The MAK Collection for Occupational Health and Safety

Di-n-butyl phthalate

MAK Value Documentation – Translation of the German version from 2017

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Di-*n*-butyl phthalate / Dibutyl benzene-1,2-dicarboxylate

MAK value documentation

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Abstract

The German Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area has re-evaluated the developmental toxicity of di-*n*-butyl phthalate. Available publications are described in detail. In prenatal developmental toxicity studies in rats, the most sensitive endpoint was altered morphometry (size and organization) of the seminiferous cords, the precursor of the seminiferous tubules, at 50 mg/kg body weight and day and above; the NOAEL was 30 mg/kg body weight and day. In rats, teratogenic effects like hypospadia and underdeveloped or absent epididymides were observed at 250 mg/kg KG body weight and day and above; the NOAEL was 100 mg/kg body weight and day.

In a two-generation reproduction toxicity study in rats, a LOAEL of 80 mg/kg body weight and day, the lowest dose, could be derived for foetotoxicity and decreased live pup weight at birth. In rats, the NOAEL for behavioural toxicity is 291 mg/kg body weight and day in male pups. From a one-generation reproduction toxicity study in mice, a NOAEL of 420 mg/kg body weight and day could be derived for foetotoxicity and decreased number of live pups. The NOAELs and the LOAEL for developmental toxicity and foetotoxicity can be scaled to concentrations of 32, 107, 84 (LOAEL) and 252 mg/m^3, respectively, at the workplace. Thus, damage to the embryo or foetus is unlikely when the MAK value of 0.58 mg/m^3 is not exceeded, and the classification in Pregnancy Risk Group C is confirmed.

Keywords

di-*n*-butyl phthalate; *n*-butyl phthalate; di(*n*-butyl) 1,2-benzenedicarboxylate; dibutyl phthalate; phthalic acid dibutyl ester; 1,2-benzenedicarboxylic acid dibutyl ester; developmental toxicity; prenatal toxicity; occupational exposure; maximum workplace concentration; MAK value; toxicity; hazardous substance

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Di-*n*-butyl phthalate

[**84-74-2**]

Supplement 2016

MAK value (2009)	**0.05 ml/m³ (ppm) ≙ 0.58 mg/m³**
Peak limitation (2009)	**Category I, excursion factor 2**
Absorption through the skin	**–**
Sensitization	**–**
Carcinogenicity (2014)	**Category 3B**
Prenatal toxicity (2009)	**Pregnancy Risk Group C**
Germ cell mutagenicity	**–**
BAT value	**–**

Developmental Toxicity

Since February 2015, the use of the plasticizers di(2-ethylhexyl) phthalate (DEHP), di-*n*-butyl phthalate (DBP), diisobutyl phthalate (DIBP) and benzylbutyl phthalate (BBP) is only permitted after authorization according to the laws regulating the use of chemicals (EU 2011).

A review by the Agency for Toxic Substances and Disease Registry (ATSDR 2001) and an EU Risk Assessment Report (ECB 2003) are available for the toxicological profile of di-*n*-butyl phthalate. These publications were already taken into consideration in the 2010 documentation (documentation "Di-*n*-butyl phthalate" 2013). A recent toxicological assessment by NICNAS (2013) and data compilations by the ECHA (2016) are also now available and have been examined in detail for studies relevant to this evaluation.

This supplement to di-*n*-butyl phthalate has become necessary as further studies of its prenatal toxicity have been published since the documentation from 2010 (documentation "Di-*n*-butyl phthalate" 2013).

Effects in humans

In a study in the offspring of 71 war veterans, an increased incidence of hypospadia ($p < 0.05$), cryptorchidism ($p < 0.05$) and breast cancer ($p < 0.05$) was found. The

former soldiers had been exposed between 1948 and 1960 to di-*n*-butyl phthalate applied daily to their clothing as an insecticide against transmitters of typhus. The authors modelled the absorption of di-*n*-butyl phthalate through the skin from the clothing, and thus calculated a theoretical dose of 64 mg/kg body weight and day. By means of questionnaires, the occurrence of cryptorchidism, penis malformations such as hypospadia, pubertas precox (only in female offspring), low sperm counts, reduced fertility, ovarian and uterine disturbances, and breast cancer was investigated. Of the 71 war veterans, 58 (81.7%) had offspring. Of the total of 155 offspring, 79 (51%) were male and 76 (49%) female. Four cases (5.1%) of cryptorchidism occurred; the incidence in the general population was 0.91% (2005) or 1.09% (2000). Two cases (2.5%) of hypospadia were found, compared with incidences of 0.33% (2000) and 0.3% (2005) in the general population; there were three cases of breast cancer (4.0%) compared with an incidence of 0.48% in the general population (2008) (Carran and Shaw 2012).

In commentaries on this publication, the following points are criticized: it is neither a cohort nor a case–control study, but a cross-sectional sampling and a cluster investigation without determining the size of the cluster. Statistically, the reported cases are meaningless. Confounders were not included. Of the war veterans asked, only 33% took part in the investigation, so that a certain bias can be assumed. The assumptions about the daily quantities absorbed are inadequate (McBride and Schep 2012). No data concerning the age of the offspring are given. The comparison with the incidences in the general population is not correct, as all the cases of breast cancer (4.0%) that occurred, which represents a cumulative incidence, are compared with a yearly incidence rate (0.48%). In actual fact, all the observed cases are within the normal range. The incidences given for hypospadia were in reality 0.65% in 2000 and 0.55% in 2005; presumably, the authors included male and female offspring in their calculation. The dates of birth (years) of the war veterans' offspring are not given. A new estimation, however, results in a relative risk of 3.90 with a 95% confidence interval of 0.99 to 15.3, which shows that although the increase in cases of hypospadia in the study is statistically significant, it is only marginal and did not occur to the extent stated in the study of Carran and Shaw (2012). It is a similar case as regards the calculations for cryptorchidism, which were corrected to 2.13% in 2000 and 1.79% in 2005, resulting in a relative risk of 2.56 with a 95% confidence interval of 0.98 to 6.65. There was thus no clear statistically significant increase. The few cases of hypospadia and cryptorchidism in the study of Carran and Shaw (2012) could also be within the range of random coincidence, and the effects of recall and selection bias as a result of using data gained exclusively from the fathers, of whom only a small number actually responded, is high (Elwood and Borman 2012).

Animal experiments and in vitro studies

Studies are described here that have appeared since the documentation from 2010 (documentation "Di-*n*-butyl phthalate" 2013). Studies with dose levels above 500 mg/kg body weight and day are not included, as they do not cover the critical dose range.

The respective studies are listed in Table 1.

Table 1 Studies with prenatal and postnatal exposure to di-*n*-butyl phthalate

Species	Exposure	Findings	References
Prenatal treatment			
rat, SD, 4–5 ♀, controls: 10 ♀	**GD 12–20**, 0.1, 1, 10, 30, 50, 100, 500 mg/kg body weight and day, gavage, vehicle: corn oil, examined on: GD 21; **for development time course: GD 12–16, 12–17, 12–18, 12–19, 12–20**, 0, 500 mg/kg body weight and day; examined on GD 17–21 daily; offspring: PND 1, PND 2; one testis (right) from 3 foetuses/litter investigated per time point	**30 mg/kg body weight**: **NOAEL** for effects on the ♂ reproductive organs; **30 mg/kg body weight and above**: foetuses: testicular cell count ↓ (reversible, normalized on PND 2 at 500 mg/kg body weight, according to the authors transient and normalized postnatally at 30 mg/kg body weight); **50 mg/kg body weight and above**: foetuses: testis volume ↓ (reversible, normalized on PND 2 at 500 mg/kg body weight), altered morphometry of seminiferous cords/tubules (size and organization; not clear whether reversible as not examined on PND 2); **100 mg/kg body weight and above**: foetuses: number of MNG in testes ↑; **500 mg/kg body weight**: clustering of Leydig cells; no data for maternal toxicity; same strain: 500 mg/kg body weight NOAEL for maternal toxicity (Barlow and Foster 2003; Mylchreest et al. 2000)	Boekelheide et al. 2009
rat, SD, 4 ♀	**GD 12–21**, 0, 10, 30, 50, 100 mg/kg body weight and day, intragastric, vehicle: corn oil, after birth: reduction of the litters to 4 ♂ and 4 ♀ offspring and rearing by a dam treated with neither DBP or corn oil; 4 ♂ animals per dose and time point examined at the age of 5, 7, 9, 14, 17 weeks	**50 mg/kg body weight**: **NOAEL** for effects on the ♂ reproductive organs; **100 mg/kg body weight**: offspring: relative testis weights ↓ (weeks 9, 14, 17, time-dependent, weeks 5 and 7 not ↓), number of Leydig cells/area ↑ (weeks 9, 14, 17, time-dependent, weeks 5 and 7 not ↑), testes: amount of sER ↓ (weeks 9, 14, 17; 5, 7 not ↓), serum: testosterone ↓ (weeks 5, 7, 9, 14, 17), serum: LH (↓ weeks 5, 7, ↑ weeks 9, 14, 17); without unusual changes: offspring: body weights, litter size, sex ratio; no data for maternal toxicity	Shirai et al. 2013

Table 1 (continued)

Species	Exposure	Findings	References
rat, SD, 4 ♀	**GD 12–21**, 0, 100 mg/kg body weight and day, intragastric, vehicle: corn oil, after birth: reduction of the litters to 4 ♂ and 4 ♀ offspring and rearing by a dam treated with neither DBP or corn oil; investigated at the age of 20 weeks	**100 mg/kg body weight**: offspring: relative testis weights ↓, serum: testosterone ↓ and LH ↑, testes: Leydig cell hyperplasia, degenerated testicular tubules, Leydig cells: atypical nuclei, an increase in free ribosomes, "stripped" rER, filaments of intermediate size, elongated cytoplasmic filopodia, atypical tight junctions and cilia formations, sER scarcely detectable; without unusual changes: dams: body weights, litter size, offspring: body weights, survival, sex ratio	Wakui et al. 2013
rat, SD Crl:CD(SD)IGS BR, 7–9 ♀	**GD 12–19**, 0, 1260–1520, 6300–7600 mg/kg diet (0, 112, 582 mg/kg body weight and day), examined on GD 19 or 20, 4 or 24 hours after terminating administration with the diet	**no NOAEL**; **112 mg/kg body weight and above**: foetuses: testes: testosterone concentration after 24 hours ↓, Leydig cell clusters ↑, number of MNG ↑, seminiferous cord diameters ↑, mRNA of different proteins or enzymes participating in cholesterol synthesis and transport as well as steroidogenesis ↓ (reversible after 4 hours and 24 hours at 112 mg/kg body weight); **583 mg/kg body weight**: foetuses: testes: testosterone concentration after 4 hours ↓, AGD ↓; no maternal toxicity	Struve et al. 2009
rat, SD, at least 3 ♀	**GD 10–19**, 0, 250, 500, 700 mg/kg body weight and day, gavage, vehicle: corn oil, examined on PND 31	**no NOAEL**; **250 mg/kg body weight and above**: offspring: ♂: preputial separation delayed; **500 mg/kg body weight and above**: offspring: ♂: absolute weights of LABC ↓, AGD/body weight ↓, retention of nipples, ERα, AR and Shh protein ↓; **700 mg/kg body weight**: offspring: ♂: body weights ↓ (PND 25, PND 31), cryptorchidism, hypospadia, absolute weights of testes, epididymis, ventral prostate, seminal vesicles, Cowper's gland, glans penis ↓, serum: dihydrotestosterone ↓, caudal agenesis in epididymis, atrophy of the ventral prostate, serum: testosterone and dihydrotestosterone ↓, 5α-reductase II ↓	Kim et al. 2010

Table 1 (continued)

Species	Exposure	Findings	References
rat, Wistar, 6–9 ♀	**GD 13–21**, 0, 100, 500 mg DBP/kg body weight and day, 150 mg DEHP/kg body weight and day, 100 mg DBP + 150 mg DEHP/kg body weight and day, gavage, vehicle: rape seed oil, examined on GD 21, PND 13, PND 90	**100 mg/kg body weight**: **NOAEL** for effects on the ♂ reproductive organs; **100 mg/kg body weight and above**: foetuses: ♂: relative AGD (related to body weight) ↓, enlarged clusters of Leydig cells (1 animal, at 500 mg/kg body weight: 2 animals, no details of how many animals examined: 3 animals per litter, but 6–9 dams per group); **500 mg DBP/kg body weight**: foetuses: testes: testosterone concentration ↓, seminiferous cord diameter ↑, percentage of seminiferous cords with MNG ↑, offspring: ♂: average number of nipples ↑ (PND 13); **100 mg DBP + 150 mg DEHP/kg body weight**: foetuses: testes: testosterone concentration ↓, seminiferous cord diameter ↑, percentage of seminiferous cords with MNG ↑; no maternal toxicity, no unusual changes, offspring: age at preputial separation, weights of testes, epididymis, seminal vesicles, LABC, number of spermatids per testis in adult rats	Martino-Andrade et al. 2009
rat, Wistar, at least 3 ♀	**GD 13–21**, 0, 100, 500 mg DBP/kg body weight and day, gavage, vehicle: corn oil, examined on PND 25	**100 mg/kg body weight and above**: offspring: absolute weights of ventral prostate ↓ (not dose-dependent); **500 mg DBP/kg body weight**: offspring: absolute testis weights ↓, absolute weights of seminal vesicles ↓, AGD ↓, penis length ↓; no details of body weights and no details of relative organ weights, study not suitable for assessing developmental toxicity because of the limited scope of the study	MacLeod et al. 2010

Table 1 (continued)

Species	Exposure	Findings	References
rat, Albino, no other details, at least 6 ♀	**GD 14–birth**, 0, 2, 10, 50 mg/kg body weight and day, gavage, vehicle: corn oil, positive controls: 6 µg DES/kg body weight and day, only ♂ offspring examined: PND 0, PND 75	**2 mg/kg body weight and above**: dams: gestation period ↑, body weight gains on GD 21 ↓; offspring: body weights on PND 21 ↓ (16%, at 10 mg/kg body weight: 13%, no dose-dependency); **10 mg/kg body weight and above**: offspring: body weights on PND 0 ↓ (3.6%; at 10 mg/kg body weight: 1.7%; no dose-dependency), **50 mg/kg body weight**: offspring: body weights on PND 75 ↓ (3.7%), absolute organ weights of epididymis, testes, prostate, seminal vesicles ↓, relative organ weights not decreased (calculated from tabular values, hence reduction in absolute organ weights attributed to reduced body weights), number of sperms ↓ (7.7%), percentage of motile sperms ↓ (3.2%), percentage of sperm abnormalities ↑ (45.6%); without unusual changes: litter size, number of live offspring, foetal mortality, sex ratio, results not plausible, as a further reduction in body weights of offspring at PND 21 compared with PND 0 is not to be expected, increased percentage of sperm abnormalities questionable in view of the high demands required of the method, study therefore not suitable for assessing developmental toxicity	Ahmad et al. 2014
rat, F344 (microarray), SD (SREBP), 3–4 ♀	**GD 12–20 (50 mg/kg body weight), GD 12–19 (500 mg/kg body weight), only GD 10 (500 mg/kg body weight, F344), GD 12–20 (SD)**, 0, 50, 500 (F344), 0, 100, 500 (SD) mg/kg body weight and day, gavage, vehicle: corn oil, examined: 1–18 hours after final dose	**F344**: **500 mg/kg body weight**: foetuses: carbohydrate, amino acid and energy metabolism ↓, lipid metabolism ↓, particularly that regulated by SREBP; **SD**: **100 mg/kg body weight and above**: foetuses: MNG ↑; **500 mg/kg body weight**: foetuses: AGD ↓, testes: testosterone and total cholesterol ↓, SREBP2 in Leydig cells ↓, not in seminiferous cords	Johnson et al. 2011

Table 1 (continued)

Species	Exposure	Findings	References
mouse, CD1, 3 ♀	**GD 14–17, only GD 18,** 0, 250 (GD 14–17), 500 (only GD 18) mg/kg body weight and day, gavage, vehicle: corn oil, examined on: GD 17 (6 hours after final dose) or GD 18 (2, 4, 8 hours after final dose)	**250 mg/kg body weight and above**: foetuses: carbohydrate, amino acid, energy metabolism ↓, lipid metabolism ↑, particularly that regulated by SREBP	Johnson et al. 2011
mouse, wild-type, p53-heterozygote, p53-null, at least 3 ♀	**GD 12–birth**, 0, 250 (only adults examined), 500 (course of development over time) mg/kg body weight and day, gavage, vehicle: corn oil, examination: only testes of ♂ foetuses/adults investigated, GD 19, PND 1, 4, 7, 10, 87–515 (only 250 mg/kg body weight and day)	**250 mg/kg body weight**: adults: no abnormal findings in germ cells of wild-type mice, DBP-treated heterozygotes and vehicle controls in p53-null mice, but abnormal germ cells in p53-null mice; **500 mg/kg body weight**: foetuses: no abnormal findings in germ cells of wild-type mice, increased formation of MNG in testes at GD 19 in p53-null (more pronounced than in p53-heterozygotes), which decreased in a time-dependent manner and were no longer detectable on PND 10; immunohistochemical staining of perinatal MNG and adult abnormal germ cells yielded negative results for octamer binding protein-3/4 and placental alkaline phosphatase (marker for primordial germ cells and CIS-cells); study not suitable for assessing developmental toxicity as knockout mice are an artificial system without workplace relevance	Saffarini et al. 2012
Prenatal and postnatal treatment			
rat, Wistar, 10 ♀	**GD 12–PND 21**, 0, 100 mg/kg body weight and day, gavage, vehicle: corn oil, examined on: GD 20, PND 1, 90	**100 mg/kg body weight**: offspring: ♂: ventral prostate: relative proportions of epithelial and stromal compartments ↑ and of luminal compartment ↓, inflammatory infiltrates in stroma with or without epithelial dysplasia and intraepithelial neoplasia, accumulation of collagen fibrils adjacent to epithelium, AR ↑ (protein), proliferation index ↑, metalloproteinase-9 activity ↑; no unusual findings: body weights (at birth, PND 90), absolute and relative weights of ventral prostate (PND 90), serum and testicular testosterone (PND 90)	Scarano et al. 2009

Table 1 (continued)

Species	Exposure	Findings	References
rat, Wistar, 10 ♀	**GD 12–PND 21**, 0, 100 mg/kg body weight and day, gavage, vehicle: corn oil, examined on: PND 90	**100 mg/kg body weight**: offspring: ♂: testes: Leydig cell clusters, MNG, interstitial compartment ↑ (GD 20); no unusual findings: AGD (PND 1), body weights (PND 90), absolute and relative weights of testes, epididymis, ventral prostate, seminal vesicles (PND 90), serum and testicular testosterone (PND 90), epididymis: proliferation index, sperm count in testes and epididymis (PND 90), sperm transit time (PND 90), sperm morphology and motility (PND 90), AR and AQP9 immunoreactivity (PND 90), epididymis: relative ratio of epithelial, stromal or luminal compartments; testes not examined histologically on PND 90	Scarano et al. 2010
rat, Wistar, 9–10 ♀	**GD 6–PND 28**, 0%, 0.037%, 0.111%, 0.333%, 1% in the diet (GD 6–21: 0, 31, 94, 291, 797 mg/kg body weight and day; PND 0–15: 0, 55, 165, 486, 1483 mg/kg body weight and day; PND 16–28: 0, 47, 140, 433, 1283 mg/kg body weight and day)	**94 mg/kg body weight**: **NOEL** for ♂ offspring AGD ↓; **291 mg/kg body weight**: **NOAEL** for behavioural effects in the offspring; **291 mg/kg body weight and above**: offspring: ♂: AGD ↓ (PND 1); **797 mg/kg body weight**: dams: gestation period ↑; offspring: ♂ and ♀: body weights ↓ (PND 0, PND 7, PND 14, PND 21, PND 28), relative liver weights ↑ (PND 28); ♂: relative testis weights ↓ (PND 28), ♂: time to standing on all four feet from supine position ↑ (PND 7), forepaw grip time ↓ (PND 10, also at 31; not at 94 and 291 mg/kg body weight); no unusual findings: number of live offspring/litter, sex ratio, offspring: ♂ and ♀: age at developmental milestones (pinna detachment, incisor eruption, eye opening), time to standing on all four feet from supine position (PND 4), landing on four feet from supine position in the air (PND 16), negative geotaxis (PND 4 or PND 7), cliff avoidance test (PND 7), open field behaviour (PND 28); contrary results from Morris water maze test (spatial learning and reference memory inhibited at low dose, increased at high dose)	Li et al. 2009

Table 1 (continued)

Species	Exposure	Findings	References
rat, Wistar, 10 ♀	**GD 6–PND 28**, 0, 25, 75, 225, 675 mg/kg body weight and day, gavage, vehicle: corn oil	**225 mg/kg body weight**: **NOAEL** for foetotoxicity; **675 mg/kg body weight**: offspring: ♂ and ♀: body weights ↓ (PND 1, PND 21), ♂: relative AGD (related to body weight) ↓ (PND 1), ♂: relative liver weights ↑ (PND 21); ♂: relative testis weights ↓ (PND 21), ♂: Morris water maze test: improvement of spatial acquisition (PND 30–33), retention of spatial memory ↑ (PND 60–62) (contradiction to study from 2009), hippocampus: mRNA and protein BDNF ↑ (PND 21); no unusual findings: number of live offspring/litter, survival of offspring, p-CREB immunocontent in hippocampus unchanged	Li et al. 2010

AGD: anogenital distance; AR: androgen receptor; AQP9: aquaporin 9; BDNF: brain derived neurotrophic factor; CIS: carcinoma in situ; CREB: cyclic adenosine monophosphate (cAMP) response element binding protein; DES: diethylstilbestrol; DBP: di-*n*-butyl phthalate; DEHP: di(2-ethylhexyl) phthalate; ER: oestrogen receptor; GD: gestation day; LABC muscle: levator ani-bulbocavernosus muscle; LH: luteinizing hormone; MNG: multinucleated germ cells; PND: postnatal day; rER: rough endoplasmic reticulum; Shh: sonic hedgehog; SD: Sprague Dawley; sER: smooth endoplasmic reticulum; SREBP: sterol regulatory element-binding protein

Prenatal treatment

The testes of male Sprague Dawley rat foetuses that had been exposed in utero from gestation days 12 to 20 to di-*n*-butyl phthalate doses of 0, 0.1, 1, 10, 30, 50, 100 or 500 mg/kg body weight and day administered to the dams by gavage were examined histopathologically and morphometrically on day 21 of gestation. A reduced number of testicular cells was found after doses of 30 mg/kg body weight and day and above, while at 50 mg/kg body weight and day and above the testicular volume was reduced and the morphometry (size and organization) of the seminiferous cords, the precursors of the seminiferous tubules, was altered. For the course of development over time, doses of 0 or 500 mg/kg body weight and day from gestation day 12 up to the day before sacrifice were used, and the testes of the male foetuses were examined on gestation days 17, 18, 19, 20 and 21, and on postnatal days 1 and 2. At the only dose tested of 500 mg/kg body weight and day, the testicular volume and number of testicular cells were reduced from day 19 of gestation onwards; both the tubular and the interstitial cells were affected. At this dose, both effects were found to be reversible. Further studies showed that di-*n*-butyl phthalate reversibly inhibits the proliferation of somatic cells (determined via bromodeoxyuridine incorporation) in the testes of foetal rats. The number of apoptotic cells in the testes detected by TUNEL (terminal deoxynucleotide transferase-mediated deoxy-UTP nick labeling) staining was not affected. This indicates that decreased proliferation is the mechanistic explanation for the reduced number of testicular cells, rather than increased apoptosis of the cells. According to the authors, the reduced number of testicular cells is transient at 30 mg/kg body weight and day and postnatally becomes normal (Boekelheide et al. 2009). In the description of the method, postnatal examination of the group given doses of 30 mg/kg body weight and day is not reported and there is no diagrammatic information in the publication. As the reduced number of testicular cells was reversible at the high dose of 500 mg/kg body weight and day, reversibility can be assumed also for the lower dose levels. The reversibility of the reduced number of cells is regarded as regeneration, and is therefore not considered adverse. The altered morphometry of the seminiferous cords at dose levels of 50 mg/kg body weight and day and above remains a critical effect. No morphometric examination of the seminiferous tubules was carried out on postnatal day 2. Thus, it cannot be excluded that the altered morphometry may be permanent, and a no observed adverse effect level (NOAEL) of 30 mg/kg body weight and day has therefore been derived for effects on the male reproductive organs.

In the male offspring of Sprague Dawley rats treated in utero from days 12 to 21 of gestation, reduced relative testis weights and Leydig cell hyperplasia were observed at 100 mg/kg body weight and day at the age of 9, 14 and 17 weeks. The extent of the reduction in testis weights and the level of Leydig cell hyperplasia increased in a time-dependent manner. The serum concentrations of testosterone were reduced time-dependently at 100 mg/kg body weight and day from the age of 5 to 17 weeks. At this dose, the serum luteinizing hormone (LH) level was reduced after 5 and 7 weeks, but subsequently increased. Electron microscopy of the Leydig cells showed that from the age of 9 weeks, at the highest dose tested of 100 mg/kg body weight and day, the smooth endoplasmic reticulum (sER) was reduced (compared with that in the control animals) with non-dilated cisternae. At the age of 17 weeks, sER

could no longer be detected by light microscopy (Shirai et al. 2013). The NOAEL for effects on the male reproductive organs is therefore 50 mg/kg body weight and day. The reduced testosterone concentrations precede Leydig cell hyperplasia; this is therefore interpreted as a compensatory response. There are no data for cytotoxicity. In another study, the rough endoplasmic reticulum (rER) was still demonstrable after doses of 100 mg/kg body weight and day (Wakui et al. 2013). While the rER is responsible for accumulating energy, steroid hormones are formed in the sER. Even if the sER is not detectable, the cell is still viable as long as the rER is not impaired.

The male offspring of Sprague Dawley rats given di-*n*-butyl phthalate intragastrically at only one dose levelof 100 mg/kg body weight and day from days 12 to 21 of gestation were found at the age of 20 weeks to have reduced relative testis weights and changes in the testes, demonstrated by light and electron microscopy, such as Leydig cell hyperplasia (Wakui et al. 2013).

In the male foetuses of Sprague Dawley rats given di-*n*-butyl phthalate with the diet from days 12 to 19 of gestation, an increase in Leydig cell clusters, an increased number of multinucleated germ cells and an increased diameter of the seminiferous cords were found at the lowest dose tested of 112 mg/kg body weight and day and above. The authors conclude that exposure to equal doses, be it with the diet or by gavage, produces similar responses in the male offspring (Struve et al. 2009). A NOAEL could not be derived.

In the male offspring of Sprague Dawley rats treated by gavage from days 10 to 19 of gestation, delayed preputial separation was observed on postnatal day 31 at the lowest dose tested of 250 mg/kg body weight and day and above (Kim et al. 2010). A NOAEL could therefore not be derived.

In male Wistar rats exposed to di-*n*-butyl phthalate in utero from days 13 to 21 of gestation, the anogenital distance was reduced in relation to the body weight at dose levels of 100 mg/kg body weight and day and above administered to the dams by gavage. In one animal at this dose and two animals given 500 mg/kg body weight, enlarged clusters of Leydig cells were found on gestation day 21. Treatment of the dams with 500 mg di-*n*-butyl phthalate/kg body weight and day did not affect the weights of the male reproductive organs or the number of spermatids per testis in adulthood. Simultaneous administration of 100 mg di-*n*-butyl phthalate/kg body weight and day and 150 mg DEHP/kg body weight and day led to reduced testosterone concentrations in the foetal testes and deformed seminiferous cords and multinucleated germ cells, while the same doses of the individual substances did not induce these effects (Martino-Andrade et al. 2009). As only one animal was found to have enlarged clusters of Leydig cells at 100 mg/kg body weight and day and the change in anogenital distance is not considered adverse, a NOAEL has been derived for effects on the male reproductive organs of 100 mg/kg body weight and day. Histopathological examination of the testes was not carried out in the adult offspring.

In a study in Wistar rats with gavage administration, only the absolute organ weights were determined. Body weights and the relative organ weights were not given (MacLeod et al. 2010). As the body weight is considered to be a confounder for the anogenital distance, it must be included in the interpretation of changes in the anogenital distance (US EPA 2005). Therefore, the study cannot be included in the evaluation.

The results of a study with albino rats (Ahmad et al. 2014) were not consistent with the other studies. A further decrease in the body weights of the offspring 21 days after the end of the exposure of the dams, which were exposed from gestation day 14 to the day of birth, is not plausible. The increased percentage of sperm abnormalities is not included in the evaluation as it is questionable whether the method used for the determination of this parameter was valid.

In male offspring of Sprague Dawley rats treated by gavage from days 12 to 20 of gestation, multinucleated germ cells were found in the testes at dose levels of 100 mg/kg body weight and day and above. A comparative study in the male offspring of rats treated in utero and in mice showed that the pathways of lipid metabolism, in particular those regulated by SREBP (sterol regulatory element-binding protein), were inhibited in rats, but induced in mice. This corresponded also with the repression of the steroidogenic pathway (Johnson et al. 2011).

In a study with male p53-null mice treated in utero, p53 was found to play a role in the perinatal apoptosis of di-*n*-butyl phthalate-induced multinucleated germ cells. In-utero treatment did not result in abnormal findings in the germ cells in the testes of wild-type mice up to doses of 500 mg/kg body weight and day. On the other hand, an increase in multinucleated germ cells was found in the testes in foetal p53-null mice; the number of multinucleated germ cells decreased with age and these were no longer demonstrable on postnatal day 10 (Saffarini et al. 2012). The knockout system is artificial and therefore has no relevance for the workplace.

Prenatal and postnatal treatment

The male offspring of Wistar rats given gavage doses of 100 mg/kg body weight and day from gestation day 12 to postnatal day 21, were found to have histopathological changes in the ventral prostate on postnatal day 90 (Scarano et al. 2009). As only one dose was used, at which effects occurred, it is not possible to derive a NOAEL. In a further study by the same research group using the same strain of rat and the same exposure scheme, Leydig cell clusters, an increase in multinucleated germ cells and an increase in interstitial compartments in the foetal testes were found at 100 mg/kg body weight and day. After the offspring had reached adulthood, no effects on the sperms and no histological effects on the epididymis were demonstrable at this dose (Scarano et al. 2010). As histological examination of the testes was not performed on postnatal day 90 in either of the studies, it is not possible to make a statement about the reversibility of the histological findings in the foetal testes.

In the male offspring of Wistar rats, a reduced anogenital distance was found at dose levels of 291 mg/kg body weight and day and above after dietary exposure of the dams from gestation day 6 to postnatal day 28. At the highest dose tested of 797 mg/kg body weight and day, systemic toxicity was found in both male and female offspring on postnatal day 7, and also behavioural changes in the male offspring, such as an increase in the time needed for righting onto all four feet from a supine position and a reduced forepaw grip time. While the low dose inhibited the spatial learning ability and reference memory in the Morris water maze test, the high dose had an enhancing effect. In this test, the two middle doses had no effect (Li et al. 2009). The contrary effects at the low and high doses in the Morris water maze test are not plausible. The reduced learning ability at the low dose could have been caused

by increased stress. Animals under stress achieve worse test results; for this reason, environmental factors leading to stress, such as temperature, light and noise, must be maintained at a constant level during the test (Bromley-Brits et al. 2011). The NOAEL for behavioural effects in the male offspring was 291 mg/kg body weight and day.

In the Morris water maze test, the male offspring of Wistar rats given gavage doses of 675 mg/kg body weight and day from gestation day 6 to postnatal day 28 exhibited improvements in spatial acquisition (comprehension of spatial position) and a better retention of spatial memory on postnatal days 30 to 33. At this dose, the body weights of the male rats were reduced by 21% on postnatal day 1, and by 14% on postnatal day 21 (Li et al. 2010). As a result of the reduced body weights in the male offspring on postnatal day 1 at the dose level 675 mg/kg body weight and day, a NOAEL for foetotoxicity of 225 mg/kg body weight and day has been derived. It is questionable whether an improvement in memory performance was obtained. In the Morris water maze test with the same strain of rat used in the study of 2009, the same research group did not find a reduction in spatial memory performance at the low dose after dietary administration (Li et al. 2009). In view of these contradictions, the results of the Morris water maze test are not included in the evaluation.

A study with Sprague Dawley rats (Hoshi and Ohtsuka 2009) is not included in the evaluation, as only two animals were used per dose group, and non-standardized tests were carried out for evaluating grooming behaviour.

A study in pregnant Wistar rats with a complex exposure scenario, a carcinogenic *N*-methyl-*N*-nitrosourea (MNU)-testosterone protocol (Peixoto et al. 2015), is not relevant for evaluating the developmental toxicity of di-*n*-butyl phthalate.

Xenograft experiments with foetal testes of mice, rats and humans in immunodeficient rats and mice

Foetal testis samples from 12 human donors (spontaneous abortions in weeks 14 to 20 of pregnancy) were xenografted into castrate male nude mice. The hosts were treated for 4 or 21 days with the vehicle, 500 mg di-*n*-butyl phthalate/kg body weight and day, or monobutyl phthalate, by gavage. All hosts were treated with human chorionic gonadotropin (hCG) to mimic normal human pregnancy. As a positive control, mice with rat foetal testis xenografts were exposed to di-*n*-butyl phthalate for 4 days. In mice with rat testis xenografts, exposure to di-*n*-butyl phthalate significantly reduced seminal vesicle weights, testis *Cyp11a1* and *Star* mRNA expression, and serum testosterone levels. In contrast, the exposure of animals to di-*n*-butyl phthalate and di-*n*-monobutyl phthalate did not affect serum testosterone concentrations or the weights of the seminal vesicles in the animals with human testis xenografts. The gene expression in the human testis xenografts was not investigated (Mitchell et al. 2012).

Foetal testes of Fischer rats (gestation day 16), C57BL/6NCrl mice and CD-1 mice (gestation day 15), and testis fragments (volume about 1 mm^3) from 26 human donors (spontaneous abortions in weeks 10 to 24 of pregnancy) were xenografted into the renal subcapsular space of rats and mice. As hosts, adult male immunodeficient Crl:NIH-Foxn1^{rnu} nude rats and BALB/c nude mice were used. Around 24 hours after surgery, the animals were given gavage doses of di-*n*-butyl phthalate of 0, 100,

250 or 500 mg/kg body weight and day in corn oil for 1, 2 or 3 days. Six hours after the final treatment, histological and steroidogenic examinations were carried out in the xenografted testes. Di-*n*-butyl phthalate induced the formation of multinucleated germ cells in the testis xenografts of rats, mice and humans. A reduction in the expression of genes responsible for the regulation of foetal testosterone biosynthesis, such as *Cyp11a1* (mitochondrial cholesterol monooxygenase), *Cyp17a1* (17α-hydroxylase/17,20-lyase/17,20-desmolase), *Scarb1* (scavenger receptor class B member 1), *Star* (steroidogenic acute regulatory protein) and *Insl3* (insulin-like 3), was found only in the testis xenografts of rats, but not in those of mice and humans. The ex-vivo testosterone production in the rat testis xenografts was reduced after exposure to di-*n*-butyl phthalate, whereas it remained unchanged in the mouse testis xenografts. The results for the ex-vivo testosterone production of human testis xenografts varied considerably. The authors explained this by the facts that the human xenografts were of different sizes and originated from different parts of the foetal testes, and covered a wide range of the gestation period (Heger et al. 2012).

The same research group carried out another study with adult male athymic Crl:NU(NCr) Foxn1^{nu} nude mice that had been castrated and xenografted with pieces of human foetal testis from 7 donors (spontaneous abortions in weeks 16 to 22 of pregnancy) into the renal subcapsular space. The hosts were subsequently treated with hCG for 4 weeks to stimulate testosterone production. Thereafter, the animals were given gavage doses of 500 mg di-*n*-butyl phthalate/kg body weight and day or 75 mg abiraterone acetate, a CYP17A1-inhibitor, for 2 weeks. While abiraterone acetate markedly reduced the testosterone concentration in serum and the weights of androgen-sensitive host organs, no effects on these androgenic end points were found with di-*n*-butyl phthalate. Di-*n*-butyl phthalate led to an increased percentage of multinucleated germ cells in the testis xenografts with borderline statistical significance. On the other hand, abiraterone acetate did not affect this end point. The substance additionally decreased the expression of genes responsible for transcription and cell differentiation while increasing the expression of genes involved in the epigenetic control of gene expression. Di-*n*-butyl phthalate induced the expression of genes coding for oxidative stress response and altered the expression of genes responsible for the actin cytoskeleton (Spade et al. 2014). Using abiraterone acetate (a CYP17A1 inhibitor) as a positive control, for the first time it was possible to demonstrate an antiandrogenic effect in a xenograft model with human foetal testes. However, di-*n*-butyl phthalate, which, like abiraterone acetate, reduces testosterone concentrations without an antagonizing effect on the androgen receptor, did not produce antiandrogenic effects in human foetal testes in this test system.

All three studies above show that di-*n*-butyl phthalate does not have antiandrogenic effects in human testis xenografts, and that the formation of multinucleated germ cells in human testis xenografts caused by di-*n*-butyl phthalate takes place independent of antiandrogenic effects.

Di-*n*-butyl phthalate-induced multinucleated germ cells are typically eliminated by postnatal day 10 in mice (Saffarini et al. 2012) and by postnatal day 16 in rats (Barlow and Foster 2003).

Generation studies

In the 2-generation study with continuous mating of Sprague Dawley rats already described in the 2010 documentation (documentation "Di-*n*-butyl phthalate" 2013), the animals were given 0%, 0.1%, 0.5% or 1.0% di-*n*-butyl phthalate with the diet (males: 0, 52, 256, 509 mg/kg body weight and day, females: 0, 80, 385, 794 mg/kg body weight and day). At and above the low dose of 52 (males) and 80 (females) mg/kg body weight and day, the number of live F1 offspring per litter was reduced in a dose-dependent manner (controls and the three dose groups in the order of increasing doses: 12.9 ± 0.2, 11.9 ± 0.3, 11.0 ± 0.5, 10.7 ± 0.4; decrease in percent per dose group compared with the number in the controls: 7.8%, 14.7%, 17%; statistically significant at the low dose and above using Dunnett's test or Shirley's test). Neither the number of live F2 offspring nor the number of live offspring from crossover mating tests, in which males and females of the high dose group (509 and 794 mg/kg body weight and day, respectively) were mated with control rats, were reduced. In the F2 generation, however, the weights at birth were decreased (controls and the three treated groups in the order of increasing doses: 5.97 ± 0.11, 5.60 ± 0.09, 5.60 ± 0.09, 5.00; decreases of 6.2%, 6.2% and 16%, respectively, compared with the number in the controls; statistically significant at the low dose and above using the chi-squared test or Shirley's test) (NTP 1995; Wine et al. 1997). Therefore, the lowest observed adverse effect level (LOAEL) for foetotoxicity is 52 mg/kg body weight and day for the male animals and 80 mg/kg body weight and day for the female animals. The dose also corresponds to the NOAEL for parental toxicity resulting from the reduced relative kidney weights in the male F0 animals. The NOAEL for effects on fertility is 256 mg/kg body weight and day for the male animals and 385 mg/kg body weight and day for the female animals (documentation "Di-*n*-butyl phthalate" 2013).

From a 1-generation study with mice already described in the 2010 documentation (documentation "Di-*n*-butyl phthalate" 2013), a NOAEL of 420 mg/kg body weight and day was obtained for parental and foetal toxicity and effects on fertility. In this study, and in fertility studies with female mice, fertility and the number of live offspring were reduced at 1410 mg/kg body weight and day (NTP 1995).

Summary

In the 2010 documentation (documentation "Di-*n*-butyl phthalate" 2013), to assess the prenatal toxicity of di-*n*-butyl phthalate at the workplace from studies with exclusively prenatal exposure and postnatal examination, a NOAEL of 50 mg di-*n*-butyl phthalate/kg body weight and day (LOAEL of 100 mg/kg body weight and day) for the retention of areola or nipples in Sprague Dawley rats was used (Mylchreest et al. 2000). In a subsequent publication, changed morphometry (size and organization) of the seminiferous cords, the precursors of the seminiferous tubules, was found in Sprague Dawley rats after in-utero exposure to doses of 50 mg/kg body weight and day and above from gestation days 12 to 20 (Boekelheide et al. 2009). Therefore, the most sensitive end point after prenatal exposure is no longer the retention of the areola or nipples, but changed morphometry of the seminiferous cords of male foetuses. The NOAEL for effects on the male reproductive organs is 30 mg di-*n*-butyl phthalate/kg body weight and day.

In two studies with mice already described in the documentation from 2010 (documentation "Di-*n*-butyl phthalate" 2013), di-*n*-butyl phthalate produced morphological changes of the germinal epithelium at dose levels of 250 mg/kg body weight and day and above (Gaido et al. 2007) and led to increased incidences of external malformations in the male offspring at 400 mg/kg body weight and day and above (ECB 2003). A dose of 100 mg/kg body weight and day was derived as the NOAEL for developmental and maternal toxicity (ECB 2003). According to ECB (2003), the description of the study is available only in the form of a summary. Because the methods used were not described and the results were not presented in detail, the study is not included in the evaluation.

When interpreting the differences in effects induced by phthalates in the various species, also the following points should be considered: (i) the large differences between the mammalian species in the organization, structure and biology of the interstitial testicular compartments, (ii) the possible age-related sensitivity range during the foetal period and after birth, which varies between the species and (iii) the intrinsic difference between the species in the timing of testis development (Albert and Jégou 2014).

Rats were found to be the animal species most sensitive to the effects induced by di-*n*-butyl phthalate on the male reproductive organs (Johnson et al. 2012; Kay et al. 2014). As a result of the species differences mentioned, doubts have been expressed regarding the extrapolation of data from studies with rodents to humans (Habert et al. 2014; Kay et al. 2014).

Manifesto (developmental toxicity)

Prenatal toxicity. The most sensitive end point of developmental toxicity in rats after prenatal exposure was found to be changed morphometry (size and organization) of the seminiferous cords, the precursors of the seminiferous tubules, at dose levels of 50 mg/kg body weight and day and above (Boekelheide et al. 2009). The NOAEL for effects on the male reproductive organs was 30 mg di-*n*-butyl phthalate/kg body weight and day. Teratogenic effects such as hypospadia and absent or partially developed epididymides occurred in rats at dose levels of 250 mg/kg body weight and day and above (Mylchreest et al. 1998, 1999). The NOAEL was 100 mg/kg body weight and day (Mylchreest et al. 1999).

In a 2-generation study with Sprague Dawley rats, at the lowest doses tested of 52 or 80 mg/kg body weight and day and above, birth weights were reduced by 5% to 8% (NTP 1995; Wine et al. 1997). Therefore, the LOAEL for foetotoxicity was 52 mg/kg body weight and day for the males and 80 mg/kg body weight and day for the females. The NOAEL for behavioural effects in the male offspring of prenatally and postnatally treated Wistar rats was 291 mg/kg body weight and day (Li et al. 2009).

From a 1-generation study in mice, a NOAEL for foetotoxicity of 420 mg/kg body weight and day was obtained. At 1410 mg/kg body weight and day, the number of live offspring was reduced (NTP 1995).

After oral administration in rodents, 60% to more than 90% of the di-*n*-butyl phthalate is rapidly absorbed (documentation "Di-*n*-butyl phthalate" 2013). Therefore, for

rats and mice, an oral absorption of 60% is assumed as the worst case. The following toxicokinetic data are taken into consideration for the extrapolation of the NOAELs or of the LOAEL for prenatal effects on male reproductive organs, teratogenicity or foetotoxicity to a concentration in workplace air: the corresponding species-specific correction values for the rat and the mouse (1:4 and 1:7), the assumed oral absorption of 60%, the body weight (70 kg) and respiratory volume (10 m^3) of the person, and the assumed 100% absorption by inhalation. The concentrations calculated from this and the differences to the MAK value of 0.58 mg/m^3 are given in Table 2. The differences between the MAK value and the calculated no observed adverse effect concentrations (NOAECs) for developmental toxicity and foetotoxicity and the lowest observed adverse effect concentration (LOAEC) for foetotoxicity are therefore sufficiently large, and the classification of di-*n*-butyl phthalate in Pregnancy Risk Group C has been confirmed.

Table 2 NOAELs for rats and mice relevant to the evaluation, toxicokinetic extrapolation of the NOAELs to a concentration in workplace air and resulting differences to the MAK value of 0.58 mg/m^3

References	Species, exposure	NOAEL: end point	Toxicokinetic extrapolation[a)] (mg/m^3)	Difference to MAK value of 0.58 mg/m^3
rat				
Boekelheide et al. 2009	prenatal, oral	30 mg/kg body weight and day: ♂ reproductive organs	32	55
Mylchreest et al. 1999		100 mg/kg body weight and day: teratogenicity	107	183
NTP 1995; Wine et al. 1997	prenatal and postnatal, oral	LOAEL: 80 mg/kg body weight and day: foetotoxicity, no NOAEL	84	145
mouse				
NTP 1995	prenatal and postnatal, oral	420 mg/kg body weight and day: foetotoxicity	252	435

[a)] (1:4 or 1:7) × 0.6 (oral absorption, animals)/1.0 (absorption by inhalation, humans)

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