0.0148    |

Model Descriptions for likelihoods calculated

- Model A1:  $Y_{ij} = \mu(i) + e(ij)$   
 $\text{Var}\{e(ij)\} = \sigma^2$
- Model A2:  $Y_{ij} = \mu(i) + e(ij)$   
 $\text{Var}\{e(ij)\} = \sigma(i)^2$
- Model R:  $Y_i = \mu + e(i)$   
 $\text{Var}\{e(i)\} = \sigma^2$

Likelihoods of Interest

| Model  | Log(likelihood) | DF | AIC        |
|--------|-----------------|----|------------|
| A1     | 15.428185       | 9  | -12.856370 |
| A2     | 17.689301       | 16 | -3.378601  |
| fitted | 15.175508       | 5  | -20.351016 |
| R      | 4.780889        | 2  | -5.561779  |

Test 1: Does response and/or variances differ among dose levels (A2 vs. R)

Test 2: Are Variances Homogeneous (A1 vs A2)

Test 3: Does the Model for the Mean Fit (A1 vs. fitted)

Tests of Interest

| Test   | $-2 \cdot \log(\text{Likelihood Ratio})$ Test | df | p-value   |
|--------|-----------------------------------------------|----|-----------|
| Test 1 | 25.8168                                       | 14 | 0.0005431 |
| Test 2 | 4.52223                                       | 7  | 0.718     |
| Test 3 | 0.505353                                      | 4  | 0.973     |

The p-value for Test 1 is less than .05. There appears to be a difference between response and/or variances among the dose levels. It seems appropriate to model the data.

The p-value for Test 2 is greater than .05. A homogeneous variance model appears to be appropriate here.

The p-value for Test 3 is greater than .05. The model chosen appears to adequately describe the data.

### Benchmark Dose Computation

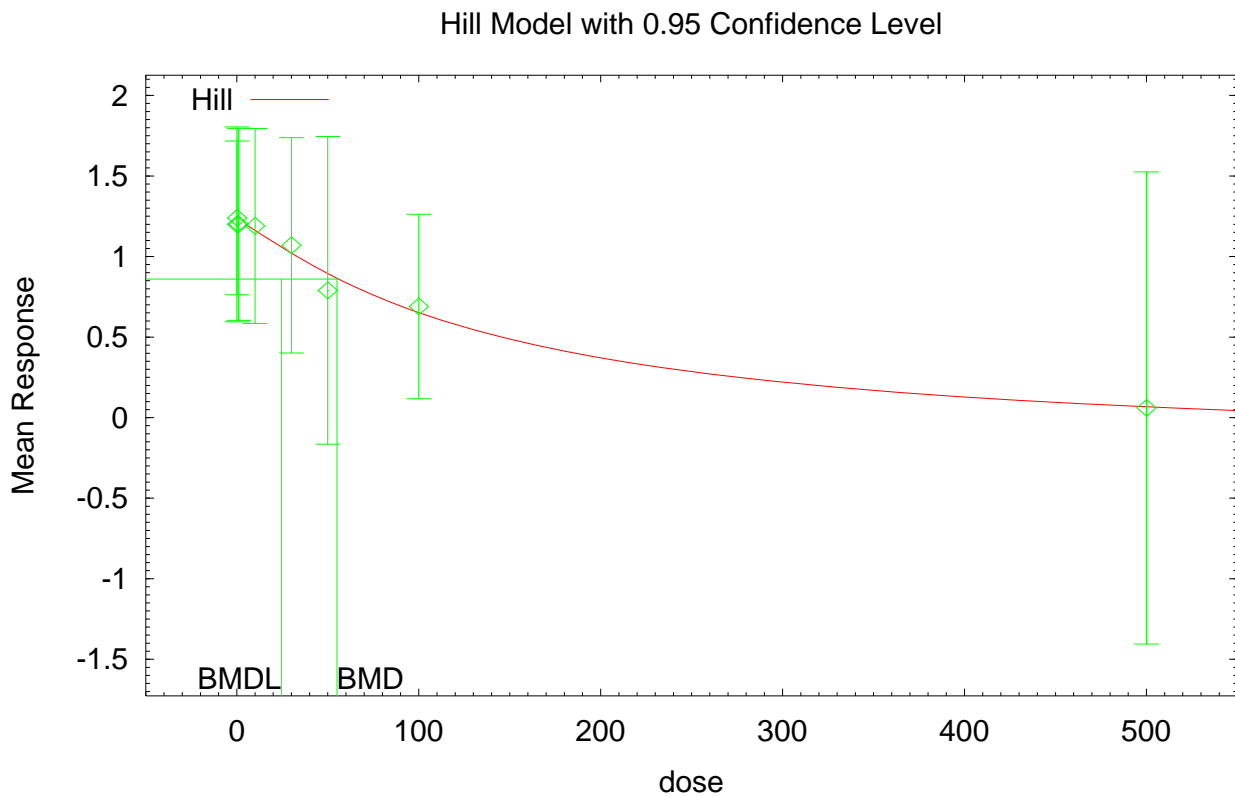
Specified effect = 1

Risk Type = Estimated standard deviations from the control mean

Confidence level = 0.95

BMD = 55.0586

BMDL = 24.415



13:15 01/02 2005

**APPENDIX C. ADDITIONAL DEVELOPMENTAL DATA**

Incidence of retained Areolas or Nipples in Male Pups (Mylchreest et al., 2000, Figure 2)

| Exposure (mg/kg-day) | Pups <sup>a</sup>   | Litters <sup>b</sup> |
|----------------------|---------------------|----------------------|
| 0                    | 9/134               | 5/19                 |
| 0.5                  | 8/119               | 5/20                 |
| 5                    | 13/103              | 8/19                 |
| 50                   | 12/120              | 10/20                |
| 100                  | 44/141 <sup>c</sup> | 16/20 <sup>c</sup>   |
| 500                  | 52/58 <sup>c</sup>  | 11/11 <sup>c</sup>   |

<sup>a</sup> Number of pups affected/total pups.

<sup>b</sup> Number of litters affected/total litters.

<sup>c</sup>  $p < 0.05$ .

Incidence of Areolas or Nipples in Male Pups/Number of Male Pups in the Litter at each exposure (Mylchreest et al., 2004)<sup>#</sup>

| Litter | 0    | 0.5 | 5    | 50  | 100  | 500   |
|--------|------|-----|------|-----|------|-------|
| 1      | 0/8  | 0/8 | 0/5  | 1/3 | 4/5  | 3/4   |
| 2      | 2/5  | 1/5 | 0/7  | 0/6 | 1/4  | 3/3   |
| 3      | 0/10 | 0/3 | 1/7  | 2/8 | 3/9  | 7/7   |
| 4      | 0/6  | 0/8 | 0/6  | 0/8 | 2/7  | 2/4   |
| 5      | 0/7  | 0/3 | 1/8  | 2/9 | 1/6  | 5/5   |
| 6      | 0/7  | 0/5 | 1/4  | 1/4 | 3/7  | 4/4   |
| 7      | 0/6  | 0/7 | 0/6  | 1/4 | 1/7  | 5/5   |
| 8      | 1/5  | 0/7 | 0/1  | 0/7 | 2/6  | 1/1   |
| 9      | 0/5  | 4/8 | 0/7  | 2/7 | 3/9  | 10/10 |
| 10     | 0/14 | 1/7 | 2/5  | 0/4 | 8/8  | 6/8   |
| 11     | 1/8  | 0/5 | 0/4  | 0/6 | 6/8  | 6/7   |
| 12     | 0/6  | 0/4 | 2/5  | 1/5 | 1/5  |       |
| 13     | 0/5  | 0/2 | 2/10 | 0/5 | 4/9  |       |
| 14     | 1/8  | 0/6 | 0/3  | 0/6 | 0/7  |       |
| 15     | 0/5  | 1/7 | 1/4  | 1/8 | 0/5  |       |
| 16     | 0/8  | 1/8 | 3/4  | 0/6 | 0/7  |       |
| 17     | 4/9  | 0/7 | 0/7  | 0/7 | 2/5  |       |
| 18     | 0/8  | 0/6 | 0/4  | 0/3 | 0/9  |       |
| 19     | 0/4  | 0/6 | 0/6  | 1/9 | 2/8  |       |
| 20     |      | 0/7 |      | 0/5 | 1/10 |       |



|          |       |       |       |       |       |       |
|----------|-------|-------|-------|-------|-------|-------|
| MEAN     | 0.068 | 0.056 | 0.133 | 0.101 | 0.311 | 0.896 |
| SEM      | 0.032 | 0.027 | 0.047 | 0.028 | 0.062 | 0.050 |
| VARIANCE | 0.019 | 0.015 | 0.042 | 0.015 | 0.076 | 0.028 |
| STDEV    | 0.138 | 0.122 | 0.204 | 0.124 | 0.276 | 0.166 |

# Individual litter data supplied from CIIT archivist to EPA. The individual litter data are not in the publication. Exposure is in mg/kg-day. Data analysis conducted by EPA. See Appendix B for benchmark dose analyses of these data.

Testosterone concentration in fetal testes (Lehmann et al., 2004)

Fetal testicular testosterone was measured by RIA using a standard curve and presented in Figure 4 of Lehmann et al. (2004). The data for each exposure were supplied to EPA by Dr. Lehmann. The testosterone concentration is expressed as  $\text{Log}_{10}$  ng/ml of homogenate using the pair of testes from each animal.

| Exposure (mg/kg-day) | #dams | #pups | Testosterone Concentration <sup>+</sup> ( $\text{Log}_{10}$ ng/ml) | SEM <sup>+</sup> |
|----------------------|-------|-------|--------------------------------------------------------------------|------------------|
| 0                    | 4     | 4     | 1.20                                                               | 0.19             |
| 0.1                  | 1     | 4     | 1.24                                                               | 0.15             |
| 1                    | 2     | 3     | 1.20                                                               | 0.14             |
| 10                   | 2     | 4     | 1.19                                                               | 0.19             |
| 30                   | 2     | 4     | 1.07                                                               | 0.21             |
| 50                   | 2     | 4     | 0.79*                                                              | 0.30             |
| 100                  | 3     | 4     | 0.69*                                                              | 0.18             |
| 500                  | 3     | 3     | 0.06*                                                              | 0.34             |

\* Statistically different from control ( $p < 0.05$ ). The statistical analysis was conducted using Dunnett's test comparing  $\text{Log}_{10}$ -transformed testosterone concentrations from each exposed group to the control. The error term for Dunnett's test was generated by a two-way ANOVA. The two factors used in this analysis were exposure and day the testosterone extract was prepared.

<sup>+</sup> Values expressed as ng/ml

| Exposure (mg/kg-day) | Testosterone Concentration (ng/ml) | SEM  |
|----------------------|------------------------------------|------|
| 0                    | 15.8                               | 1.55 |
| 0.1                  | 17.4                               | 1.41 |
| 1                    | 15.8                               | 1.38 |
| 10                   | 15.5                               | 1.55 |
| 30                   | 11.7                               | 1.62 |
| 50                   | 6.2                                | 2.00 |
| 100                  | 4.9                                | 1.51 |
| 500                  | 1.1                                | 2.19 |

The decrease at 30 mg/kg-day is 26%  $[(15.8-11.7)/(15.8) = 0.26]$  and the decrease at 50 mg/kg-day is 61%  $[(15.8-6.2)/(15.8) = 0.61]$ .

APPENDIX D. SELECTED DATA FROM NTP (1995)

E-10

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TABLE E2 Litter Data and Body Weights of F<sub>1</sub> Sprague-Dawley Rat Pups in the Continuous Breeding Study of Dibutyl Phthalate<sup>1</sup>

|                                           | 0 ppm                  | 1,000 ppm                | 5,000 ppm                | 10,000 ppm                 |
|-------------------------------------------|------------------------|--------------------------|--------------------------|----------------------------|
| <b>Litter 1</b>                           |                        |                          |                          |                            |
| Number of pairs delivering                | 40                     | 20                       | 19                       | 20                         |
| Live pups/litter <sup>2</sup>             | 14.9 ± 0.3             | 13.2 ± 0.8               | 12.8 ± 0.9*              | 13.1 ± 0.6*                |
| Live pups/litter (%)                      | 99 ± 0                 | 96 ± 3                   | 98 ± 1                   | 93 ± 3                     |
| Sex ratio <sup>3</sup> (%)                | 51 ± 2                 | 47 ± 5                   | 50 ± 4                   | 41 ± 4*                    |
| Male pup weight (g)                       | 5.71 ± 0.06            | 5.83 ± 0.10 <sup>4</sup> | 5.69 ± 0.08 <sup>5</sup> | 5.29 ± 0.11 <sup>4,4</sup> |
| Female pup weight (g)                     | 5.36 ± 0.06            | 5.51 ± 0.09              | 5.45 ± 0.10              | 5.01 ± 0.10*               |
| Total live pup weight (g)                 | 5.54 ± 0.06            | 5.66 ± 0.09              | 5.59 ± 0.08              | 5.13 ± 0.09*               |
| Adjusted live pup weight <sup>6</sup> (g) | 5.59 ± 0.06            | 5.63 ± 0.08              | 5.52 ± 0.08              | 5.11 ± 0.08*               |
| <b>Litter 2</b>                           |                        |                          |                          |                            |
| Number of pairs delivering                | 40                     | 20                       | 19                       | 20                         |
| Live pups/litter                          | 13.5 ± 0.5             | 13.9 ± 0.4               | 12.6 ± 0.9               | 12.0 ± 0.7                 |
| Live pups/litter (%)                      | 98 ± 1                 | 99 ± 1                   | 98 ± 1                   | 92 ± 4                     |
| Sex ratio (%)                             | 48 ± 2                 | 54 ± 3                   | 54 ± 4                   | 43 ± 4                     |
| Male pup weight (g)                       | 6.22 ± 11 <sup>7</sup> | 6.09 ± 0.11              | 5.74 ± 0.13*             | 5.48 ± 0.13 <sup>4</sup>   |
| Female pup weight (g)                     | 5.87 ± 0.10            | 5.66 ± 0.10              | 5.53 ± 0.15 <sup>5</sup> | 5.24 ± 0.11*               |
| Total live pup weight (g)                 | 6.04 ± 0.10            | 5.90 ± 0.10              | 5.66 ± 0.14*             | 5.37 ± 0.12*               |
| Adjusted live pup weight (g)              | 6.06 ± 0.09            | 5.94 ± 0.12              | 5.62 ± 0.12*             | 5.33 ± 0.12*               |
| <b>Litter 3</b>                           |                        |                          |                          |                            |
| Number of pairs delivering                | 40                     | 20                       | 19                       | 20                         |
| Live pups/litter                          | 13.6 ± 0.4             | 11.8 ± 0.6*              | 10.4 ± 0.8*              | 10.5 ± 0.7*                |
| Live pups/litter (%)                      | 97 ± 1                 | 94 ± 2                   | 97 ± 2                   | 98 ± 1                     |
| Sex ratio (%)                             | 51 ± 2                 | 51 ± 2                   | 51 ± 3                   | 42 ± 4*                    |
| Male pup weight (g)                       | 6.22 ± 0.09            | 6.22 ± 0.09              | 6.12 ± 0.13              | 5.47 ± 0.14 <sup>4</sup>   |
| Female pup weight (g)                     | 5.88 ± 0.09            | 5.80 ± 0.11              | 5.82 ± 0.13              | 5.29 ± 0.14*               |
| Total live pup weight (g)                 | 6.04 ± 0.09            | 6.01 ± 0.09              | 5.95 ± 0.12              | 5.40 ± 0.13*               |
| Adjusted live pup weight (g)              | 6.19 ± 0.08            | 6.02 ± 0.10              | 5.79 ± 0.11*             | 5.24 ± 0.11*               |
| <b>Litter 4</b>                           |                        |                          |                          |                            |
| Number of pairs delivering                | 38                     | 20                       | 19                       | 20                         |
| Live pups/litter                          | 11.1 ± 0.5             | 10.1 ± 0.5               | 10.1 ± 0.8               | 9.3 ± 0.6*                 |
| Live pups/litter (%)                      | 94 ± 1                 | 96 ± 2                   | 96 ± 2                   | 98 ± 1                     |
| Sex ratio (%)                             | 48 ± 2                 | 45 ± 3                   | 48 ± 4                   | 45 ± 4                     |
| Male pup weight (g)                       | 6.51 ± 0.11            | 6.45 ± 0.13              | 6.01 ± 0.13*             | 5.71 ± 0.18 <sup>4,4</sup> |
| Female pup weight (g)                     | 6.14 ± 0.09            | 6.25 ± 0.13              | 5.72 ± 0.12*             | 5.53 ± 0.12*               |
| Total live pup weight (g)                 | 6.31 ± 0.09            | 6.34 ± 0.12              | 5.86 ± 0.11*             | 5.61 ± 0.14*               |
| Adjusted live pup weight (g)              | 6.43 ± 0.08            | 6.30 ± 0.10              | 5.82 ± 0.10*             | 5.46 ± 0.10*               |
| <b>Litter 5</b>                           |                        |                          |                          |                            |
| Number of pairs delivering                | 34                     | 19                       | 18                       | 18 <sup>8</sup>            |
| Live pups/litter                          | 10.8 ± 0.5             | 10.4 ± 0.5               | 9.2 ± 0.6                | 8.6 ± 0.9*                 |
| Live pups/litter (%)                      | 93 ± 2                 | 95 ± 2                   | 95 ± 3                   | 93 ± 6                     |
| Sex ratio (%)                             | 51 ± 2                 | 52 ± 3                   | 51 ± 4                   | 50 ± 3                     |
| Male pup weight (g)                       | 6.46 ± 0.12            | 6.52 ± 0.16              | 6.09 ± 0.15*             | 5.85 ± 0.18*               |
| Female pup weight (g)                     | 6.15 ± 0.11            | 6.17 ± 0.13              | 5.96 ± 0.15              | 5.67 ± 0.22*               |
| Total live pup weight (g)                 | 6.31 ± 0.11            | 6.34 ± 0.13              | 6.02 ± 0.13              | 5.76 ± 0.19*               |
| Adjusted live pup weight (g)              | 6.41 ± 0.10            | 6.38 ± 0.13              | 5.92 ± 0.13*             | 5.61 ± 0.14*               |

**TABLE E2 Litter Data and Body Weights of F<sub>1</sub> Sprague-Dawley Rat Pups  
In the Continuous Breeding Study of Dibutyl Phthalate (continued)**

|                               | 0 ppm       | 1,000 ppm               | 5,000 ppm                | 10,000 ppm               |
|-------------------------------|-------------|-------------------------|--------------------------|--------------------------|
| <b>Litters 1 through 5</b>    |             |                         |                          |                          |
| Average live pups/litter      | 12.9 ± 0.2  | 11.9 ± 0.3 <sup>1</sup> | 11.0 ± 0.5 <sup>1</sup>  | 10.7 ± 0.4 <sup>1</sup>  |
| Average live pups/litter (%)  | 97 ± 1      | 96 ± 1                  | 97 ± 1                   | 95 ± 1                   |
| Average sex ratio (%)         | 50 ± 1      | 50 ± 1                  | 51 ± 2                   | 45 ± 2                   |
| Average male pup weight (g)   | 6.12 ± 0.07 | 6.15 ± 0.07             | 5.84 ± 0.07 <sup>2</sup> | 5.53 ± 0.11 <sup>2</sup> |
| Average female pup weight (g) | 5.81 ± 0.06 | 5.82 ± 0.06             | 5.63 ± 0.07              | 5.26 ± 0.10 <sup>2</sup> |
| Total live pup weight (g)     | 5.96 ± 0.06 | 5.99 ± 0.06             | 5.74 ± 0.07 <sup>2</sup> | 5.38 ± 0.10 <sup>2</sup> |
| Adjusted live pup weight (g)  | 6.04 ± 0.06 | 5.99 ± 0.08             | 5.66 ± 0.08 <sup>2</sup> | 5.30 ± 0.08 <sup>2</sup> |

<sup>1</sup> Data for live pups/litter, pups/breeding pair, sex ratios, and pup weights are given as mean ± standard error. Differences from the control group for percent live pups/litter are not significant by Dunn's or Shirley's test.

<sup>2</sup> Mean of average number of live pups per litter for each fertile pair.

<sup>3</sup> Live male pups/live pups.

<sup>4</sup> n=19.

<sup>5</sup> n=18.

<sup>6</sup> Least-squares estimate of mean pup weight adjusted for average litter size.

<sup>7</sup> n=39.

<sup>8</sup> Because no live pups were born in one litter, n=17 for pup weights and sex ratio.

<sup>9</sup> Significantly different (P<0.05) from the control group by Dunnett's (adjusted live pup weights only) or Shirley's test.

**TABLE E7 Fertility, Reproductive Performance, and Body Weight Data for F<sub>1</sub> and F<sub>2</sub> Sprague-Dawley Rats in the Offspring Assessment Phase of the Continuous Breeding Study of Dibutyl Phthalate<sup>1</sup>**

|                                           | 0 ppm        | 1,000 ppm    | 5,000 ppm    | 10,000 ppm     |
|-------------------------------------------|--------------|--------------|--------------|----------------|
| <b>F<sub>1</sub> Adult Data</b>           |              |              |              |                |
| Mating index <sup>2</sup>                 | 20/20 (100%) | 19/20 (95%)  | 18/20 (90%)  | 6/20 (30%)*    |
| Pregnancy index <sup>3</sup>              | 19/20 (95%)  | 17/20 (85%)  | 17/20 (85%)  | 1/20 (5%)*     |
| Fertility index <sup>4</sup>              | 19/20 (95%)  | 17/19 (89%)  | 17/18 (94%)  | 1/6 (17%)*     |
| Dam weight at delivery (g)                | 323 ± 6      | 307 ± 8      | 317 ± 9      | 279            |
| Days to litter                            | 22.3 ± 0.2   | 21.6 ± 0.1   | 21.9 ± 0.1   | 21.0           |
| <b>F<sub>2</sub> Pup Data</b>             |              |              |              |                |
| Live male pups/litter                     | 7.1 ± 0.5    | 7.5 ± 0.4    | 5.9 ± 0.7    | 6.0            |
| Live female pups/litter                   | 6.9 ± 0.6    | 8.1 ± 0.5    | 6.8 ± 0.5    | 7.0            |
| Total live pups/litter                    | 14.0 ± 0.8   | 15.5 ± 0.4   | 12.8 ± 0.8   | 13.0           |
| Live pups/litter (%)                      | 98 ± 1       | 100 ± 0      | 99 ± 1       | 100            |
| Sex ratio <sup>5</sup> (%)                | 52 ± 3       | 49 ± 3       | 45 ± 4       | 46             |
| Male pup weight (g)                       | 6.13 ± 0.13  | 5.79 ± 0.11  | 5.83 ± 0.11  | 5.10           |
| Female pup weight (g)                     | 5.81 ± 0.11  | 5.43 ± 0.08* | 5.42 ± 0.08* | 4.91           |
| Total live pup weight (g)                 | 5.97 ± 0.11  | 5.60 ± 0.09* | 5.60 ± 0.09* | 5.00           |
| Adjusted live pup weight <sup>6</sup> (g) | 5.98 ± 0.08  | 5.69 ± 0.09* | 5.50 ± 0.09* | — <sup>7</sup> |

<sup>1</sup> Days to litter, live pups/litter, sex ratio, and body weight data are given as mean ± standard error. Differences from the control group for dam and male pup body weights, days to litter, live pups/litter, and sex ratio are not significant by Dunn's test.

<sup>2</sup> Females with sperm plug/cohabiting pairs.

<sup>3</sup> Pregnant females/cohabiting pairs.

<sup>4</sup> Pregnant females/females with sperm plug.

<sup>5</sup> Live male pups/live pups.

<sup>6</sup> Least-squares estimate of mean pup weight adjusted for average litter size.

<sup>7</sup> Because only one litter was produced in this exposure group, no adjusted live pup weight was calculated.

\* Significantly different (P<0.05) from the control group by the chi-square test (reproductive indexes) or Shirley's test.