NTP Technical Report on the Toxicology and Carcinogenesis Studies of Di-*n*-butyl Phthalate (CASRN 84-74-2) Administered in Feed to Sprague Dawley (Hsd:Sprague Dawley® SD®) Rats and B6C3F1/N Mice

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Foreword

The National Toxicology Program (NTP), established in 1978, is an interagency program within the Public Health Service of the U.S. Department of Health and Human Services. Its activities are executed through a partnership of the National Institute for Occupational Safety and Health (part of the Centers for Disease Control and Prevention), the Food and Drug Administration (primarily at the National Center for Toxicological Research), and the National Institute of Environmental Health Sciences (part of the National Institutes of Health), where the program is administratively located. NTP offers a unique venue for the testing, research, and analysis of agents of concern to identify toxic and biological effects, provide information that strengthens the science base, and inform decisions by health regulatory and research agencies to safeguard public health. NTP also works to develop and apply new and improved methods and approaches that advance toxicology and better assess health effects from environmental exposures.

The Technical Report series began in 1976 with carcinogenesis studies conducted by the National Cancer Institute. In 1981, this bioassay program was transferred to NTP. The studies described in the NTP Technical Report series are designed and conducted to characterize and evaluate the toxicological potential, including carcinogenic activity, of selected substances in laboratory animals (usually two species, rats and mice). Substances (e.g., chemicals, physical agents, and mixtures) selected for NTP toxicity and carcinogenicity studies are chosen primarily on the basis of human exposure, level of commercial production, and chemical structure. The interpretive conclusions presented in NTP Technical Reports are derived solely from the results of these NTP studies, and extrapolation of the results to other species, including characterization of hazards and risks to humans, requires analyses beyond the intent of these reports. Selection for study per se is not an indicator of a substance's carcinogenic potential.

NTP conducts its studies in compliance with its laboratory health and safety guidelines and Food and Drug Administration Good Laboratory Practice Regulations and meets or exceeds all applicable federal, state, and local health and safety regulations. Animal care and use are in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals. Studies are subjected to retrospective quality assurance audits before they are presented for public review. Draft reports undergo external peer review before they are finalized and published.

The NTP Technical Reports are available free of charge on the NTP website and cataloged in PubMed, a free resource developed and maintained by the National Library of Medicine (part of the National Institutes of Health). Data for these studies are included in NTP's Chemical Effects in Biological Systems database.

For questions about the reports and studies, please email NTP or call 984-287-3211.

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Explanation of Levels of Evidence of Carcinogenic Activity

The National Toxicology Program (NTP) describes the results of individual experiments on a chemical agent and notes the strength of the evidence for conclusions regarding each study. Negative results, in which the study animals do not have a greater incidence of neoplasia than control animals, do not necessarily mean that a chemical is not a carcinogen, in as much as the experiments are conducted under a limited set of conditions. Positive results demonstrate that a chemical is carcinogenic for laboratory animals under the conditions of the study and indicate that exposure to the chemical has the potential for hazard to humans. Other organizations, such as the International Agency for Research on Cancer, assign a strength of evidence for conclusions based on an examination of all available evidence, including animal studies such as those conducted by NTP, epidemiologic studies, and estimates of exposure. Thus, the actual determination of risk to humans from chemicals found to be carcinogenic in laboratory animals requires a wider analysis that extends beyond the purview of these studies.

Five categories of evidence of carcinogenic activity are used in the Technical Report series to summarize the strength of evidence observed in each experiment: two categories for positive results **(clear evidence** and **some evidence)**; one category for uncertain findings **(equivocal evidence)**; one category for no observable effects **(no evidence)**; and one category for experiments that cannot be evaluated because of major flaws **(inadequate study)**. These categories of interpretative conclusions were first adopted in June 1983 and then revised on March 1986 for use in the Technical Report series to incorporate more specifically the concept of actual weight of evidence of carcinogenic activity. For each separate experiment (male rats, female rats, male mice, female mice), one of the following five categories is selected to describe the findings. These categories refer to the strength of the experimental evidence and not to potency or mechanism.

- **Clear evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a dose-related (i) increase of malignant neoplasms, (ii) increase of a combination of malignant and benign neoplasms, or (iii) marked increase of benign neoplasms if there is an indication from this or other studies of the ability of such tumors to progress to malignancy.
- **Some evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a chemical-related increased incidence of neoplasms (malignant, benign, or combined) in which the strength of the response is less than that required for clear evidence.
- **Equivocal evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a marginal increase of neoplasms that may be chemical related.
- **No evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing no chemical-related increases in malignant or benign neoplasms.
- **Inadequate study** of carcinogenic activity is demonstrated by studies that, because of major qualitative or quantitative limitations, cannot be interpreted as valid for showing either the presence or absence of carcinogenic activity.

For studies showing multiple chemical-related neoplastic effects that if considered individually would be assigned to different levels of evidence categories, the following convention has been adopted to convey completely the study results. In a study with clear evidence of carcinogenic activity at some tissue sites, other responses that alone might be deemed some evidence are indicated as "were also related" to chemical exposure. In studies with clear or some evidence of carcinogenic activity, other responses that alone might be termed equivocal evidence are indicated as "may have been" related to chemical exposure.

When a conclusion statement for a particular experiment is selected, consideration must be given to key factors that would extend the actual boundary of an individual category of evidence. Such consideration should allow for incorporation of scientific experience and current understanding of long-term carcinogenesis studies in laboratory animals, especially for those evaluations that may be on the borderline between two adjacent levels. These considerations should include:

- adequacy of the experimental design and conduct;
- occurrence of common versus uncommon neoplasia;
- progression (or lack thereof) from benign to malignant neoplasia as well as from preneoplastic to neoplastic lesions;
- some benign neoplasms have the capacity to regress but others (of the same morphologic type) progress. At present, it is impossible to identify the difference. Therefore, where progression is known to be a possibility, the most prudent course is to assume that benign neoplasms of those types have the potential to become malignant;
- combining benign and malignant tumor incidence known or thought to represent stages of progression in the same organ or tissue;
- latency in tumor induction;
- multiplicity in site-specific neoplasia;
- metastases;
- supporting information from proliferative lesions (hyperplasia) in the same site of neoplasia or other experiments (same lesion in another sex or species);
- presence or absence of dose relationships;
- statistical significance of the observed tumor increase;
- concurrent control tumor incidence as well as the historical control rate and variability for a specific neoplasm;
- survival-adjusted analyses and false positive or false negative concerns;
- structure-activity correlations; and
- in some cases, genetic toxicology.

Peer Review

The National Toxicology Program (NTP) convened a virtual external ad hoc panel to peer review the draft *NTP Technical Report on the Toxicology and Carcinogenesis Studies of Di-n-butyl Phthalate (CASRN 84-74-2) Administered in Feed to Sprague Dawley (Hsd:Sprague Dawley® SD®) Rats and B6C3F1/N Mice* on April 2, 2021. NTP announced the peer-review meeting in the Federal Register (X FR. XXXX. DATE). The public could view the proceedings online, and opportunities were provided for submission of written and oral public comments. The selection of panel members and conduct of the peer review were in accordance with federal policies and regulations. The panel was charged to:

(1) Review and evaluate the scientific and technical elements of each study and its presentation.

(2) Determine whether each study's experimental design, conduct, and findings support the NTP's conclusions regarding the conditions of each study.

NTP carefully considered the panel's recommendations in finalizing the report. The peer-review report is provided in Appendix D. Other meeting materials are available on the NTP website (https://ntp.niehs.nih.gov/go/meeting).

Peer Reviewers

[to come]

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Abstract

Di-*n*-butyl phthalate (DBP) is a phthalate used in the manufacture of consumer products such as plastics and personal care products. Widespread exposure in the population occurs throughout life, including during pregnancy and lactation. Because limited data are available in both animals and humans to evaluate DBP as a human carcinogen, the National Toxicology Program conducted 2-year studies of DBP in rats and mice. Time-mated female Sprague Dawley (Hsd:Sprague Dawley® SD®) rats were exposed to 0, 300, 1,000, 3,000, or 10,000 ppm DBP in feed during gestation and lactation. Postweaning, F_1 offspring consumed diets with the same exposure concentrations as the dam for 2 years (n = 50/sex/exposure group). Male and female adult B6C3F1/N mice were exposed to 0, 1,000, 3,000, or 10,000 ppm DBP in feed for 2 years (n = 50/sex/exposure group). Estimated average chronic chemical consumption was 16–17, 54–57, 152–169, and 510–600 mg DBP/kg body weight/day (mg/kg/day) in rats in the 300, 1,000, 3,000, and 10,000 ppm groups, respectively, and 105–112, 329–347, and 1,306–1,393 mg/kg/day in mice in the 1,000, 3,000, and 10,000 ppm groups, respectively.

Two-year Studies

In rats, no exposure-related effect in mortality between exposed and control groups was observed. DBP did not affect rat reproductive or littering parameters. Marginal F_0 weight effects (≤6% difference from the control group) were observed during gestation and by the end of lactation; F_1 male and female pup weights in the 10,000 ppm group were significantly decreased by 12 and 13%, respectively, compared to the control groups upon weaning. Throughout the postweaning period, F_1 body weights were approximately within 20% of the control animals. At 2 years, the incidence of pancreatic acinus adenomas was slightly higher in the 10,000 ppm group compared to the control group in male rats only. Because pancreatic acinus adenomas and carcinomas are associated with peroxisome proliferator-activated receptor alpha activation—and have been observed with exposure to other phthalates—the marginal increase observed could have been related to chemical exposure. The male reproductive tract was a primary target system of DBP in rats. A high incidence of small or absent organs of the male reproductive tract and undescended testes occurred only in the 10,000 ppm group. Some gross lesions correlated with microscopic lesions in the testes (germinal epithelium atrophy), epididymis (hypospermia), and prostate and seminal vesicles (decreased secretory fluid) in the 10,000 ppm groups. Additional microscopic lesions in the reproductive tract in rats included seminiferous tubule dysgenesis, testicular interstitial cell hyperplasia, testicular edema, and fibrosis and granuloma of the rete testis.

In mice, there were no exposure-related effects on survival, and mean body weights were lower only in the 10,000 ppm groups compared to the control groups. There was no exposure-related increase in neoplasms. No gross lesions were observed in the male reproductive tract, but significantly increased incidences of germinal epithelium degeneration in the testes and exfoliated germ cells in the epididymal duct were observed microscopically. The lesions generally occurred only in the 10,000 ppm group and were fewer and less severe in mice compared to rats.

Other nonneoplastic lesions observed were generally limited to the 10,000 ppm group. These lesions were found in the liver (hepatocyte cytoplasmic alteration in male and female rats and mice and multinucleated hepatocytes in male mice), in the pituitary gland (pars distalis

hypertrophy and hyperplasia in male rats), and in the kidney (renal tubule hyperplasia in female mice).

Conclusions

Under the conditions of these 2-year feed studies, there was *equivocal evidence of carcinogenic activity* of di-*n*-butyl phthalate (DBP) in male Hsd:Sprague Dawley® SD® rats based on marginal increases in the incidence of pancreatic acinus adenomas. There was *no evidence of carcinogenic activity* of DBP in female Hsd:Sprague Dawley® SD® rats at exposure concentrations of 300, 1,000, 3,000, or 10,000 ppm.

There was *no evidence of carcinogenic activity* of DBP in male or female B6C3F1/N mice at exposure concentrations of 1,000, 3,000, or 10,000 ppm.

Exposure to DBP increased incidences of gross lesions in the male reproductive system in rats and of nonneoplastic lesions in the male reproductive system (rats and mice), liver (male and female rats and mice), pituitary gland pars distalis (male rats), and kidney (female mice).

Synonyms: dibutyl benzene-1,2-dicarboxylate; 1,2-benzenedicarboxylic acid, dibutyl ester; di-*n*-butylorthophthalate

Summary of the Perinatal and Two-year Carcinogenesis Studies of Di-*n*-butyl Phthalate

	Male Sprague Dawley Rats	Female Sprague Dawley Rats	Male B6C3F1/N Mice	Female B6C3F1/N Mice
Concentrations in Feed	0, 300, 1,000, 3,000, or 10,000 ppm	0, 300, 1,000, 3,000, or 10,000 ppm	0, 1,000, 3,000, or 10,000 ppm	0, 1,000, 3,000, or 10,000 ppm
Survival Rates	27/49, 38/50, 31/50, 34/50, 33/50	29/50, 37/50, 27/50, 32/50, 29/50	44/50, 40/50, 36/50, 43/50	42/50, 42/50, 44/50, 47/50
Body Weights	10,000 ppm group 3.5% less than the control group	10,000 ppm group 10.6% less than the control group	10,000 ppm group 23.1% less than the control group	10,000 ppm group 34.5% less than the control group
Gross Lesions	Testis: small (1/48, 0/49, 4/48, 2/49, 36/46); enlarged (0/48, 0/49, 1/48, 1/49, 1/46); fluid or blood filled (1/48, 0/49, 0/48, 1/49, 3/46); right, not present (0/48, 0/49, 0/48, 0/49, 2/46); right or left; abdominal; undescended (1/48, 3/49, 2/48, 2/49, 29/46); right or left; inguinal; undescended (1/48, 0/49, 0/48, 0/49, 7/46); right or left; abdominal or inguinal; undescended (2/48, 3/49, 2/48, 2/49, 32/46) Epididymis: small (0/48, 0/49, 3/48, 0/49, 27/46); right, agenesis (0/48, 0/49, 0/48, 0/49, 2/46) Prostate gland: small (0/48, 0/49, 1/48, 2/49, 4/46); right or left, ventral, dorsal or lateral, agenesis, (0/48, 0/49, 0/48, 1/49, 2/46) Seminal vesicle: small (1/48, 2/49, 2/48, 1/49, 7/46)	None	None	None

	Male Sprague Dawley Rats	Female Sprague Dawley Rats	Male B6C3F1/N Mice	Female B6C3F1/N Mice
	Vas deferens: right, not present (0/48, 0/49, 0/48, 0/49, 2/46) Gubernaculum: right, not present (0/48, 0/49, 0/48, 0/49, 1/46)			
Nonneoplastic Effects	Testis: edema (includes bilateral) (2/49, 3/50, 2/50, 2/47, 18/50); germinal epithelium, atrophy (includes bilateral) (8/49, 21/50, 11/50, 10/47, 42/50); interstitial cell, hyperplasia, diffuse, bilateral (0/49, 0/50, 1/50, 0/47, 9/50); interstitial cell, hyperplasia, focal (includes bilateral) (1/49, 7/50, 5/50, 3/47, 11/50); rete testis, fibrosis (includes bilateral) (0/49, 0/50, 0/50, 0/47, 11/50); rete testis, sperm granuloma (0/49, 0/50, 0/50, 0/47, 2/50); seminiferous tubule, dysgenesis (includes bilateral) (0/49, 0/50, 0/50, 1/47, 9/50) Epididymis: hypospermia (includes bilateral) (4/49, 7/50, 10/50, 9/50, 40/50) Prostate gland: decreased secretory fluid (5/49, 8/50, 5/50, 5/50, 18/50)	Liver: hepatocyte, cytoplasmic alteration (0/50, 0/50, 0/50, 1/50, 40/50); bile duct, hyperplasia (5/50, 11/50, 6/50, 6/50, 12/50)	Testis: germinal epithelium, degeneration (includes bilateral) (6/50, 15/50, 9/50, 15/50) Epididymis: duct, exfoliated germ cell (includes bilateral) (6/50, 10/50, 5/50, 13/50) Liver: hepatocyte, cytoplasmic alteration (0/50, 0/50, 0/50, 36/50); hepatocyte, multinucleated (8/50, 11/50, 25/50, 42/50)	Liver: hepatocyte, cytoplasmic alteration (0/50, 0/50, 0/49, 48/49) Kidney: renal tubule, hyperplasia (0/50, 0/50, 0/49, 47/49)

	Male Sprague Dawley Rats	Female Sprague Dawley Rats	Male B6C3F1/N Mice	Female B6C3F1/N Mice
	Seminal vesicle: decreased secretory fluid (6/49, 7/50, 9/50, 6/50, 15/50) Pituitary gland: pars distalis, hypertrophy (0/48, 0/50, 0/50, 0/50, 29/50); pars distalis, hyperplasia (15/48, 13/50, 13/50, 18/50, 22/50) Liver: hepatocyte, cytoplasmic alteration (0/49, 0/50, 0/50, 0/50, 39/50)			
Neoplastic Effects	None	None	None	None
Equivocal Findings	Pancreas: acinus adenoma (4/49, 4/50, 3/50, 1/50, 10/49)	None	None	None
Level of Evidence of Carcinogenic Activity	Equivocal evidence	No evidence	No evidence	No evidence

Overview

Phthalates are plasticizers typically used to provide flexibility to products composed of polyvinyl chloride plastic or vinyl chloride resins. Significant concerns have been raised about in utero and early-life phthalate exposure and resultant adverse reproductive, developmental, and carcinogenic effects.

Previous hazard assessments of phthalates typically did not assess the effects of exposure throughout the perinatal period (gestation and lactation), and no carcinogenicity assessments of phthalates have used a lifetime exposure paradigm that includes the perinatal period. Thus, whether developmental exposure would alter lifetime phthalate-associated carcinogenic risk is unknown. Exposure during these critical periods of development and growth might be relevant for the evaluation of lifetime toxicological and carcinogenic risk.

In response to these data gaps, the National Toxicology Program initiated a broad program of work to provide toxicity data and a cancer hazard assessment for lifetime exposure to environmental phthalates. The program would also provide critical data to inform cumulative and aggregate risk characterization efforts for multiple phthalates including di(2-ethylhexyl) phthalate, di-isobutyl phthalate, and di-*n*-butyl phthalate.

Introduction

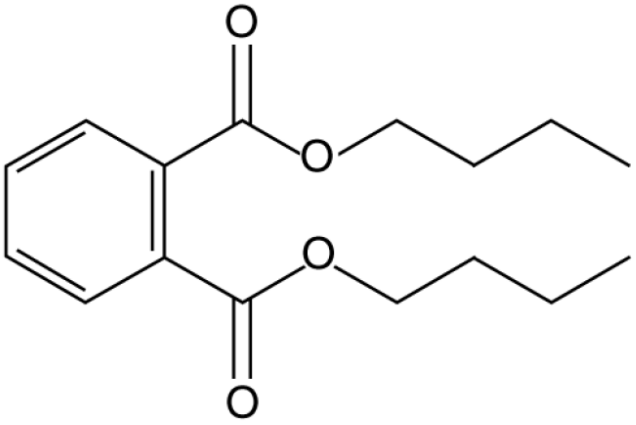


Figure 1. Di-*n*-butyl Phthalate (CASRN 84-74-2; Chemical Formula C16H22O4; Molecular Weight: 278.34)

Synonyms: dibutyl benzene-1,2-dicarboxylate; 1,2-benzenedicarboxylic acid, dibutyl ester; di-*n*-butylorthophthalate

Chemical and Physical Properties

Di-*n*-butyl phthalate (DBP) is a colorless, oily liquid at room temperature. It is soluble in most solvents but minimally so in water, having a K_{ow} of 3.70 to 4.72. DBP has a melting point of −35°C and a boiling point of 340°C.[1] At 25°C, its vapor pressure is approximately 2.67 mPa.[2]

Production, Use, and Human Exposure

DBP is a phthalate ester and is produced primarily in Europe, the United States, Asia, and Pacific Rim countries.[3] Between 1980 and 1994, U.S. production of DBP, including di-*iso*-butyl phthalate, ranged from 6.5 to 11.5 million kg, according to the U.S. International Trade Commission.[1] The national imported volume of DBP reported in the U.S. Environmental Protection Agency's (EPA's) Chemical Data Reporting database was approximately 7 million pounds in 2011.[4]

DBP is primarily used as a plasticizer, which increases the malleability of plastics, particularly in nitrocellulose plastics that can be used as food packaging materials. Lower molecular weight phthalates, like DBP, also are used in solvents, inks, and latex adhesives.[5-7] DBP is the primary plasticizer in nail polish and has been detected in cosmetics and personal care products such as perfume and hair spray.[8; 9; 6; 10] DBP also is used as an enteric coating for some pharmaceutical products to modulate drug absorption.[11; 12]

Due to the widespread use of phthalates, metabolites of phthalates are detectable in humans (reviewed in Wang et al.[13]). The primary metabolite of DBP, mono-*n*-butyl phthalate (MBP), has been detected in human urine with median concentrations ranging from 0 to 2,540 μg/L.[13] In biomonitoring data from the National Health and Nutrition Evaluation Survey (NHANES) for 2013–2014, urinary MBP in the U.S. population ranged from 0.3 to 489.6 μg/L.[14] In workers at plastic manufacturing plants in Slovakia, urinary MBP concentrations ranged from 11.9 to 1,221.8 μg/L.[15]

Concentrations of MBP in serum tend to be lower than those in urine.[13] In a study of Danish men, serum MBP concentration was two orders of magnitude lower than urinary MBP (0.4 versus 42.5 μg/L).[16] Additionally, MBP has been detected in cord blood, amniotic fluid, and breast milk, suggesting gestational and lactational transfer. Concentrations of MBP in these matrices are lower than urinary MBP concentrations. Median maternal plasma concentration of MBP was 4.37 μg/L, whereas median cord plasma concentration was 5.15 μg/L.[17] MBP was detected in approximately 93% of amniotic fluid samples collected in the United States, and concentrations ranged from below the limit of detection to 0.264 μg/L.[18] Median breast milk concentrations of MBP from various cohorts ranged from 0.5 to 12 μg/L.[19-21]

Human exposure to DBP occurs primarily through consumption of DBP-contaminated food, typically dairy, fish, or seafood products.[1] Because phthalates do not bind to plastic, they are prone to leaching into foods from the packaging products to which they have been added. Estimated exposure through food, based on surveys of phthalates in various food products, is 0–7 μg DBP/kg body weight/ day (μg/kg/day),[22] although some estimates suggest a maximum likely food intake of DBP of approximately 31 μg/kg/day.[6] Actual exposure depends highly on food consumption patterns and usage of food packaging, as some food products tend to have higher DBP concentrations than others.[23] Exposure to DBP through oral pharmaceuticals in a Danish population was estimated at 0.5–32.8 mg DBP per defined daily dose.[11] Many pharmaceutical products, assuming an intake of one defined dose per day, exceeded the maximum daily exposure limit of 0.01 mg/kg/day for DBP.[11]

DBP exposure via inhalation and dermal routes is also pertinent, both for the general population and for certain occupations. Estimated inhalation of DBP in indoor air by the general adult population—due to off-gassing of household products and/or inhalation of dust containing DBP—ranged from 0.01 to 6.18 μg/m^3.[23; 6; 24] Inhalation exposures for children tended to be higher.[23] For workers in phthalate manufacturing, one exposure estimate via inhalation was 143 μg/kg/day.[22] Manicurists at nail salons are also exposed via inhalation and dermal absorption; use of exhaust systems and gloves decreased urinary MBP concentrations measured after work.[25] Dermal exposure to DBP in the general population occurs through use of personal care products containing phthalates. An estimate of the maximal daily DBP dermal dose in Canadian women, based on detected concentrations in 252 products, was 0.36 μg/kg/day.[26]

Regulatory Status

Six phthalate chemicals, including DBP, were banned from use in the United States in children's toys at concentrations >0.1% by the Consumer Product Safety Commission, starting in 2008.[27] The final rule was published in 2017.[28]

The U.S. Food and Drug Administration (FDA) has not published a safety assessment of DBP but did release industry guidance in 2012 that recommended not using it as an excipient.[29] Additionally, in 2018, FDA proposed an amendment to the Code of Federal Regulations to remove 26 phthalates, including DBP, from food-additive and food-contact use.[30]

DBP is listed as a hazardous air pollutant under the Clean Air Act, it is listed in the Toxic Substances Control Act Chemical Substance Inventory, and it is reportable to the Toxic Release Inventory. As such, EPA has established an oral reference dose (RfD) for DBP: 0.1 mg/kg/day based on increased mortality with a no-observed-adverse-effect level of 125 mg/kg/day.[31; 32]

Confidence in this RfD is low, however, due to limitations of the Smith et al. study; animals of only one sex were used, and mortality was observed early in the study with no indication of the cause of death.[32] The ATSDR minimal risk level (MRL), an estimate of exposure that is likely without appreciable risk of adverse, noncancer health effects, for acute oral exposure is 0.5 mg/kg/day.[33] This value was based on a no-observed-adverse-effect level of 50 mg/kg/day for developmental effects. In addition, EPA has classified DBP as a group D carcinogen, not classifiable as to human carcinogenicity, due to the lack of adequate studies.[32] DBP has not been evaluated for human carcinogenicity by the International Agency for Research on Cancer.

Absorption, Distribution, Metabolism, and Excretion

Experimental Animals

Following oral administration, DBP is hydrolyzed to MBP by lipases and esterases in the gastrointestinal tract; hence, the available data primarily concerns MBP.[34-37] MBP is rapidly absorbed and distributed in the body after oral exposure to DBP. In Wistar and Sprague Dawley rats, most of an oral dose (ranging from 0 to 250 mg/kg) is accounted for in urine within 24 hours after dosing.[38; 39; 34; 40] MBP is distributed to tissues with minimal bioaccumulation.[39; 34; 41; 40] In male Sprague Dawley rats given 30 mg/kg intravenously, the half-life of DBP was approximately 3.6 hours.[38]

MBP and its glucuronide are the primary metabolites of DBP, eliminated mostly in urine.[34] Some fecal elimination does occur,[38; 39; 41; 40] and enterohepatic recirculation has been reported.[39; 40] In plasma, tissues, and urine, most MBP exists as unconjugated MBP.[42; 39; 41] MBP can be metabolized further through oxidation to produce 3-hydroxybutyl phthalate (3OH-MBP), 4-hydroxybutyl phthalate (4OH-MBP), 3-ketobutyl phthalate, or 4-carboxypropyl phthalate.[1]

Gestational transfer of DBP has been evaluated in two studies.[39; 41] Pregnant Sprague Dawley rats given a single dose of DBP (50, 100, or 250 mg/kg) on gestational day 20 via oral gavage had a maternal plasma T_{max} of MBP and MBP-glucuronide of 0.5 and 2 hours, respectively.[39] MBP was detected in fetal plasma; C_{max} was 2.4- to 4-fold higher in maternal plasma, but the area under the curve was only 1.4- to 1.7-fold higher. The ratio of MBP:MBP-glucuronide in maternal plasma was higher than that in the fetal plasma.[39] At higher doses of DBP (500 and 1,500 mg/kg), the plasma T_{max} of MBP was similar to that observed with lower doses, ranging from 1.4 to 2.6 hours.[41] Concentrations of MBP in embryos and the placenta were <33% of maternal plasma concentrations; MBP-glucuronide was lower than MBP.[41] MBP and MBP-glucuronide were detected in the amniotic fluid at concentrations similar to those in embryos. Overall, these studies indicate fetal exposure to MBP and MBP-glucuronide occurs, although the concentrations are lower than those observed in the dam. This exposure could occur through transfer of metabolites across the placenta and/or by fetal metabolism.

Humans

As in animals, DBP in humans is converted to MBP in the gastrointestinal tract before it is absorbed. Human and rodent lipases show similar rates of conversion of DBP to MBP.[43] Three studies have evaluated human absorption, distribution, metabolism, and excretion of DBP after oral consumption. In one study, one male volunteer ingested 60 μg/kg body weight of DBP.[44] In the second study, 17 volunteers consumed one capsule of an herbal drug containing 3,600 μg of

DBP.[45] In the third study, 24 volunteers consumed phthalates, including DBP (255 and 510 μg), spiked into margarine and administered on toast.[46]

As shown for animals, most of the dose administered to humans was excreted as MBP in the urine within the first 24 hours.[46; 44; 45] Hydroxylated monoester DBP metabolites were detected in urine after DBP consumption in Koch et al.[44] In urine, MBP was the dominant metabolite, comprising 84% of the metabolites, followed by 3OH-MBP (7%); other excreted metabolites included 2-hydroxybutyl phthalate (2OH-MBP), 4OH-MBP, and mono-carboxy-propyl phthalate (MCPP), which contributed <1% each.[44] In plasma, MBP, 3OH-MBP, and MCPP were detected at 196, 13.5, and 2.8 μg/L, respectively.[44] Unlike rodent studies, in which most MBP is unconjugated, most MBP in human urine and plasma is conjugated with glucuronide. This pattern has been observed in urine and plasma samples from NHANES studies,[47] in urine after oral ingestion of DBP[45], and in urine after topical exposure to DBP.[48]

The urinary half-life of MBP reported for an individual who ingested DBP was 2.6 hours; 3OH-MBP and 4OH-MBP had half-lives similar to that of MBP, but the other detected metabolites had longer (approximately 6 hours) half-lives.[44]

Toxicity

Experimental Animals

Several studies have been conducted to evaluate the toxicity of DBP following oral exposure. NTP has conducted both subchronic and reproductive and developmental studies on DBP; these findings are reported in NTP Toxicity Report 30.[49] Generally, oral DBP exposure is well tolerated in animal models, as indicated by some studies reporting an acute oral LD_{50} (median lethal dose) in rodents >20,000 mg/kg.[50; 51] NTP Toxicity Report 30 studies in rats and mice show minimal differences in survival after 3 months of exposure of up to approximately 4,278 mg/kg/day.[49] Toxicity to the male reproductive tract is the primary endpoint studied with DBP exposure; these effects are summarized in the reproductive and developmental toxicity section below. Neurotoxicity, particularly following in utero DBP exposure, has been reported in some rodent studies[52-54] but not others.[55] Other target organs include the liver and the hematopoietic system.

Increased liver weight has been observed in rats following exposure to DBP for 7 or more days,[56; 57; 49] which might be related to proliferation of peroxisomes. Some phthalates are known peroxisome proliferator-activated receptor (PPAR) agonists[58]; DBP and its metabolites weakly activate the three PPAR receptor subtypes in vitro.[59; 60] In the NTP 13-week study of rats administered DBP in feed, peroxisomal enzyme activity was induced at exposure concentrations of ≥356 mg/kg/day.[49] Hepatic cytoplasmic alteration was noted in male and female rats exposed to ≥720 mg/kg/day and in male and female mice exposed to ≥1,601 mg/kg/day and ≥4,278 mg/kg/day, respectively. Decreases in serum triglyceride and/or cholesterol were observed in the NTP 3-month studies and in other studies[61; 56; 49] and could be due to hepatic toxicity because the liver is the site of cholesterol synthesis. These changes also could be due to PPAR activation, which could lead to impaired lipid and glucose homeostasis.

Decreases in erythrocyte, hemoglobin, and hematocrit levels were observed in male rats given DBP at 752 mg/kg/day.[55] In the NTP 13-week rodent studies, dietary exposure to DBP resulted

in minimally severe anemia characterized by decreases in erythrocyte, hemoglobin, and hematocrit levels in male rats only at exposure concentrations >359 mg/kg/day.[49] Decreased hematocrit was observed in female mice following exposure to 4,278 mg/kg/day.[49]

Humans

Studies that followed humans after ingestion of up to 5.4 mg DBP reported no adverse effects after acute exposure.[46; 44; 45] Epidemiological studies have focused on associations of phthalate exposure with asthma, reproductive health, metabolic disease, neurodevelopmental disorders, and cancer (particularly breast cancer due to potential estrogen binding of phthalates) (reviewed in Benjamin et al. [62]). Specifically, MBP exposure was significantly associated with metabolic disease and with spermatotoxicity in some studies.[62]

Reproductive and Developmental Toxicity

Experimental Animals

Reproductive and developmental toxicity is the main toxicity associated with phthalate exposure in rodents (reviewed in Kavlock et al.[22]). Developmental abnormalities in the male rodent reproductive tract following in utero DBP exposure include malformations of the male reproductive tract, undescended testes, and decreased anogenital distance.[63; 64; 22] In addition to small and atrophic testes, hypoplastic epididymides, and decreased size or absence of prostate, seminal vesicles, and vas deferens, male rats exposed to 100 or 500 mg/kg/day via gavage in utero (gestation day [GD] 12 to 21) were diagnosed with testicular dysgenesis.[63] These lesions were characterized by areas of malformed seminiferous tubules surrounded by Leydig cell aggregates.[63] The testicular dysgenesis lesions did not increase over time, suggesting these lesions were of developmental origin. Low testosterone and decreased insulin-like peptide 3 (*Insl3*) levels have been observed after in utero exposure in some studies,[65; 49] which are proposed mechanisms of phthalate toxicity.[66-70] Delayed puberty was observed in male rats,[71] but not in females, following exposure to DBP.[72]

Other developmental effects observed with in utero DBP exposure (generally >600 mg/kg/day) include increased resorptions, decreased pup body weight, delayed ossification, and fetal malformations.[1] External (e.g., cleft palate) and skeletal (e.g., fused sternebrae, deformed vertebral column) malformations have been reported at doses >500 mg/kg/day and depend on the window of exposure during gestation.[73-76; 41] Administration of MBP has elicited similar developmental impairments, suggesting that the metabolite might be responsible for these effects.[77; 78; 41]

Exposure to DBP impaired fertility and decreased the number of live pups per litter in multiple rodent studies.[71; 49; 79] Acute and subchronic oral exposure (feed and gavage) to DBP causes reproductive toxicity in male rodents, guinea pigs, and rabbits, characterized by malformations of the reproductive tract, reduced sperm count, and testicular damage and atrophy.[80; 71; 81; 65; 49; 79] The effects of DBP on female reproduction are less clear and have been reviewed by Kay et al.[72] Minimal effects on the ovary or estrous cycling were observed in Sprague Dawley and Long Evans rats[82; 49; 79]) and in CD-1 mice.[49] A crossover breeding study in CD-1 mice, however, suggests some effect of DBP on female fertility, as decreases in fertility and in the number of pups per litter occurred in exposed females mated with control males.[49] Furthermore, treatment

of Long Evans rats with 500 mg/kg/day DBP increased midterm abortions, and F_1 female offspring in the study displayed reduced fecundity.[82; 83] The embryonic loss after DBP exposure could be mediated in part by reduced uterine decidualization and/or by disruptions in hormones during gestation.[84; 82]

Humans

The epidemiological data on phthalate exposure and male and female reproductive health have been reviewed by Radke et al.[85; 86] Some evidence supports the relationship between increasing DBP exposure and adverse male reproductive effects, including decreased semen quality, decreased anogenital distance, and decreased fecundability.[85] Ten studies evaluated testosterone concentrations and DBP exposure. Of those, five reported decreased testosterone with increasing DBP exposure[85]; the association was statistically significant in only one study.[87] Concerning female reproductive health, no significant associations of DBP exposure with time to pregnancy, spontaneous abortion, or pubertal development were found.[86] Six studies evaluated DBP exposure and preterm birth; three showed a positive association (significant in two), and three showed no or a negative association.[86]

Immunotoxicity

Experimental Animals

Hansen et al.[88] have reviewed the immunotoxicity of phthalates. Generally, experimental studies indicate that phthalates can affect immune cells and modify cytokine expression; however, the effect is inconsistent across studies and phthalates. Only a few studies have evaluated DBP specifically. Exposure of murine peritoneal exudate macrophages to DBP (1–100 μM) resulted in a dose-dependent decrease in expression of some cell surface markers, decreased phagocytosis, decreased cytokine production, and impaired antigen-presenting capacity.[89] Testicular macrophages isolated from Sprague Dawley rats after a 90-day exposure to 250 mg/kg/day DBP had higher interleukin (IL)-1β protein expression.[90] Another study in female Wistar rats found that DBP exposure exacerbated chronic lymphocytic thyroiditis, increased thyroid auto-antibodies, disrupted T helper Type 1 (Th1) and Th2 balances, and activated proinflammatory cytokines.[91] DBP did not elicit these effects in animals without chronic lymphocytic thyroiditis.[91]

Humans

To date, epidemiological studies evaluating phthalate exposure and immune function have focused on asthma. Overall, these studies suggest a possible correlation between phthalate exposure and childhood asthma but are limited by the cross-sectional study design and the use of polyvinyl chloride materials or di(2-ethylhexyl)phthalate dust as surrogates of exposure (summarized in Bornehag and Nanberg[92]). One in vitro study stimulated human whole blood with DBP and found that DBP might exert immunosuppressive effects.[93]

Carcinogenicity

Experimental Animals

Two rodent studies have examined the carcinogenic effects of chronic DBP exposure. The first exposed 10 male rats/group to 0, 0.01, 0.05, 0.25, and 1.25% DBP in feed for 1 year.[31] High

mortality occurred at the highest exposure concentration (approximately 625 mg/kg/day) within the first week of the study; the cause of death or whether the deaths were exposure-related was not reported. No hematological or gross pathological changes were observed.[31] The second study evaluated male Sprague Dawley rats at 6, 12, and 18 months of age after in utero-only exposure to DBP.[63] Pregnant Sprague Dawley rats were administered 0, 100, or 500 mg/kg/day DBP via oral gavage from GD 12 to 21. Leydig cell adenomas, categorized according to the morphological criteria of the Society of Toxicologic Pathology, were observed only in the 100 mg/kg/day dose group at 18 months of age.[63] Areas composed of Leydig cell aggregates surrounding malformed, contorted seminiferous tubules, defined as testicular dysgenesis, however, were observed consistently in the high dose group. Low incidences of other neoplasms (e.g., pituitary adenoma, basal cell adenoma, sarcoma) also were reported. Female animals were not evaluated.

Humans

Carcinogenicity of DBP in humans has not been evaluated by the International Agency for Research on Cancer, and EPA has been unable to classify it due to a lack of adequate studies, both in animal and in epidemiological or human studies. Phthalates have been implicated in breast cancer due to their reactivity with the estrogen receptor in vitro. However, DBP binds weakly to the estrogen receptor in mammalian in vitro screens.[94] In six epidemiological studies, MBP was not found to be associated with breast cancer (reviewed in Zuccarello et al.[95]). Other epidemiological studies have examined associations of DBP with uterine tumors and gastrointestinal cancers. Urinary MBP was associated with uterine leiomyomas,[96] but DBP exposure was not associated with increased gastric cancers,[97] although sample sizes for both studies were small. Cumulative phthalate exposure from drug excipients had an overall protective effect against colorectal cancer; exclusion of nonsteroidal anti-inflammatory drug users from the analysis reversed the relationship.[98]

Genetic Toxicity

The genetic toxicity of DBP has been evaluated in a variety of in vitro and in vivo assays, and results are mixed. In bacterial mutation assays that examined reversion to histidine prototrophy, DBP was reported to be negative or weakly positive in a variety of *Salmonella typhimurium* tester strains, both with and without exogenous metabolic activation (S9).[99-102] In a bacterial forward mutation assay, DBP (0.18–0.45 mM) induced a weak but dose-related increase in 8-azaguanine resistance in *S. typhimurium* strain TA100 in the absence of, but not the presence of, S9.[103] DBP at concentrations of 10 or 20 μL/mL did not increase gene reversion frequencies in the yeast *Saccharomyces cerevesiae* strain XV185-14c after 48 hours of exposure, with or without S9.[104; 105]

In contrast to the mostly negative results in bacteria and yeast, positive results were obtained in most genotoxicity assays in cultured mammalian cells. DBP (46–54 μg/mL) induced mutations in mouse lymphoma L5178Y$^{tk+/-}$ cells treated in the absence of S9 at concentrations that induced high levels of cytotoxicity; it was not tested with S9.[49] In a second mouse lymphoma cell gene mutation assay that used concentrations of DBP ranging up to 0.15 μL/mL, a significant increase in mutations was reported in the presence of S9 and at concentrations that induced marked cytotoxicity (relative total growth <20%).[106] DBP (354 mM for 60 minutes) induced DNA damage in cultured human oropharyngeal mucosal cells, human nasal mucosal cells, human

mucosal cells of the upper aerodigestive tract, and human lymphocytes, as measured by the alkaline comet assay.[107; 108] Negative results were reported for DBP (1 μM–1 mM) in tests for induction of chromosomal aberrations and sister chromatid exchanges in Chinese hamster DON cells.[109]

Conflicting results were obtained in vivo in genotoxicity tests conducted in mice. No increases in the frequencies of micronucleated normochromatic erythrocytes were observed in peripheral blood samples obtained from male and female B6C3F1/N mice after 3 months of exposure to DBP (1,250–20,000 ppm or 163–4,278 mg/kg/day) in dosed feed.[49] In contrast, significant increases in the percentages of chromosomally aberrant splenic lymphocytes and micronucleated peripheral blood erythrocytes were observed in male and female B6C3F1 mice following 16 to 52 weeks of exposure to 5,000 ppm DBP in dosed feed.[110] In addition, increases in *hprt* transversion mutations in splenic T-cells of these male and female mice exposed to DBP (5,000 ppm) for 32 or 52 weeks were reported.[111] Recently, DBP was reported to disrupt meiotic prophase, disrupt homologous recombination, and increase the number of γH2AX foci (indicative of DNA double-strand breaks) in oocytes of fetal mice following exposure of the dam via oral administration of 10 or 100 mg/kg/day for 3 days.[112]

Study Rationale

Available studies to evaluate DBP carcinogenicity are limited to two rodent studies that evaluated a small number of male animals with adult-only exposure. Given widespread exposure to DBP in the general population and that previous literature indicates developmental toxicities, a perinatal exposure was included in the rat study to provide a hazard assessment of lifetime exposure to environmental phthalates. To emulate the primary route of human exposure, the animals in this study were exposed via feed. In addition, this study is part of a larger body of work that aims to inform cumulative and aggregate risk characterization efforts for a range of environmental phthalates.

Materials and Methods

Procurement and Characterization of Di-*n*-butyl Phthalate

Di-*n*-butyl phthalate (DBP) was obtained from Sigma-Aldrich (St. Louis, MO) in a single lot (lot MKBB8432) and was used in the perinatal and 2-year studies. Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory at RTI International (Research Triangle Park, NC) (Appendix A). Reports on analyses performed in support of the DBP studies are on file at the National Institute of Environmental Health Sciences (NIEHS).

Lot MKBB8432 of the chemical, a clear liquid, was identified as DBP by infrared (IR), ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy, and gas chromatography (GC) with mass spectrometry (MS) detection. The IR spectrum was in good agreement with a reference spectrum, and the structure was consistent with DBP. Both ^{1}H and ^{13}C NMR spectra were consistent with reference and predicted spectra. The GC/MS spectrum correlated well with the structure of DBP. The boiling point, elemental analysis, and precise mass determination of lot MKBB8432 matched those of DBP.

Karl Fischer titration was used to determine the water content of lot MKBB8432, which was 0.204%. Ultra-performance liquid chromatography (UPLC) with photodiode array detection (PDA) and GC with flame ionization detection (FID) were used to determine a purity of 99.9%. The UPLC/PDA analysis identified one minor impurity peak with <0.1% of the total response. The overall purity of lot MKBB8432 was determined to be >99%.

Accelerated stability studies, performed on an additional lot of DBP (lot 91997PJ) obtained from Sigma-Aldrich (St. Louis, MO), confirmed that it was stable for at least 2 weeks when stored in sealed glass vials at temperatures from 5°C to 60°C. The bulk chemical of lot MKBB8432 was homogenized by mixing for 15 minutes and transferred to 1-gallon amber storage bottles, which were stored at room temperature (approximately 25°C). Periodic reanalysis of the bulk chemical was performed during the perinatal and 2-year studies by the study laboratory using high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection, and no degradation was detected.

Preparation and Analysis of Dose Formulations

Dose formulations were prepared approximately monthly by mixing DBP with NIH-07 or NTP-2000 feed (Table A-2). The rat perinatal and 2-year study used dose formulations of 300, 1,000, 3,000, and 10,000 ppm, whereas the mouse 2-year study used dose formulations of 1,000, 3,000, and 10,000 ppm. Formulations were stored in sealed, plastic bag-lined containers at room temperature (approximately 25°C) for up to 42 days. The plastic bags used by the study laboratory in the preparation and storage of blank and dosed feed were determined to have no DBP above the limit of detection of the assay (1.47 ppm).

Homogeneity studies of the dose formulations in a 72 kg NIH-07 feed batch (300 and 1,000 ppm), a 92 kg NTP-2000 feed batch (300 ppm), and a 60 kg NTP-2000 feed batch (1,000 and 10,000 ppm) were performed prior to animal studies by the study laboratory with HPLC/UV (Table A-1). Additional homogeneity studies of the 300 ppm dose formulation in a 60 kg NTP-2000 batch size and of the 10,000 ppm dose formulation in a 72 kg NTP-2000 batch size

were performed during the 2-year studies by the study laboratory. Formulations were determined to be homogenous and stable for 42 days at room temperature.

Periodic analyses of the dose formulations of DBP were conducted by the study laboratory using HPLC/UV to determine purity (Table A-3, Table A-4). All preadministration dose formulations were within 10% of the target concentrations. In the perinatal and 2-year rat studies, five samples collected from the residual food in the feeders and one sample from the storage barrel were below 90% of the target concentrations (% relative error, RE: −10.7% to −13.4%). In the 2-year mouse study, three samples collected from the residual food in the feeders and one sample from the storage barrel were below 90% of the target concentrations (%RE: −11.1% to −13.4%). All other postadministration values were within 10% of the target concentrations.

Animal Source

Time-mated (F_0) female Sprague Dawley (Hsd:Sprague Dawley® SD®) rats were obtained from Envigo (formerly Harlan Laboratories, Inc., Indianapolis, IN). Male and female B6C3F1/N mice were obtained from Taconic Biosciences, Inc. (Germantown, NY).

Animal Welfare

Animal care and use are in accordance with the Public Health Service Policy on Humane Care and Use of Animals. All animal studies were conducted in an animal facility accredited by AAALAC International. Studies were approved by the Battelle (Columbus, OH) Animal Care and Use Committee and conducted in accordance with all relevant National Institutes of Health and National Toxicology Program (NTP) animal care and use policies and applicable federal, state, and local regulations and guidelines.

Two-year Studies

Exposure Concentration Selection Rationale

Studies summarized in NTP Toxicity Report 30[49] established 10,000 ppm as the highest safe exposure concentration of DBP that could be used in rat and mouse reproductive studies; at 20,000 ppm, pups died and the body weight of postweaning and adult rodents was reduced by more than 10% relative to the control groups. At 10,000 ppm, a significant increase in the incidence of nonneoplastic lesions of the liver and testis and alterations in organ weights were observed. For these reasons, 10,000 ppm was chosen as the highest exposure concentration for both rats and mice for the 2-year bioassay.

Study Design for Rats

F_0 female rats were 11 to 14 weeks old upon receipt. Evidence of mating is defined as gestation day (GD) 1; F_0 females were received on GD 2 and held for 4 days. F_0 females were randomly assigned to one of five exposure groups on GD 5. Randomization was stratified by body weight that produced similar group mean weights using PATH/TOX SYSTEM software (Xybion Medical Systems Corporation, Cedar Knolls, NJ).

F_0 females were quarantined for 11 days after receipt. Ten nonmated females received with the time-mated females were designated for disease monitoring 11 days after arrival; samples were

collected for serological analyses, and the rats were euthanized, necropsied, and examined for the presence of disease or parasites. The health of the F_1 rats was monitored during the study according to the protocols of the NTP Sentinel Animal Program (Appendix C). Pinworms (*Syphacia* spp.) were diagnosed in sentinel animals during routine health monitoring evaluations. Infected animals did not display clinical signs, and no pathological lesions were noted in relation to the presence of the pinworms. As a follow-up to this finding, NTP, in coordination with the testing laboratory, developed and implemented a successful plan of pinworm containment and eradication. NTP required the testing laboratories to monitor animals actively to ensure the continued exclusion of pinworms from all subsequent studies. All other test results were negative.

Beginning on GD 6, groups of 45 (0, 300, 1,000, and 3,000 ppm) or 47 (10,000 ppm; includes two extra rats received in the animal order) F_0 time-mated female rats were fed diets containing 0, 300, 1,000, 3,000, or 10,000 ppm DBP, respectively, throughout gestation and lactation. Groups of 50 F_1 rats per sex per exposure concentration continued in the study after weaning and were fed diets containing the same respective DBP concentrations for 2 years.

F_0 female rats were housed individually during gestation and with their respective litters during lactation. Dosed feed and water were available ad libitum. F_0 female body weights were recorded on GDs 5, 6, 9, 12, 15, 18, and 21 and on lactation days (LDs) 1, 4, 7, 10, 14, and 21. During gestation, feed consumption was continuously measured over 3-day intervals from GD 6 through GD 21 (GDs 6–9, 9–12, 12–15, 15–18, and 18–21). The day of parturition was considered LD 0 or postnatal day (PND) 0. On apparent GD 27, all time-mated female rats that failed to deliver were euthanized, and the uteri were examined and stained for evidence of implantation. Total litter weight and litter weights by sex were collected on PND 1. Individual F_1 pup weights were recorded on PNDs 4, 7, 14, and 21. Clinical observations and survival were evaluated throughout lactation. During lactation, feed consumption was measured over 3-day intervals from PND 1 through PND 21 (PNDs 1–4, 4–7, 7–10, 10–14, 14–17, and 17–21).

Select dams and their litters were removed on GD 18 and PND 4 to quantify mono-*n*-butyl phthalate (MBP) concentrations. On GD 18, blood was collected from the retroorbital sinus of randomly selected dams (n = 5 per exposure group). Blood samples were collected into tubes containing tripotassium ethylene diamine tetraacetic acid (K_3 EDTA) and centrifuged, and the plasma was harvested. Amniotic fluid was collected and pooled by dam. Each dam's fetuses were collected, pooled by litter, and flash frozen in liquid nitrogen. On PND 4, randomly selected dams (n = 5 per exposure group) and their pups not selected to continue in the study were used for biological sampling. Plasma was collected from dams in the same manner as on GD 18. Up to four pups were collected from each dam (two per sex when possible) and flash frozen in liquid nitrogen. All samples were stored frozen at approximately −20°C before shipment to RTI International (Research Triangle Park, NC) for analysis. All samples were analyzed using a previously validated method.[113]

F_1 litters were standardized on PND 4 to eight pups/litter, with at least two pups of each sex and a preference for four males and four females each. Litters that did not meet the minimum of eight pups (or had fewer than two pups of either sex) were removed from the study. For continuation of exposure after weaning, two males and two females per litter were randomly selected from 28 (0 ppm), 25 (300 ppm), 27 (1,000 and 10,000 ppm), and 30 (3,000 ppm) litters. Before weaning, on the day the last litter reached PND 19, 25 litters per exposure group were randomly

selected, and pups (generally two/sex/litter) were randomly assigned to the 2-year study. On the day the last litter reached PND 21, dams were removed from the cages, and the pups were weaned. Weaning marked the beginning of the 2-year study.

After weaning, F_1 rats were housed up to two (males) or four (females) per cage. Water and dosed feed were available ad libitum. Feed consumption was measured weekly for the first 3 months, then for one 7-day period every 4 weeks thereafter, and at study termination. Cages were changed at least once weekly through PND 4, then changed at least twice weekly. Racks were changed and rotated at least every 2 weeks. Further details of animal maintenance are given in Table 1.

Two diets were used in the rat studies: (1) NIH-07 during the perinatal phase and (2) NTP-2000 during the postnatal phase. NIH-07 is a higher protein diet that supports reproduction and lactation in rodents, whereas NTP-2000 is a lower protein diet that decreases the incidence of chronic nephropathy in adult rats. Information on feed composition and contaminants for both diets is provided in Appendix B.

Study Design for Mice

Male and female B6C3F1/N mice were approximately 3 to 4 weeks old upon receipt and were quarantined for 11 (females) or 12 (males) days before study start. Mice were randomly assigned to one of four exposure groups (n = 50 mice/sex/group). Randomization was stratified by body weight that produced similar group mean weights using PATH/TOX SYSTEM software (Xybion Medical Systems Corporation, Cedar Knolls, NJ). Mice were fed diets containing 0, 1,000, 3,000, or 10,000 ppm DBP for 2 years.

Five male and five female mice were randomly selected for parasite evaluation and gross observation of disease. The health of the mice was monitored during the study according to the protocols of the NTP Sentinel Animal Program (Appendix C). All test results were negative.

Mice were housed individually (males) or up to four (females) per cage. Water and dosed feed were available ad libitum. Feed consumption was measured weekly for the first 3 months, then for one 7-day period every 4 weeks thereafter. Cages were changed at least once weekly (males) or twice weekly (females) and rotated every 2 weeks. Racks were changed and rotated every 2 weeks. Further details of animal maintenance are given in Table 1. Information on feed composition and contaminants is given in Appendix B.

Clinical Examinations and Pathology

In the 2-year studies of rats and mice, animals were observed twice daily for signs of morbidity and moribundity and were weighed before dosed feed exposure on day 1, weekly for the next 3 months, every 4 weeks thereafter, and at study termination. Clinical observations were recorded every 4 weeks beginning on day 29 (rats) or day 1 (mice) and at study termination.

For determination of internal dose in rats, plasma, amniotic fluid, and fetal tissue samples were analyzed using a previously validated method.[113]

Complete necropsies and microscopic examinations were performed on all F_1 rats and all mice at the end of the 2-year study. At necropsy, all organs and tissues were examined for grossly visible lesions, and all major tissues were fixed and preserved in 10% neutral buffered formalin except

for eyes, which were first fixed in Davidson's solution, and testes, vaginal tunics, and epididymides, which were first fixed in modified Davidson's solution. Tissues were processed and trimmed, embedded in paraffin, sectioned to a thickness of 4 to 6 μm, and stained with hematoxylin and eosin (H&E) for microscopic examination. For all paired organs (e.g., adrenal gland, kidney, ovary), samples from each organ were examined. Three transverse sections of each testis were taken: approximately one-third of the way down from the cranial pole containing the rete testis, at the midpoint, and approximately one-third of the way up from the caudal pole. Two transverse sections of the uterus, one from approximately the midpoint of each horn, were collected. Tissues examined microscopically are listed in Table 1.

Microscopic evaluations were completed by the study laboratory pathologist, and the pathology data were entered into the Toxicology Data Management System. The report, slides, paraffin blocks, residual wet tissues, and pathology data were sent to the NTP Archives for inventory, slide/block match, wet tissue audit, and storage. The slides, individual animal data records, and pathology tables were evaluated by a quality assessment (QA) pathologist at a pathology laboratory independent of the study laboratory. The individual animal records and tables were compared for accuracy, the slide and tissue counts were verified, and the histotechnique was evaluated. For the 2-year studies, a QA pathologist evaluated slides from all tumors and all potential target organs, which included the liver of rats and mice; the testis, epididymis, prostate gland, seminal vesicles, and pituitary gland of male rats and male mice; the kidney of male and female mice; the exocrine pancreas of rats; and the Harderian gland of female rats.

The QA report and the reviewed slides were submitted to the NTP Pathology Working Group (PWG) coordinator, who reviewed the selected tissues and addressed any inconsistencies in the diagnoses made by the laboratory and QA pathologists. Representative histopathology slides containing examples of lesions related to chemical administration, examples of disagreements in diagnoses between the laboratory and QA pathologists, or lesions of general interest were presented by the coordinator to the PWG for review. The PWG consisted of the QA pathologist and other pathologists experienced in rodent toxicological pathology. This group examined the tissues without any knowledge of exposure groups. When the PWG consensus differed from that of the laboratory pathologist, the diagnosis was changed. Final diagnoses for reviewed lesions represent a consensus between the laboratory pathologist, reviewing pathologist(s), and the PWG. Details of these review procedures have been described, in part, by Maronpot and Boorman[114] and Boorman et al.[115] For subsequent analyses of the pathology data, the decision to evaluate the diagnosed lesions for each tissue type separately or combined was generally based on the guidelines of McConnell et al.[116]

Table 1. Experimental Design and Materials and Methods in the Perinatal and Two-year Feed Studies of Di-*n*-butyl Phthalate

Rats	Mice
Study Laboratory	
Battelle (Columbus, OH)	Same as rat study
Strain and Species	
Sprague Dawley (Hsd:Sprague Dawley® SD®)	B6C3F1/N
Animal Source	
Envigo (formerly Harlan Laboratories, Inc., Indianapolis, IN)	Taconic Biosciences, Inc. (Germantown, NY)
Time Held Before Studies	
F_0 females: 11 days	11 (females) or 12 (males) days
Average Age When Studies Began	
F_0 females: 11 to 14 weeks	5 to 6 weeks
Date of First Exposure	
F_0 females: July 23, 2010	August 16 (females) or 17 (males), 2010
F_1: August 30, 2010 (males and females)	
Duration of Exposure	
F_0 females: GD 6 to LD 21	2 years
F_1: Perinatal plus 2 years	
Date of Last Exposure	
F_0 females: August 30, 2010	August 15 (females) or 17 (males), 2012
F_1: August 29 (males) or 31 (females), 2012	
Necropsy Dates	
F_1: August 27–29 (males) or 29–31 (females), 2012	August 13 to 15 (females) or 15 to 17 (males), 2012
Average Age at Necropsy	
F_1: 2 years	2 years
Size of Study Groups	
F_0 females: 45 (0, 300, 1,000, 3,000 ppm) or 47 (10,000 ppm)	50/sex
F_1 (2-year study): 50/sex	
Method of Distribution	
Animals were distributed randomly into groups of approximately equal initial mean body weights	Same as rat study
Animals per Cage	
F_0 females: 1 (with litter)	Males: 1
F_1: 2 (males) or 4 (females)	Females: Up to 4

Rats	Mice
Method of Animal Identification	
F_0 female: Cage card and tail marking with permanent pen	Cage card and tail tattoo
F_1 (pups): Limb tattoo	
F_1 (2-year study): Cage card and tail tattoo	
Diet	
Irradiated NIH-07 meal feed (perinatal phase) or irradiated NTP-2000 meal feed (2-year study) (Zeigler Brothers, Inc., Gardners, PA), available ad libitum, changed twice weekly	Irradiated NTP-2000 meal feed (Zeigler Brothers, Inc., Gardners, PA), available ad libitum, changed twice weekly
Water	
Tap water (Columbus, OH municipal supply) via automatic watering system (Edstrom Industries, Inc., Waterford, WI), available ad libitum	Same as rat study
Cages	
Solid polycarbonate (Lab Products, Inc., Seaford, DE), changed weekly through PND 4, then twice weekly, rotated every 2 weeks	Solid polycarbonate (Lab Products, Inc., Seaford, DE), changed weekly (males) or twice weekly (females), rotated every 2 weeks
Bedding	
Irradiated Sani-Chips® (P.J. Murphy Forest Products Corporation, Montville, NJ), changed with cage changes	Same as rat study
Rack Filters	
Spun-bonded polyester (Snow Filtration Company, Cincinnati, OH or National Filter Media Corporation, Olive Branch, MS), changed every 2 weeks	Spun-bonded polyester (Snow Filtration Company, Cincinnati, OH), changed every 2 weeks
Racks	
Stainless steel (Lab Products, Inc., Seaford, DE), changed and rotated every 2 weeks	Same as rat study
Animal Room Environment	
Temperature: 72°F ± 3°F Relative humidity: 50% ± 15% Room fluorescent light: 12 hours/day Room air changes: 10/hour	Same as rat study
Exposure Concentrations	
0, 300, 1,000, 3,000, or 10,000 ppm in feed	0, 1,000, 3,000, or 10,000 ppm in feed
Type and Frequency of Observation	
F_0 females: Observed twice daily. Weighed on GDs 5, 6, 9, 12, 15, 18, and 21 and on LDs 1, 4, 7, 10, 14, and 21. Feed consumption was measured continuously over 3-day intervals from GD 6 to LD 21.	Observed twice daily. Weighed initially, weekly for the first 13 weeks, every 4 weeks thereafter, and at study termination. Clinical observations were recorded every 4 weeks beginning at week 6 and at study termination. Feed consumption was measured weekly for the first 3 months and then at 4-week intervals thereafter.

Rats	Mice
F_1: Observed twice daily. Litter data (litter count by sex, litter weights by sex, and litter observations) were recorded on PND 1. Pups per litter were recorded on PNDs 2 and 3. Pups were weighed on PNDs 4, 7, 14, and 21, weekly for the first 13 weeks, every 4 weeks thereafter, and at study termination. Clinical observations were recorded every 4 weeks beginning on day 29 and at study termination. Feed consumption was recorded weekly for 3 months, and then at 4-week intervals thereafter.	
Method of Euthanasia	
Carbon dioxide	Same as rat study
Necropsy	
Necropsies were performed on all F_1 rats.	Necropsies were performed on all mice.
Histopathology	
Complete histopathology was performed on all F_1 rats. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, brain, clitoral gland, esophagus, eyes, femur, Harderian gland, heart and aorta, large intestine (cecum, colon, and rectum), small intestine (duodenum, jejunum, and ileum), kidneys, liver, lung, lymph nodes (mandibular and mesenteric), mammary gland, nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, seminal vesicle, spleen, stomach (forestomach and glandular), testis with epididymis, thymus, thyroid gland, trachea, urinary bladder, and uterus.	Complete histopathology was performed on all mice. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, brain, clitoral gland, esophagus, eyes, femur, gallbladder, Harderian gland, heart and aorta, large intestine (cecum, colon, and rectum), small intestine (duodenum, jejunum, and ileum), kidneys, liver, lung, lymph nodes (mandibular and mesenteric), mammary gland, nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, seminal vesicle, spinal cord, spleen, stomach (forestomach and glandular), testis with epididymis, thymus, thyroid gland, trachea, urinary bladder, uterus.
Internal Dose Assessment	
Maternal plasma, amniotic fluid (pooled by litter), and fetuses (pooled by litter) (n = 5) were collected at GD 18, and maternal plasma (n = 5) and whole pup (n = 2/sex) were collected at PND 4 to determine mono-*n*-butyl phthalate concentration to estimate gestational and lactational transfer, respectively.	None

1 PND = postnatal day; GD = gestation day. LD = lactation day.

Statistical Methods

Survival Analyses

The probability of survival was estimated by the product-limit procedure of Kaplan and Meier[117] and is presented graphically. Animals surviving to the end of the observation period are treated as censored observations, as are animals dying from unnatural causes within the observation period. Animals dying from natural causes are included in analyses and are treated as uncensored observations. For the 2-year mouse study, exposure concentration-related trends are identified with Tarone's life table test,[118] and pairwise exposure concentration-related effects are assessed using Cox's method.[119] For the rat perinatal study, exposure concentration-related trends and pairwise exposure concentration-related effects on survival are assessed using a Cox proportional hazards model[119] with a random litter effect. All reported p values for the survival analyses are two-sided.

Calculation of Incidence

The incidence of neoplasms or nonneoplastic lesions is presented as the numbers of animals bearing such lesions at a specific anatomic site. For calculation of incidence rates, the denominator for most neoplasms and all nonneoplastic lesions is the number of animals where the site was examined microscopically. When macroscopic examination was required to detect neoplasms in certain tissues (e.g., mesentery, pleura, peripheral nerve, skeletal muscle, tongue, tooth, Zymbal's gland) before microscopic evaluation, however, the denominator consists of the number of animals that had a gross abnormality. When neoplasms had multiple potential sites of occurrence (e.g., leukemia or lymphoma), the denominator consists of the number of animals on which a necropsy was performed. Additional study data also give the survival-adjusted neoplasm rate for each group and each site-specific neoplasm. This survival-adjusted rate (based on the Poly-3 method described below) accounts for differential mortality by assigning a reduced risk of neoplasm, proportional to the third power of the fraction of time on study, only to site-specific, lesion-free animals that do not reach terminal euthanasia.

Analysis of Neoplasm and Nonneoplastic Lesion Incidence

Statistical analyses of neoplasm and nonneoplastic lesion incidence considered two features of the data. Some animals did not survive the entire 2 years of the study, so survival differences between groups had to be considered. In addition, up to two animals per sex were randomly selected from each litter to participate in the study. The statistical analysis of lesion incidence used the Poly-3 test to account for survival differences, with a Rao-Scott adjustment for litter effects, as described below.

The Poly-k test[120-122] was used to assess neoplasm and nonneoplastic lesion prevalence. This test is a survival-adjusted quantal-response procedure that modifies the Cochran-Armitage linear trend test to account for survival differences. More specifically, this method modifies the denominator in the quantal estimate of lesion incidence to approximate more closely the total number of animal years at risk. For analysis of a given site, each animal is assigned a risk weight. This value is 1 if the animal had a lesion at that site or if it survived until terminal euthanasia; if the animal died before terminal euthanasia and did not have a lesion at that site, its risk weight is the fraction of the entire study time that it survived, raised to the kth power.

This method yields a lesion prevalence rate that depends only on the choice of a shape parameter for a Weibull hazard function describing cumulative lesion incidence over time.[120] Unless otherwise specified, a value of k = 3 was used in the analysis of site-specific lesions. This value was recommended by Bailer and Portier[120] after an evaluation of neoplasm onset time distributions for a variety of site-specific neoplasms in control Fischer 344 rats and B6C3F1 mice.[123] Bailer and Portier[120] showed that the Poly-3 test gave valid results if the true value of k was within the range of 1 to 5. A further advantage of the Poly-3 method is that it does not require lesion lethality assumptions. Variation introduced by the use of risk weights, which reflect differential mortality, was accommodated by adjusting the variance of the Poly-3 statistic as recommended by Bieler and Williams.[124] Poly-3 tests used the continuity correction described by Nam.[125]

Littermates tend to be more like each other than like fetuses or pups in other litters. Failure to account for correlation within litters leads to underestimates of variance in statistical tests, resulting in higher probabilities of Type I errors ("false positives"). Because up to two pups per sex per litter were present in the core rat study, the Poly-3 test was modified to accommodate litter effects using the Rao-Scott approach.[126] The Rao-Scott approach accounts for litter effects by estimating the ratio of the variance in the presence of litter effects to the variance in the absence of litter effects. This ratio is then used to adjust the sample size downward to yield the estimated variance in the presence of litter effects. The Rao-Scott approach was implemented in the Poly-3 test as recommended by the Fung et al.[127] formula $\bar{\tau}_{RS2}$.

Tests of significance included pairwise comparisons of each exposed group with control groups and a test for an overall exposure concentration-related trend. Continuity-corrected Rao-Scott-adjusted Poly-3 tests were used in the analysis of lesion incidence and reported p values are one-sided. The significance of a lower incidence or negative trend in lesions is approximated as 1−p with the letter N added (e.g., p = 0.99 is presented as p = 0.01N). For neoplasms and nonneoplastic lesions observed without litter structure (e.g., at the interim evaluation), Poly-3 tests that included the continuity correction, but without adjustment for potential litter effects, were used for trend and pairwise comparisons to the control group.

To evaluate incidence rates by litter, the proportions of litters affected by each lesion type were tested among groups. Cochran-Armitage trend tests and Fisher's exact tests[128] were used to test for trends and pairwise differences from the control group, respectively.

Analysis of Continuous Variables

Before statistical analysis, extreme values identified by the outlier test of Dixon and Massey[129] for small samples (n < 20) and Tukey's outer fences method[130] for large samples (n ≥ 20) were examined by NTP personnel, and implausible values were eliminated from the analysis. Body weight measurements, which historically have approximately normal distributions, were analyzed with the parametric multiple comparison procedures of Dunnett[131] and Williams.[132; 133] Dam gestational and lactational feed consumption, litter sizes, pup survival, implantations, number of resorptions, and proportions of male pups per litter for all studies were analyzed using the nonparametric multiple comparison methods of Shirley,[134] as modified by Williams,[135] and Dunn,[136] given that these endpoints typically have skewed distributions. For all quantitative endpoints unaffected by litter structure, the Jonckheere test[137] was used to assess the significance of the exposure concentration-related trends and to determine at the 0.01 level of significance

whether a trend-sensitive test (the Williams or Shirley test) was more appropriate for pairwise comparisons than a test that does not assume a monotonic exposure concentration-related trend (the Dunnett or Dunn test).

Postweaning body weights were measured on two pups per sex per litter in the 2-year study; more than two pups per sex per litter were possible in preweaning body weight measurements. The analyses of pup mean body weights and mean body weights adjusted for litter size (described below) of these animals took litter effects into account using a mixed model with litter as a random effect. To adjust for multiple comparisons, a Dunnett-Hsu adjustment was used.[138] Dam mean body weights during gestation and lactation were analyzed with the parametric multiple comparison procedures of Dunnett[131] and Williams,[132; 133] depending on whether the Jonckheere test indicated the use of a trend-sensitive test. P values for these analyses are two-sided.

Analysis of Gestational and Fertility Indices

Cochran-Armitage trend tests were used to test the significance of trends in gestational and fertility indices across exposure groups. Fisher's exact test was used to conduct pairwise comparisons of each exposed group with the control group. P values for these analyses are two-sided.

Body Weight Adjustments

Preweaning pup body weights were adjusted for live litter size as follows: A linear model was fit to body weights as a function of exposure concentration and litter size. The estimated coefficient of litter size was then used to adjust each pup body weight based on the difference between its litter size and the mean litter size. Prestandardization PND 4 body weights were adjusted for PND 1 litter size, and body weights measured between PND 4 poststandardization and PND 21 were adjusted for PND 4 poststandardization litter size. After adjustment, mean body weights were analyzed with a linear mixed model with a random litter effect.

Historical Control Data

The concurrent control group is the most valid comparison to the exposed groups and is the only control group analyzed statistically in NTP bioassays. Historical control data are often helpful in interpreting potential exposure-related effects, however, particularly for uncommon or rare neoplasm types. For meaningful comparisons, the conditions for studies in the historical control data must be generally similar. Significant factors affecting the background incidence of neoplasms at a variety of sites are diet, sex, strain/stock, and route of exposure. The NTP historical control database contains all 2-year studies for each species, sex, and strain/stock with histopathological findings in control animals completed within the most recent 5-year period,[139-141] including the concurrent control for comparison across multiple technical reports. In general, the historical control data for a given study includes studies using the same route of administration, and the overall incidence of neoplasms in control groups for all routes of administration are included for comparison, including the current study.

Quality Assurance Methods

The 2-year studies were conducted in compliance with U.S. Food and Drug Administration Good Laboratory Practice Regulations.[142] In addition, the 2-year study reports were audited retrospectively by an independent QA contractor against study records submitted to the NTP Archives. Separate audits covered completeness and accuracy of the pathology data, pathology specimens, final pathology tables, and a draft of this NTP Technical Report. Audit procedures and findings are presented in the reports and are on file at NIEHS. The audit findings were reviewed and assessed by NTP staff, and all comments were resolved or otherwise addressed during the preparation of this Technical Report.

Results

Data Availability

The National Toxicology Program (NTP) evaluated all study data. Data relevant for evaluating toxicological findings are presented here. All study data are available in the NTP Chemical Effects in Biological Systems (CEBS) database: https://doi.org/10.22427/NTP-DATA-TR-600.[143]

Rats

Two-year Study (Perinatal Phase)

No effects related to di-*n*-butyl phthalate (DBP) exposure were observed on the pregnancy status, maternal survival, gestation length, or number of dams that littered (Table 2).

Table 2. Summary of the Disposition of F_0 Female Rats during Perinatal Exposure in the Perinatal and Two-year Feed Study of Di-*n*-butyl Phthalate

Reproductive Performance	0 ppm	300 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Time-mated Females (GD 6)	45	45	45	45	47[a]
Females Pregnant (%)[b]	40 (88.9)	34 (75.6)	39 (86.7)	38 (84.4)	37 (78.7)
Females Not Pregnant (%)	5 (11.1)	11 (24.4)	6 (13.3)	7 (15.6)	10 (21.3)
Dams Not Delivering with Evidence of Pregnancy (%)	5 (12.5)	7 (20.6)	6 (15.4)	3 (7.9)	5 (13.5)
Dams with Litters on LD 0 (%)	35 (87.5)	27 (79.4)	33 (84.6)	35 (92.1)	32 (86.5)
Gestation Length (Days)[c,d]	22.2 ± 0.1	22.1 ± 0.1	22.0 ± 0.0	22.2 ± 0.1	22.1 ± 0.1
Litters Poststandardization (LD 4)[e]	28	25	27	30	27
Weaned Males/Females	112/112	97/102	104/111	120/120	104/111

GD = gestation day; LD = lactation day.
[a]Two additional time-mated animals were received for the study and were placed in the 10,000 ppm group.
[b]Statistical analysis performed by the Cochran-Armitage (trend) and Fisher's exact (pairwise) tests.
[c]Statistical analysis performed by the Jonckheere (trend) and Shirley or Dunn (pairwise) tests.
[d]Gestation length calculated for sperm-positive females that delivered a litter. Data are presented as mean ± standard error.
[e]Standardization to eight pups per litter (four pups/sex) when possible.

Maternal mean body weights and body weight gains of exposed dams during gestation were comparable to those of the control group (Table 3). During lactation, minimal differences in mean body weights were noted (<6%) between the highest exposure group (10,000 ppm) and control group, with sporadic differences observed in body weight gains across all exposure groups.

Table 3. Summary of Mean Body Weights and Body Weight Gains of F_0 Female Rats during Gestation and Lactation in the Perinatal and Two-year Feed Study of Di-*n*-butyl Phthalate

Parameter[a,b]	0 ppm	300 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Gestation Day					
6	230.1 ± 2.6 (40)	231.7 ± 2.0 (34)	231.4 ± 1.8 (39)	232.5 ± 1.7 (38)	226.8 ± 3.1 (37)
9	240.1 ± 3.2* (40)	245.0 ± 2.2 (34)	244.6 ± 1.6 (39)	242.8 ± 1.7 (38)	236.2 ± 2.4 (37)
12	259.2 ± 2.3* (40)	262.1 ± 2.3 (34)	261.5 ± 1.7 (39)	261.0 ± 1.4 (38)	254.1 ± 2.2 (37)
15	278.0 ± 2.4 (40)	279.6 ± 3.4 (34)	281.9 ± 1.9 (39)	279.8 ± 2.0 (38)	275.1 ± 2.5 (37)
18	314.7 ± 2.7 (40)	318.6 ± 3.5 (34)	317.2 ± 2.7 (39)	320.3 ± 1.9 (38)	315.7 ± 3.4 (37)
21[c]	355.5 ± 5.0 (35)	366.6 ± 6.1 (28)	361.8 ± 4.7 (34)	366.2 ± 2.5 (34)	353.6 ± 4.9 (30)
Gestation Weight Change					
Gestation Day Interval					
6–9	10.0 ± 0.9** (40)	13.3 ± 1.0 (34)	13.2 ± 0.8 (39)	10.3 ± 0.8 (38)	9.4 ± 1.3 (37)
9–12	19.1 ± 1.4 (40)	17.1 ± 1.3 (34)	16.9 ± 0.6 (39)	18.2 ± 1.0 (38)	17.9 ± 1.0 (37)
12–15	18.7 ± 0.6* (40)	17.5 ± 2.0 (34)	20.4 ± 0.6 (39)	18.8 ± 1.4 (38)	21.0 ± 0.7 (37)
15–18	36.8 ± 1.1* (40)	39.0 ± 2.3 (34)	35.2 ± 1.4 (39)	40.5 ± 1.6 (38)	40.6 ± 1.3 (37)
18–21	42.2 ± 3.4** (35)	49.1 ± 2.7 (28)	45.3 ± 2.0 (34)	46.9 ± 1.2 (34)	39.7 ± 1.3 (30)
6–21	126.4 ± 4.6 (35)	136.3 ± 5.8 (28)	129.6 ± 4.4 (34)	133.7 ± 2.4 (34)	127.3 ± 3.7 (30)
Lactation Day					
1	271.5 ± 2.6 (35)	277.5 ± 3.3 (27)	274.4 ± 1.9 (33)	274.4 ± 1.6 (35)	270.7 ± 3.1 (32)
4	286.6 ± 2.5 (35)	291.6 ± 2.8 (27)	294.5 ± 2.2 (33)	293.7 ± 1.9 (35)	282.9 ± 2.9 (32)
7[d]	297.0 ± 2.6** (28)	299.6 ± 2.9 (25)	298.7 ± 2.1 (27)	294.9 ± 1.9 (30)	286.4 ± 2.9** (27)
10	304.4 ± 3.1** (28)	311.6 ± 2.7 (25)	307.4 ± 2.3 (27)	305.6 ± 2.0 (30)	291.8 ± 3.3** (27)
14	318.4 ± 3.0** (28)	322.1 ± 3.1 (25)	317.4 ± 2.3 (27)	313.0 ± 2.2 (30)	299.5 ± 2.5** (27)
21	299.0 ± 2.9* (28)	301.9 ± 2.9 (25)	301.5 ± 2.6 (27)	290.7 ± 4.3 (30)	290.8 ± 3.1 (27)
Lactation Weight Change					
Lactation Day Interval					
1–4	15.1 ± 1.3 (35)	14.1 ± 1.4 (27)	20.1 ± 1.4* (33)	19.3 ± 1.1 (35)	12.2 ± 1.2 (32)
4–7	11.0 ± 1.3** (28)	7.4 ± 1.1* (25)	3.4 ± 1.3** (27)	2.2 ± 1.1 (30)**	3.0 ± 1.2** (27)
7–10	7.4 ± 1.3 (28)	12.0 ± 1.0 (25)	8.7 ± 1.3 (27)	10.7 ± 1.4 (30)	5.4 ± 1.4 (27)
10–14	14.0 ± 1.9** (28)	10.5 ± 1.4 (25)	10.0 ± 1.5 (27)	7.4 ± 1.3 (30)**	7.7 ± 1.7** (27)
14–21	−19.4 ± 1.9** (28)	−20.2 ± 1.9 (25)	−15.9 ± 2.0 (27)	−22.3 ± 3.8 (30)	−8.7 ± 1.5** (27)
1–21	26.8 ± 2.2 (28)	23.2 ± 1.8 (25)	26.7 ± 2.1 (27)	17.0 ± 4.5 (30)	19.6 ± 2.4 (27)

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group.
Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
[a]Data are presented as mean ± standard error (number of dams). Body weight data are presented in grams.
[b]Each exposure group was compared to the vehicle control group with the Williams test when a trend was present ($p \leq 0.01$ from the Jonckheere trend test) or with the Dunnett test when no trend was present.
[c]Decreased number of dams at gestation day 21 reflects animals removed at gestation day 18 for internal dose assessment.
[d]Decreased number of dams at lactation day 7 reflects animal removal at postnatal day 4 for sample collection to determine gestational and lactational transfer.

Feed consumption during gestation was significantly higher (5–6%) only in the 10,000 ppm group compared to the control group during gestation day (GD) 12–15 and GD 15–18 intervals (Table 4). Feed consumption in all exposed groups, except the 10,000 ppm group, was significantly increased (1–9%) compared to the control group from lactation day (LD) 1 to 4 (Table 4). Feed consumption for LD 10–14 and LD 17–21 intervals, and overall during lactation in the 10,000 ppm group, was 4–9% less than in control animals. Chemical intake throughout gestation and lactation was generally proportional to the exposure concentration and was higher during lactation than gestation. Chemical intake from LD 14 to 21 was not calculated because the entire litter ate feed; therefore, an accurate assessment could not be made for F_0 or F_1 consumption.

Table 4. Summary of Feed and Di-*n*-butyl Phthalate Consumption by F_0 Female Rats during Gestation and Lactation in the Perinatal and Two-year Feed Study

Parameter[a]	0 ppm	300 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Gestation Day Interval[b,c]					
6–9	16.1 ± 0.6 (40)	17.6 ± 0.3 (34)	17.6 ± 0.3 (39)	17.2 ± 0.3 (38)	18.0 ± 0.7 (36)
9–12	18.9 ± 0.3 (40)	19.7 ± 0.3 (34)	19.7 ± 0.3 (39)	19.5 ± 0.3 (37)	19.1 ± 0.3 (34)
12–15	18.6 ± 0.2** (40)	18.5 ± 0.4 (34)	19.1 ± 0.2 (39)	18.7 ± 0.3 (38)	19.6 ± 0.3** (37)
15–18	20.8 ± 0.2* (40)	21.7 ± 0.3 (34)	21.0 ± 0.3 (39)	21.4 ± 0.3 (38)	22.1 ± 0.4* (37)
18–21	22.1 ± 0.5 (35)	22.9 ± 0.4 (29)	22.6 ± 0.3 (34)	22.5 ± 0.3 (34)	23.0 ± 0.5 (30)
6–21	19.3 ± 0.2 (35)	20.1 ± 0.3 (29)	20.0 ± 0.2 (34)	19.9 ± 0.2 (33)	20.0 ± 0.3 (28)
Lactation Day Interval[b,c]					
1–4	33.24 ± 0.56 (35)	36.30 ± 0.96* (27)	36.20 ± 0.76** (33)	36.39 ± 0.59** (35)	33.50 ± 0.66 (32)
4–7	39.89 ± 0.50* (28)	40.36 ± 0.69 (25)	40.41 ± 0.44 (27)	40.12 ± 0.52 (30)	38.06 ± 0.45 (27)
7–10	47.45 ± 0.92 (28)	49.54 ± 0.78 (25)	49.55 ± 0.55 (27)	49.89 ± 0.56 (30)	46.44 ± 0.59 (27)
10–14	56.81 ± 0.77** (27)	58.55 ± 0.87 (25)	57.12 ± 0.61 (27)	56.10 ± 0.71 (30)	53.12 ± 0.58** (27)
14–17	58.00 ± 0.88 (28)	58.14 ± 0.83 (25)	57.53 ± 0.79 (27)	58.64 ± 0.91 (30)	57.83 ± 0.73 (27)
17–21	72.03 ± 1.29** (28)	72.80 ± 1.14 (25)	72.31 ± 1.27 (27)	66.38 ± 1.74* (30)	65.58 ± 1.08** (27)
1–14	45.45 ± 0.64** (27)	47.21 ± 0.61 (25)	46.87 ± 0.49 (27)	46.38 ± 0.51 (30)	43.63 ± 0.43* (27)
Chemical Intake (mg/kg/day)[d,e]					
GD 6–21	0.00 ± 0.00 (35)	21.62 ± 0.18 (29)	71.60 ± 0.64 (34)	213.5 ± 2.14 (33)	739.9 ± 12.57 (28)
LD 1–14	0.00 ± 0.00 (27)	46.61 ± 0.45 (25)	155.4 ± 1.36 (27)	465.6 ± 4.84 (30)	1,514 ± 15.81 (27)

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group.
Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
GD = gestation day; LD = lactation day.
[a]Data are presented as mean ± standard error (number of dams).
[b]Feed consumption data are presented as grams/animal/day.
[c]Each exposure group was compared to the vehicle control group with the Shirley test when a trend was present ($p \leq 0.01$ from the Jonckheere trend test) or with the Dunn test when no trend was present.
[d]Chemical intake calculated as: ([exposure concentration × feed consumption]/[average body weight of day range]).
[e]No statistical analysis performed on the chemical intake data.

In the lowest exposure group (300 ppm), total and live litter size were significantly increased (12%) relative to the control group on postnatal day (PND) 1 and PND 4 prestandardization (Table 5). On PND 21, all groups had similar live litter sizes. No significant effects on live litter size occurred in other exposed groups at any time point. No differences in sex ratio or survival during lactation were found.

Table 5. Summary of Mean Litter Size and Survival Ratio of F_1 Male and Female Rats during Lactation in the Perinatal and Two-year Feed Study of Di-*n*-butyl Phthalate

Parameter[a]	0 ppm	300 ppm	1,000 ppm	3,000 ppm	10,000 ppm
PND 1[b,c]					
Total	11.49 ± 0.35 (35)	12.85 ± 0.39* (26)	12.21 ± 0.34 (33)	12.30 ± 0.25 (33)	12.09 ± 0.44 (32)
Live	11.37 ± 0.34 (35)	12.77 ± 0.39* (26)	12.18 ± 0.34 (33)	12.12 ± 0.26 (33)	11.91 ± 0.46 (32)
% Male per Litter	50.4 ± 3.1 (31)	47.7 ± 3.2 (23)	46.0 ± 3.1 (32)	48.1 ± 1.7 (32)	46.1 ± 2.5 (20)
% Male[d,e]	50.7 (355)	47.4 (293)	46.5 (389)	48.2 (388)	46.1 (243)
Male[b,c]					
PND 1	5.81 ± 0.40 (31)	6.04 ± 0.46 (23)	5.66 ± 0.42 (32)	5.84 ± 0.26 (32)	5.60 ± 0.48 (20)
PND 4 Prestandardization	5.77 ± 0.40 (31)	6.04 ± 0.46 (23)	5.66 ± 0.42 (32)	5.84 ± 0.26 (32)	5.60 ± 0.48 (20)
PND 4 Poststandardization	4.00 ± 0.10 (28)	3.88 ± 0.09 (25)	3.89 ± 0.15 (27)	4.00 ± 0.00 (30)	3.85 ± 0.12 (27)
PND 21	4.00 ± 0.10 (28)	3.88 ± 0.09 (25)	3.85 ± 0.16 (27)	4.00 ± 0.00 (30)	3.85 ± 0.12 (27)
Female[b,c]					
PND 1	5.65 ± 0.37 (31)	6.70 ± 0.46 (23)	6.50 ± 0.38 (32)	6.28 ± 0.25 (32)	6.55 ± 0.51 (20)
PND 4 Prestandardization	5.65 ± 0.37 (31)	6.70 ± 0.46 (23)	6.47 ± 0.38 (32)	6.28 ± 0.25 (32)	6.55 ± 0.51 (20)
PND 4 Poststandardization	4.00 ± 0.10 (28)	4.12 ± 0.09 (25)	4.11 ± 0.15 (27)	4.00 ± 0.00 (30)	4.15 ± 0.12 (27)
PND 21	4.00 ± 0.10 (28)	4.08 ± 0.10 (25)	4.11 ± 0.15 (27)	4.00 ± 0.00 (30)	4.11 ± 0.10 (27)
Male and Female[b,c]					
PND 4 Prestandardization	11.34 ± 0.34 (35)	12.77 ± 0.39* (26)	12.15 ± 0.35 (33)	12.12 ± 0.26 (33)	11.88 ± 0.46 (32)
PND 4 Poststandardization	8.00 ± 0.00 (28)	8.00 ± 0.00 (25)	8.00 ± 0.00 (27)	8.00 ± 0.00 (30)	8.00 ± 0.00 (27)
PND 21	8.00 ± 0.00 (28)	7.96 ± 0.04 (25)	7.96 ± 0.04 (27)	8.00 ± 0.00 (30)	7.96 ± 0.04 (27)

Parameter[a]	0 ppm	300 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Survival per Litter					
Total Dead: PND 1–4[e,f]	5 (35)	2 (26)	2 (33)	6 (33)	7 (32)
Total Dead: PND 4–21[e,f]	0 (28)	1 (25)	1 (27)	0 (30)	1 (27)
Dead: PND 1–4[b,c,g]	0.14 ± 0.07 (35)	0.08 ± 0.05 (26)	0.06 ± 0.04 (33)	0.18 ± 0.11 (33)	0.22 ± 0.10 (32)
Dead: PND 4–21[b,c,g]	0.00 ± 0.00 (28)	0.04 ± 0.04 (25)	0.04 ± 0.04 (27)	0.00 ± 0.00 (30)	0.04 ± 0.04 (27)
Survival Ratio: PND 1–4[b,c,h]	0.998 ± 0.002 (35)	1.000 ± 0.000 (26)	0.997 ± 0.003 (33)	1.000 ± 0.000 (33)	0.997 ± 0.003 (32)
Survival Ratio: PND 4–21[b,c,i]	1.000 ± 0.000 (28)	0.995 ± 0.005 (25)	0.995 ± 0.005 (27)	1.000 ± 0.000 (30)	0.995 ± 0.005 (27)

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group.
*Statistically significant at $p \leq 0.05$.
PND = postnatal day.
[a]Litters in which the male/female pup counts were inconsistent between PND 1 and PND 4 were excluded from the male- or female-specific endpoints.
[b]Each exposure group was compared to the vehicle control group with the Shirley test when a trend was present ($p \leq 0.01$ from the Jonckheere trend test) or with the Dunn test when no trend was present.
[c]Data are presented as mean ± standard error (number of dams).
[d][100 × (number of live males in dietary exposure group)/(number of live males and females in dietary exposure group)] (number of pups).
[e]No statistics performed on this endpoint.
[f]Total number of dead pups in exposure group (number of dams).
[g]Number dead per litter.
[h]Survival per litter: Number of pups prestandardization on PND 4/number of live pups on PND 1.
[i] Survival per litter: Number of live pups on PND 21/number of live pups poststandardization on PND 4.

Male and female pup mean body weight on PND 1 was ≤4% in the 10,000 ppm group compared to the control group (Table 6). By the end of the lactation period (PND 21), significant decreases in male and female pup weight occurred only in the 10,000 ppm groups, approximately 12% and 13% lower than their respective control groups (Table 6).

Table 6. Summary of Preweaning F_1 Male and Female Rat Pup Mean Body Weights Following Perinatal Exposure to Di-*n*-butyl Phthalate

Parameter[a]	0 ppm	300 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Male (g)					
PND 1[b,c,d]	7.28 ± 0.12 (31)	7.38 ± 0.11 (23)	7.33 ± 0.09 (32)	7.48 ± 0.08 (32)	7.00 ± 0.13 (20)
PND 4[e,f,g,h]	10.57 ± 0.17** (202/35)	10.90 ± 0.14 (159/26)	10.86 ± 0.14 (185/33)	10.89 ± 0.12 (193/33)	10.16 ± 0.13 (178/32)
PND 7[e,i]	16.32 ± 0.29 (112/28)	16.29 ± 0.27 (97/25)	16.61 ± 0.29 (105/27)	17.04 ± 0.22 (120/30)	16.76 ± 1.07 (104/27)
PND 14[e,i]	33.35 ± 0.50** (112/28)	34.50 ± 0.36 (97/25)	34.28 ± 0.48 (105/27)	34.39 ± 0.38 (119/30)	31.56 ± 0.39* (104/27)
PND 21[e,i]	54.15 ± 0.86** (112/28)	55.77 ± 0.67 (97/25)	55.34 ± 0.87 (104/27)	53.27 ± 0.72 (120/30)	47.85 ± 0.61** (104/27)
Female (g)					
PND 1[b,c,d]	6.96 ± 0.13 (31)	7.09 ± 0.15 (23)	6.96 ± 0.10 (32)	7.17 ± 0.08 (32)	6.77 ± 0.14 (20)
PND 4[e,f,g,h]	10.19 ± 0.16** (195/35)	10.39 ± 0.14 (173/26)	10.39 ± 0.15 (216/33)	10.40 ± 0.12 (207/33)	9.76 ± 0.11 (202/32)
PND 7[e,i]	15.93 ± 0.28* (112/28)	15.56 ± 0.27 (103/25)	15.82 ± 0.32 (111/27)	16.30 ± 0.24 (120/30)	15.05 ± 0.25 (111/27)
PND 14[e,i]	32.79 ± 0.54** (112/28)	33.39 ± 0.33 (102/25)	33.02 ± 0.49 (111/27)	33.10 ± 0.37 (120/30)	30.54 ± 0.35** (110/27)
PND 21[e,i]	52.65 ± 0.90** (112/28)	52.72 ± 0.54 (102/25)	52.16 ± 0.88 (111/27)	50.25 ± 0.79 (120/30)	45.88 ± 0.58** (111/27)
Male and Female (g)					
PND 1[b,c,d]	7.12 ± 0.10 (35)	7.15 ± 0.10 (26)	7.16 ± 0.08 (33)	7.34 ± 0.08 (33)	6.77 ± 0.09* (32)
PND 4[e,f,g,h]	10.40 ± 0.16** (397/35)	10.60 ± 0.12 (332/26)	10.64 ± 0.13 (401/33)	10.64 ± 0.11 (400/33)	9.96 ± 0.11 (380/32)
PND 7[e,i]	16.12 ± 0.28 (224/28)	15.91 ± 0.25 (200/25)	16.23 ± 0.28 (216/27)	16.67 ± 0.22 (240/30)	15.89 ± 0.55 (215/27)
PND 14[e,i]	33.05 ± 0.50** (224/28)	33.94 ± 0.32 (199/25)	33.68 ± 0.41 (216/27)	33.74 ± 0.35 (239/30)	31.03 ± 0.33** (214/27)
PND 21[e,i]	53.34 ± 0.82** (224/28)	54.22 ± 0.54 (199/25)	53.78 ± 0.72 (215/27)	51.76 ± 0.72 (240/30)	46.86 ± 0.55** (215/27)

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group.
Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
PND = postnatal day.
[a]Statistical analysis performed using mixed models with random litter effect for both trend and pairwise tests, using the Dunnett-Hsu adjustment for multiple comparisons.
[b]Data are presented as mean ± standard error (number of dams).
[c]Each exposure group was compared to the vehicle control group with the Williams test when a trend was present ($p \leq 0.01$ from the Jonckheere trend test) or with the Dunnett test when no trend was present.
[d]Total pup weight at PND 1 divided by number of live pups at PND 1.
[e]Data are presented as mean of litter means ± standard error (number of pups/number of dams).
[f]PND 4 prestandardization.
[g]Individual pup weights first adjusted for live litter size on PND 1.

[h]Litters in which the male/female pup counts were inconsistent between PND 1 and PND 4 were excluded from the male- or female-specific endpoints.
[i]Individual pup weights first adjusted for live litter size on PND 4 poststandardization.

Mono-*n*-butyl phthalate (MBP) concentrations were measured using validated analytical methods in samples taken on GD 18 and PND 4 (Table 7).[113] Low concentrations were detected in some matrices in control groups, likely due to a combination of background levels from sample collection and from the analytical assay. At GD 18, MBP concentrations increased more than proportionally to the exposure concentration, despite a linear increase in chemical consumption. There was an approximately 137-fold and 83-fold increase in dam plasma and fetus MBP concentrations, respectively, in the 10,000 ppm group compared to the 300 ppm group, but exposure concentration increased only approximately 33-fold. Concentrations in fetuses were approximately 17–29% that of dam plasma, demonstrating moderate gestational transfer of MBP (Table 7). MBP was also measured in amniotic fluid at concentrations lower than those found in fetuses (Table 7).

MBP concentrations in exposed dams on PND 4 were slightly higher than concentrations detected at GD 18 in respective exposure groups likely due to higher feed and, subsequently, chemical consumption (Table 7). Similar to what was found in dams on GD 18, MBP concentrations in dam plasma on PND 4 increased more than proportionally to the exposure concentration, despite a linear increase in chemical consumption, with an approximately 99-fold increase in concentration when exposure concentration increased approximately 33-fold. MBP concentrations in whole pups increased proportionally to the exposure concentration. Concentrations in whole pups were approximately 3–11% that of dams, demonstrating low lactational transfer of MBP in rats.

Table 7. Summary of Internal Dose Data for Rats in the Perinatal and Two-year Feed Study of Di-*n*-butyl Phthalate

	0 ppm	300 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Mono-*n*-butyl Phthalate Concentration[a,b]					
n	5	5	5	3[c]	5
Gestation Day 18					
Dam plasma (ng/mL)[d]	9.2 ± 4.0**	1,148.0 ± 48.3**	4,372.0 ± 567.1**	19,633.3 ± 3,077.5**	156,800.0 ± 3,878.1**
Amniotic fluid (ng/mL)	BD[e]	84.5 ± 13.7	403.8 ± 34.5	1,693.3 ± 153.4	22,760.0 ± 2,183.3
Fetuses (ng/g)[f]	BD	323.6 ± 17.8	1,270.6 ± 127.7	3,930.0 ± 705.0	26,840.0 ± 2,763.6
n	5	2[g]	5	5	5
Postnatal Day 4					
Dam plasma (ng/mL)	BD	1,625.0 ± 75.0	6,234.0 ± 897.2	25,580.0 ± 2,272.1	161,200.0 ± 17,690.1
Pups (ng/g)[h,i]	8.6 ± 1.3**	146.6 ± 103.7	350.8 ± 70.8**	2,743.2 ± 725.5**	4,721.5 ± 1,044.6**

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group.
Statistical significance for the vehicle control group indicates a significant trend test.
**Statistically significant at $p \leq 0.01$.
BD = below detection; group did not have more than 20% of its values above the limit of detection (LOD).
[a]Data are presented as mean ± standard error except for pup concentrations, which are displayed as mean ± standard error of the litter means.
[b]Values below the LOD (6.9 ng/mL in plasma, 21.8 ng/mL in amniotic fluid, 9.4 ng/g in fetuses) were substituted with one-half the LOD value.
[c]Three of the five dams designated for biological sampling were determined not to be pregnant. Although the replacement dams for this group were pregnant, only three complete sample sets (dam plasma, amniotic fluid, fetuses) were able to be collected.
[d]Statistical analysis performed by the Jonckheere (trend) and the Shirley or Dunn (pairwise) tests.
[e]If 80% or more of the values in the vehicle control group were below the LOD, no mean or standard error were calculated and no statistical analysis was performed.
[f]All fetuses from the dam were used and weights recorded.
[g]Only one extra litter (beyond the 25 minimum used for the 2-year study) met the collection criteria for this exposure group. An additional litter consisting of two male pups and one female pup (that would have been standardized due to unacceptable sex split) was used for biological sample collection.
[h]Statistical analysis for pup concentrations was performed using a bootstrapped Jonckheere trend test, and pairwise comparisons used the Datta-Satten modified Wilcoxon test with a Hommel adjustment for multiple comparisons.
[i]The number of pups evaluated was 20 in all groups (2 male pups and 2 female pups per dam) except for the 300 ppm group in which only 7 pups were able to be evaluated. Data reported are for male and female pup data combined.

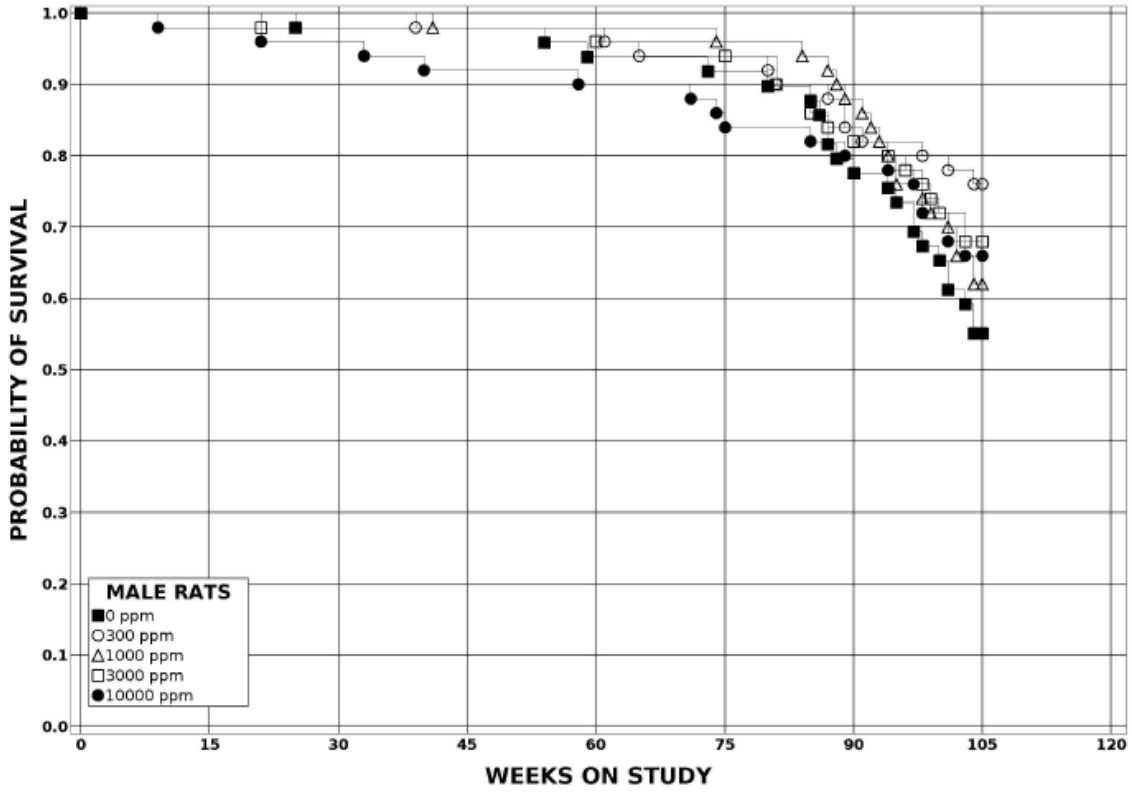
Two-year Study (Postweaning Phase)

Survival of all male and female exposure groups was similar to that of the control groups (Table 8; Figure 2). There were no exposure-related clinical observations (Appendix D).

Table 8. Summary of Survival of Male and Female Rats in the Perinatal and Two-year Feed Study of Di-*n*-butyl Phthalate

	0 ppm	300 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Male					
Animals Initially in Study	49[a]	50	50	50	50
Moribund	9	4	10	9	10
Natural Deaths	13	8	9	7	7
Animals Surviving to Study Termination	27[b]	38	31	34	33[b]
Percent Probability of Survival at End of Study[c]	55.1	76.0	62.0	68.0	66.0
Mean Survival (Days)[d]	674	691	694	685	655
Survival Analysis[e]	p = 0.948	p = 0.029N	p = 0.354N	p = 0.168N	p = 0.359N
Female					
Animals Initially in Study	50	50	50	50	50
Moribund	9	8	14	11	13
Natural Deaths	12	5	9	7	8
Animals Surviving to Study Termination	29[f]	37	27[b]	32	29
Percent Probability of Survival at End of Study	58.0	74.0	54.0	64.0	58.0
Mean Survival (Days)	669	679	645	671	648
Survival Analysis	p = 0.490	p = 0.137N	p = 0.540	p = 0.590N	p = 0.803

[a]One pup was missexed at the beginning of the study and was removed.
[b]Includes one animal that died naturally during the last week of the study.
[c]Kaplan-Meier determinations.
[d]Mean of litter means of all deaths (uncensored, censored, and study termination).
[e]The result of the Cox proportional hazards trend test is in the vehicle control group column, and the results of the Cox proportional hazards pairwise comparisons with the vehicle control group are in the exposed group columns. A negative trend or lower mortality in an exposure group is indicated by N.
[f]Includes one animal that was euthanized moribund during the last week of the study.



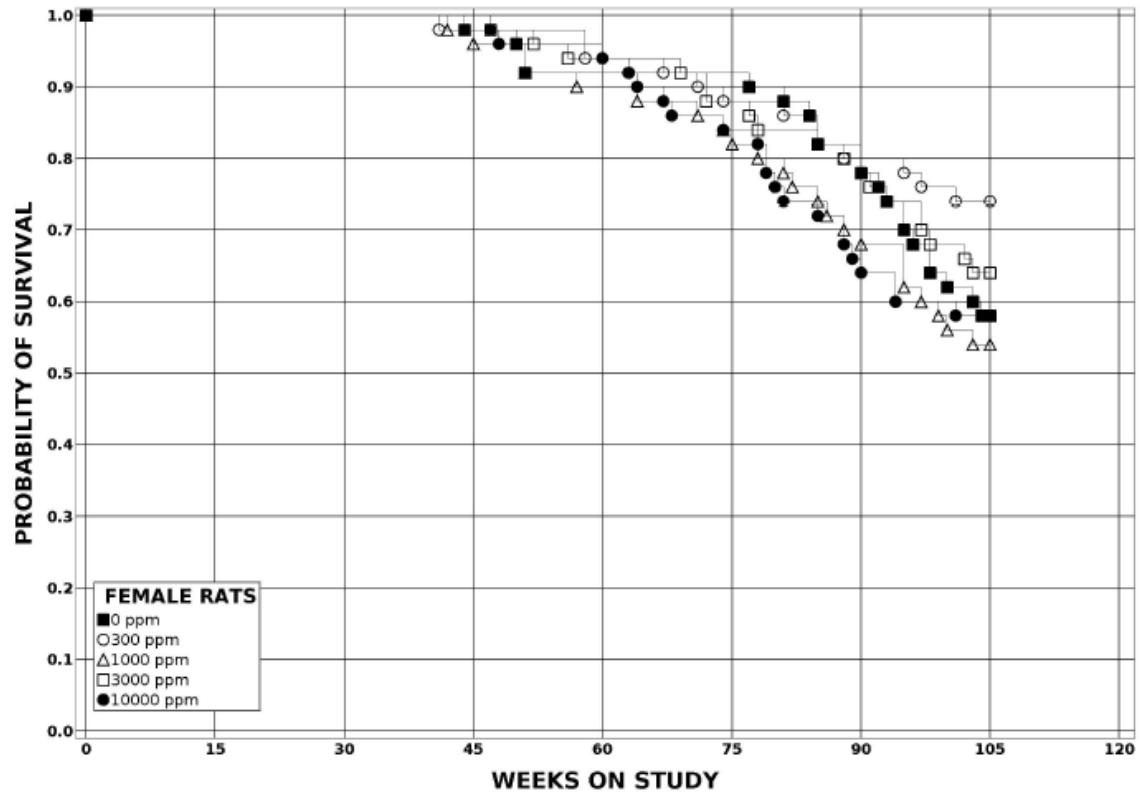


Figure 2. Kaplan-Meier Survival Curves for Rats Exposed to Di-*n*-butyl Phthalate in Feed for Two Years

Exposure-related significant decreases in group mean body weight were observed throughout the study in both males and females in the 10,000 ppm groups (Table 9, Table 10; Figure 3). At study termination, group mean body weights of the 10,000 ppm group were lower than those of the control groups by 3.5% in males and by 10.6% in females. Group mean feed consumption over the course of the study was similar across exposed and control groups, except that males in the 10,000 ppm group tended to consume less than control males (1–25% less) (Table 11, Table 12; Appendix D). Group mean chemical consumption increased proportionally to exposure concentration and was similar across male and female animals. Average daily chemical consumption (mg/kg/day) by the 300, 1,000, 3,000, and 10,000 ppm groups was 16.4, 53.6, 152.3, and 510.4 mg/kg/day, respectively, for males and 17.2, 57.4, 168.8, and 599.9 mg/kg/day, respectively, for females.

Table 9. Summary of Survival and Mean Body Weights of Male Rats in the Perinatal and Two-year Feed Study of Di-*n*-butyl Phthalate

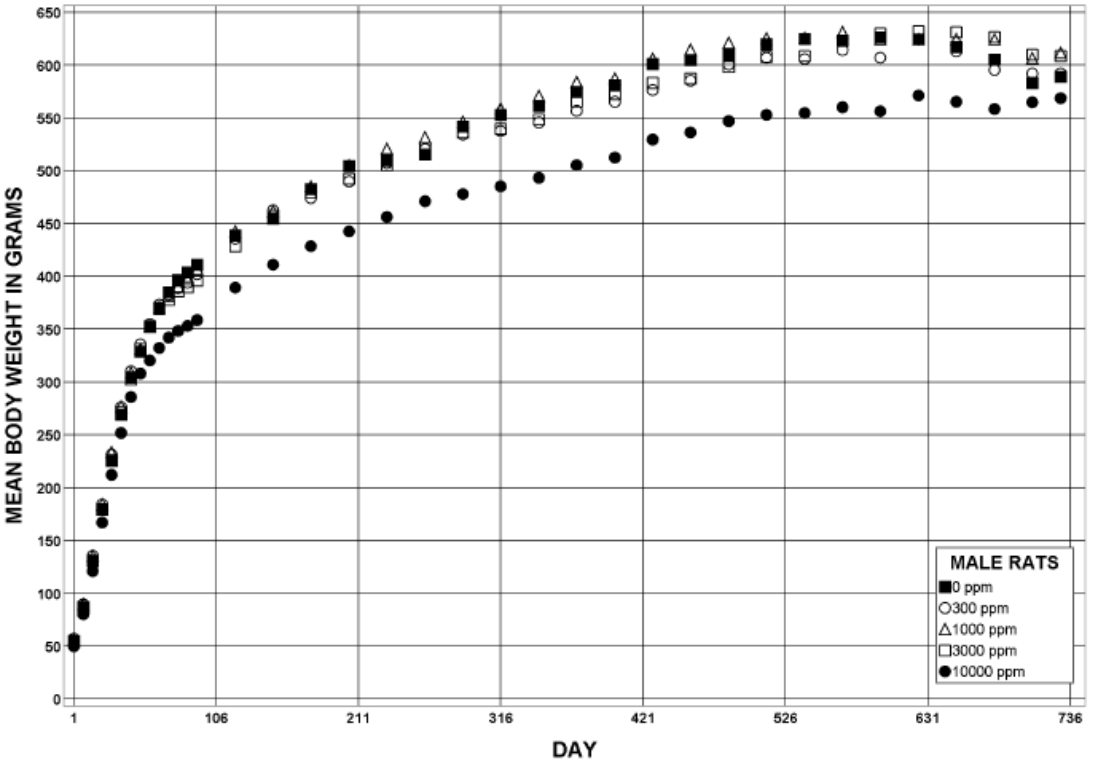
	0 ppm		300 ppm			1,000 ppm			3,000 ppm			10,000 ppm		
Study Day[a]	Av. Wt. (g)[b]	No. of Litters	Av. Wt. (g)	Wt. (% of Controls)	No. of Litters	Av. Wt. (g)	Wt. (% of Controls)	No. of Litters	Av. Wt. (g)	Wt. (% of Controls)	No. of Litters	Av. Wt. (g)	Wt. (% of Controls)	No. of Litters
1	55.1	25	57.3	104.0	25	56.7	103.0	25	53.7	97.4	25	50.0	90.7	25
8	87.0	25	89.5	102.9	25	90.1	103.6	25	86.6	99.6	25	80.0	91.9	25
15	130.4	25	135.5	103.9	25	135.5	103.9	25	131.2	100.6	25	120.9	92.7	25
22	179.0	25	184.1	102.6	25	184.3	102.8	25	179.9	100.3	25	166.9	93.1	25
29	226.3	25	231.3	102.2	25	233.5	103.2	25	225.5	99.6	25	212.0	93.7	25
36	269.4	25	276.2	102.5	25	276.8	102.7	25	271.7	100.8	25	252.0	93.6	25
43	304.5	25	310.3	101.9	25	309.0	101.5	25	302.5	99.3	25	285.8	93.9	25
50	328.9	25	335.6	102.0	25	331.3	100.7	25	331.5	100.8	25	308.0	93.7	25
57	352.6	25	354.7	100.6	25	353.2	100.2	25	352.5	100.0	25	320.3	90.8	25
64	370.4	25	373.1	100.7	25	371.2	100.2	25	369.4	99.7	25	332.2	89.7	25
71	385.0	25	382.5	99.3	25	381.4	99.1	25	378.0	98.2	25	342.1	88.9	25
78	396.8	25	388.9	98.0	25	390.8	98.5	25	385.8	97.2	25	348.5	87.8	25
85	404.0	25	394.1	97.5	25	397.3	98.3	25	389.8	96.5	25	353.0	87.4	25
92	411.1	25	402.1	97.8	25	407.0	99.0	25	396.2	96.4	25	358.7	87.3	25
120	438.9	25	435.8	99.3	25	442.5	100.8	25	428.5	97.6	25	389.2	88.7	25
148	454.8	25	462.8	101.8	25	461.7	101.5	25	458.1	100.7	25	411.1	90.4	25
176	482.5	25	474.2	98.3	25	485.2	100.6	25	479.4	99.4	25	428.6	88.8	25
204	504.4	25	490.0	97.1	25	505.2	100.2	25	492.4	97.6	25	442.4	87.7	25
232	509.7	25	507.2	99.5	25	521.0	102.2	25	505.0	99.1	25	456.2	89.5	25
260	515.6	25	520.5	100.9	25	531.5	103.1	25	521.6	101.2	25	471.2	91.4	25
288	541.9	25	534.0	98.5	25	546.1	100.8	25	536.3	99.0	25	477.9	88.2	25
316	552.7	25	537.6	97.3	25	559.0	101.1	25	539.5	97.6	25	485.0	87.7	25
344	561.4	25	545.8	97.2	25	571.0	101.7	25	548.8	97.8	25	493.1	87.8	25
372	574.7	25	556.6	96.9	25	583.8	101.6	25	565.5	98.4	25	505.1	87.9	25
400	580.8	25	565.4	97.4	25	587.4	101.1	25	572.6	98.6	25	512.3	88.2	25
428	601.1	25	576.2	95.9	25	606.0	100.8	25	583.2	97.0	25	529.4	88.1	25
456	604.9	25	585.4	96.8	25	614.5	101.6	25	587.3	97.1	25	536.2	88.7	25
484	609.6	25	600.7	98.5	25	621.1	101.9	25	598.5	98.2	25	546.9	89.7	25
512	619.7	25	606.9	97.9	25	625.3	100.9	25	607.9	98.1	25	552.9	89.2	25
540	624.8	25	605.9	97.0	25	625.6	100.1	25	608.7	97.4	25	554.7	88.8	25
568	623.1	25	614.3	98.6	24	631.1	101.3	25	623.0	100.0	25	560.2	89.9	25
596	626.2	25	607.2	97.0	24	624.5	99.7	25	630.1	100.6	25	556.1	88.8	25
624	624.6	25	624.2	99.9	24	624.4	100.0	25	632.3	101.2	25	571.3	91.5	24
652	617.4	25	612.8	99.3	24	624.8	101.2	25	631.4	102.3	25	565.1	91.5	24
680	605.4	24	595.3	98.3	24	624.5	103.2	25	626.5	103.5	25	558.5	92.3	24
708	583.3	22	591.9	101.5	23	606.5	101.5	25	610.0	104.6	24	565.0	96.9	23
EOS	**589.1**	**20**	**592.0**	**100.5**	**23**	**611.6**	**103.8**	**23**	**608.9**	**103.4**	**23**	**568.5**	**96.5**	**23**

EOS = end of study; No. of litters = number of litters represented in weight average.
[a]Study day 1 is the day animals were placed on study after pups were weaned.
[b]Average weights shown are means of litter means.

Table 10. Summary of Survival and Mean Body Weights of Female Rats in the Perinatal and Two-year Feed Study of Di-*n*-butyl Phthalate

Study Day[a]	0 ppm		300 ppm			1,000 ppm			3,000 ppm			10,000 ppm		
	Av. Wt. (g)[b]	No. of Litters	Av. Wt. (g)	Wt. (% of Controls)	No. of Litters	Av. Wt. (g)	Wt. (% of Controls)	No. of Litters	Av. Wt. (g)	Wt. (% of Controls)	No. of Litters	Av. Wt. (g)	Wt. (% of Controls)	No. of Litters
1	56.5	25	56.8	100.5	25	54.8	96.9	25	54.2	95.9	25	48.7	86.2	25
8	86.0	25	85.5	99.4	25	84.2	97.9	25	83.6	97.2	25	77.3	89.9	25
15	120.4	25	120.7	100.3	25	119.6	99.4	25	118.3	98.2	25	111.0	92.1	25
22	151.2	25	149.7	99.0	25	148.6	98.3	25	149.0	98.5	25	141.1	93.3	25
29	170.2	25	165.6	97.3	25	168.5	99.0	25	169.7	99.7	25	162.5	95.5	25
36	190.3	25	190.7	100.2	25	187.9	98.7	25	188.3	98.9	25	179.6	94.4	25
43	203.9	25	204.5	100.3	25	197.3	96.8	25	203.3	99.7	25	194.8	95.5	25
50	217.0	25	218.1	100.5	25	212.8	98.1	25	215.1	99.1	25	204.2	94.1	25
57	224.4	25	227.3	101.3	25	221.7	98.8	25	223.6	99.6	25	215.3	96.0	25
64	233.3	25	235.8	101.1	25	231.1	99.0	25	233.6	100.1	25	223.9	96.0	25
71	238.8	25	239.7	100.4	25	237.2	99.3	25	240.2	100.6	25	228.4	95.6	25
78	243.8	25	244.0	100.1	25	241.4	99.0	25	241.5	99.1	25	233.3	95.7	25
85	249.6	25	249.0	99.8	25	247.0	99.0	25	248.3	99.5	25	237.9	95.3	25
92	255.0	25	253.6	99.4	25	249.6	97.9	25	251.9	98.8	25	242.9	95.3	25
120	268.6	25	267.4	99.6	25	264.6	98.5	25	266.2	99.1	25	256.4	95.5	25
148	276.7	25	275.5	99.6	25	273.7	98.9	25	272.9	98.6	25	262.9	95.0	25
176	288.4	25	287.1	99.6	25	284.8	98.8	25	286.7	99.4	25	269.6	93.5	25
204	297.3	25	292.7	98.4	25	290.1	97.6	25	292.2	98.3	25	276.7	93.1	25
232	303.8	25	301.5	99.2	25	296.6	97.6	25	298.8	98.4	25	278.9	91.8	25
260	311.0	25	307.4	98.9	25	306.7	98.6	25	306.3	98.5	25	284.3	91.4	25
288	313.8	25	311.5	99.3	25	311.9	99.4	25	312.6	99.6	25	292.0	93.1	25
316	322.9	25	316.7	98.1	25	320.9	99.4	25	319.6	99.0	25	294.8	91.3	25
344	329.5	25	322.6	97.9	25	325.5	98.8	25	324.3	98.4	25	295.9	89.8	25
372	327.4	25	328.0	100.2	25	335.0	102.3	25	329.8	100.7	25	298.9	91.3	25
400	329.5	25	336.1	102.0	25	338.0	102.6	25	328.6	99.7	25	299.0	90.7	25
428	341.4	25	338.0	99.0	25	347.7	101.9	25	339.4	99.4	25	307.2	90.0	25
456	343.8	25	342.5	99.6	25	345.9	100.6	25	347.1	101.0	25	304.2	88.5	25
484	354.5	25	350.3	98.8	25	352.7	99.5	25	353.0	99.6	25	309.0	87.2	25
512	360.5	25	350.8	97.3	25	360.8	100.1	25	356.6	98.9	25	312.1	86.6	25
540	363.9	25	356.8	98.1	25	370.0	101.7	25	366.1	100.6	24	320.0	88.0	25
568	372.7	25	359.0	96.3	25	369.0	99.0	24	367.5	98.6	24	326.5	87.6	24
596	378.9	25	366.0	96.6	24	371.0	97.9	24	374.4	98.8	24	321.3	84.8	23
624	393.7	24	368.9	93.7	24	376.5	95.6	23	381.0	96.8	23	328.5	83.4	22
652	397.4	24	371.9	93.6	24	380.1	95.6	22	375.6	94.5	22	330.3	83.1	21
680	390.4	23	363.5	93.1	24	379.2	97.1	21	388.4	99.5	21	331.5	84.9	20
708	391.3	23	366.0	93.5	23	375.0	95.8	19	386.8	98.9	21	339.0	86.6	19
EOS	**390.3**	**22**	**374.7**	**96.0**	**23**	**375.5**	**96.2**	**18**	**393.4**	**100.8**	**20**	**348.8**	**89.4**	**19**

EOS = end of study; No. of litters = number of litters represented in weight average.
[a]Study day 1 is the day animals were placed on study after pups were weaned.
[b]Average weights shown are means of litter means.



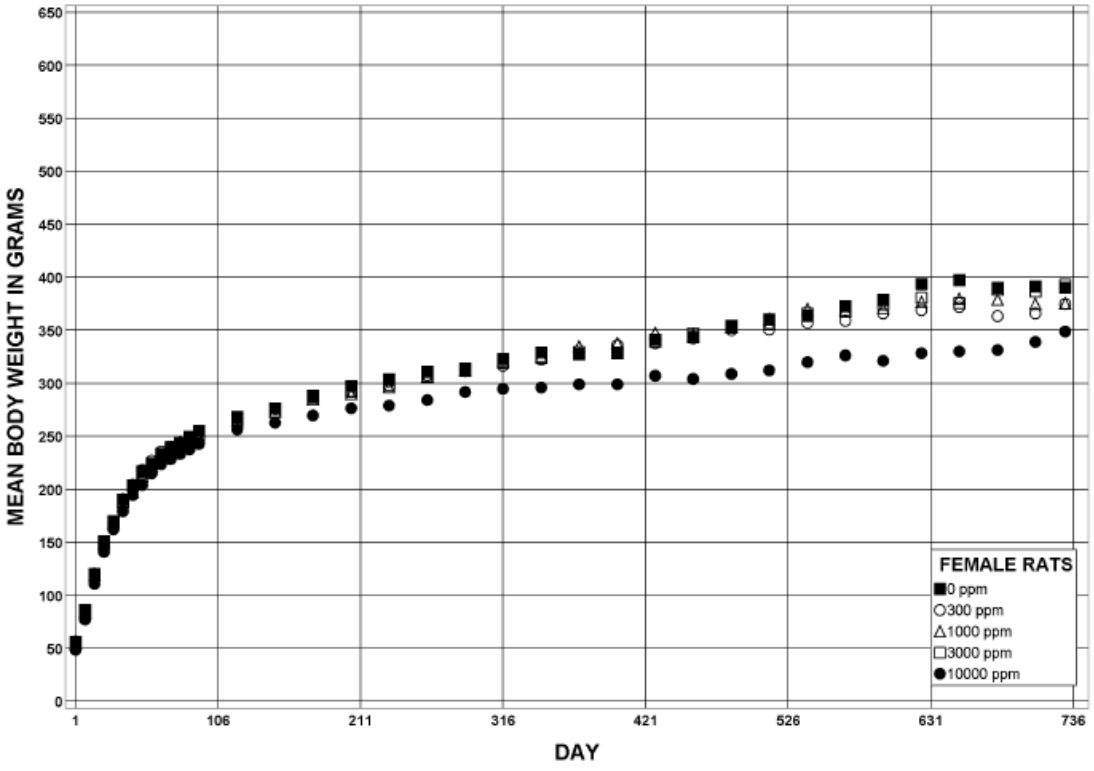


Figure 3. Growth Curves for Rats Exposed to Di-*n*-butyl Phthalate in Feed for Two Years

Table 11. Summary of Feed and Di-*n*-butyl Phthalate Consumption of Male Rats in the Perinatal and Two-year Feed Study

Week	0 ppm	300 ppm		1,000 ppm		3,000 ppm		10,000 ppm	
	Feed (g/day)[a]	Feed (g/day)	Dose (mg/kg/day)[b]	Feed (g/day)	Dose (mg/kg/day)	Feed (g/day)	Dose (mg/kg/day)	Feed (g/day)	Dose (mg/kg/day)
1	9.0	9.1	47.7	9.2	162.2	8.8	492.0	7.8	1,560.5
13	24.3	23.9	18.2	24.0	60.4	21.5	165.5	19.9	562.7
54	27.2	27.6	14.8	27.6	47.3	26.4	140.0	22.7	450.4
102	21.4	24.6	12.4	22.7	37.3	23.0	112.9	22.1	391.4

[a]Grams of feed consumed per animal per day.
[d]Milligrams of di-*n*-butyl phthalate consumed per kilogram body weight per day.

Table 12. Summary of Feed and Di-*n*-butyl Phthalate Consumption of Female Rats in the Perinatal and Two-year Feed Study

Week	0 ppm	300 ppm		1,000 ppm		3,000 ppm		10,000 ppm	
	Feed (g/day)[a]	Feed (g/day)	Dose (mg/kg/day)[b]	Feed (g/day)	Dose (mg/kg/day)	Feed (g/day)	Dose (mg/kg/day)	Feed (g/day)	Dose (mg/kg/day)
1	9.6	9.2	48.6	9.7	177.0	9.6	531.2	8.6	1,764.6
13	15.6	15.3	18.4	15.2	61.5	15.5	187.3	15.0	630.5
54	16.8	16.4	15.0	17.1	51.7	16.7	152.1	16.3	544.7
102	19.9	19.4	15.8	19.2	51.6	19.4	149.8	20.2	600.3

[a]Grams of feed consumed per animal per day.
[b]Milligrams of di-*n*-butyl phthalate consumed per kilogram body weight per day.

Pathology

This section describes statistically significant or biologically noteworthy changes in the incidences of neoplasms and/or nonneoplastic lesions of the pancreas, testis, epididymis, prostate gland, seminal vesicle, preputial gland, pituitary gland, liver, and uterus. Summaries of the incidences of neoplasms and nonneoplastic lesions, individual animal tumor diagnoses, statistical analysis of primary neoplasms that occurred with an incidence of at least 5% in at least one animal group, and historical incidences for the biologically significant neoplasms mentioned in this section are presented as supplemental data in Appendix D.

Exocrine Pancreas: The incidence of exocrine pancreatic acinus adenomas in the male rats was slightly higher in the 10,000 ppm group compared to the control group, but only the positive trend was statistically significant (Table 13). Two pancreatic acinus carcinomas occurred in the control group and none in the exposed groups (Table 13). The incidence of pancreatic acinus adenomas in the 10,000 ppm group was within the historical control range (0% to 28%) for studies in Sprague Dawley (Hsd:Sprague Dawley® SD®) rats on the NTP-2000 diet, all exposure routes, and all vehicles. The incidence of pancreatic acinus carcinomas in the control group exceeded the previous historical control range (0% to 4%). The incidence of pancreatic acinus hyperplasia was not significantly increased in any of the exposure groups (Table 13).

The adenomas of the exocrine pancreas were discrete masses of neoplastic acinar cells that often compressed the adjacent tissue. The masses were >3 mm in diameter and largely devoid of islets

of Langerhans, with the exception of the occasional entrapped islet at the edge of the mass. As opposed to the carcinomas, the adenomas lacked a fibrous capsule, were not invasive, and the neoplastic cells did not exhibit atypia. Mitotic figures were rare. Acinus hyperplasia was similar to adenoma but was smaller (<3 mm in diameter) and generally lacked compression of adjacent tissue.

Table 13. Incidences of Neoplastic and Nonneoplastic Lesions of the Pancreas in Male Rats in the Perinatal and Two-year Feed Study of Di-*n*-butyl Phthalate

	0 ppm	300 ppm	1,000 ppm	3,000 ppm	10,000 ppm
n[a]	49	50	50	50	49
Acinus, Hyperplasia[b]	19 (2.3)[c]	21 (2.1)	18 (2.1)	23 (2.0)	18 (2.1)
Acinus, Adenoma, Multiple	2	1	0	0	2
Acinus, Adenoma (Includes Multiple)[d]					
Overall rate[e]	4/49 (8%)	4/50 (8%)	3/50 (6%)	1/50 (2%)	10/49 (20%)
Rate per litters[f]	4/25 (16%)	4/25 (16%)	3/25 (12%)	1/25 (4%)	9/25 (36%)
Adjusted rate[g]	9.7%	8.9%	6.8%	2.3%	24.1%
Terminal rate[h]	2/27 (7%)	3/38 (8%)	3/31 (10%)	1/34 (3%)	8/33 (24%)
First incidence (days)	676	565	729 (T)	729 (T)	684
Rao-Scott-adjusted Poly-3 test[i]	p = 0.010	p = 0.595N	p = 0.472N	p = 0.192N	p = 0.094
Acinus, Carcinoma[j]	2	0	0	0	0
Acinus, Adenoma or Carcinoma (Combined)[l]					
Overall rate	6/49 (12%)	4/50 (8%)	3/50 (6%)	1/50 (2%)	10/49 (20%)
Rate per litters	6/25 (24%)	4/25 (16%)	3/25 (12%)	1/25 (4%)	9/25 (36%)
Adjusted rate	14.3%	8.9%	6.8%	2.3%	24.1%
Terminal rate	2/27 (7%)	3/38 (8%)	3/31 (10%)	1/34 (3%)	8/33 (24%)
First incidence (days)	611	565	729 (T)	729 (T)	684
Rao-Scott-adjusted Poly-3 test	p = 0.042	p = 0.349N	p = 0.243N	p = 0.072N	p = 0.214

(T) = terminal euthanasia.
[a]Number of animals with tissue examined microscopically.
[b]Number of animals with lesion.
[c]Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.
[d]Historical control incidence for all routes of 2-year studies (mean ± standard deviation): 60/488 (11.58% ± 9.25%); range: 0–28%.
[e]Number of animals with neoplasm per number of animals necropsied.
[f]Number of litters with tumor-bearing animals per number of litters examined at anatomical site.
[g]Poly-3-estimated neoplasm incidence after adjustment for intercurrent mortality.
[h]Observed incidence at study termination.
[i]Beneath the control incidence is the p value associated with the trend test. Beneath the exposed group incidences are the p values corresponding to pairwise comparisons between the control group and that exposed group. The Rao-Scott test adjusts the Poly-3 test, which accounts for differential mortality in animals that do not reach study termination, for within-litter correlation. A negative trend or a lower incidence in an exposure group is indicated by N.
[j]Historical control incidence: 4/488 (0.8% ± 1.42%); range: 0–4%.
[k]Not applicable; no neoplasms in animal group.
[l]Historical control incidence: 62/488 (12.03% ± 9.16%); range: 0–28%.

Male Reproductive Tract: Several exposure-related gross lesions occurred in the reproductive tract of male rats (Table 14). These lesions included undescended testes; fluid- or blood-filled testes; missing or small testes, epididymides, prostate glands, seminal vesicles, or vas deferens; and missing gubernaculum or gubernaculum with a length >20 mm. These lesions occurred with the greatest frequency in the 10,000 ppm group. Of a total of 46 animals examined in this group, 36 had small testes, 27 had small epididymides, 4 had small prostate glands, 7 had small seminal vesicles, 19 (of the 40 measured) had a gubernaculum length >20 mm, and 36 had undescended testes.

In general, the diagnosis of small testis correlated microscopically to germinal epithelial atrophy, enlarged testis correlated to interstitial cell adenoma and/or interstitial edema, and fluid-filled testis correlated to interstitial edema. Small testes tended to also be undescended (either abdominal or inguinal locations). In the two male rats exposed to 10,000 ppm DBP with right testis not present (synonymous with agenesis), both also had the right vas deferens not present, and agenesis of the right epididymis (caput, corpus, and cauda) and ventral prostate gland (right and left). In addition, the left testis in both animals was undescended (abdominal location) and small. A single animal was exposed to 3,000 ppm DBP with agenesis of the right side of the prostate (ventral, dorsal, or lateral lobes). There did not appear to be differences in laterality of other male reproductive tract lesions; therefore, combined incidences are shown.

In general, small epididymis correlated to hypospermia, and small seminal vesicle and small prostate gland both correlated to decreased secretory fluid. There were low occurrences of other gross lesions in the male and female reproductive tracts, but they were considered sporadic and unrelated to DBP exposure (Appendix E).

Table 14. Summary of Gross Lesions in the Reproductive Tract of Male Rats in the Perinatal and Two-year Feed study of Di-*n*-butyl Phthalate

	0 ppm	300 ppm	1,000 ppm	3,000 ppm	10,000 ppm
n[a]	48	49	48	49	46
Testis[b]					
Size, small	1[c] (1)**[d]	0	4 (4)	2 (2)	36 (23)**
Size, enlarged (or swelling)	0	0	1 (1)	1 (1)	1 (1)
Fluid or blood filled	1 (1)	0	0	1 (1)	3 (3)
Right, not present	0	0	0	0	2 (2)
Right or left; abdominal; undescended	1 (1)	3 (3)	2 (2)	2 (2)	29 (21)**
Right or left; inguinal; undescended	1 (1)	0	0	0	7 (7)
Right or left; abdominal or inguinal; undescended	2 (2)	3 (3)	2 (2)	2 (2)	32 (23)**
Epididymis[b]					
Size, small	0**	0	3 (3)	0	27 (19)**
Right, agenesis	0	0	0	0	2 (2)

	0 ppm	300 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Prostate Glands[b]					
Size, small	0*	0	1 (1)	2 (2)	4 (4)
Right or left, ventral, dorsal, or lateral, agenesis	0	0	0	1 (1)	2 (2)
Seminal Vesicles[b]					
Size, small	1 (1)*	2 (2)	2 (2)	1 (1)	7 (6)
Vas Deferens[b]					
Right, not present	0	0	0	0	2 (2)
Gubernaculum[e,f]					
Right, not present	0	0	0	0	1 (1)
Left, length (mm)	14.24 ± 0.83 33 (23)[g]	14.73 ± 0.71 41 (24)	13.18 ± 0.78 39 (25)	14.78 ± 0.89 38 (23)	30.92 ± 4.09 35 (24)
Right, length (mm)	14.04 ± 0.71 33 (23)	14.52 ± 0.70 41 (24)	13.84 ± 0.85 39 (25)	14.80 ± 0.75 38 (23)	33.86 ± 4.80 33 (22)

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group.
Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$; **$p \leq 0.01$.
[a]Number of animals examined for each tissue.
[b]Statistical analysis performed by the Cochran-Armitage test with a Rao-Scott modification for the random effect due to litter. All trend and pairwise p values are reported as one-sided.
[c]Number of animals affected given each observation.
[d]Number of litters with observations for F_1 animals. F_1 litter incidence based on the number of F_0 dams.
[e]Statistical analysis performed using a bootstrapped Jonckheere test for trend and a Datta-Satten modified Wilcoxon test with Hommel adjustment for pairwise comparisons.
[f]Data are presented as mean ± standard error.
[g]Number of animals examined (number of litters represented).

Testis: Several lesions were observed in the testis involving the seminiferous tubules, the interstitium, or the rete testis (Table 15). There were significant increases in the incidences of the combination of unilateral and bilateral edema, bilateral germinal epithelial atrophy, the combination of unilateral and bilateral germinal epithelial atrophy, diffuse interstitial cell hyperplasia (always bilateral when it was diagnosed), the combination of unilateral and bilateral focal interstitial cell hyperplasia, the combination of unilateral and bilateral rete testis fibrosis, and the combination of unilateral and bilateral seminiferous tubule dysgenesis (Table 15, Appendix D). The incidences of these lesions were all significantly increased in the 10,000 ppm group compared to the control group, and the positive trends also were statistically significant. The incidences of germinal epithelial atrophy and focal interstitial cell hyperplasia also were significantly increased in the 300 ppm group. The average severities of germinal epithelium atrophy were higher in the 1,000, 3,000, and 10,000 ppm groups than in the control group. The incidence of sperm granuloma in the rete testis also was higher in the 10,000 ppm group; although not statistically significant, it was considered related to DBP exposure as sperm granuloma is thought to be the precursor lesion to rete testis fibrosis. The incidences of the combination of unilateral and bilateral germinal epithelium degeneration were not higher compared to the control group (Table 15). The average severity of this lesion was higher in the exposed groups. This is a common background change in laboratory rodents, but the lower incidence of degeneration in the 10,000 ppm group is likely due to exacerbation by DBP

exposure, resulting in progression to atrophy, which was clearly exposure-related (degeneration is considered to be on a continuum with atrophy, with atrophy the end stage of ongoing degeneration).

Table 15. Incidences of Nonneoplastic Lesions of the Testis, Epididymis, Prostate Gland, and Seminal Vesicle in Male Rats in the Perinatal and Two-year Feed Study of Di-*n*-butyl Phthalate

	0 ppm	300 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Testis[a]	49	50	50	47	50
Edema (includes bilateral)[b]	2** (3.0)[c]	3 (2.0)	2 (2.5)	2 (3.0)	18** (3.5)
Germinal epithelium, atrophy (includes bilateral)	8** (2.3)	21** (1.8)	11 (2.5)	10 (2.9)	42** (3.9)
Germinal epithelium, degeneration (includes bilateral)	8 (1.1)	7 (2.6)	12 (2.2)	7 (2.0)	3 (2.0)
Interstitial cell, hyperplasia, diffuse, bilateral[d]	0**	0	1 (2.0)	0	9** (2.2)
Interstitial cell, hyperplasia, focal (includes bilateral)	1* (3.0)	7* (1.6)	5 (1.2)	3 (1.7)	11** (1.5)
Rete testis, fibrosis (includes bilateral)	0**	0	0	0	11** (2.5)
Rete testis, sperm granuloma (includes bilateral)	0	0	0	0	2
Seminiferous tubule, dysgenesis (includes bilateral)	0**	0	0	1 (2.0)	9* (1.9)
Epididymis	49	50	50	50	50
Hypospermia (includes bilateral)	4** (3.5)	7 (3.1)	10 (3.0)	9 (3.2)	40** (3.9)
Prostate Gland	49	50	50	50	50
Decreased secretory fluid[e]	5**	8	5	5	18**
Seminal Vesicle	49	50	50	50	49
Decreased secretory fluid[e]	6**	7	9	6	15*

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group.
Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant ($p \leq 0.05$) from the vehicle control group by the Rao-Scott adjusted Poly-3 test; **$p \leq 0.01$.
[a]Number of animals with tissue examined microscopically.
[b]Incidence reported is the combination of unilateral and bilateral lesions. Severity grade for these types of lesions = sum of unilateral and bilateral severity scores/number of unilateral and bilateral incidences.
[c]Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.
[d]This lesion was observed only as a bilateral lesion.
[e]This lesion was not graded.

Microscopically, germinal epithelium atrophy was seen as seminiferous tubules devoid of germ cells and lined only by Sertoli cells. Germinal epithelium degeneration was observed as several degenerative features, frequently co-occurring, including tubular vacuolation, focal to segmental germ cell dropout, general depletion of germ cells, degeneration of germ cells not restricted to stage or cell type, and disorganization of the germinal epithelium.

Seminiferous tubule dysgenesis (Figure 4) was observed microscopically as malformed, anastomosing seminiferous tubules. Generally, lesions of dysgenesis consisted of a single focus per testis, but, occasionally, up to three foci were observed. Tubules in dysgenetic lesions were not active in spermatogenesis and contained immature-appearing Sertoli cells with small, elongated nuclei and a less prominent nucleolus than observed in mature Sertoli cells. The basement membranes were thickened and convoluted and appeared discontinuous in some areas. Small "islands" of entrapped Leydig cells were occasionally present within the malformed tubules. Dysgenetic lesions often were surrounded by angular aggregates of Leydig cells, which were smaller, spindle-shaped, and contained less cytoplasm than typically observed in focal Leydig cell hyperplasia or adenomas. Dysgenesis was usually observed in only one of the three sections of a single testis and was generally located in a section of testis that also contained the rete testis.

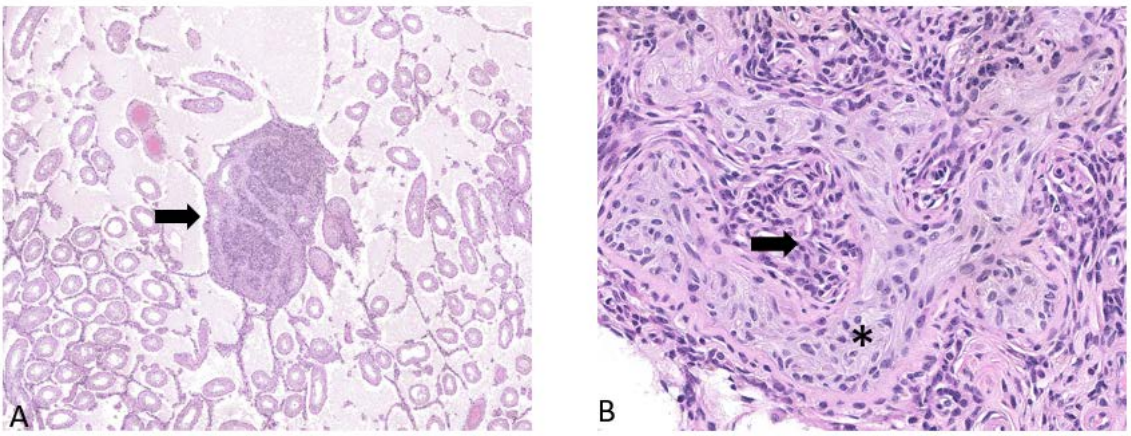


Figure 4. Representative Images Depicting Seminiferous Tubule Dysgenesis in a Male Rat in the Perinatal and Two-year Feed Study of Di-*n*-butyl Phthalate

A) Seminiferous tubule dysgenesis (arrow) in a 10,000 ppm male rat. There is also germinal epithelial atrophy in all seminiferous tubules, as well as interstitial edema. B) Higher magnifications of the dysgenesis lesion in panel A showing the convoluted, malformed seminiferous tubules (asterisk) with immature-appearing Sertoli cells and thickened basement membranes, surrounded by aggregated Leydig cells (arrow). Original magnification: A = 4x, B = 20x.

Edema and interstitial cell hyperplasia were lesions present in the testicular interstitium in DBP-exposed male rats. Microscopically, testicular edema (Figure 4) appeared as increased amounts of eosinophilic fluid within the interstitium. Another lesion of the interstitium was hyperplasia of Leydig cells, both diffuse and focal. Diffuse interstitial cell hyperplasia (Figure 5) was observed as increased numbers of Leydig cells with bridging strands several layers thick between seminiferous tubules, generally involving a large portion of the testis. Focal interstitial cell hyperplasia was seen as a single angular or rounded Leydig cell aggregate between seminiferous tubules with a diameter less than or equal to three seminiferous tubules.

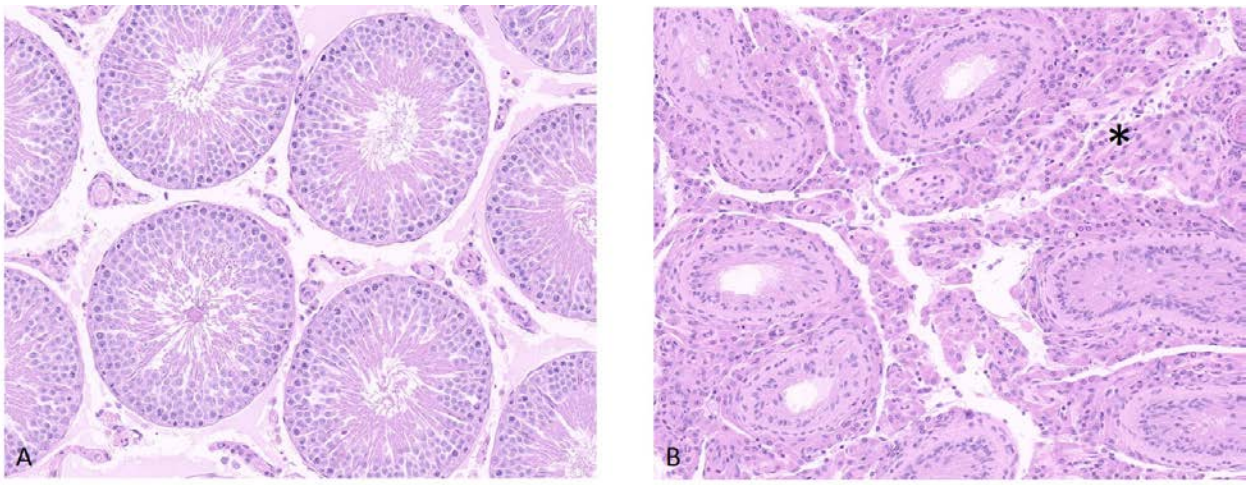


Figure 5. Representative Images of the Testis from Male Rats in the Perinatal and Two-year Feed Study of Di-*n*-butyl Phthalate

A) Normal testis and Leydig cell population from a control male rat. B) Diffuse interstitial (Leydig) cell hyperplasia (asterisk) from a 10,000 ppm male rat with seminiferous tubule atrophy. Original magnification: A = 20x, B = 20x.

In the rete testis region, two lesions were observed microscopically. Rete testis granuloma (Figure 6) was seen as expansion of a profile in the rete testis region, subjacent to the capsule at the cranial pole of the testis, by an accumulation of abundant spermatozoa and occasional cholesterol clefts. These spermatozoa and cholesterol clefts were surrounded by a granulomatous inflammatory response, including numerous multinucleated giant cells, which was in turn surrounded by fibrous connective tissue with interspersed macrophages and fibroblasts. Rete testis fibrosis (Figure 7) was observed as a lesion similar in magnitude and location as the rete testis granulomas but consisted of disorganized fibrous connective tissue containing occasional macrophages and fibroblasts and numerous, apparently anastomosing channels, which were lined by cuboidal epithelium. Three sections of a single testis were examined histologically; of the 11 males diagnosed with rete testis fibrosis, 2 also had a rete testis sperm granuloma present in a different section of testis.

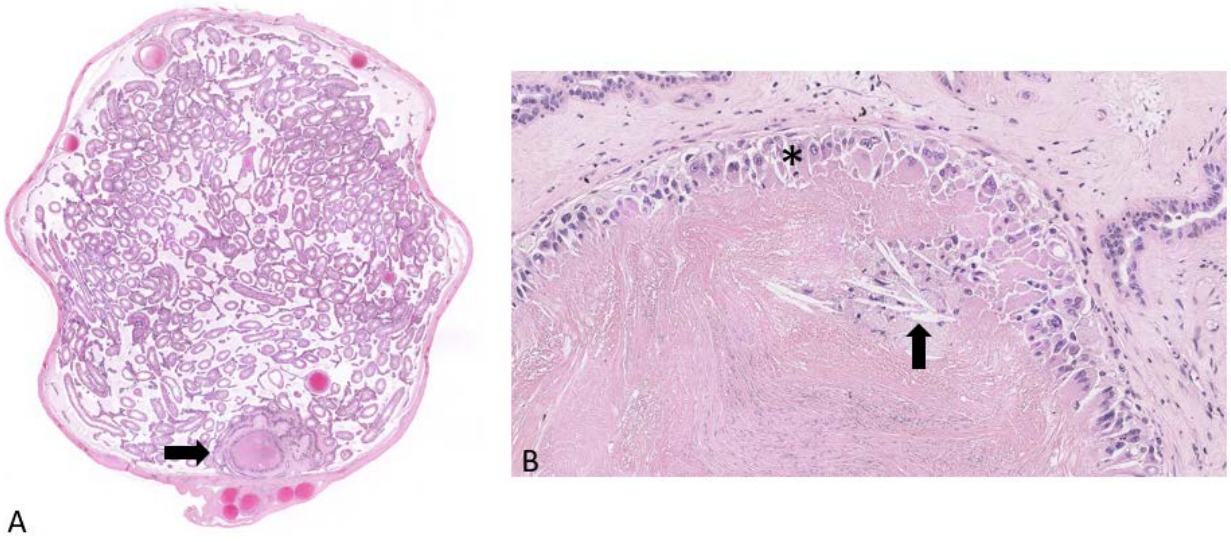


Figure 6. Representative Images Depicting Sperm Granuloma in the Rete Testis of a Male Rat in the Perinatal and Two-year Feed Study of Di-*n*-butyl Phthalate

A) Sperm granuloma in the rete testis (arrow) in a 10,000 ppm male rat. There is also germinal epithelial atrophy and interstitial edema. B) Higher magnification of the lesion in panel A showing the macrophages at the periphery of the lesion (asterisk) and surrounding the cholesterol clefts (arrow). There are degenerating spermatozoa in the center of the lesion and fibrosis surrounding the lesion. Original magnification: A = 1.4x, B = 20x.

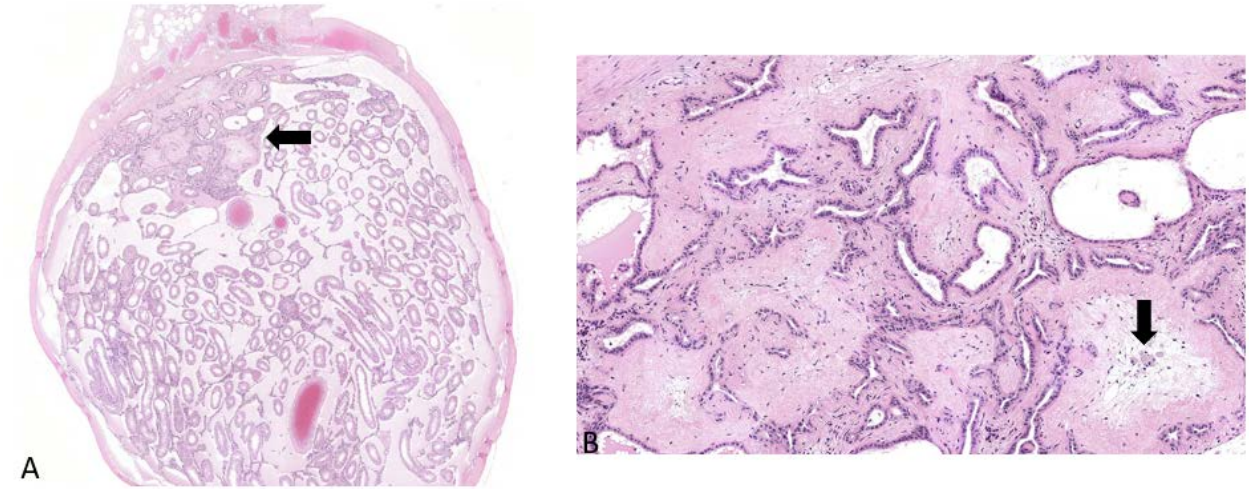


Figure 7. Representative Images Depicting Fibrosis in the Rete Testis of a Male Rat in the Perinatal and Two-year Feed Study of Di-*n*-butyl Phthalate

A) Fibrosis in the rete testis in a 10,000 ppm male rat (arrow). There is an increased number of tubular profiles within the lesion. There is also germinal epithelial atrophy and interstitial edema. B) Higher magnification of the lesion in panel A showing the fibrosis separating the tubular profiles. The epithelium in the tubular profiles is hyperplastic. There are several macrophages within the lesion (arrow) suggesting progression of a sperm granuloma. Original magnification: A = 2.5x, B = 10x.

Epididymis: In the epididymis, the incidence of the combination of unilateral and bilateral hypospermia was significantly increased in the 10,000 ppm group compared to the control group (Table 15). The incidence of bilateral hypospermia alone was also significantly increased in the high exposure group compared to the control group (Appendix D). The incidence of unilateral hypospermia alone also was significantly increased in the 10,000 ppm group compared to the control group (Appendix D).

Hypospermia was observed microscopically as reduced density or absence of sperm in the epididymal duct lumen throughout the epididymis. Due to the reduced sperm and fluid volume, hypospermia was generally accompanied by ductal atrophy, which was not diagnosed separately. Epididymides with hypospermia usually had generalized narrowing of the duct lumen, with some intraductal folding of epithelium.

Accessory Sex Organs: The incidences of decreased secretory fluid in the prostate gland and seminal vesicle were significantly increased in the 10,000 ppm group compared to the control group (Table 15). These lesions were not graded.

Microscopically, decreased secretory fluid in the prostate gland and seminal vesicle was seen as reduced acinar or vesicle lumina with reduced luminal secretion. In the seminal vesicle, decreased secretory fluid also was seen as crowding of the intravesicle epithelial folds.

Preputial Gland: The incidences of chronic inflammation (7/49, 10/49, 2/50, 0/49, 0/50), chronic active inflammation (27/49, 8/49, 1/50, 1/49, 2/50), and duct dilation (35/49, 16/49, 3/50, 2/49, 3/50) in the preputial gland were significantly decreased compared to the control group (Appendix D). Duct dilation is a common age-related change. The pathogenesis of the decreased incidences of these lesions in exposed male rats is unclear, but it could be related to hormonal disturbance as preputial gland secretion is influenced by testosterone and pituitary hormones.[144]

Pituitary Gland: In the males, the incidence of hypertrophy of endocrine cells in the pars distalis was significantly increased in only the 10,000 ppm group compared to the control group. The incidence of this lesion in the high exposure group was significant as was the positive trend (Table 16). A significant positive trend in the incidence of hyperplasia of cells of the pars distalis also occurred.

The hypertrophy (Figure 8) was characterized by increased amounts of pale-staining, eosinophilic cytoplasm and, in many of the cells, by the presence of multiple small vacuoles or a single large vacuole that displaced the nucleus peripherally. The lesion affected individual cells often clustered in the pars distalis. The hyperplasia was characterized by increased numbers of cells, and the lesion tended to be focal.

Table 16. Incidences of Nonneoplastic Lesions of the Pituitary Gland and Liver in Male and Female Rats in the Perinatal and Two-year Feed Study of Di-*n*-butyl Phthalate

	0 ppm	300 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Male					
Pituitary Gland[a]	48	50	50	50	50
Pars distalis, hypertrophy[b]	0**	0	0	0	29** (1.0)[c]
Pars distalis, hyperplasia	15* (1.8)	13 (2.1)	13 (1.7)	18 (1.6)	22 (1.7)

	0 ppm	300 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Liver	49	50	50	50	50
Hepatocyte, cytoplasmic alteration	0**	0	0	0	39** (1.0)
Bile duct, hyperplasia	15 (1.1)	14 (1.1)	14 (1.0)	9 (1.0)	9 (1.0)
Female					
Pituitary Gland	50	50	50	50	50
Pars distalis, hypertrophy	1	0	0	1 (1.0)	1 (1.0)
Pars distalis, hyperplasia	24 (2.3)	15 (2.2)	14 (2.6)	15 (2.5)	18 (1.9)
Liver	50	50	50	50	50
Hepatocyte, cytoplasmic alteration	0**	0	0	1 (1.0)	40** (1.0)
Bile duct, hyperplasia	5 (1.0)	11 (1.1)	6 (1.2)	6 (1.2)	12* (1.3)

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group.
Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant ($p \leq 0.05$) from the vehicle control group by the Rao-Scott adjusted Poly-3 test; **$p \leq 0.01$.
[a]Number of animals examined microscopically.
[b]Number of animals with lesion.
[c]Average severity grade of observed lesion in affected animals: 1 = minimal; 2 = mild; 3 = moderate; 4 = marked.

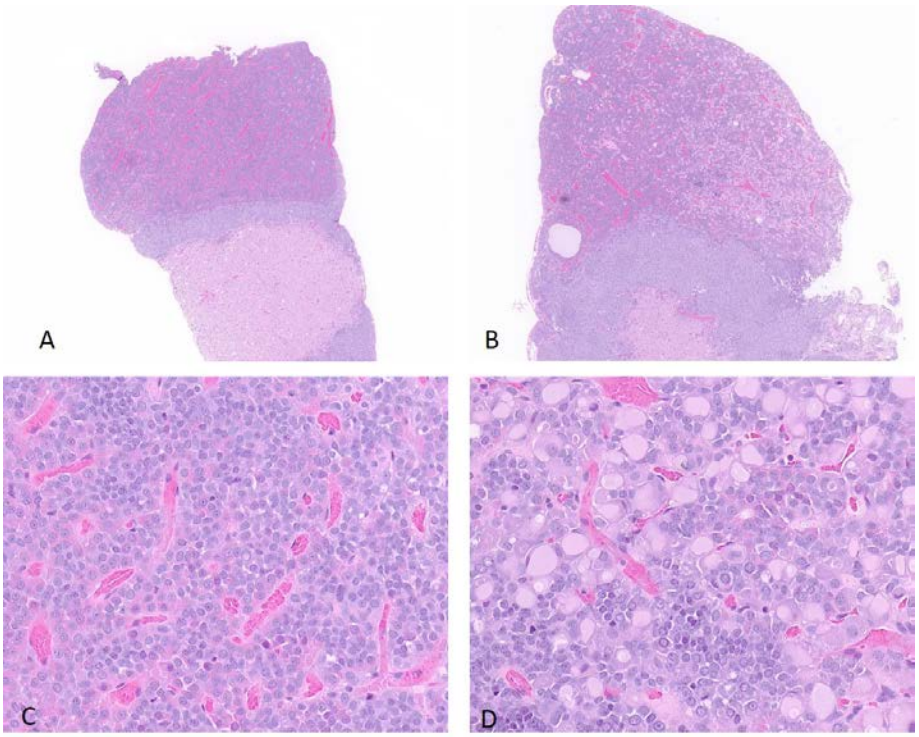


Figure 8. Representative Images of the Pituitary Gland from Male Rats in the Perinatal and Two-year Feed Study of Di-*n*-butyl Phthalate

A) Pituitary gland from a control male. B) Pituitary gland with hypertrophy of the pars distalis endocrine cells in a male exposed to 10,000 ppm DBP. C) Higher magnification of the pars distalis in panel A. D) Higher magnification of the pars distalis in exposed male seen in panel B. Some of the endocrine cells of the pars distalis are enlarged and have a few to one large vacuole with pale eosinophilic cytoplasm. Original magnification: A = 4x, B = 4x, C = 40x, D = 40x.

Liver: Significantly increased incidences of cytoplasmic alteration of hepatocytes occurred in the 10,000 ppm groups of both males and females. This lesion was not observed in any other groups (except in one 3,000 ppm female), including the control groups (Table 16). In addition, a slight increase in the incidence of bile duct hyperplasia occurred in the 300 and 10,000 ppm groups of female rats but was significant only in the 10,000 ppm group.

The cytoplasmic alteration (Figure 9) was characterized by increased amounts of cytoplasm with fine eosinophilic granules, mainly in the centrilobular regions in the liver. The bile duct hyperplasia was characterized by increased numbers of bile duct profiles in some of the portal tracts accompanied by increased numbers of inflammatory cells (mainly lymphocytes).

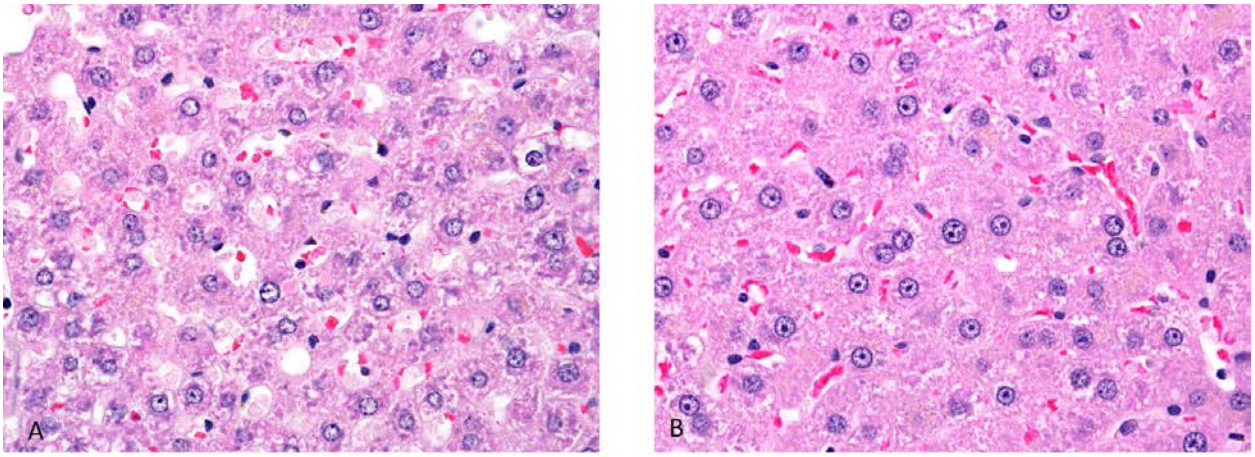


Figure 9. Representative Images of the Liver from Female Rats in the Perinatal and Two-year Feed Study of Di-*n*-butyl Phthalate

A) Normal liver from a control female rat. B) Cytoplasmic alteration in a 10,000 ppm female rat liver. The hepatocytes are enlarged by increased amounts of cytoplasm with fine eosinophilic granules, mainly in the centrilobular regions of the liver. Original magnification: A = 40x, B = 40x.

Uterus: The incidence of stromal polyps in the uterus was higher in the 10,000 ppm group compared to the control group (5/50, 6/50, 5/50, 5/50, 10/50) (Appendix D). The positive trend was significant, but the incidences of these lesions were not significantly increased compared to the control group. In NTP studies on cell-phone radiation that used the same uterine evaluation in Sprague Dawley rats, the incidence of stromal polyps in control animals was 16/90 (18%).[145] Because there is no supportive evidence from the companion mouse study that this is an exposure-related response and because this lesion and exposure to DBP have no known association (as opposed to the pancreatic adenomas), the positive trend was not considered related to DBP exposure.

Other Lesions: Several lesions were observed in the male rats with lower incidences in exposed groups compared to the control group (Appendix D). These included keratoacanthomas of the skin, the combined incidences of epithelial neoplasms in the skin (basal or squamous cell carcinoma, carcinoma, basosquamous tumor [malignant or benign], basal cell adenoma, adenoma, papilloma, squamous papilloma, keratoacanthoma, and trichoepithelioma), adenomas of the pars distalis of the pituitary gland, focal hyperplasia of the adrenal cortex, and focal hyperplasia of the adrenal medulla. The biological relevance of the lower incidences of these nonneoplastic lesions is not clear.

Mice

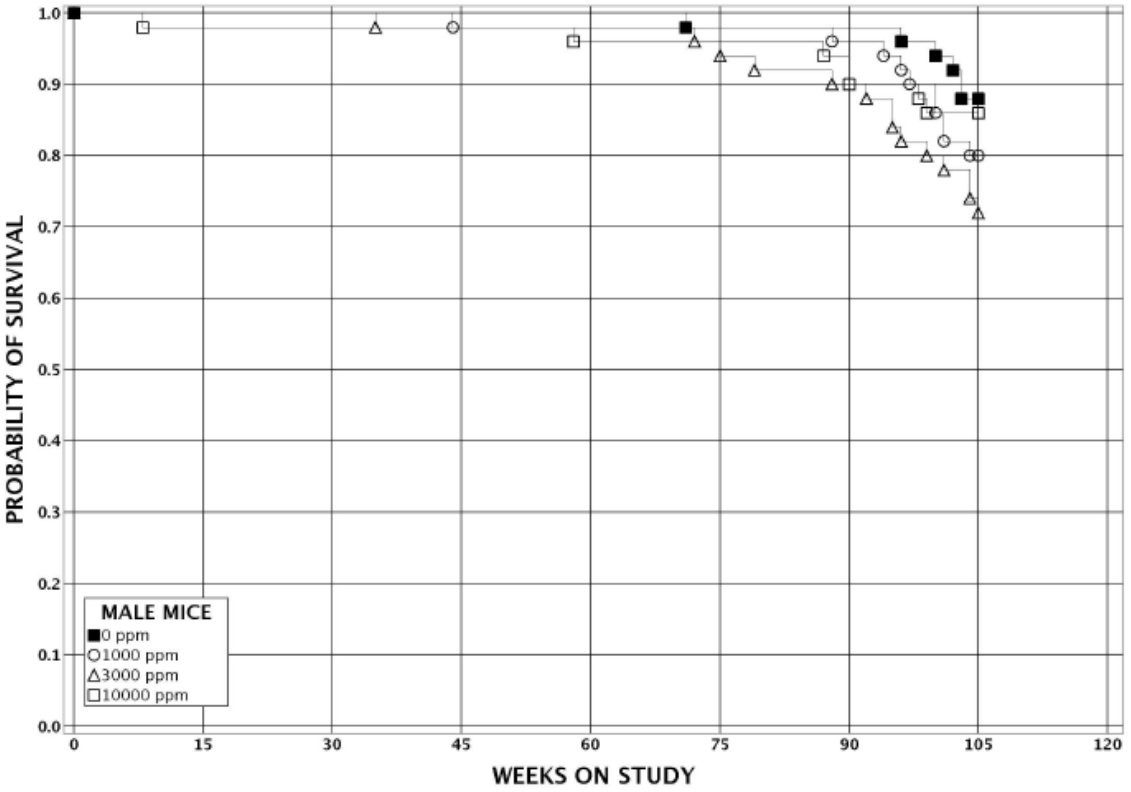
Two-year Study

Survival of all exposed groups was similar to that of the control group for both males and females (Table 17; Figure 10). There were no exposure-related clinical observations (Appendix D).

Table 17. Summary of Survival of Male and Female Mice in the Two-year Feed Study of Di-*n*-butyl Phthalate

	0 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Male				
Animals Initially in Study	50	50	50	50
Moribund	2	2	3	2
Natural Deaths	4	8	11	5
Animals Surviving to Study Termination	44	40[a]	36	43
Percent Probability of Survival at End of Study[b]	88.0	80.0	72.0	86.0
Mean Survival (Days)[c]	722	713	698	701
Survival Analysis[d]	p = 0.927N	p = 0.386	p = 0.070	p = 0.921
Female				
Animals Initially in Study	50	50	50	50
Moribund	1	1	0	0
Natural Deaths	7	7	6	3
Animals Surviving to Study Termination	42	42	44	47
Percent Probability of Survival at End of Study	84.0	84.0	88.0	94.0
Mean Survival (Days)	713	709	714	722
Survival Analysis	p = 0.126N	p = 1.000	p = 0.775N	p = 0.198N

[a]Includes one animal that died naturally during the last week of the study.
[b]Kaplan-Meier determinations.
[c]Mean of litter means of all deaths (uncensored, censored, and study termination).
[d]The result of the Tarone trend test is in the vehicle control group column, and the results of the Cox proportional hazards pairwise comparisons with the vehicle control group are in the exposed group columns. A negative trend or lower mortality in an exposure group is indicated by N.



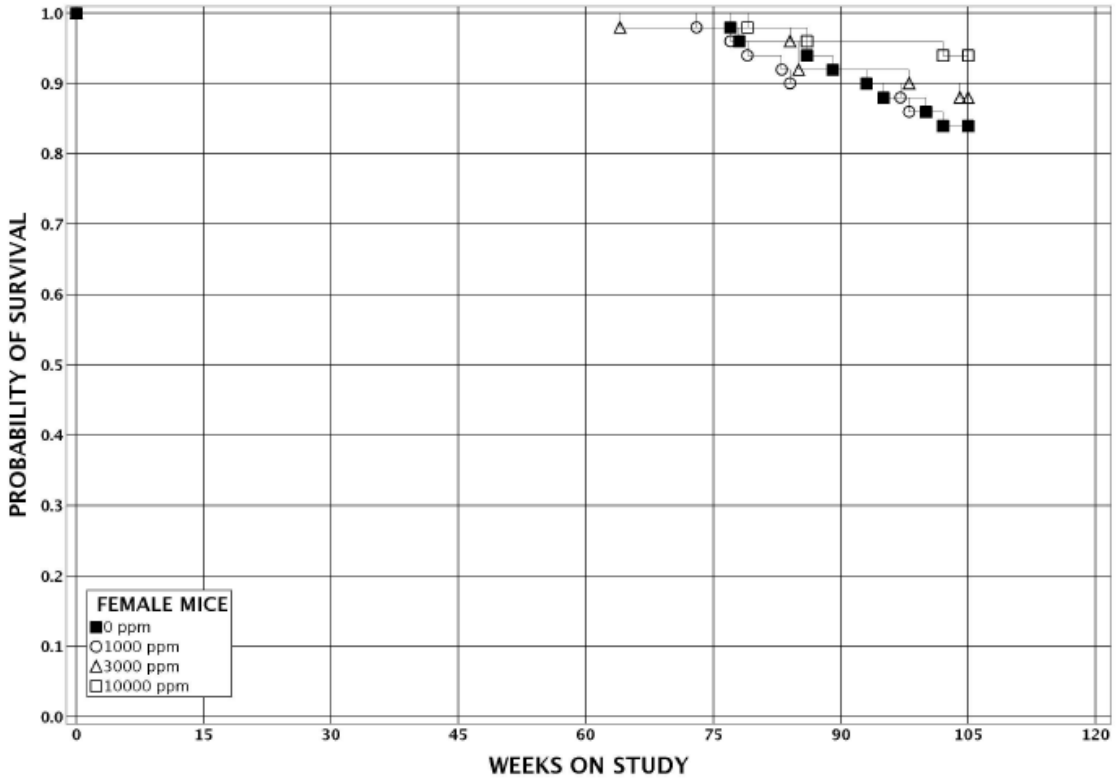


Figure 10. Kaplan-Meier Survival Curves for Mice Exposed to Di-*n*-butyl Phthalate in Feed for Two Years

Exposure-related significant decreases of group mean body weights were observed in the highest exposure group (10,000 ppm) throughout the study in both males and females (Table 18, Table 19; Figure 11). At study termination, group mean body weights for the 10,000 ppm groups were lower than those of the control group by 23.1% for males and by 34.5% for females. Group mean feed consumption over the course of the study was similar across exposed and control groups (Table 20, Table 21). Group mean chemical consumption increased proportionally with exposure concentration and was similar among males and females. Daily chemical consumption by the 1,000, 3,000, and 10,000 ppm groups averaged 111.6, 346.5, and 1,306.0 mg/kg/day, respectively, for males and 105.2, 329.4, and 1,393.3 mg/kg/day, respectively, for females.

Table 18. Summary of Survival and Mean Body Weights of Male Mice in the Two-year Feed Study of Di-*n*-butyl Phthalate

Study Day[a]	0 ppm		1,000 ppm			3,000 ppm			10,000 ppm		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors
1	20.5	50	20.5	100.4	50	20.4	99.9	50	20.6	100.6	50
8	21.7	50	21.8	100.4	50	21.4	98.6	50	20.9	96.2	50
15	22.3	50	22.5	100.7	50	22.1	98.7	50	21.2	94.7	50
22	22.9	50	23.2	101.3	50	23.0	100.2	50	22.0	95.7	50
29	23.8	50	24.1	101.5	50	23.9	100.5	50	22.6	95.0	50
36	24.8	50	24.9	100.4	50	24.5	98.6	50	23.0	92.7	50
43	25.7	50	25.9	100.8	50	25.4	98.7	50	23.7	92.5	50
50	26.3	50	26.6	101.2	50	26.0	98.9	50	24.0	91.2	50
57	26.7	50	27.3	102.3	50	26.4	99.0	50	24.4	91.3	49
64	27.0	50	28.0	103.7	50	27.0	100.0	50	25.0	92.7	49
71	27.3	50	28.4	104.0	50	26.9	98.6	50	25.0	91.6	49
78	28.5	50	29.0	101.7	50	28.3	99.2	50	25.7	90.3	49
85	29.7	50	30.4	102.4	50	29.3	98.8	50	26.4	89.0	49
92	30.4	50	30.9	101.7	50	29.9	98.2	50	26.0	85.5	49
120	32.3	50	33.8	104.5	50	31.9	98.6	50	27.9	86.4	49
148	35.2	50	37.1	105.5	50	34.7	98.6	50	29.1	82.8	49
176	38.5	50	40.1	104.3	50	37.5	97.5	50	30.6	79.5	49
204	39.4	50	41.7	105.8	50	38.9	98.6	50	31.9	80.8	49
232	41.8	50	43.5	104.1	50	40.8	97.6	50	32.7	78.4	49
260	43.3	50	44.6	102.9	50	42.6	98.3	49	33.5	77.4	49
288	44.3	50	45.2	102.0	50	43.7	98.7	49	34.5	77.9	49
316	45.2	50	46.4	102.6	49	44.1	97.5	49	34.8	76.9	49
344	46.2	50	47.2	102.2	49	45.1	97.7	49	35.6	77.0	49
372	46.3	50	47.3	102.1	49	45.5	98.1	49	36.2	78.0	49
400	46.1	50	47.6	103.3	49	45.5	98.8	49	35.9	77.9	48
428	46.7	50	47.7	102.1	49	46.4	99.4	49	36.8	78.8	48
456	47.6	50	48.6	102.1	49	46.0	96.6	49	37.5	78.7	48
484	47.9	50	49.3	103.1	49	47.2	98.7	49	37.8	79.0	48
512	48.3	49	49.7	102.9	49	47.1	97.6	48	37.6	77.8	48
540	48.8	49	50.5	103.5	49	47.3	96.9	47	38.2	78.4	48
568	48.7	49	49.5	101.6	49	46.9	96.3	46	38.1	78.2	48
596	48.2	49	48.9	101.5	49	46.3	96.1	46	37.7	78.2	48
624	48.9	49	50.0	102.2	48	47.1	96.4	45	38.1	77.9	45
652	48.7	49	48.8	100.2	47	45.6	93.6	44	37.4	76.9	45
680	47.5	48	47.9	100.8	45	45.9	96.7	41	37.4	78.8	44
708	47.3	47	47.9	101.1	41	45.9	97.0	39	37.0	78.1	43
EOS	**47.7**	**44**	**47.6**	**99.8**	**39**	**46.9**	**98.4**	**36**	**36.7**	**76.9**	**43**

EOS = end of study.

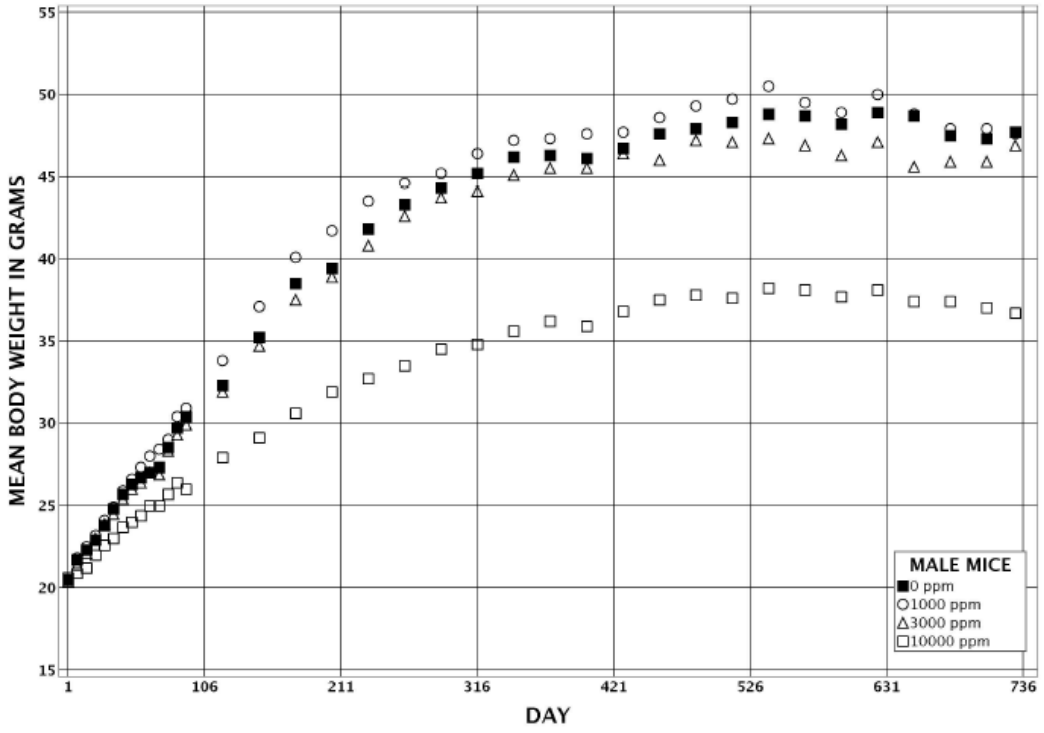
[a]Study day 1 is the day animals were placed on study.

Table 19. Summary of Survival and Mean Body Weights of Female Mice in the Two-year Feed Study of Di-*n*-butyl Phthalate

Study Day[a]	Control		1,000 ppm			3,000 ppm			10,000 ppm		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of Controls)	No. of Survivors
1	16.5	50	16.7	101.1	50	16.8	101.6	50	16.6	100.1	50
8	17.5	50	17.5	100.4	50	17.4	99.4	50	16.7	95.8	50
15	18.4	50	18.5	100.4	50	18.4	99.6	50	17.3	94.1	50
22	19.4	50	19.3	99.4	50	19.4	99.8	50	17.6	90.6	50
29	20.1	50	20.3	101.1	50	20.1	99.7	50	18.3	90.7	50
36	21.1	50	21.1	100.1	50	20.8	98.7	50	18.8	89.2	50
43	21.2	50	21.2	99.7	50	21.3	100.2	50	19.2	90.3	50
50	22.0	50	21.8	99.5	50	21.7	99.0	50	19.3	87.8	50
57	22.2	50	22.3	100.6	50	21.8	98.1	50	19.6	88.2	50
64	23.5	50	23.1	98.5	50	22.9	97.4	50	20.3	86.6	50
71	23.8	50	23.8	99.8	50	23.1	96.9	50	20.8	87.5	50
78	24.5	50	24.4	99.4	50	23.7	96.7	50	21.4	87.4	50
85	24.4	50	24.7	101.3	50	24.3	99.6	50	21.3	87.4	50
92	25.2	50	25.0	99.5	50	24.7	98.1	50	21.9	86.9	50
120	27.5	50	27.2	98.7	50	26.4	95.7	50	22.9	83.0	50
148	29.5	50	30.0	101.4	50	28.7	97.1	50	23.5	79.5	50
176	32.6	50	33.4	102.5	50	31.7	97.3	50	24.4	74.7	50
204	34.0	50	34.7	102.0	50	33.3	97.9	50	25.1	73.8	50
232	36.2	50	37.2	102.9	50	35.1	97.1	50	26.2	72.3	50
260	38.6	50	38.7	100.5	50	35.7	92.6	50	26.9	69.7	50
288	39.4	50	40.3	102.2	50	37.8	95.8	50	27.6	70.1	50
316	41.7	50	42.2	101.1	50	39.8	95.4	50	28.3	67.8	50
344	42.7	50	43.3	101.5	50	41.2	96.6	50	28.8	67.6	50
372	44.6	50	45.6	102.4	50	43.2	96.9	50	30.5	68.4	50
400	45.5	50	46.3	101.7	50	44.1	96.8	50	30.3	66.7	50
428	46.5	50	47.5	102.3	50	44.8	96.5	50	31.1	67.0	50
456	48.2	50	49.5	102.7	50	46.1	95.7	49	31.9	66.2	50
484	48.1	50	51.0	105.9	50	46.7	97.0	49	32.7	67.9	50
512	50.4	50	52.1	103.4	49	48.0	95.3	49	32.1	63.8	50
540	50.9	49	51.6	101.4	48	48.1	94.5	49	31.9	62.6	50
568	49.6	48	50.9	102.6	47	44.3	89.3	49	31.6	63.8	49
596	49.3	48	52.0	105.5	45	45.3	91.8	46	32.4	65.7	49
624	51.0	46	52.8	103.6	45	48.1	94.2	46	32.6	63.9	48
652	51.5	45	53.4	103.6	45	48.7	94.6	46	33.8	65.7	48
680	51.4	44	51.1	99.5	44	47.9	93.2	46	33.4	64.9	48
708	51.4	43	50.9	98.9	43	48.2	93.7	45	33.7	65.5	47
EOS	**51.7**	**42**	**50.7**	**98.0**	**42**	**48.6**	**94.0**	**44**	**33.9**	**65.5**	**47**

EOS = end of study.

[a]Study day 1 is the day animals were placed on study.



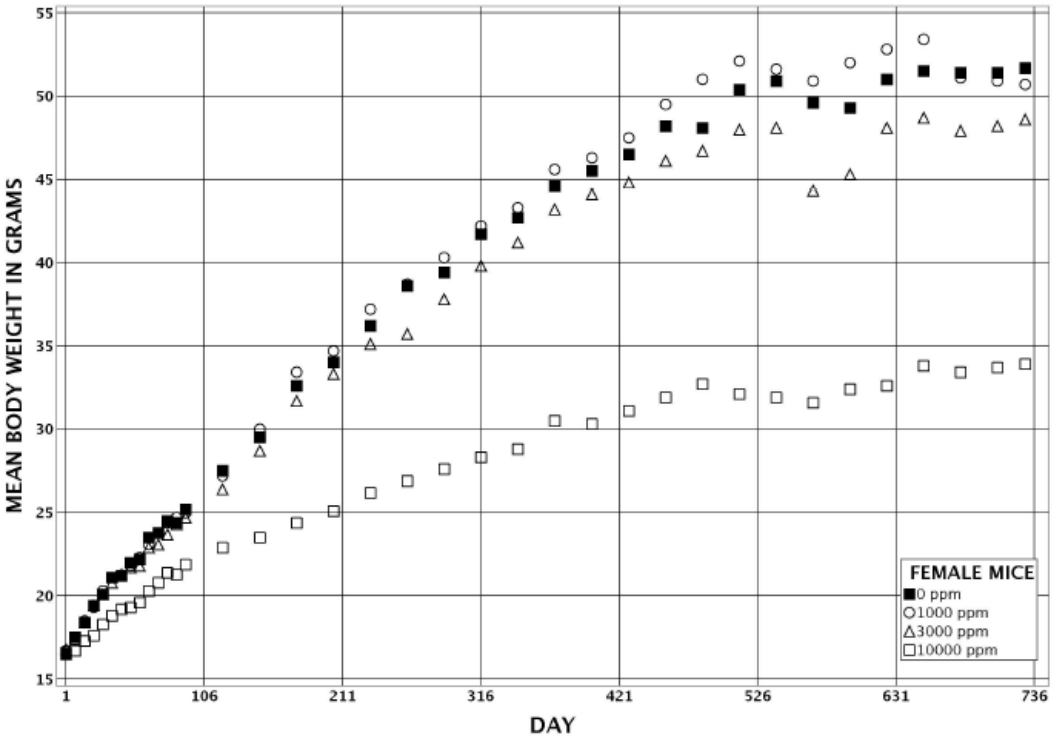


Figure 11. Growth Curves for Mice Exposed to Di-*n*-butyl Phthalate in Feed for Two Years

Table 20. Summary of Feed and Di-*n*-butyl Phthalate Consumption of Male Mice in the Two-year Feed Study

Week	0 ppm	1,000 ppm		3,000 ppm		10,000 ppm	
	Feed (g/day)[a]	Feed (g/day)	Dose (mg/kg/day)[b]	Feed (g/day)	Dose (mg/kg/day)	Feed (g/day)	Dose (mg/kg/day)
2	3.6	3.5	160.7	3.7	518.9	3.9	1,869.2
13	4.3	4.4	144.8	4.3	440.0	4.3	1,629.2
54	4.5	4.4	93.0	4.3	283.8	4.1	1,134.1
102	4.9	5.1	106.5	4.7	307.2	4.4	1,189.9

[a]Grams of feed consumed per animal per day.
[b]Milligrams of di-*n*-butyl phthalate consumed per kilogram body weight per day.

Table 21. Summary of Feed and Di-*n*-butyl Phthalate Consumption of Female Mice in the Two-year Feed Study

Week	0 ppm	1,000 ppm		3,000 ppm		10,000 ppm	
	Feed (g/day)[a]	Feed (g/day)	Dose (mg/kg/day)[b]	Feed (g/day)	Dose (mg/kg/day)	Feed (g/day)	Dose (mg/kg/day)
1	3.1	3.2	191.3	3.4	606.6	4.4	2,656.7
13	3.4	3.5	141.8	3.6	445.1	3.5	1,643.8
54	4.2	4.2	92.1	3.9	271.1	3.9	1,278.8
102	4.7	4.7	92.4	4.8	298.9	4.6	1,366.4

[a]Grams of feed consumed per animal per day.
[b]Milligrams of di-*n*-butyl phthalate consumed per kilogram body weight per day.

Pathology

This section describes statistically significant or biologically noteworthy changes in the incidences of nonneoplastic lesions in the testis, epididymis, coagulating gland, seminal vesicle, liver, and kidney of mice. Summaries of the incidences of neoplasms and nonneoplastic lesions, individual animal tumor diagnoses, statistical analysis of primary neoplasms that occurred with an incidence of at least 5% in at least one animal group, and historical incidences for the biologically significant neoplasms mentioned in this section are presented in Appendix D. No neoplasms or gross pathology lesions in male or female mice were considered to be related to DBP exposure.

Testis: In the testis, the incidences of the combination of unilateral and bilateral degeneration of the germinal epithelium were higher in all exposed groups compared to the control group, but the increases were significant only in the 1,000 and 10,000 ppm groups (Table 22). The incidence of bilateral (only) germinal epithelial degeneration was also higher in all exposed groups compared to the control group but was statistically significant only in the 10,000 ppm group (Appendix D).

Microscopically, degeneration of the germinal epithelium (Figure 12) consisted variably of scattered loss of germ cells (round and/or elongating spermatids), seminiferous tubules with vacuoles (variable in size and number), degenerating germ cells with eosinophilic cytoplasm, disorganization of germinal epithelium, occasional sloughing of germ cells, and occasional multinucleated germ cells.

Table 22. Incidences of Nonneoplastic Lesions of the Testis, Epididymis, Coagulating Gland, and Seminal Vesicle in Male Mice in the Two-year Feed Study of Di-*n*-butyl Phthalate

	0 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Testis[a]	50	50	50	50
Germinal epithelium, degeneration (includes bilateral)[b]	6 (2.2)[c]	15* (1.8)	9 (2.0)	15* (2.2)
Epididymis	50	50	50	50
Duct, exfoliated germ cell (includes bilateral)	6* (1.3)	10 (1.3)	5 (2.0)	13* (1.6)
Hypospermia (includes bilateral)	1 (3.0)	4 (3.5)	3 (3.3)	3 (3.0)
Duct, atrophy, bilateral[d]	0	1 (3.0)	3 (3.0)	1 (3.0)
Coagulating Gland[e]	0	1	4	1
Atrophy, bilateral[d]	0	1 (3.0)	3 (3.0)	1 (3.0)
Seminal Vesicle	50	50	50	50
Atrophy, bilateral[d]	1 (3.0)	1 (3.0)	3 (3.0)	1 (3.0)

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group.
Statistical significance for the vehicle control group indicates a significant trend test.
*Statistically significant at $p \leq 0.05$ by the Poly-3 test.
[a]Number of animals with tissue examined microscopically.
[b]Incidence reported is the combination of unilateral and bilateral lesions. Severity grade for these types of lesions = sum of unilateral and bilateral severity scores/number of unilateral and bilateral incidences.
[c]Average severity grade of observed lesion in affected animals: 1 = minimal; 2 = mild; 3 = moderate; 4 = marked.
[d]This lesion was observed only as a bilateral lesion.
[e]Only tissues from animals with gross lesions of the indicated organ were collected at necropsy for histopathological evaluation.

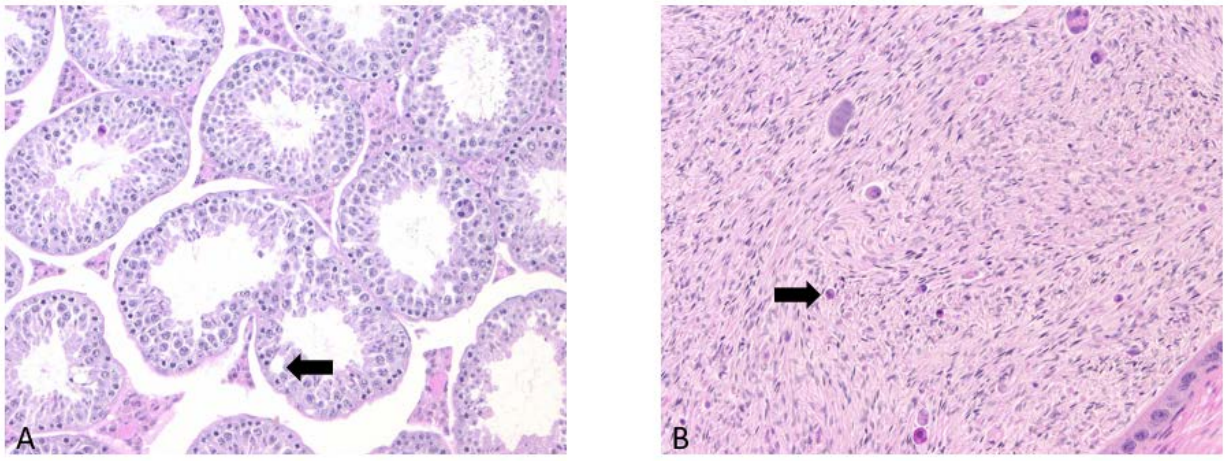


Figure 12. Representative Images Depicting Degeneration of the Germinal Epithelium of the Testis in a Male Mouse in the Two-year Feed Study of Di-*n*-butyl Phthalate

A) Degeneration of the germinal epithelium of the testis from a 10,000 ppm male mouse. The germinal epithelium is vacuolated (arrow). B) Exfoliated germ cells in the epididymal duct lumen from the same mouse in panel A. Exfoliated germ cells (arrow) and debris are present among the spermatozoa. Original magnification: A = 10x, B = 20x.

Epididymis: In the epididymal duct, the combined incidence of unilateral and bilateral exfoliated germ cells was higher in the 1,000 and 10,000 ppm groups compared to the control group, but

the incidence was statistically significant only in the 10,000 ppm group (Table 22). The incidence of bilateral (only) exfoliated germ cells was higher in all exposed groups compared to the control group, but none of the increases were statistically significant (Appendix D). The incidences of hypospermia and duct atrophy in the epididymis were also higher in all exposed groups compared to the control group (Table 22). Although these changes were small and not statistically significant and although severity did not increase with increasing exposure concentration, they were considered to be related to DBP exposure.

The microscopic lesion of exfoliated germ cells in the epididymis (Figure 12) was characterized by the presence of degenerating germ cells and cell debris in the epididymal lumen. Exfoliated germ cells were diagnosed when numbers were increased relative to the low concentration occurring in the control animals. When germinal epithelial damage to the testes occurs, it is often accompanied by the presence of exfoliated germ cells in the epididymides. In most mice diagnosed with testicular germinal epithelium degeneration, corroborating lesions of increased exfoliating germ cells and/or low incidences of hypospermia in the epididymis that reinforced a testicular effect were seen. Hypospermia was characterized microscopically by reduced sperm content throughout the epididymis and often was accompanied by reduced size of the duct lumen and exfoliated germ cells. Hypospermia coincided with diagnoses of germinal epithelium degeneration in the testis in all cases. In fewer cases, hypospermia also was diagnosed with seminal vesicle and/or coagulating gland atrophy or epididymal duct atrophy. Epididymis duct atrophy appeared as a diffuse collapse of the duct lumen, generally empty duct profiles, decreased epithelial height, and increased and prominent mesenchymal stroma. The animals with epididymal atrophy also had concomitant testicular degeneration and interstitial cell atrophy, hypospermia, and seminal vesicle and coagulating gland atrophy.

Accessory Sex Organs: The incidence of bilateral atrophy of the coagulating gland was slightly higher in the exposed groups compared to the control group, and the incidence of bilateral atrophy of the seminal vesicle was slightly higher in the 3,000 ppm group compared to the control group (Table 22). Although these changes were small and not statistically significant and although severity did not increase with increasing exposure concentration, they were considered to be related to DBP exposure.

Microscopically, atrophy of the seminal vesicle or coagulating gland was characterized by reduced lumina and crowding of intravesicular epithelial folds, reduced or absent luminal secretory material, and increased stroma. The atrophy was considered of moderate severity for both tissues and was always bilateral. These lesions are commonly seen with chronic androgen depletion.[146]

Liver: Cytoplasmic alteration of hepatocytes was observed only in the 10,000 ppm males and females, and the incidences of these lesions were significantly increased compared to the control groups (Table 23). In all cases, the severity of this lesion was considered minimal. The cytoplasmic alteration was characterized by increased amounts of cytoplasm with fine eosinophilic granules, mainly in the centrilobular regions of the liver. In the male mice, the incidences of multinucleated hepatocytes (Figure 13) were significantly increased in all exposed groups compared to the control group (Table 23). Only the incidences in the 3,000 and 10,000 ppm groups were statistically significant. Multinucleated hepatocytes with up to three or, rarely, four nuclei can be seen as a background lesion in mice, but the multinucleated hepatocytes considered related to DBP exposure were much larger and contained up to 20 nuclei.

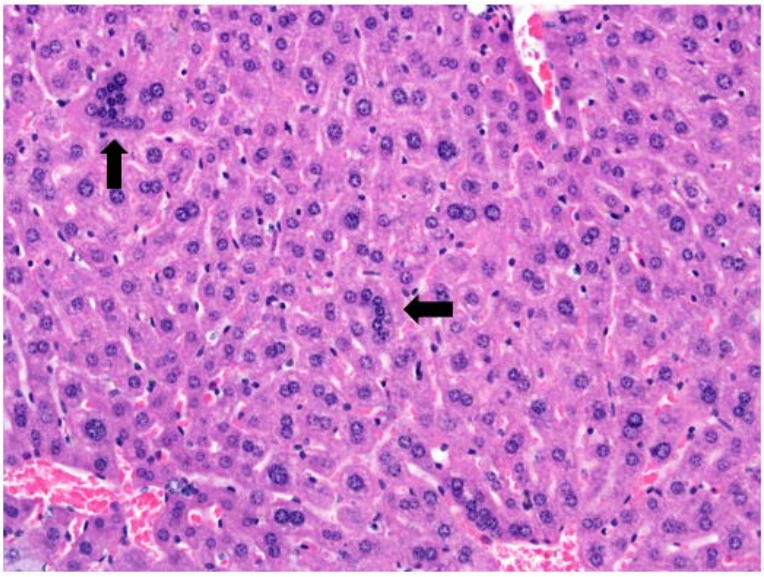


Figure 13. Representative Image Depicting Multinucleated Hepatocytes in the Liver of a Male Mouse in the Two-year Feed Study of Di-*n*-butyl Phthalate

Multinucleated hepatocytes in the liver from a 10,000 ppm male mouse (arrows). Original magnification: 20x.

Table 23. Incidences of Nonneoplastic Lesions of the Liver and Kidney in Male and Female Mice in the Two-year Feed Study of Di-*n*-butyl Phthalate

	0 ppm	1,000 ppm	3,000 ppm	10,000 ppm
Male				
Liver[a]	50	50	50	50
Hepatocyte, cytoplasmic alteration[b]	0**	0	0	36** (1.0)[c]
Hepatocyte, multinucleated	8** (1.0)	11 (1.0)	25** (1.0)	42** (1.0)
Female				
Liver	50	50	49	49
Hepatocyte, cytoplasmic alteration	0**	0	0	48** (1.0)
Kidney	50	50	49	49
Renal tubule, hyperplasia	0**	0	0	47** (1.0)

Statistical significance for an exposure group indicates a significant pairwise test compared to the vehicle control group.
Statistical significance for the vehicle control group indicates a significant trend test.
**Statistically significant at $p \leq 0.01$ by the Poly-3 test.
[a]Number of animals with tissue examined microscopically.
[b]Number of animals with lesion.
[c]Average severity grade of observed lesion in affected animals: 1 = minimal; 2 = mild; 3 = moderate; 4 = marked.

Kidney: In the female mice, hyperplasia of the renal tubule epithelium was observed, and the incidences were significantly increased in the 10,000 ppm group; this lesion was not seen in any other group, including the control group (Table 23). In all cases, the severity was considered minimal. This lesion is unique and is distinct from the tubular epithelial hyperplasia found in association with chronic progressive nephropathy. It is characterized by segmental hyperplasia of the epithelium in tubules at the corticomedullary junction (Figure 14). Affected renal tubules

often were dilated and lined by tightly crowded epithelial cells. The cells also were slightly enlarged with increased height and cytoplasmic volume. Some cells also had slightly enlarged nuclei, and some were multinucleated.

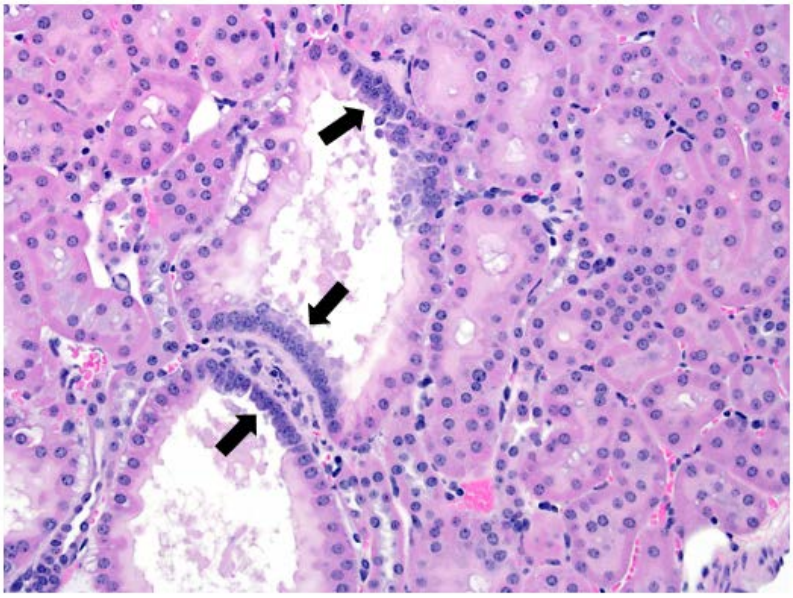


Figure 14. Representative Image Depicting Renal Tubule Hyperplasia in the Kidney in a Female Mouse in the Two-year Feed Study of Di-*n*-butyl Phthalate

Renal tubule hyperplasia in the kidney of a 10,000 ppm female mouse. There are crowded, basophilic epithelial cells lining portions of the dilated renal tubules (arrows). Original magnification: 20x.

Discussion

The use of di-*n*-butyl phthalate (DBP) in the manufacture of various consumer products has resulted in widespread exposure, as evidenced by its detection in human urine, plasma, and umbilical cord blood. DBP and other phthalates have well-documented effects on the male reproductive system, particularly with perinatal exposure.[147] Data to evaluate DBP as a human carcinogen, however, are inadequate. To address this knowledge gap, the National Toxicology Program (NTP) conducted a 2-year bioassay in mice and rats. Given the widespread exposure and known effects of phthalates on male reproduction, perinatal exposure was included in the rat study. To emulate the primary route of exposure in humans, animals were exposed to DBP via feed.

Chemical consumption of DBP was calculated on the basis of feed consumption measurements. Few differences were found in feed consumption between control and exposed groups in rats and mice. Chemical consumption between sexes was similar in both species and increased with increasing exposure concentration in a generally proportional manner. Mice were exposed to twice as much DBP as rats fed the same concentration in feed due to differences in feed consumption.

Even though chemical consumption was linearly related to the exposure concentrations, mono-*n*-butyl phthalate (MBP) concentrations measured in biological matrices in rats did not increase linearly or in proportion to exposure concentration. For example, MBP concentrations in dam plasma on gestation day (GD) 18 in the 3,000 ppm group were 17-fold higher than concentrations in the 300 ppm group; in the 10,000 ppm group, dam plasma MBP was 36-fold higher than that in the 1,000 ppm group. Because DBP is primarily converted to MBP through first-pass metabolism, the nonproportional increase in MBP concentrations suggests a saturation of phase 2 metabolism and clearance processes at higher exposure concentrations (i.e., less MBP-glucuronide formed) and/or an induction of absorption process transporters. In this study, only unconjugated forms of MBP were assessed. Measuring both unconjugated and conjugated forms of MBP would help to inform the relationship between internal and external dose.

Although no DBP was administered via feed to control animals, MBP was detected in the plasma of some control rats. Because phthalates are ubiquitous in food packaging and many plastics, the presence of MBP in control animals could be due to exposure to background levels of DBP in the feed (although all measurements of DBP in the control diet were below the limit of quantitation). Another possibility is contamination during sample collection or analysis. The concentrations of MBP in control animals, however, were very low compared to those in exposed animals and are likely not a confounder in the study.

The exposure concentration of DBP and the internal concentration of MBP in rats in this study were much higher than those observed in humans. Given that estimated human intake of DBP is 0–7 μg/kg body weight/day (μg/kg/day)[22] and that gestational and lactational DBP consumption in this study ranged from 19 to 66 mg/kg/day in the lowest exposure group (300 ppm), the exposure multiple comparing rats to humans is at least 3,000. Similarly, concentrations of MBP measured in the rat plasma were much higher than concentrations observed in human plasma. The GD 18 maternal plasma concentration in rats at the lowest exposure concentration was 263-fold higher than plasma concentrations observed in pregnant Czech women.[17] Gestational

transfer of DBP and/or MBP was observed in this study, consistent with previous studies on gestational transfer in rodents.[39; 41] Lactational transfer also was observed, although less was transferred during lactation than during gestation.

Transfer during gestation and lactation is suggested in humans, as MBP has been detected in human amniotic fluid and breast milk.[19; 20; 148; 21; 18] In samples taken from 54 women during routine amniocentesis, the median MBP concentration in amniotic fluid was 5.8 ng/mL with a highest measured value of 263.9 ng/mL.[18] In another study, amniotic fluid samples from 100 women in Crete, Greece, contained, on average, 12 ng/mL MBP with a maximum of 24.5 ng/mL.[148] Exposure in this group of women was estimated to be 0.9 µg DBP/kg/day based on questionnaires and on measured urinary metabolite concentrations. Therefore, despite the much higher intake of DBP in rats, the exposure of the rodent fetus to MBP via amniotic fluid from dams in the lowest exposure group (84.5 ng/mL) is within an order of magnitude of the concentrations to which some human fetuses could be exposed.

In the rats, exposure to DBP during gestation had minimal effects on dam mean body weight, dam survival, and littering parameters. During lactation, there were minor decreases in dam mean body weight, mean body weight gain, and feed consumption in the 10,000 ppm group. The lower feed consumption in the later days of lactation could be due to less feed consumption by the pups. Chemical consumption by the pups might have contributed to the lower pup mean body weight at postnatal day (PND) 14 and PND 21 in the 10,000 ppm group, given the higher chemical-to-body-weight concentration in young animals. Findings here are similar to those from perinatal subchronic studies previously reported in NTP Toxicity Report 30 in which F_0 fertility and gestation length of rats and mice were not affected by exposure at concentrations up to 10,000 ppm, but pup number, dam mean body weight, and pup mean body weight were significantly decreased in the 10,000 ppm group compared to the control group.[49] These effects were observed in lower exposure groups (1,000 and 5,000 ppm) in the NTP reproductive and continuous breeding studies, particularly in later litters and in the F_1 generation, suggesting a compounding effect of long-term DBP exposure on reproductive capacity.[79]

During the 2-year study phase, there were no decreases in survival related to DBP exposure. The only differences in mean body weight were observed in the highest dietary exposure groups (10,000 ppm) in rats and mice. In both species, mean body weights of animals in the highest exposure groups were affected, with exposed females having marginally larger deficits in mean body weight than males compared to the respective control groups. Furthermore, female mice had the largest discrepancy in mean body weight compared to the control group. Two other studies have evaluated the chronic effects of DBP exposure (≥1 year) in adult male rats[31] and in male rats exposed only in utero.[63] In both studies, no clear dose-dependent changes in mortality were found. In Smith et al.[31] in which adult rats were administered DBP for 1 year, no differences in body weight due to DBP were reported. In Barlow et al.,[63] body weight was not reported. Studies in female animals are not available for comparison. Findings from this study suggest that female mice could be more susceptible to DBP effects on body weight than male mice.

DBP exposure might have resulted in an increase in the incidence of pancreatic acinus adenomas in male rats, as a statistically significant positive trend was found. Although the incidence in the 10,000 ppm group was within the historical control range and there was no concurrent increase in the incidence of pancreatic acinus hyperplasia, this positive trend is consistent with reported

effects of other phthalates. Pancreatic acinus adenomas and acinus carcinomas have been observed with exposure to other phthalates and peroxisome proliferator-activated receptor alpha (PPARα) agonists. Di-(2-ethylhexyl) phthalate (DEHP) and butyl benzyl phthalate (BBP) induced pancreatic acinus adenomas and acinus carcinomas in male Fischer 334/N rats.[149; 150] These neoplasms and/or acinus hyperplasia were also reported following chronic exposure to other PPARα agonists, such as perfluorooctanoic acid,[151] Wyeth-14,643,[151] and nafenopin.[152] The marginal increase in the incidence of pancreatic acinus adenomas was considered equivocal evidence of carcinogenicity in male rats because of observations of similar pancreatic neoplasms with exposure to other phthalates and other agonists of the proposed mechanistic pathway.

The mode of action underlying the increase in the incidence of pancreatic acinus adenomas is not certain, but studies suggest that PPARα agonists alter bile acid synthesis or decrease bile flow, leading to hepatic cholestasis. The altered bile acids or decreased bile acid flow lead to increased secretion of cholecystokinin, which acts as a growth factor on pancreatic acinar cells in rats.[153-156] Sustained release of cholecystokinin might lead to increased proliferation of acinar cells. Indeed, phthalates like DEHP, DBP, BBP, and their metabolites (mono(2-ethylhexyl) phthalate [MEHP], MBP, and monobenzyl phthalate [MBzP], respectively) bind to PPARα nuclear receptors.[157; 60] MEHP is the most effective of the phthalate esters in activating mouse and human PPARα, followed by MBzP, and then MBP.[157] Docking studies have also found DEHP and BBP to have stronger binding affinities to PPARs than DBP.[158] Therefore, the small increase in the incidence of pancreatic acinus adenomas observed with DBP compared to stronger agonists could be due to the relatively lower binding affinity of DBP for PPARα.

In previous studies conducted by NTP, DBP exposure for 3 months appeared to activate hepatic PPARα in rats. At 5,000 and 10,000 ppm DBP, male and female rats had elevated peroxisome enzyme activity, increased liver weight, and hepatocellular cytoplasmic alterations at the end of the study.[49] Although peroxisome enzyme activity was not measured in the present study, the increased incidences of hepatocyte cytoplasmic alteration in male and female animals at the highest exposure concentration (10,000 ppm) suggests PPARα activation by DBP. This activation, however, was apparently insufficient to stimulate hepatic neoplasm growth in this study. In support of this hypothesis, chronic exposure to BBP—a weaker PPARα agonist relative to DEHP but stronger than DBP—did not produce hepatic neoplasms in rats.[149] Hepatic neoplasms have been reported with exposure to DEHP.[150]

In rats and mice of both sexes, cytoplasmic alteration of hepatocytes was present in nearly all animals in the 10,000 ppm groups. This subtle lesion, visible as fine, eosinophilic granules in affected hepatocytes, was graded as minimal severity in all animals. Similar cytoplasmic alteration of hepatocytes was previously reported in the 3-month DBP dosed feed study, and ultrastructural examination suggested the presence of increased numbers of peroxisomes in the cytoplasm.[49] An increase in the number and size of multinucleated hepatocytes was also observed in the livers of exposed male mice, but the biological relevance of this lesion is unclear.

In utero exposure to DBP and other phthalates has been shown to induce developmental malformations in male rats, collectively referred to as the "phthalate syndrome."[159] Phthalate syndrome is characterized by malformations in reproductive tissues and is considered to be androgen-dependent or *Insl3*-dependent.[160] *Insl3* is a peptide hormone synthesized by fetal Leydig cells. Phthalate syndrome includes malformations of the epididymis, penis (hypospadia), prostate, seminal vesicles, and vas deferens; reduced anogenital distance; retention of

nipples/areolae (all considered androgen-dependent), gubernacular abnormalities (considered to be *Insl3*-dependent), and undescended testes (considered both androgen- and *Insl3*-dependent).[63; 160; 70]

Characteristics of phthalate syndrome were observed in this study. Gross pathology findings at necropsy generally were limited to the male reproductive tract in rats (Table 14). These findings included decreased size of testis, epididymis, seminal vesicle, or prostate gland; gubernacular length exceeding 20 mm; segmental or complete agenesis of the epididymis (caput, corpus, or cauda); agenesis of the prostate gland (ventral lobes); testis not present (at necropsy and synonymous with agenesis); vas deferens not present; and undescended (cryptorchid) testes. The most frequently occurring gross findings related to DBP exposure in the rat were undescended testes and small testes, both of which were present in most of the 10,000 ppm male rats. Undescended testes were more frequently seen in abdominal locations than in inguinal ones. Increased gubernacular length, observed in about half the 10,000 ppm males, is associated with failure of testes to fully descend. The undescended testes observed in DBP-exposed male rats were consistent with previously reported decreased androgens and *Insl3* in DBP-exposed males; both are involved in testis descent.[63; 160; 70] The undescended testes were generally smaller and corresponded to decreased testis weights and, histologically, with germinal epithelial atrophy. In this 2-year study with perinatal exposure in the rats, ascertaining whether the decreased size of testes seen at necropsy is due to testicular atrophy, which is the decrease in size after having attained full size, or from hypoplasia, in which the tissue never developed properly, is difficult. The small epididymides observed grossly corresponded to reduced epididymis weights and hypospermia microscopically. As with the testis, however, the possibility of epididymis hypoplasia cannot be ruled out, especially given the male reproductive tract malformations observed in rats.

The male reproductive tract was a target of DBP exposure in both rats and mice. Lesions were generally more severe in rats than in mice. For example, at 10,000 ppm, 42 of 50 male rats had testicular germinal epithelial atrophy (seminiferous tubules containing only Sertoli cells and few or no germ cells), which is the end stage of degeneration. In contrast, the main testicular lesion in male mice at 10,000 ppm was germinal epithelial degeneration (15/50), with far fewer incidences of atrophy and epididymal hypospermia in the mice compared to the rats. In rats, testicular germinal epithelial atrophy was generally concomitant with epididymal hypospermia (42/50 and 40/50, respectively, at 10,000 ppm). In mice, testicular germinal epithelial degeneration was generally concomitant with exfoliated germ cells in the epididymal duct (15/50 and 13/50, respectively, at 10,000 ppm), with few incidences of epididymal hypospermia in mice. In rats, 18 of 42 animals with germinal epithelial atrophy also had edema in the testis, which was observed at a significantly increased incidence in 10,000 ppm male rats than in control animals. Testicular edema was not observed in male mice. This species effect of increased sensitivity of rats compared to mice for testicular toxicity of phthalates has been demonstrated previously.[81]

In addition to testicular atrophy (rats) or degeneration (mice) and their corroborating epididymal lesions, additional microscopic lesions in the male reproductive tracts of rats and mice likely were due to DBP exposure. Incidences of decreased secretory fluid in the prostate gland or seminal vesicles were significantly increased in the 10,000 ppm rats compared to the control rats. Similar incidences of atrophy in the coagulating gland or seminal vesicle were observed in a few exposed mice, but they were not significantly increased compared to the control group. Decreased incidences of secretory fluid or atrophy of the prostate gland or seminal vesicles are

commonly found with chronic androgen depletion.[146] Again, hypoplasia of the accessory glands cannot be ruled out in the perinatal rat study because the diagnostic features of hypoplasia and decreased secretory fluid are similar. In general, however, the overall structure of the prostate glands and seminal vesicles was considered normal rather than malformed; therefore, the diagnosis was decreased secretory fluid rather than hypoplasia.

Lesions suggestive of systemic hormonal disturbance, including disturbance of the hypothalamus-pituitary-gonad axis, included diffuse interstitial (Leydig) cell hyperplasia in the testis and hypertrophy in the pars distalis of the pituitary gland in male rats exposed to DBP. Diffuse testicular interstitial hyperplasia, which was always present in both testes when diagnosed, was seen in one male rat at 1,000 ppm and nine male rats at 10,000 ppm. The pathogenesis of diffuse interstitial cell hyperplasia is generally related to a compensatory physiological response to hormonal imbalance.[146] Specifically, decreased systemic testosterone can cause a decrease in the normal negative feedback of testosterone on the hypothalamus-pituitary-gonad axis. This, in turn, leads to increased release of gonadotropin-releasing hormone by the hypothalamus and, subsequently, increased luteinizing hormone and follicle-stimulating hormone release by gonadotrophs in the pars distalis of the pituitary gland. This stimulation of gonadotrophs in the pars distalis might have led to the development of the lesion of hypertrophied and, often, vacuolated cells in the pituitary gland pars distalis ("gonadectomy" or "castration" cells) observed in 29 of 50 male rats exposed to 10,000 ppm DBP. Increased luteinizing hormone, owing to lack of the normal negative feedback of testosterone on the pars distalis, might have resulted in a stimulatory response of the Leydig cells, which produce testosterone, resulting microscopically in diffuse interstitial (Leydig) cell hyperplasia. Phthalates have been shown to decrease testosterone production by fetal Leydig cells.[69] Reduced intratesticular testosterone production by fetal Leydig cells might result in compromised adult Leydig cell function. In utero exposure of rats to DBP has been demonstrated to reduce the number of adult Leydig stem cells by approximately 40% at birth to adulthood and affect their function by inducing compensated adult Leydig cell failure (low/normal serum testosterone and elevated luteinizing hormone).[161]

Certain microscopic lesions observed in the 2-year perinatal exposure study of DBP in rats are considered developmentally induced lesions resulting from in utero exposure. These lesions, which were not observed in mice, include dysgenesis of the seminiferous tubules and granuloma and fibrosis of the rete testis. These lesions have also been reported in a rat perinatal 2-year study of DEHP.[150] In the current study of DBP, incidences of testicular seminiferous tubule dysgenesis were significantly increased at 10,000 ppm (9/50), with an additional occurrence at 3,000 ppm (Table 15). Testicular dysgenesis has been described in the literature as having occurred after in utero exposure to various phthalates during the masculinization programming window (embryonic day [E] 15.5–18.5 in rats).[63; 162-165] Seminiferous tubule dysgenesis is characterized as a developmental malformation observed microscopically as aberrant or misshapen seminiferous tubules, either with no lumens or dilated lumens, which are often surrounded by focal Leydig cell aggregates. The Leydig cell aggregates within foci of dysgenesis differ morphologically from the Leydig cells in adenomas. The Leydig cells in the foci of dysgenesis appear to be poorly differentiated, are spindle-shaped, and resemble embryonic Leydig cells, and they do not have the abundant eosinophilic or vacuolated cytoplasm often apparent in hyperplasia or adenoma.[63] Dysgenetic lesions can occur as one or more small foci per testis, which tend to be located near the center of the testis or may occupy the entire testis.[63] The

malformed tubules can appear to form anastomotic networks. The dysgenetic tubules contain poorly differentiated Sertoli cells, with small, elongated, and sometimes cleaved nuclei and less prominent nucleoli than the typically prominent tripartite nucleoli of mature Sertoli cells. Spermatogenesis is absent in these foci of dysgenesis but can be present elsewhere in the testis. Dysgenetic foci can be present in one or both testes and can be more severe in undescended testes than scrotal testes.[163; 165] In human males, similar microscopic dysgenetic foci have been reported in undescended testes,[166] in testes also containing testicular cancer (both scrotal and undescended testes[167]), and from testicular biopsies from the contralateral testis in men undergoing orchiectomy for testicular cancer.[168]

The microscopic lesion of dysgenesis appears to originate during gestation but is not evident in the embryonic testis; it becomes evident as malformed tubules only in early postnatal life.[163] In rats exposed in utero to phthalates, immunohistochemical markers have been used to demonstrate Sertoli cells and gonocytes at ectopic locations within the interstitium and to identify Leydig cells at ectopic locations inside the malformed seminiferous tubules.[163; 169; 164; 165] Rats exposed in utero to DBP showed normal formation of seminiferous cords between E 13.5 and E 14.5, followed by abnormal fetal Leydig cell aggregation at E 17.5.[169] At E 18.5, based on triple staining for Sertoli cells, gonocytes, and peritubular myoid cells, the seminiferous cords in exposed rats appeared to rupture, releasing their contents (i.e., Sertoli cells and gonocytes) into the interstitium. The cords appeared normally formed at one end, with a normal smooth muscle, actin positive, and peritubular myoid layer, and appeared ruptured at the other end, with loss of smooth muscle actin staining.[169] The ectopic Sertoli cells and gonocytes appear in late gestation and disappear early in postnatal life, but the ectopic intratubular Leydig cells can persist throughout postnatal life.

Additional lesions considered related to in utero DBP exposure include rete testis fibrosis and rete testis sperm granuloma. In this and other DBP studies, sperm stasis in the rete testis appears to lead to formation of sperm granulomas and, subsequently, fibrosis in the rete testis region.[63; 170] Fibrosis of the rete testis is a unique lesion that presumably begins as a sperm granuloma and resolves into a fibrotic lesion with increased fibrous connective tissue containing numerous embedded epithelial-lined profiles of the rete testis.[63; 170] The mechanism is unknown but could occur as a result of toxicity and efferent duct occlusion. The efferent ducts, however, were not evaluated in the current study. The sperm granulomas or fibrosis of the rete testis, which did not always occur together, were co-incident with the lesion of seminiferous tubule dysgenesis in five of the nine animals with dysgenesis, so the rete testis and dysgenetic lesions might be linked to developmental exposure to DBP.

DBP exposure also resulted in a unique renal tubular hyperplastic lesion in female mice but not in male mice or in male or female rats. This lesion has been reported in a previous NTP study on peroxisome proliferators.[171] Ozaki et al.[171] reported this lesion in male rats and mice exposed to Wyeth-14,643 (a prototypical peroxisome proliferator) or 2,4-dichlorophenoxyacetic acid (2,4-D, a weak peroxisome proliferator). In contrast to the current study, in which the lesion was not observed in rats, Ozaki et al.[171] reported that rats were more sensitive to the renal tubular effects of the two peroxisome proliferators tested. Similar to DBP, 2,4-D did not cause hepatic lesions typical of peroxisome proliferators.

Conclusions

Under the conditions of these 2-year feed studies, there was *equivocal evidence of carcinogenic activity* of di-*n*-butyl phthalate (DBP) in male Hsd:Sprague Dawley® SD® rats based on marginal increases in the incidence of pancreatic acinus adenomas. There was *no evidence of carcinogenic activity* of DBP in female Hsd:Sprague Dawley® SD® rats at exposure concentrations of 300, 1,000, 3,000, or 10,000 ppm.

There was *no evidence of carcinogenic activity* of DBP in male or female B6C3F1/N mice at exposure concentrations of 1,000, 3,000, or 10,000 ppm.

Exposure to DBP increased incidences of gross lesions in the male reproductive system in rats and of nonneoplastic lesions in the male reproductive system (rats and mice), liver (male and female rats and mice), pituitary gland pars distalis (male rats), and kidney (female mice).

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Appendix A. Chemical Characterization and Dose Formulation Studies

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Tables

Figures

A.1. Procurement and Characterization of Di-*n*-butyl Phthalate

Di-*n*-butyl phthalate (DBP) was obtained from Sigma-Aldrich (St. Louis, MO) in a single lot (lot MKBB8432). Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory at RTI International (Research Triangle Park, NC). Reports on analyses performed in support of the DBP studies are on file at the National Institute of Environmental Health Sciences.

The appearance, a clear liquid, and density of lot MKBB8432 (1.05 g/mL at 21.7°C) matched that of DBP (1.043 g/mL at 25°C). The boiling point of lot MKBB8432 (335°C) matched that of DBP (340°C), and elemental analysis confirmed the anticipated relative ratios; both analyses were performed by Galbraith Laboratories (Knoxville, TN). A precise molecular mass was measured using a research-grade high-resolution mass spectrometer (HRMS) at the University of South Carolina Mass Spectrometry Facility (Columbia, SC). The observed mass values (278.1514) were within acceptable limits (≤5 ppm) of the calculated mass (278.1518).

The lot was identified using infrared (IR) spectroscopy. In addition, the lot was analyzed using ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy and gas chromatography (GC) with mass spectrometry (MS) detection. The IR spectrum was in good agreement with the structure of DBP and with the reference spectrum of DBP from Sigma-Aldrich (Product No. 524980, accessed December 22, 2009) (Figure A-1). ^{1}H and ^{13}C NMR spectra were consistent with the structure of DBP and the prediction from Advanced Chemistry Development's Spectral Prediction Program (Version 10.02, Toronto, Ontario, Canada) (Figure A-2, Figure A-3). GC/MS identified the major peak of lot MKBB8432 as DBP using fragmentation pattern and comparison with the National Institute of Standards and Technology (Gaithersburg, MD) reference spectrum (No. 312145) for DBP (Table A-1; System A).

The moisture content of lot MKBB8432 was determined by Karl Fischer titration. The purity of lot MKBB8432 was determined using ultra-performance liquid chromatography (UPLC) with photodiode array detection (PDA) and GC with flame ionization detection (FID) (Table A-1; Systems B and C, respectively). The Karl Fischer titration yielded a water content of 0.204%. UPLC analysis demonstrated one major peak accounting for 99.9% and no minor peaks >0.1% of the total integrated area, although one minor impurity (<0.1%) was observed. GC/FID analysis also found one major peak accounting for 99.9% and no minor peaks >0.1%. The overall purity of lot MKBB8432 was determined to be >99%.

Accelerated stability studies were conducted on samples of DBP by the analytical chemistry laboratory using lot 91997PJ from Sigma-Aldrich (St. Louis, MO) stored at ambient temperature (approximately 22°C), refrigerated temperature (approximately 5°C), and elevated temperature (approximately 60°C) in amber vials. After 14 days, samples were analyzed by GC/FID (Table A-1; System C). Stability of DBP was confirmed for at least 2 weeks when stored in sealed glass vials at temperatures from 5°C to 60°C. Upon receipt, the bulk chemical of lot MKBB8432 was homogenized by mixing for 15 minutes and transferred to 1-gallon amber storage bottles stored at room temperature. Periodic analyses of the bulk chemical lot MKBB8432 were performed prior to and during the animal studies by the laboratory using high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection (Table A-1; System D), and no degradation of the test chemical was detected.

A.2. Preparation and Analysis of Dose Formulations

The base diet was meal feed purchased from Zeigler Brothers, Inc. (Gardners, PA). The 2-year rat and mouse studies used NTP-2000 feed (24 lots milled June 2010 through June 2012), whereas NIH-07 feed (two lots milled May and June 2010) also was used during the perinatal phase of the rat study. No analysis was performed on the feed beyond determining suitability for feeding the animals.

Dose formulations were prepared monthly by mixing DBP with feed (Table A-2). For the perinatal and 2-year rat study, formulations were prepared in NIH-07 feed at concentrations of 0, 300, 1,000, 3,000, and 10,000 ppm (July 8 and July 29, 2010) and in NTP-2000 feed at concentrations of 0, 300, 1,000, 3,000, and 10,000 ppm (27 formulations; August 2010 to August 2012). For the 2-year mouse study, formulations were prepared in NTP-2000 feed at concentrations of 0, 1,000, 3,000, and 10,000 ppm (27 formulations; August 2010 to August 2012).

Homogeneity studies of the 300 and 10,000 ppm dose formulations in 72 kg NIH-07 feed batch sizes, 300 ppm dose formulation in a 92 kg NTP-2000 feed batch size, and 1,000 and 10,000 ppm dose formulations in 60 kg NTP-2000 feed batch sizes were performed before the animal studies by the study laboratory using HPLC/UV (Table A-1; System D). Additional homogeneity studies for different batch sizes were performed during the 2-year studies by the study laboratory with the same HPLC/UV system: batch size of 60 kg for the 300 ppm dose formulation in NTP-2000 feed (December 2010) and batch size of 72 kg for the 10,000 ppm dose formulation in NTP-2000 feed (July 2012). All formulations analyzed were determined homogeneous and of appropriate concentration. Stability was confirmed for 42 days at room temperature (approximately 25°C).

The plastic bags used for preparation and storage of animal feed and dose formulations were analyzed for the presence of 11 different phthalates commonly found in plastics, including DBP, by the analytical chemistry laboratory at RTI International (Research Triangle Park, NC). Analysis of the extracts from plastic bags used by the study laboratory in the preparation and storage of control and dosed feed showed no DBP above the limit of detection (1.47 ppm) (Table A-1; System B). The developed method suggested no significant contribution of DBP from the plastic storage bags.

Control and treated formulations were stored in individual plastic bag-lined containers at room temperature (approximately 25°C). The formulations were used within 42 days of preparation.

Periodic analyses of the preadministration dose formulations of DBP were conducted by the study laboratory every 1 to 3 months to determine purity, whereas postadministration (animal room) samples were analyzed every 1 to 8 months (Table A-3, Table A-4). All preadministration formulations for rats and mice were within 10% of the target concentrations. In the perinatal and 2-year rat study, all postadministration barrel samples were within 10% of target concentration, except for the 10,000 ppm formulation prepared on August 9, 2010, which was 13.2% below the target concentration. Postadministration samples collected from residual feed in the feeders prepared July 8, 2010 (10,000 ppm), August 9, 2010 (300, 1,000, and 10,000 ppm), and February 21, 2011 (10,000 ppm) ranged from 10.7% to 13.4% below the target concentrations. All other postadministration values were within 10% of the target concentration. In the 2-year

mouse study, postadministration samples of the 1,000 and 10,000 ppm formulations prepared on August 9, 2010, collected from residual feed in the feeders, were up to 13.4% below the target concentrations. Additionally, the 10,000 ppm barrel sample was 13.2% below the target concentration. The postadministration barrel sample from the 10,000 ppm formulation prepared on February 21, 2011 was 12.1% below the target concentration. All other postadministration values were within 10% of the target concentration.

Table A-1. Chromatography Systems Used in the Perinatal and Two-year Feed Studies of Di-*n*-butyl Phthalate

Chromatography	Detection System	Column	Mobile Phase
System A			
Gas chromatography	Mass selective detector	J&W DB-1 (25 m × 0.32 mm ID, 0.25 μm film thickness)	Helium, 1.65 mL/min flow rate
System B			
Ultra-performance liquid chromatography	Photodiode array detector (205 to 400 nm, extracted at 225 nm)	Waters Acquity UPLC BEH Phenyl (2.1 mm ID × 50 mm, 1.7 μm particle size), with Waters Acquity In-Line Filter (0.2 μm)	A: Methanol B: Water Gradient program: A:B 25:75 to 75:25 in 3 min, hold at 78:22 for 1 min, ramp to 100:0 in 1 min, hold at 100:0 for 1 min, reverse to 25:75 in 0.5 min, hold at 25:75 for 1.5 min 0.6 mL/min flow rate
System C			
Gas chromatography	Flame ionization detection (325°C)	J&W HP-5 (30 m × 0.32 mm ID, film thickness 0.25 μm)	Helium, 1 mL/min flow rate
System D			
High-performance liquid chromatography	Ultraviolet (225 nm)	Thermo Scientific Hypersil Phenyl (250 mm × 4.6 mm ID, 5 μm) with Hypersil Phenyl guard (5 μm),	A: Methanol B: ASTM Type I Water Gradient program: A:B 70:30 to 85:15 in 5 min, ramp to 100:0 in 4 min, hold at 100:0 for 4 min, reverse to 70:30 in 0.1 min, hold at 70:30 for 10.9 min 1.0 mL/min flow rate

UPLC = ultra-performance liquid chromatography; BEH = Ethylene Bridged Hybrid; ID = internal diameter; ATSM = American Society for Testing and Materials.

Table A-2. Preparation and Storage of Dose Formulations in the Perinatal and Two-year Feed Studies of Di-*n*-butyl Phthalate

Preparation
Stock solutions of di-*n*-butyl phthalate were created by weighing an appropriate amount of lot MKBB8432 and adding it to a volumetric flask. Acetone was used to bring the solution to volume. Flasks of stock solutions were sealed and shaken until the chemical was dissolved (at least 10 inversions). An initial formulation premix was created by weighing an appropriate amount of feed (NIH-07 or NTP-2000) into a mixing bowl. The stock di-*n*-butyl phthalate solution was slowly poured onto the feed while the mixture was stirred using a Hobart mixer. The premix formulations were mixed for approximately 1 hour and acetone was used twice to rinse the sides of the bowl and incorporate any residuals. The entire procedure was conducted under a nitrogen stream to encourage cyclonic flow and ensure acetone fully evaporated. In a twin shell blender, half the remaining untreated feed was evenly covered with the premix. The sides were "rinsed" with the remaining untreated feed in two increments. The final formulation was mixed in the blender for 15 minutes. The dose formulations were prepared approximately every 4 weeks.
Chemical Lot Number
MKBB8432
Maximum Storage Time
42 days
Storage Conditions
Stored in sealed plastic bag-lined container at ~25°C
Study Laboratory
Battelle (Columbus, OH)

Table A-3. Results of Analyses of Dose Formulations Administered to Rats in the Perinatal and Two-year Feed Study of Di-*n*-butyl Phthalate

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration (ppm)[a]	Difference from Target (%)
July 8, 2010	July 9, 2010	0	BLOQ	NA
		300	288 ± 10	−4.0
		1,000	970 ± 10	−3.0
		3,000	2,910 ± 80	−3.0
		10,000	9,920 ± 500	−0.8
August 9, 2010	August 10–12, 2010	0	BLOQ	NA
		300	304 ± 2	1.2
		1,000	997.0	−0.3
		3,000	2,775.0	−7.5
		10,000	9,060.0	−9.4

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration (ppm)[a]	Difference from Target (%)
October 4, 2010	October 5, 2010	0	BLOQ	NA
		300	299 ± 7	−0.5
		1,000	968.5	−3.2
		3,000	2,875.0	−4.2
		10,000	9,645.0	−3.6
December 20, 2010	December 20, 2010	0	BLOQ	NA
		300	300.5	0.2
		1,000	1,025.0	2.5
		3,000	3,040.0	1.3
		10,000	10,650.0	6.5
February 21, 2011	February 21, 2011	0	BLOQ	NA
		300	304	1.3
		1,000	1,050.0	5.0
		3,000	3,045.0	1.5
		10,000	9,970.0	−0.3
May 16, 2011	May 16, 2011	0	BLOQ	NA
		300	294.5	−1.8
		1,000	976.5	−2.4
		3,000	2,980.0	−0.7
		10,000	9,045.0	−9.6
August 8, 2011	August 9, 2011	0	BLOQ	NA
		300	293	−2.3
		1,000	989.5	−1.1
		3,000	3,015.0	0.5
		10,000	9,210.0	−7.9
October 3, 2011	October 3, 2011	0	BLOQ	NA
		300	295.5	−1.5
		1,000	990.5	−1.0
		3,000	3,020.0	0.7
		10,000	9,625.0	−3.8
November 28, 2011	November 30, 2011	0	BLOQ	NA
		300	291.5	−2.8
		1,000	957.0	−4.3
		3,000	2,975.0	−0.8
		10,000	9,410.0	−5.9
February 20, 2012	February 21, 2012	0	BLOQ	NA
		300	303.5	1.2
		1,000	964.5	−3.6

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration (ppm)[a]	Difference from Target (%)
		3,000	2,865.0	−4.5
		10,000	9,500.0	−5.0
May 14, 2012	May 14, 2012	0	BLOQ	NA
		300	295.5	−1.5
		1,000	991.5	−0.9
		3,000	3,035.0	1.2
		10,000	9,675.0	−3.3
July 9, 2012	July 10, 2012	0	BLOQ	NA
		300	301.5	0.5
		1,000	973.0	−2.7
		3,000	3,005.0	0.2
		10,000	9,665.0	−3.4
Animal Room Samples				
July 8, 2010	August 17, 2010 (feeder)	0	BLOQ	NA
		300	284 ± 4	−5.3
		1,000	923 ± 15	−7.7
		3,000	2,710 ± 40	−9.8
		10,000	8,820 ± 190	−11.8
	August 17, 2010 (barrel)	0	BLOQ	NA
		300	282 ± 3	−6.0
		1,000	918 ± 14	−8.2
		3,000	2,710 ± 30	−9.8
		10,000	9,050 ± 110	−9.5
August 9, 2010	September 15, 2010 (feeder)	0	BLOQ	NA
		300	268 ± 5	−10.7
		1,000	866 ± NA[b]	−13.4
		3,000	2,830 ± 230	−5.7
		10,000	8,890 ± 310	−11.1
	September 15, 2010 (barrel)	0	BLOQ	NA
		300	288 ± l	−4.1
		1,000	971 ± 25	−2.9
		3,000	2,940 ± 290	−2.0
		10,000	8,680 ± 40	−13.2
October 4, 2010	November 9, 2010 (feeder)	0	BLOQ	NA
		300	285 ± 6	−5.0
		1,000	953 ± 6	−4.7
		3,000	2,730 ± 60	−8.9
		10,000	9,100 ± 120	−9.0

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration (ppm)[a]	Difference from Target (%)
	November 9, 2010 (barrel)	0	BLOQ	NA
		300	284 ± 8	−5.3
		1,000	1,010 ± 10	0.6
		3,000	2,840 ± 10	−5.2
		10,000	9,730 ± 230	−2.7
February 21, 2011	March 31, 2011 (feeder)	0	BLOQ	NA
		300	292 ± 6	−2.7
		1,000	973 ± 5	−2.7
		3,000	2,960 ± 30	−1.2
		10,000	8,790 ± 50	−12.1
	March 31, 2011 (barrel)	0	BLOQ	NA
		300	298 ± l	−0.8
		1,000	1,020 ± 10	1.7
		3,000	2,990 ± 80	−0.4
		10,000	9,740 ± 200	−2.6
October 3, 2011	November 9, 2011 (feeder)	0	BLOQ	NA
		300	289 ± 4	−3.6
		1,000	973 ± 4	−2.7
		3,000	2,840 ± 70	−5.4
		10,000	9,260 ± 120	−7.4
	November 9, 2011 (barrel)	0	BLOQ	NA
		300	294 ± 3	−1.9
		1,000	989 ± 7	−1.1
		3,000	3,010 ± 20	0.4
		10,000	9,470 ± 420	−5.3
May 14, 2012	June 20, 2011 (feeder)	0	BLOQ	NA
		300	290 ± 2	−3.2
		1,000	993 ± 8	−0.7
		3,000	2,990 ± 20	−0.2
		10,000	9,240 ± 100	−7.6
	June 20, 2011 (barrel)	0	BLOQ	NA
		300	298 ± 1	−0.7
		1,000	999 ± 11	−0.1
		3,000	3,020 ± 50	0.8
		10,000	9,210 ± 70	−7.9

BLOQ = below the limit of quantification; NA = not applicable.

[a]Preadministration samples are an average of triplicate analysis on two preparations from the same sample or an average and standard deviation of triplicate analysis of a single sample. Animal room samples are an average and standard deviation of triplicate analysis of a single sample.

[b]Third replicate value Q-tested out, so standard deviation was not calculated. Precision of duplicates (POD) was 1.01.

Table A-4. Results of Analyses of Dose Formulations Administered to Mice in the Two-year Feed Study of Di-*n*-butyl Phthalate

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration (ppm)[a]	Difference from Target (%)
August 9, 2010	August 10–12, 2010	0	BLOQ	NA
		1,000	997.0	−0.3
		3,000	2,775.0	−7.5
		10,000	9,060.0	−9.4
October 4, 2010	October 5, 2010	0	BLOQ	NA
		1,000	968.5	−3.2
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December 20, 2010	December 20, 2010	0	BLOQ	NA
		1,000	1,025.0	2.5
		3,000	3,040.0	1.3
		10,000	10,650.0	6.5
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		1,000	1,050.0	5.0
		3,000	3,045.0	1.5
		10,000	9,970.0	−0.3
May 16, 2011	May 16, 2011	0	BLOQ	NA
		1,000	976.5	−2.4
		3,000	2,980.0	−0.7
		10,000	9,045.0	−9.6
August 8, 2011	August 9, 2011	0	BLOQ	NA
		1,000	989.5	−1.1
		3,000	3,015.0	0.5
		10,000	9,210.0	−7.9
October 3, 2011	October 3, 2011	0	BLOQ	NA
		1,000	990.5	−1.0
		3,000	3,020.0	0.7
		10,000	9,625.0	−3.8
November 28, 2011	November 30, 2011	0	BLOQ	NA
		1,000	957.0	−4.3
		3,000	2,975.0	−0.8
		10,000	9,410.0	−5.9

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration (ppm)[a]	Difference from Target (%)
February 20, 2012	February 21, 2012	0	BLOQ	NA
		1,000	964.5	−3.6
		3,000	2,865.0	−4.5
		10,000	9,500.0	−5.0
May 14, 2012	May 14, 2012	0	BLOQ	NA
		1,000	991.5	−0.9
		3,000	3,035.0	1.2
		10,000	9,675.0	−3.3
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		1,000	1,005.0	0.5
		3,000	3,005.0	0.2
		10,000	9,665.0	−3.4
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August 9, 2010	September 15, 2010 (feeder)	0	BLOQ	NA
		1,000	866 ± NA[b]	−13.4
		3,000	2,830 ± 230	−5.7
		10,000	8,890 ± 310	−11.1
	September 15, 2010 (barrel)	0	BLOQ	NA
		1,000	971 ± 25	−2.9
		3,000	2,940 ± 290	−2.0
		10,000	8,680 ± 40	−13.2
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	November 9, 2010 (barrel)	0	BLOQ	NA
		1,000	1,010 ± 10	0.6
		3,000	2,840 ± 10	−5.2
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		1,000	973 ± 5	−2.7
		3,000	2,960 ± 30	−1.2
		10,000	8,790 ± 50	−12.1
	March 31, 2011 (barrel)	0	BLOQ	NA
		1,000	1,020 ± 10	1.7
		3,000	2,990 ± 80	−0.4
		10,000	9,740 ± 200	−2.6
October 3, 2011	November 9, 2011 (feeder)	0	BLOQ	NA
		1,000	973 ± 4	−2.7
		3,000	2,840 ± 70	−5.4
		10,000	9,260 ± 120	−7.4
	November 9, 2011 (barrel)	0	BLOQ	NA
		1,000	989 ± 7	−1.1
		3,000	3,010 ± 20	0.4
		10,000	9,470 ± 420	−5.3
May 14, 2012	June 20, 2012 (feeder)	0	BLOQ	NA
		1,000	993 ± 8	−0.7
		3,000	2,990 ± 20	−0.2
		10,000	9,240 ± 100	−7.6
	June 20, 2012 (barrel)	0	BLOQ	NA
		1,000	999 ± 11	−0.1
		3,000	3,020 ± 50	0.8
		10,000	9,210 ± 70	−7.9

BLOQ = below the limit of quantification; NA = not applicable.
[a]Preadministration samples are an average of triplicate analysis on two preparations from the same sample. Animal room samples are an average and standard deviation of triplicate analysis of a single sample.
[b]Third replicate value Q-tested out, so standard deviation was not calculated. Precision of duplicates (POD) was 1.01.

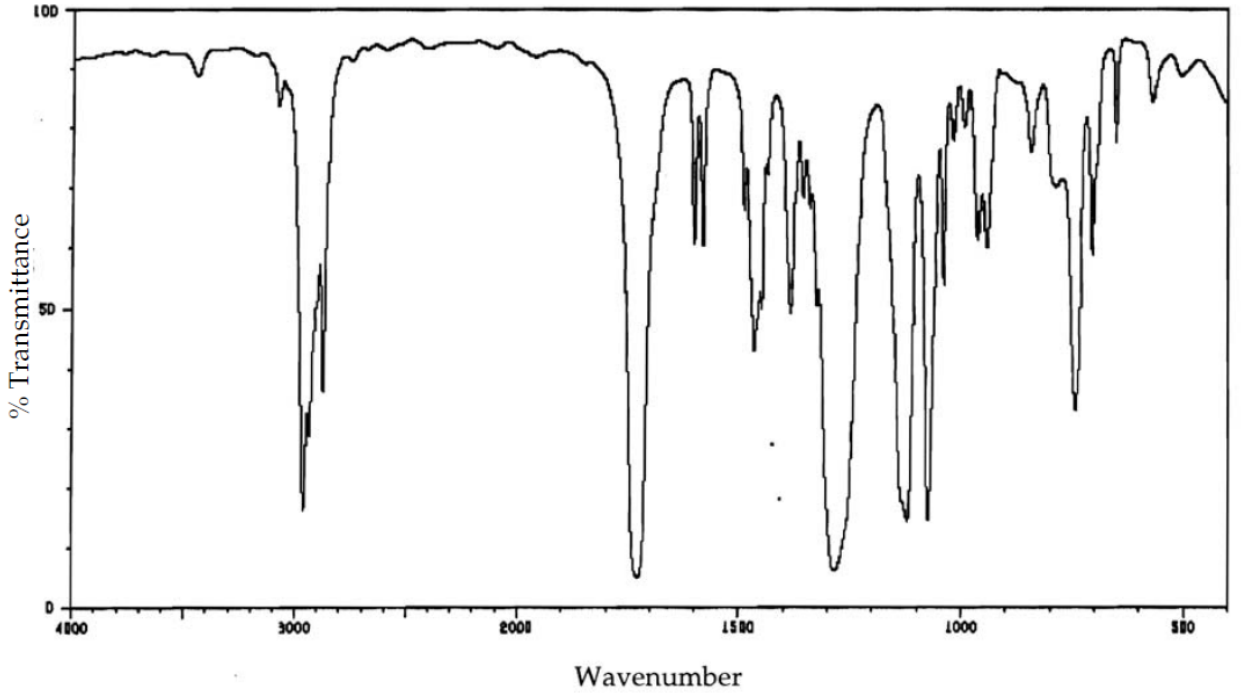

Figure A-1. Reference Fourier Transformed Infrared Absorption Spectrum of Di-*n*-butyl Phthalate

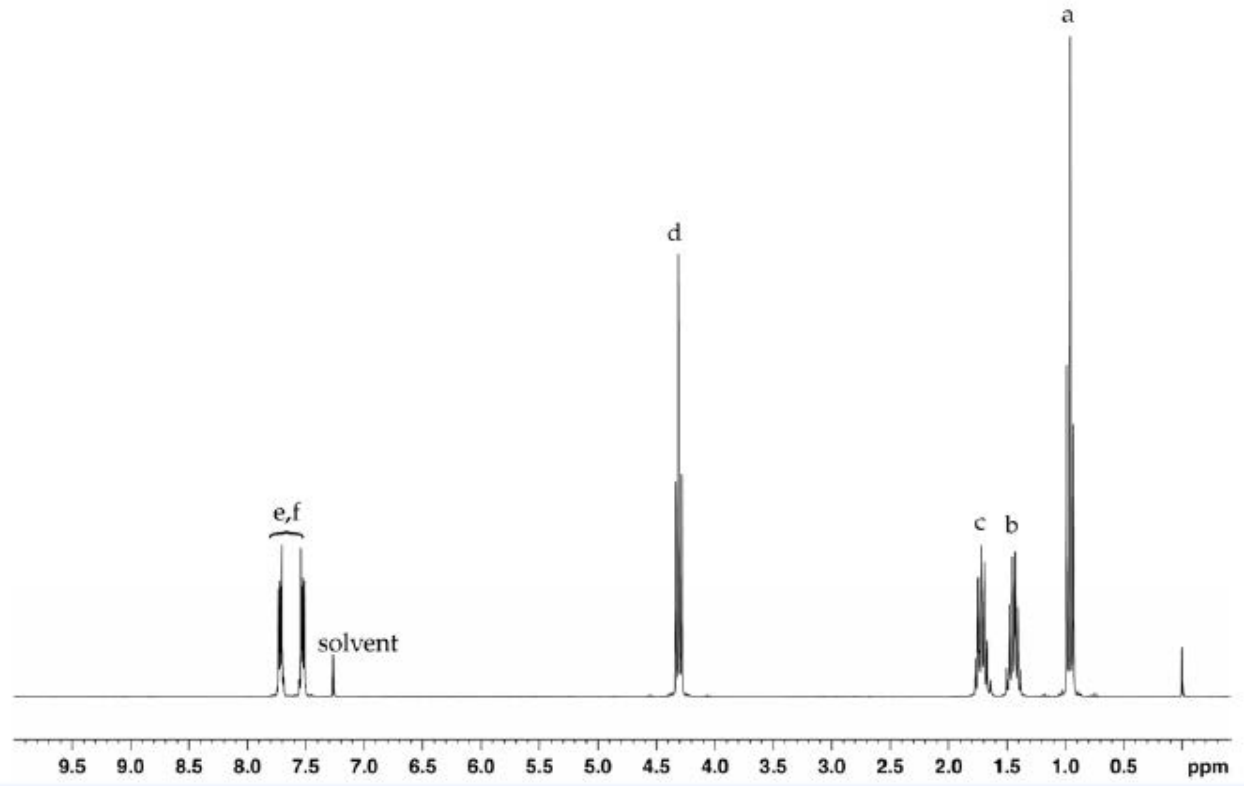

Figure A-2. ^{1}H NMR Spectrum of Sample of Di-*n*-butyl Phthalate (Lot MKBB8432)

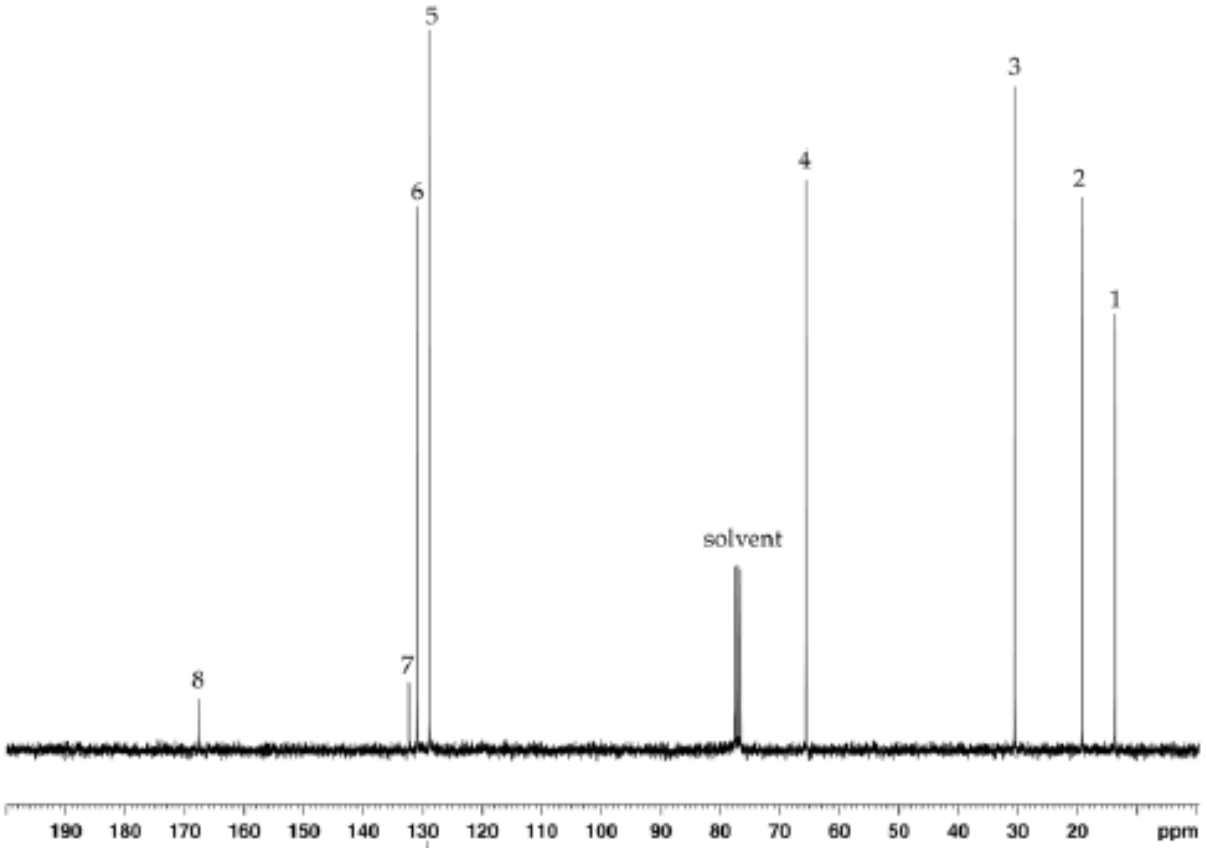

Figure A-3. ^{13}C NMR Spectrum of Sample of Di-*n*-butyl Phthalate (Lot MKBB8432)

Appendix B. Ingredients, Nutrient Composition, and Contaminant Levels in NIH-07 and NTP-2000 Rat and Mouse Ration

Tables

Table B-1. Ingredients of NIH-07 Rat Ration

Ingredients	Percent by Weight
Ground Hard Winter Wheat	23.00
Ground #2 Yellow Shelled Corn	24.25
Wheat Middlings	10.0
Oat Hulls	0.0
Alfalfa Meal (Dehydrated, 17% Protein)	4.0
Purified Cellulose	0.0
Soybean Meal (47% Protein)	12.0
Fish Meal (62% Protein)	10.0
Corn Oil (without Preservatives)	0.0
Soy Oil (without Preservatives)	2.5
Dried Brewer's Yeast	2.0
Calcium Carbonate (USP)	0.5
Vitamin Premix[a]	0.25
Mineral Premix[b]	0.15
Calcium Phosphate, Dibasic (USP)	1.25
Sodium Chloride	0.5
Choline Chloride (70% Choline)	0.10
Dried Skim Milk	5.00
Dried Molasses	1.50
Corn Gluten Meal (60% Protein)	3.00
Methionine	0.00

USP = United States Pharmacopeia.
[a]Wheat middlings as carrier.
[b]Calcium carbonate as carrier.

Table B-2. Vitamins and Minerals in NIH-07 Rat Ration

	Amount[a]	Source
Vitamins		
Vitamin A	6,062 IU	Stabilized vitamin A palmitate or acetate
Vitamin D	5,070 IU	D-activated animal sterol
Vitamin K	3.1 mg	Menadione sodium bisulfite complex
Vitamin E	22 IU	α-Tocopheryl acetate
Niacin	33 mg	
Folic Acid	2.4 mg	
d-Pantothenic Acid	19.8 mg	d-Calcium pantothenate
Riboflavin	3.8 mg	

	Amount[a]	Source
Thiamine	11 mg	Thiamine mononitrate
B_{12}	50 µg	
Pyridoxine	6.5 mg	Pyridoxine hydrochloride
Biotin	0.15 mg	d-Biotin
Minerals		
Iron	132 mg	Iron sulfate
Zinc	18 mg	Zinc oxide
Manganese	66 mg	Manganese oxide
Copper	4.4 mg	Copper sulfate
Iodine	2.0 mg	Calcium iodate
Cobalt	0.44 mg	Cobalt carbonate

[a]Per kg of finished diet.

Table B-3. Nutrient Composition of NIH-07 Rat Ration

Nutrient	Mean ± Standard Deviation	Range	Number of Samples
Protein (% by Weight)	24.25 ± 1.485	23.2–25.3	2
Crude Fat (% by Weight)	5.5 ± 0.424	5.2–5.8	2
Crude Fiber (% by Weight)	3.515 ± 0.191	3.38–3.65	2
Ash (% by Weight)	6.135 ± 0.021	6.12–6.15	2
Amino Acids (% of Total Diet)			
Arginine	1.380 ± 0.06	1.3–1.49	10
Cystine	0.322 ± 0.031	0.274–0.372	10
Glycine	1.150 ± 0.070	1.06–1.31	10
Histidine	0.518 ± 0.024	0.497–0.553	10
Isoleucine	0.984 ± 0.024	0.952–1.03	10
Leucine	2.018 ± 0.067	1.93–2.13	10
Lysine	1.243 ± 0.051	1.13–1.32	10
Methionine	0.488 ± 0.016	0.468–0.515	10
Phenylalanine	1.097 ± 0.022	1.07–1.12	10
Threonine	0.918 ± 0.031	0.883–0.961	10
Tryptophan	0.277 ± 0.020	0.265–0.326	10
Tyrosine	0.860 ± 0.037	0.785–0.894	10
Valine	1.134 ± 0.025	1.11–1.17	10
Essential Fatty Acids (% of Total Diet)			
Linoleic	2.30 ± 0.219	1.99–2.59	10
Linolenic	0.25 ± 0.275	0.217–0.296	10

Nutrient	Mean ± Standard Deviation	Range	Number of Samples
Vitamins			
Vitamin A (IU/kg)	4,085 ± 161.9	2,940–5,230	2
α-Tocopherol (ppm)	6,704 ± 21,045	40.3–66,600	10
Thiamine (ppm)[a]	10.6 ± 0.283	10.4–10.8	2
Riboflavin (ppm)	14.47 ± 3.352	10.0–19.8	10
Niacin (ppm)	99.33 ± 8.235	87.0–112.0	10
Pantothenic Acid (ppm)	44.38 ± 3.806	38.2–51.1	10
Pyridoxine (ppm)[a]	12.876 ± 3.171	9.63–19.7	10
Folic Acid (ppm)	2.482 ± 0.487	1.68–3.09	10
Biotin (ppm)	0.3283 ± 0.172	0.0–0.638	10
B_{12} (ppb)	49.4 ± 6.83	41.8–61.6	10
Choline (as Chloride) (ppm)	1,821 ± 197.5	1,570–2,200	10
Minerals			
Calcium (%)	1.140 ± 0.014	1.13–1.15	2
Phosphorus (%)	0.955 ± 0.006	0.951–0.959	2
Potassium (%)	0.830 ± 0.036	0.769–0.88	10
Chloride (%)	0.652 ± 0.106	0.441–0.8	10
Sodium (%)	0.378 ± 0.46	0.318–0.469	10
Magnesium (%)	0.187 ± 0.014	0.17–0.218	10
Iron (ppm)	385.1 ± 54.9	276.0–469.0	10
Manganese (ppm)	90.81 ± 7.566	80.7–104.0	10
Zinc (ppm)	64.15 ± 10.07	52.4–89.2	10
Copper (ppm)	14.13 ± 2.57	11.9–21.1	10
Iodine (ppm)	1.811 ± 0.992	0.54–3.45	10
Chromium (ppm)	3.946 ± 0.036	3.89–4.0	8
Cobalt (ppm)	0.5155 ± 0.267	0.01–0.963	10

[a]As hydrochloride.

Table B-4. Contaminant Levels in NIH-07 Rat Ration

	Mean ± Standard Deviation	Range	Number of Samples
Contaminants			
Arsenic (ppm)	0.355 ± 0.010	0.348–0.362	2
Cadmium (ppm)	0.0445 ± 0.005	0.041–0.048	2
Lead (ppm)	0.0885 ± 0.018	0.076–0.101	2
Mercury (ppm)	0.022 ± 0.001	0.021–0.023	2
Selenium (ppm)	0.5125 ± 0.046	0.48–0.545	2
Aflatoxins (ppb)[a]	5	–	2
Nitrate Nitrogen (ppm)[b]	15 ± 3.677	12.4–17.6	2
Nitrite Nitrogen (ppm)[a,b]	<0.61	–	2
BHA (ppm)[a,c]	<1.0	–	2
BHT (ppm)[a,c]	<1.0	–	2
Aerobic Plate Count (CFU/gm)	<10	–	2
Coliform (MPN/gm)	<3	–	2
E. coli (MPN/gm)	<10	–	2
Salmonella (MPN/gm)	Negative	–	2
Total Nitrosamines (ppb)[d]	1.8 ± 2.546	0.0–3.6	2
N-Nitrosodimethylamine (ppb)[d]	1.8 ± 2.546	0.0–3.6	2
N-Nitrosopyrrolidine (ppb)[d]	0	–	2
Pesticides (ppm)			
α-BHC[a]	<0.01	–	2
β-BHC[a]	<0.02	–	2
γ-BHC[a]	<0.01	–	2
δ-BHC[a]	<0.01	–	2
Heptachlor[a]	<0.01	–	2
Aldrin[a]	<0.01	–	2
Heptachlor Epoxide[a]	<0.01	–	2
DDE[a]	<0.01	–	2
DDD[a]	<0.01	–	2
DDT[a]	<0.01	–	2
HCB[a]	<0.01	–	2
Mirex[a]	<0.01	–	2
Methoxychlor[a]	<0.05	–	2
Dieldrin[a]	<0.01	–	2
Endrin[a]	<0.01	–	2

	Mean ± Standard Deviation	Range	Number of Samples
Telodrin[a]	<0.01	–	2
Chlordane[a]	<0.05	–	2
Toxaphene[a]	<0.10	–	2
Estimated PCBs[a]	<0.20	–	2
Ronnel[a]	<0.01	–	2
Ethion[a]	<0.02	–	2
Trithion[a]	<0.05	–	2
Diazinon[a]	<0.10	–	2
Methyl Chlorpyrifos	0.067 ± 0.067	0.02–0.114	2
Methyl Parathion[a]	<0.02	–	2
Ethyl Parathion[a]	<0.02	–	2
Malathion	0.153 ± 0.002	0.151–0.154	2
Endosulfan I[a]	<0.01	–	2
Endosulfan II[a]	<0.01	–	2
Endosulfane Sulfate[a]	<0.03	–	2

All samples were irradiated. BHA = butylated hydroxyanisole; BHT = butylated hydroxytoluene; CFU = colony-forming units; MPN = most probable number; BHC = hexachlorocyclohexane or benzene hexachloride; DDE = dichlorodiphenyldichloroethylene; DDD = dichlorodiphenyldichloroethane; DDT = dichlorodiphenyltrichloroethane; HCB = hexachlorobenzene; PCB = polychlorinated biphenyl.
[a]All values were below the detection limit. The detection limit is given as the mean.
[b]Sources of contamination include alfalfa, grains, and fish meal.
[c]Sources of contamination include soy oil and fish meal.
[d]All values were corrected for percent recovery.

Table B-5. Ingredients of NTP-2000 Rat and Mouse Ration

Ingredients	Percent by Weight
Ground Hard Winter Wheat	22.26
Ground #2 Yellow Shelled Corn	22.18
Wheat Middlings	15.0
Oat Hulls	8.5
Alfalfa Meal (Dehydrated, 17% Protein)	7.5
Purified Cellulose	5.5
Soybean Meal (49% Protein)	5.0
Fish Meal (60% Protein)	4.0
Corn Oil (without Preservatives)	3.0
Soy Oil (without Preservatives)	3.0
Dried Brewer's Yeast	1.0
Calcium Carbonate (USP)	0.9

Ingredients	Percent by Weight
Vitamin Premix[a]	0.5
Mineral Premix[b]	0.5
Calcium Phosphate, Dibasic (USP)	0.4
Sodium Chloride	0.3
Choline Chloride (70% Choline)	0.26
Methionine	0.2

USP = United States Pharmacopeia.
[a]Wheat middlings as carrier.
[b]Calcium carbonate as carrier.

Table B-6. Vitamins and Minerals in NTP-2000 Rat and Mouse Ration

	Amount[a]	Source
Vitamins		
Vitamin A	4,000 IU	Stabilized vitamin A palmitate or acetate
Vitamin D	1,000 IU	D-activated animal sterol
Vitamin K	1.0 mg	Menadione sodium bisulfite complex
α-Tocopheryl Acetate	100 IU	
Niacin	23 mg	
Folic Acid	1.1 mg	
d-Pantothenic Acid	10 mg	d-Calcium pantothenate
Riboflavin	3.3 mg	
Thiamine	4 mg	Thiamine mononitrate
B_{12}	52 µg	
Pyridoxine	6.3 mg	Pyridoxine hydrochloride
Biotin	0.2 mg	d-Biotin
Minerals		
Magnesium	514 mg	Magnesium oxide
Iron	35 mg	Iron sulfate
Zinc	12 mg	Zinc oxide
Manganese	10 mg	Manganese oxide
Copper	2.0 mg	Copper sulfate
Iodine	0.2 mg	Calcium iodate
Chromium	0.2 mg	Chromium acetate

[a]Per kg of finished product.

Table B-7. Nutrient Composition of NTP-2000 Rat and Mouse Ration

Nutrient	Mean ± Standard Deviation	Range	Number of Samples
Protein (% by Weight)	14.81 ± 0.544	14.2–16.8	25
Crude Fat (% by Weight)	8.72 ± 0.367	8.0–9.7	25
Crude Fiber (% by Weight)	9.38 ± 0.442	8.34–10.1	25
Ash (% by Weight)	5.3 ± 0.187	4.6–14.2	25
Amino Acids (% of Total Diet)			
Arginine	0.805 ± 0.075	0.67–0.97	29
Cystine	0.220 ± 0.021	0.15–0.025	29
Glycine	0.702 ± 0.038	0.62–0.80	29
Histidine	0.342 ± 0.070	0.27–0.68	29
Isoleucine	0.549 ± 0.040	0.43–0.66	29
Leucine	1.100 ± 0.063	0.96–1.24	29
Lysine	0.700 ± 0.104	0.31–0.86	29
Methionine	0.409 ± 0.042	0.26–0.49	29
Phenylalanine	0.623 ± 0.047	0.471–0.72	29
Threonine	0.513 ± 0.041	0.43–0.61	29
Tryptophan	0.155 ± 0.027	0.11–0.2	29
Tyrosine	0.422 ± 0.066	0.28–0.54	29
Valine	0.666 ± 0.040	0.55–0.73	29
Essential Fatty Acids (% of Total Diet)			
Linoleic	3.94 ± 0.235	3.49–4.55	29
Linolenic	0.30 ± 0.064	0.005–0.368	29
Vitamins			
Vitamin A (IU/kg)	3,821 ± 80.2	2,030–5,290	25
α-Tocopherol (ppm)	2,456 ± 12,817	13.6–69,100	29
Thiamine (ppm)[a]	8.47 ± 1.958	3.9–12.5	25
Riboflavin (ppm)	8.17 ± 2.841	4.2–17.5	29
Niacin (ppm)	78.66 ± 8.11	66.4–98.2	29
Pantothenic Acid (ppm)	26.42 ± 11.05	17.4–81.0	29
Pyridoxine (ppm)[a]	9.75 ± 2.045	6.44–14.3	29
Folic Acid (ppm)	1.58 ± 0.43	1.15–3.27	29
Biotin (ppm)	0.323 ± 0.093	0.2–0.704	29
B_{12} (ppb)	50.41 ± 34.89	18.3–174	29
Choline (as Chloride) (ppm)	2,593 ± 633.8	1,160–3,790	29
Minerals			
Calcium (%)	0.911 ± 0.046	0.831–1.03	25
Phosphorus (%)	0.547 ± 0.105	0.0531–0.613	25
Potassium (%)	0.668 ± 0.029	0.626–0.733	29
Chloride (%)	0.392 ± 0.044	0.3–0.517	29

Nutrient	Mean ± Standard Deviation	Range	Number of Samples
Sodium (%)	0.195 ± 0.027	0.16–0.283	29
Magnesium (%)	0.217 ± 0.054	0.185–0.49	29
Iron (ppm)	191.6 ± 36.18	135–311	29
Manganese (ppm)	50.11 ± 9.42	21–73.1	29
Zinc (ppm)	57.3 ± 25.54	43.3–184	29
Copper (ppm)	7.57 ± 2.49	3.21–16.3	29
Iodine (ppm)	0.513 ± 0.221	0–0.972	29
Chromium (ppm)	1.02 ± 1.04	0.33–3.97	28
Cobalt (ppm)	0.222 ± 0.152	0.0857–0.864	27

[a]As hydrochloride.

Table B-8. Contaminant Levels in NTP-2000 Rat and Mouse Ration

	Mean ± Standard Deviation	Range	Number of Samples
Contaminants			
Arsenic (ppm)	0.22 ± 0.066	0.149–0.385	25
Cadmium (ppm)	0.054 ± 0.013	0.038–0.094	25
Lead (ppm)	0.132 ± 0.104	0.064–0.474	25
Mercury (ppm)	0.014 ± 0.009	0.01–0.049	25
Selenium (ppm)	0.16 ± 0.031	0.029–0.209	25
Aflatoxins (ppb)[a]	<5.0	–	25
Nitrate Nitrogen (ppm)[b]	15.04 ± 4.97	10.0–28.1	25
Nitrite Nitrogen (ppm)[a,b]	<0.61	–	25
BHA (ppm)[a,c]	<1.00	–	25
BHT (ppm)[c]	1.09 ± 0.428	1.0–3.14	25
Aerobic Plate Count (CFU/gm)	<10.0	–	25
Coliform (MPN/gm)	<3	–	25
E. coli (MPN/gm)	<10.0	–	25
Salmonella (MPN/gm)	Negative	–	25
Total Nitrosamines (ppb)[d]	11.7 ± 5.58	3.2–24.5	25
N-Ndimethylamine (ppb)[d]	2.6 ± 1.66	1.0–6.8	25
N-Npyrrolidine (ppb)[d]	9.2 ± 5.16	2.1–20.0	25
Pesticides (ppm)			
α-BHC[a]	<0.01	–	25
β-BHC[a]	<0.02	–	25
γ-BHC[a]	<0.01	–	25
δ-BHC[a]	<0.01	–	25

	Mean ± Standard Deviation	Range	Number of Samples
Heptachlor[a]	<0.01	–	25
Aldrin[a]	<0.01	–	25
Heptachlor Epoxide[a]	<0.01	–	25
DDE[a]	<0.01	–	25
DDD[a]	<0.01	–	25
DDT[a]	<0.01	–	25
HCB[a]	<0.01	–	25
Mirex[a]	<0.01	–	25
Methoxychlor[a]	<0.05	–	25
Dieldrin[a]	<0.01	–	25
Endrin[a]	<0.01	–	25
Telodrin[a]	<0.01	–	25
Chlordane[a]	<0.05	–	25
Toxaphene[a]	<0.10	–	25
Estimated PCBs[a]	<0.20	–	25
Ronnel[a]	<0.01	–	25
Ethion[a]	<0.02	–	25
Trithion[a]	<0.05	–	25
Diazinon[a]	<0.10	–	25
Methyl Chlorpyrifos	0.09 ± 0.073	0.02–0.315	25
Methyl Parathion[a]	<0.02	–	25
Ethyl Parathion[a]	<0.02	–	25
Malathion	0.094 ± 0.09	0.02–0.355	25
Endosulfan I[a]	<0.01	–	25
Endosulfan II[a]	<0.01	–	25
Endosulfane Sulfate[a]	<0.03	–	25

All samples were irradiated. BHA = butylated hydroxyanisole; BHT = butylated hydroxytoluene; CFU = colony-forming units; MPN = most probable number; BHC = hexachlorocyclohexane or benzene hexachloride; DDE = dichlorodiphenyldichloroethylene; DDD = dichlorodiphenyldichloroethane; DDT = dichlorodiphenyltrichloroethane; HCB = hexachlorobenzene; PCB = polychlorinated biphenyl.

[a]All values were below the detection limit. The detection limit is given as the mean.

[b]Sources of contamination include alfalfa, grains, and fish meal.

[c]Sources of contamination include soy oil and fish meal.

[d]All values were corrected for percent recovery.

Appendix C. Sentinel Animal Program

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Tables

C.1. Methods

Rodents used in the National Toxicology Program are produced in optimally clean facilities to eliminate potential pathogens that might affect study results. The Sentinel Animal Program is part of the periodic monitoring of animal health that occurs during the toxicological evaluation of test compounds. Under this program, the disease state of the rodents is monitored via sera or feces from extra (sentinel) or exposed animals in the study rooms. The sentinel animals and the study animals are subject to identical environmental conditions. Furthermore, the sentinel animals come from the same production source and weanling groups as the animals used for the studies of test compounds.

For these toxicology and carcinogenesis studies, blood samples were collected from each sentinel animal, allowed to clot, and the serum was separated. Additionally, fecal samples were collected and tested for endoparasites and *Helicobacter* species. All samples were processed appropriately with serology testing sent to IDEXX BioResearch (formerly Rodent Animal Diagnostic Laboratory [RADIL], University of Missouri), Columbia, MO, for determination of the presence of pathogens. Evaluation for endo- and ectoparasites was performed in-house by the testing laboratory.

The laboratory methods and agents for which testing was performed are tabulated in Table C-1 and Table C-2 below; the times at which samples were collected during the studies are also listed.

C.2. Results

Rats: Positive for endoparasites, pinworms (*Syphacia* spp.). All other test results were negative.

Mice: All test results were negative.

Table C-1. Methods and Results for Sentinel Animal Testing in Male and Female Rats

Collection Time Points	Quarantine[a]	Perinatal[b]	6 Months	12 Months	17 Months	18 Months	End of Study
Number Examined (Males/Females)	0/10	0/10	5/5	5/5	0/1	5/6	5/5
Method/Test							
Multiplex Fluorescent Immunoassay (MFI)							
Kilham rat virus (KRV)	–	–	–	–	NT	–	–
Mycoplasma pulmonis	–	–	–	–	NT	–	–
Pneumonia virus of mice (PVM)	–	–	–	–	NT	–	–
Rat coronavirus/sialodacryoadenitis virus (RCV/SDA)	–	–	–	–	NT	–	–
Rat minute virus (RMV)	–	–	–	–	NT	–	–
Rat parvo virus (RPV)	–	–	–	–	NT	–	–
Rat theilovirus (RTV)	–	–	–	–	NT	–	–
Sendai	–	–	–	–	NT	–	–

Collection Time Points	Quarantine[a]	Perinatal[b]	6 Months	12 Months	17 Months	18 Months	End of Study
Theiler's murine encephalomyelitis virus (TMEV)	–	–	–	–	NT	–	–
Toolan's H-1	–	–	–	–	NT	–	–
In-house Evaluation							
Endoparasites (evaluation of cecal content)	NT	NT	NT	NT	+	+	NT
Ectoparasites (evaluation of perianal surface)	NT	NT	NT	NT	+	+	NT

– = negative; + = positive; NT = not tested.
[a]Age matched nonpregnant females.
[b]Time-mated females that did not have a litter; 3.5 weeks after arrival.

Table C-2. Methods and Results for Sentinel Animal Testing in Male and Female Mice

Collection Time Points	1 Month	6 Months	12 Months	18 Months	End of Study
Number Examined (Males/Females)	5/5	5/5	5/5	5/5	5/5
Method/Test					
Multiplex Fluorescent Immunoassay (MFI)					
Ectromelia virus	–	–	–	–	–
Epizootic diarrhea of infant mice (EDIM)	–	–	–	–	–
Lymphocytic choriomeningitis virus (LCMV)	–	–	–	–	–
Mycoplasma pulmonis	–	–	–	–	–
Mouse hepatitis virus (MHV)	–	–	–	–	–
Mouse norovirus (MNV)	–	–	–	–	–
Mouse parvovirus (MPV)	–	–	–	–	–
Minute virus of mice (MVM)	–	–	–	–	–
Pneumonia virus of mice (PVM)	–	–	–	–	–
Reovirus (REO3)	–	–	–	–	–
Sendai	–	–	–	–	–
Theiler's murine encephalomyelitis virus (TMEV) GDVII	–	–	–	–	–
Immunofluorescence Assay (IFA)					
Epizootic diarrhea of infant mice (EDIM)	NT	NT	–	NT	NT
Ectromelia virus	NT	NT	–	NT	NT
Polymerase Chain Reaction (PCR)					
Helicobacter species	NT	NT	NT	–	NT

– = negative; NT = not tested.

Appendix D. Peer-review Report

[The peer-review report will appear in a future draft of this report.]

Appendix E. Supplemental Data

Tables with supplemental data can be found here: https://doi.org/10.22427/NTP-DATA-TR-600.[143]

E.1. Perinatal and Two-year Study in Rats

E01 – Animal Removal Summary by Treatment Group

E02 – Animals Removed from Experiment

E03 – Growth Curves

E04 – Mean Body Weights and Survival Table

E05 – Clinical Observations Summary

E08 – Feed Water and Compound Consumption Table

Gestational and Lactational Chemical Consumption

Gestational Body Weights

Gestational Food Consumption

Lactational Body Weights

Lactational Food Consumption

P03 – Incidence Rates of Non-Neoplastic Lesions by Anatomic Site

P04 – Neoplasms by Individual Animal

P05 – Incidence Rates of Neoplasms by Anatomic Site (Systemic Lesions Abridged)

P08 – Statistical Analysis of Primary Tumors

P09 – Non-Neoplastic Lesions by Individual Animal

P10 – Statistical Analysis of Non-Neoplastic Lesions

P11 – Statistical Analysis of Survival Data

P14 – Individual Animal Pathology Data

P17 – Neoplasms by Individual Animal (Systemic Lesions Abridged)

P18 – Incidence Rates of Non-Neoplastic Lesions by Anatomic Site with Average Severity Grades

P22 – Cause of Death Summary

P40 – Survival Curves

PA46R – Summary of Gross Pathology with Litter Incidence

PA48 – Summary of Tissue Concentration

PND 1 Litter Data

PND 4 and 21 Live Litter Size and Survival

Pup Body Weights

R02 – Reproductive Performance Summary

R23 – Gubernaculum Length Summary

E.2. Individual Animal Data for Perinatal and Two-year Study in Rats

Female Individual Animal Body Weight Data All Animals

Female Individual Animal Pathology Data Neoplastic

Female Individual Animal Pathology Data Non-Neoplastic

Female Individual Animal Survival Data

Female Individual Animal Terminal Body Weight Data

Female Individual Clinical Observations

Gubernaculum and Urogenital Findings Data

Individual Animal DamID and PupID Data

Individual Animal Reproductive Performance Data

Individual Animal Tissue Concentration Data

Male Individual Animal Body Weight Data All Animals

Male Individual Animal Clinical Observations

Male Individual Animal Neoplastic Pathology Data

Male Individual Animal Organ Weight Data

Male Individual Animal Non-Neoplastic Pathology Data

Male Individual Animal Survival Data

E.3. Two-year Study in Mice

E01 – Animal Removal Summary by Treatment Group

E02 – Animals Removed from Experiment

E03 – Growth Curves

E04 – Mean Body Weights and Survival Table

E05 – Clinical Observations Summary

E08 – Feed Water and Compound Consumption Table

P03 – Incidence Rates of Non-Neoplastic Lesions by Anatomic Site

P04 – Neoplasms by Individual Animal

P05 – Incidence Rates of Neoplasms by Anatomic Site (Systemic Lesions Abridged)

P08 – Statistical Analysis of Primary Tumors

P09 – Non-Neoplastic Lesions by Individual Animal

P10 – Statistical Analysis of Non-Neoplastic Lesions

P11 – Statistical Analysis of Survival Data

P14 – Individual Animal Pathology Data

P17 – Neoplasms by Individual Animal (Systemic Lesions Abridged)

P18 – Incidence Rates of Non-Neoplastic Lesions by Anatomic Site with Average Severity Grades

P22 – Cause of Death Summary

P40 – Survival Curves

E.4. Individual Data for Two-year Study in Mice

Female Individual Animal Body Weight Data

Female Individual Animal Clinical Observations

Female Individual Animal Organ Weight Data

Female Individual Animal Pathology Data Neoplastic

Female Individual Animal Pathology Data Non-Neoplastic

Female Individual Animal Survival Data

Male Individual Animal Body Weight Data

Male Individual Animal Clinical Observations

Male Individual Animal Organ Weight Data

Male Individual Animal Pathology Data Neoplastic

Male Individual Animal Pathology Data Non-Neoplastic

Male Individual Animal Survival Data