The MAK Collection for Occupational Health and Safety

Nitrobenzene

MAK Value Documentation, addendum – Translation of the German version from 2017

A. Hartwig[1,*], MAK Commission[2,*]

[1] *Chair of the Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area, Deutsche Forschungsgemeinschaft, Institute of Applied Biosciences, Department of Food Chemistry and Toxicology, Karlsruhe Institute of Technology (KIT), Adenauerring 20a, Building 50.41, 76131 Karlsruhe, Germany*

[2] *Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area, Deutsche Forschungsgemeinschaft, Kennedyallee 40, 53175 Bonn, Germany*

* *email: A. Hartwig (andrea.hartwig@kit.edu), MAK Commission (arbeitsstoffkommission@dfg.de)*

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Nitrobenzene[1)]

MAK Value Documentation

A. Hartwig[1, *], MAK Commission[2, *]

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Abstract

The German Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area has re-evaluated carcinogenicity of nitrobenzene [98-95-3], considering all endpoints. Available publications and unpublished study reports are described in detail.

The genotoxic potential of nitrobenzene is low and might be a result of reactive oxygen species. Long-term studies with exposure by inhalation resulted in a significant increase in liver and kidney adenomas in rats, and lung and mammary adenomas in mice. In severely toxic concentrations a nonsignificant increase in carcinomas was observed, probably resulting from a chronic toxic mechanism, supported by secondary genotoxic or DNA-damaging effects at high doses. In view of other aromatic amines and the known mechanisms of action via phenylhydroxylamine, nitrobenzene is considered to be carcinogenic. Because of the non-linear dose-response relationship of the tumour incidences and as genotoxic effects play a minor part, nitrobenzene is classified in Carcinogen Category 4.

Eye and skin irritation from nitrobenzene is low. The critical effect is the bronchialisation in the lungs of mice in a long-term inhalation study. However, the incidence of bronchialisation at the lowest tested concentration of 5 ml/m³ is too high to calculate a NAEC. One long-term inhalation study in Sprague-Dawley rats resulted in a NOAEC of 1 ml/m³, another one in F344 rats showed minimal extramedullary haematopoesis in the spleen of males at the lowest tested concentration of 1 ml/m³. Using this concentration as point of departure, a MAK value of 0.1 ml/m³ is calculated, which is far below the LOAEC of the critical effect in the mouse lung. As the MAK value is derived from a systemic effect, nitrobenzene is assigned to Peak Limitation Category II. Because of the initial half-life of 5 hours, the excursion factor of 4 is set.

Skin contact may contribute significantly to systemic toxicity; therefore, the "H" notation is retained.

Damage to the embryo or foetus is unlikely when the MAK value is observed and thus, the substance is classified in Pregnancy Risk Group C.

Sensitization is not expected from the limited data.

Keywords

nitrobenzene; mechanism of action; toxicokinetics; metabolism; (sub)acute toxicity; (sub)chronic toxicity; irritation; allergenic effects; reproductive toxicity; fertility; developmental toxicity; genotoxicity; carcinogenicity; peak limitation; prenatal toxicity; germ cell mutagenicity; absorption through the skin; sensitization; occupational exposure; maximum workplace concentration; MAK value; toxicity; hazardous substance

Author Information

[1] Chair of the Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area, Deutsche Forschungsgemeinschaft, Department of Food Chemistry and Toxicology, Institute of Applied Biosciences, Karlsruhe Institute of Technology (KIT), Adenauerring 20a, Building 50.41, 76131 Karlsruhe, Germany

[2] Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area, Deutsche Forschungsgemeinschaft, Kennedyallee 40, 53175 Bonn, Germany

* Email: A. Hartwig (andrea.hartwig@kit.edu), MAK Commission (arbeitsstoffkommission@dfg.de)

1) The substance can occur simultaneously as vapour and aerosol.

Nitrobenzene[1)]

[98-95-3]

Supplement 2016

MAK value (2016)	**0.1 ml/m³ (ppm) ≙ 0.510 mg/m³**
Peak limitation (2016)	**Category II, excursion factor 4**
Absorption through the skin (1958)	**H**
Sensitization	**–**
Carcinogenicity (2016)	**Category 4**
Prenatal toxicity (2016)	**Pregnancy Risk Group C**
Germ cell mutagenicity	**–**
BLW (2016)	**100 µg aniline (released from haemoglobin conjugate)/l blood**
1 ml/m³ (ppm) ≙ 5.108 mg/m³	**1 mg/m³ ≙ 0.195 ml/m³ (ppm)**

Documentation for nitrobenzene is available from 1998 (documentation "Nitrobenzene" 2003). For the derivation of a MAK value and classification in Carcinogen Category 4, the necessary data were not available at the time. As a result of new data, for example regarding the mechanism of action and genotoxicity of nitrobenzene, it has become necessary to revise the classification of the substance and examine whether it is possible to derive a MAK value.

Toxic Effects and Mode of Action

Nitrobenzene has a low genotoxic potential, presumably caused by the formation of reactive oxygen species, as is the case with aniline (supplement "Aniline" 2010). This is confirmed by new investigations of its genotoxicity. In mammalian cells in vitro, nitrobenzene was found to have clastogenic potential at high concentrations (≥ 7 µg/ml) and aneugenic effects at markedly lower concentrations (≥ 0.012 µg/ml). Nitrobenzene is not mutagenic in Salmonella typhimurium. In rats, after oral administration, clastogenic effects occur in vivo, especially at high, toxic doses. No such

1) The substance can occur simultaneously as vapour and aerosol.

effects are induced in the bone marrow at low doses after intraperitoneal injection or inhalation in rats and mice.

After inhalation exposure at concentrations of 25 mg/m³ and above, nitrobenzene caused adenomas and in some cases carcinomas in the liver, kidneys and thyroid gland of rats and in the lungs and mammary gland of mice. As already described in the documentation from 1998 (documentation "Nitrobenzene" 2003), the tumours are not caused primarily by genotoxic mechanisms, but by cytotoxicity or systemic toxicity. As a result, also chronic toxic effects are found in the affected organs (see documentation "Nitrobenzene" 2003) at lower concentrations than those which induce tumours. Here also, the mechanism of action for tumour formation is similar to that of aniline; the tumours occur, however, in different organs, as is frequently the case with this class of substances.

After long-term inhalation exposure, bronchiolarization, centrilobular hepatocytomegaly, multinucleated hepatocytes and pigmentation of the olfactory epithelium occurred in mice at concentrations of 5 ml/m³ and above, and, in female animals, also degeneration of the olfactory epithelium. In F344 rats exposed to 1 ml/m³, pigmentation of the olfactory epithelium and, in the males, pigmentation and extramedullary haematopoiesis in the spleen occurred. At this concentration, no effects were seen in Sprague Dawley rats; at the concentration of 5 ml/m³, centrilobular hepatocytomegaly and pigmentation of the olfactory epithelium occurred.

In a large number of studies, toxic effects on the testes were found. Exposure to nitrobenzene initially causes a reduced sperm count and reduced sperm motility, leading to the complete loss of the germinal epithelium and reduced fertility after exposure for more than 2 weeks. In a 2-generation study with male F1 offspring, the fertility index, testis and epididymis weights and the sperm count were reduced after inhalation exposure to 40 ml/m³. Developmental toxicity was not observed in rats after exposure to 40 ml/m³, the highest concentration tested. In rabbits, at the highest concentration tested of 100 ml/m³, an increased number of resorptions was found, which was, however, close to that in historical controls.

There are no data available for the sensitizing effects of the substance on the skin and airways in humans. A valid local lymph node assay in mice yielded negative results.

Mechanism of Action

Methaemoglobin formation

In rats, after inhalation exposure for 90 days, concentration-dependent methaemoglobin formation was observed at concentrations of 5 ml/m³ and above. The NOAEC (no observed adverse effect concentration) was 1 ml/m³ (CIIT 1984). The formation of methaemoglobin (metHb) is caused mainly by the metabolite phenylhydroxylamine produced during the reduction of nitrobenzene (documentation "Nitrobenzene" 2003). In the erythrocytes, the reverse reaction of phenylhydroxylamine to nitrosobenzene takes place as part of a coupled process in which methaemoglobin is formed (Greim and Lehnert 1995).

The formation of metHb by the metabolite phenylhydroxylamine takes place in analogy to aniline. With aniline, phenylhydroxylamine is formed via oxidation, with nitrobenzene via reduction (documentation "Monocyclic aromatic amino and nitro compounds" 2005; Neumann 2007). With a haemoglobin-binding index (binding [mmol/mol Hb] per dose [mmol/kg body weight]) of 79, nitrobenzene seems to have stronger effects than aniline, which has a haemoglobin-binding index of 22 (documentation "Monocyclic aromatic amino and nitro compounds" 2005).

Reactive oxygen species

Free radicals are formed (see Figure 1) in the reductive metabolism of nitrobenzene to phenylhydroxylamine. At high doses, these reactive oxygen species can no longer be adequately captured by the cells.

Type I nitroreductase (oxygen-insensitive) is responsible for the two-electron reduction. In contrast, type II nitroreductase is responsible for the one-electron reduction, which produces a nitro radical anion; this in turn can be oxidized back to the initial substance (Ask et al. 2004). The quantitative participation of type I or type II nitroreductases in the metabolism of nitrobenzene has not been investigated. As has been described for aniline (supplement "Aniline" 2010) and other aromatic nitrogen compounds (documentation "Monocyclic aromatic amino and nitro compounds" 2005), redox active iron is released in erythrocytes exposed to high levels of methaemoglobin (metHb), which, via the Fenton and Haber-Weiss reactions, contributes as a catalyst for auto-oxidation reactions to the further formation of oxygen radicals. This causes a disturbance in the redox balance of erythrocytes. Possible sequelae are membrane changes as a result of lipid peroxidation, disturbance of the reversible interactions between the thiol groups of haemoglobin and those of glutathione, and the binding of haemoglobin to the erythrocyte membrane.

Protein binding

The intermediate nitrosobenzene is chemically reactive, and with glutathione and the thiol groups of proteins containing cysteine can form glutathione conjugates or protein conjugates (documentation "Nitrobenzene" 2003). It is assumed that the relatively stable glutathione-nitrosobenzene conjugates are transported throughout the whole body, can be cleaved homolytically to form the reactive glutathiyl radical and are then reduced to phenylhydroxylamine, or that the aniline can be cleaved after transformation to glutathione sulfinamide (Eyer 1979; Eyer and Lierheimer 1980; Eyer and Ascherl 1987; Maples et al. 1990).

Haemoglobin adducts are detectable for example in the erythrocytes of mice treated with nitrobenzene (Li et al. 2003).

Genotoxicity

The aneugenic effects of nitrobenzene are probably caused by damage to the spindle apparatus. In a cell-free system, nitrobenzene affected the tubulin-kinesin motor system (NOAEC 7.5 μM) and induced complete inhibition at the concentration of

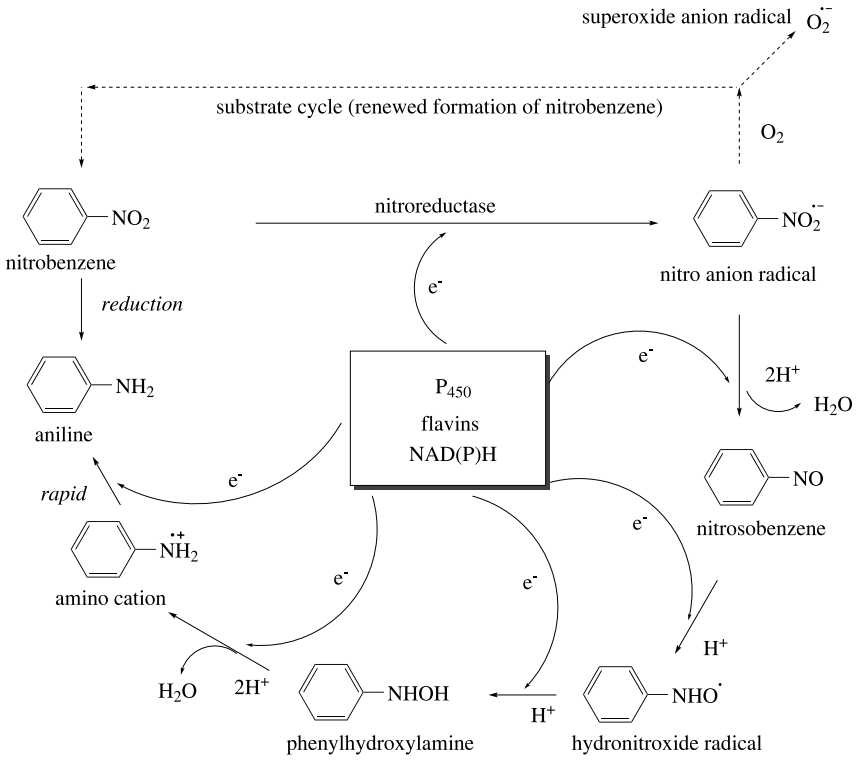

Figure 1 Mechanism of microsomal nitrobenzene reduction (US EPA 2009)

30 µM. Nitrobenzene inhibits the aggregation of tubulin at 1 mM, resulting in protein denaturation at 3 mM. In V79-cells, the cytoskeleton is not damaged up to the solubility limit of 15 mM (Bonacker et al. 2004).

The available results regarding the genotoxicity of nitrobenzene are similar to those obtained with aniline (supplement "Aniline" 2010).

The extent to which the clastogenic effects induced by the direct reaction of nitrobenzene or its metabolites with the DNA are caused by secondary erythrocyte toxicity or increased medullary erythropoiesis, has not been investigated in detail for nitrobenzene. For aniline, the following investigations indicate a relationship between DNA damage and erythrocyte toxicity, the release of redox active iron and increased erythropoiesis (supplement "Aniline" 2010):

The clastogenic effects occur only at aniline doses which are clearly toxic to the erythrocytes and the haematopoietic system in rats. Possible causes for the increased formation of micronuclei are, on the one hand, the reduced generation time and more rapid maturation (greater number of mitotic divisions) of the erythroblasts, which leads to a decrease in DNA repair efficiency, and, on other hand, the

increased occurrence of spontaneous DNA damage due to an increase in cell proliferation. In addition, the coupled co-oxidation reaction of the iron can result in oxidative damage in the bone marrow. Although the evidence suggests that the clastogenic effects induced by aniline are of secondary origin, it could not be excluded that very high doses induce chromosomal aberrations (supplement "Aniline" 2010). New, mechanistic studies have in the meantime confirmed that the oxidative stress caused by aniline leads to secondary genotoxic effects, which explains the tumour formation induced at high doses (Ma et al. 2008, 2011, 2013; Wang et al. 2010).

Carcinogenicity

Genotoxic effects occur only at high dose levels, at which usually also chronic inflammatory, degenerative or proliferative changes occur in the tissues developing tumours. Therefore, tumour formation can also be considered to be the result of chronic damage (documentation "Nitrobenzene" 2003; SCOEL 2002).

This applies to all aromatic amines, for which acute and chronic toxicity plays an important role especially in the promotion of tumours. In particular, proliferation is increased as a result of the toxic effects in the typical target organs, the spleen, liver and kidneys. Also in the long-term studies with nitrobenzene, chronic toxicity was observed in all tissues in which tumours were induced, accompanied by inflammation and increased proliferation. It is not yet clear to what extent this is the result of non-specific cytotoxicity, for example due to the increased degradation of erythrocytes, or whether specific, biochemical mechanisms, for example the inhibition of apoptosis or increased apoptosis, play a role (documentation "Monocyclic aromatic amino and nitro compounds" 2005).

In the case of aniline, it is assumed that the toxic effects are induced by the *N*-oxidation products phenylhydroxylamine and nitrosobenzene, and oxidative stress is a major factor in spleen damage. The formation of free radicals has been described in several studies, and confirmed for other aromatic amino and nitro compounds. After prolonged exposure to aniline, iron is continually released, which produces an increase in free iron in the spleen and in the Kupffer cells of the liver. The formation of haemoglobin thiyl radicals, as a result of the conversion of phenylhydroxylamine and oxyhaemoglobin, has been suggested as the cause of the haemolysis or the premature ageing of the erythrocytes. Accordingly, the release of iron within the erythrocytes would be the cause of this damage and not the result of the degradation of the damaged erythrocytes; this would agree with the occurrence of haemosiderosis in different parts of the organism. The fact that the tumour formation does not correlate with the extent of methaemoglobin (metHb) formation speaks against the notion of "spleen overload" due to erythrocyte degradation. Thus, nitrobenzene produces markedly more metHb than aniline, and also the corresponding effects on the spleen are observed; unlike aniline, nitrobenzene does not induce tumours in the spleen, but in various other tissues (documentation "Monocyclic aromatic amino and nitro compounds" 2005).

As before, it is difficult to understand the causal chain of metHb formation, spleen toxicity and tumour induction. The tumours are formed presumably at the sites with the greatest cytotoxicity; in the case of aniline and nitrobenzene these differ greatly, despite them sharing some of the same metabolites.

The free radicals produced during the reductive metabolism to phenylhydroxylamine can, at high doses, no longer be adequately captured by the cells. This indicates the occurrence of oxidative stress (see Figure 1, Section "Reactive oxygen species") in the target organs.

The species differences in tumour localization are presumably due to the differences in enzyme patterns (Ask et al. 2004), inducibility (Wang et al. 1992) and metabolic pathways (see Section "Toxicokinetics and Metabolism").

For nitrobenzene, nitroreductase activity has been demonstrated for all tumour localizations (i.e. the target organs) in rats (Ask et al. 2004). Also in mice, the activity of nitroreductases in the liver was demonstrated, although with a different substrate sensitivity (to the test substance 4-nitroquinoline 1-oxide) than in rats (Benson 1993). Another investigation revealed that the nitroreductases are induced in the lungs, but not in the liver, of mice exposed to polycyclic aromatic hydrocarbons (PAHs) (Wang et al. 1992). An earlier study confirmed also the activity of nitroreductases in the human mammary gland. It amounts to 1/8 of the activity in the human liver (Green et al. 1956).

Nitroreductase activity in humans has been demonstrated in the erythrocytes (Belisario et al. 1996), in the liver (Wen et al. 2008), and, in an earlier study, mainly in the intestines and liver, as well as in the ovaries, gallbladder, muscles, thyroid gland, kidneys, blood, mammary gland, brain and at low levels also in the spleen, pancreas and stomach (Green et al. 1956). It can therefore be assumed that the mechanism of action described above is relevant to humans.

To summarize, the carcinogenicity of nitrobenzene is based on two mechanisms: firstly, the cytotoxicity in various organs of different species (the target organs of carcinogenicity in humans are not known), and secondly, erythrocyte damage and the formation of superoxide radicals. As it is an aromatic amine, forms the same metabolites as aniline and has a higher haemoglobin binding index than aniline, nitrobenzene—in an overall assessment of the tumours involved—is regarded as a carcinogen. As, in addition, an effect threshold is recognizable, nitrobenzene can be evaluated in the same way as aniline, which is classified in Carcinogen Category 4 (supplement "Aniline" 2010).

The above-mentioned assumptions regarding the different tumour localizations are discussed below:

Liver

The liver tumours in male rats (carcinomas not significantly increased) were significantly increased only at the high concentration of 25 ml/m^3 (Cattley et al. 1994; CIIT 1993). The light-brown foci about 1 mm^2 in size occurring on the surface of the liver in a 90-day inhalation study in rats at concentrations of 50 ml/m^3 and above were interpreted as necrosis (CIIT 1984). In the 2-year inhalation study with F344 and Sprague Dawley rats, no hepatocellular necrosis or degeneration was described, although the incidence of preneoplastic foci in the eosinophilic cells of the liver increased in a dose-dependent manner in the same way as the incidence of liver tumours (CIIT 1993; Cattley et al. 1994). Liver hypertrophy and changes in liver function, such as delayed bromosulfophthalein excretion, yellow discoloration (icterus) in a slightly increased number of animals and an increase in indirect bili-

rubin were also found in a woman who had inhaled nitrobenzene (WHO 2003), so that it can be assumed that the liver is a target organ also in humans.

The question remains as to whether the organs affected by tumours are also the target organs for genotoxicity, and whether the latter participates in tumour formation. The following data are available for the liver:

No evidence of DNA repair synthesis (UDS) could be detected either in vitro in the hepatocytes of rats or humans (Butterworth et al. 1989) or in vivo in the liver of rats after the ingestion of up to 500 mg/kg body weight (Mirsalis et al. 1982). DNA damage was, however, found in the comet assay after single oral doses of 310 mg/kg body weight in rats (Mattioli et al. 2006).

One mechanism, which would explain the increased incidences of preneoplastic foci and liver tumours in rats, could take place via the reduction of the nitro group and the formation of phenylhydroxylamine. Reduction of the nitro group, but also oxidative metabolites and the combination of free iron and reactive oxygen species (another disturbance in the redox balance) and binding to macromolecules are assumed to be non-genotoxic causes of tumour formation (Hsu et al. 2007). Binding to macromolecules (DNA) in the liver of mice after intraperitoneal administration has been shown (Li et al. 2003). However, it was not clarified whether adducts were formed or not.

However, in the long-term inhalation study, unlike rats, mice were found to have neither tumours nor preneoplastic changes such as eosinophilic foci (CIIT 1993; Cattley et al. 1994). This difference in sensitivity between the species and also strains can be explained by the various metabolic pathways found in F344 rats, Sprague Dawley rats and mice (see Figure 2, Section "Toxicokinetics and Metabolism") or also differences between the species or even between the sexes as regards the inducibility of the enzymes. In mice, for example, nitroreductase cannot be induced in the liver by PAHs, but it can be induced in the lungs (Wang et al. 1992). Differences between the sexes are found also in the rat. Unlike in male rats, centrilobular hepatocytomegaly (with the correlating thyroid findings) was not found in female rats. It appears that enzyme induction does not occur in female rats; this can be a cause of centrilobular hepatocytomegaly. One reason for the fact that no tumours are produced in female rats could also be that the nitroreductase activity is lower in this sex (Aguilar et al. 1987).

With the nitro compound nilutamide (5,5-dimethyl-3-[4-nitro 3-(trifluoromethyl) phenyl]-2,4-imidazolidinedione), the formation of free nitro anion radicals in the rat liver and the formation of superoxide anions and hydrogen peroxide in rat liver microsomes, were found only in male Sprague Dawley rats (Hsu et al. 2007).

Conclusions: The liver tumours in the male rats (carcinomas not significantly increased) are presumably the result of two effects. One of these is the formation of reactive oxygen species, which, at high concentrations, are no longer captured by the cell and can then induce genotoxic effects. In addition, due to the enzyme-inducing effect of nitrobenzene, centrilobular hepatocytomegaly occurs. Both these effects are sex and species-specific, which is presumably a result of differences in metabolism. In addition, there are differences between F344 rats and Sprague Dawley rats with regard to metabolism and tumour incidence. The latter is pronounced only in F344 rats.

As in both, humans and mice, *p*-aminophenol and its conjugates have been identified in the urine (US EPA 2009), the human metabolism could be similar to that of mice, in which species no liver tumours were found. In the rat, *p*-aminophenol could not be detected in the urine (see also Figure 2, Section "Toxicokinetics and Metabolism"). The relevance for humans of the liver tumours is unclear, in spite of the fact that also in humans the liver is the target organ for the toxic effects of nitrobenzene.

Kidneys

Renal adenomas (carcinomas not significantly increased) occurred in male F344 rats only at the high concentration of 25 ml/m^3, but not in Sprague Dawley rats. In addition, in this 2-year inhalation study by Cattley et al. (1994), chronic nephropathy was found in almost all male F344 and Sprague Dawley rats in the control and treated groups, and also in most of the female F344 rats.

Chronic progressive nephropathy (CPN) is a spontaneous, age-related disease in rats, with a predisposition for its development in male animals; the precise etiology and pathogenesis of the disease is not known. In addition to age, diet seems to be an influencing factor, as a reduced incidence is found with a calorie-reduced diet. The glomerular dysfunction is accompanied by a high incidence of cell proliferation in the affected tubules, which is considered to be an increased risk for tubular neoplasms. Because there is no human equivalent for the CPN observed in rats, an increase in tubular tumours in combination with an increase in CPN is not considered relevant for humans (Weber et al. 2011). The dose-dependent occurrence, with a positive trend, of tubular epithelial hyperplasia was demonstrated in male F344 rats, in which also the number of renal tumours was significantly increased (Cattley et al. 1994).

As renal tumours occur only in male rats, these could have been formed as a result of the α_{2u}-globulin mechanism (US EPA 1991). This is, however, not relevant for humans (Swenberg 1993) and was not investigated in the case of nitrobenzene. In favour of this hypothesis is the fact that α_{2u}-globulin-induced tumour formation has been well investigated for 1,3,5-trinitrobenzene and confirmed. Against this, it can be argued that not all criteria for α_{2u}-globulin-induced tumour formation were actually met (Hsu et al. 2007).

The kidneys were found to be the target organ also for a possible genotoxic effect at high doses: in primary kidney cells of humans and rats, nitrobenzene caused the formation of micronuclei, and a positive result was obtained in the comet assay (Robbiano et al. 2004). In vivo, the oral administration of nitrobenzene likewise led to DNA damage in the comet assay (Mattioli et al. 2006; Robbiano et al. 2004) and the formation of micronuclei (Robbiano et al. 2004) in Sprague Dawley rats. In the study carried out by Mattioli et al. (2006), male rats were used; this is unclear in the study of Robbiano et al. (2004).

In the exposed male F344 rats, but also in the females, the occurrence of eosinophilic droplets in the tubules was increased in a concentration-dependent manner (Cattley et al. 1994). This effect is found also in the case of α_{2u}-globulin nephropathy, although not in female animals. This means that in the female F344 rats the renal toxicity does not lead to tumour formation. The fact that tumours were not found in male Sprague Dawley rats could be explained by the above-mentioned differenc-

es in metabolism; the genotoxicity study with Sprague Dawley rats yielded positive results in the kidney cells.

A further mechanism could take place via the highly active prostaglandin H synthases located in the renal cortex. As a result of their hydroperoxidase activity, generally co-oxidation of aromatic amines can take place. They are then converted into the corresponding nitroso and nitro derivatives, which, via a coupled redox reaction, in turn leads to the super oxide anion and possibly also to 8-oxo-7,8-dihydroguanine (Hsu et al. 2007). However, here too, there is no explanation why only male F344 rats develop tumours.

Conclusions: The tumour formation in the kidneys of male F344 rats is presumably the result of oxidative stress, combined with chronic progressive nephropathy (CPN). Nitrobenzene is also toxic to the kidneys of male Sprague Dawley rats, but does not induce tumours, although genotoxicity was observed in the kidney cells of male Sprague Dawley rats. The strain, sex and species sensitivity could, as in the liver, be enhanced by differences in metabolism.

As a result of the age-dependent occurrence of CPN, rats are more susceptible to effects on the kidneys than other species. As there is no clear correlate for CPN in humans, an increase in tubular tumours, together with increased CPN, is not considered relevant for humans (Weber et al. 2011).

Thyroid gland

Tumours of the thyroid gland are found only in male F344 rats. There was a statistically significant increase in the total incidence of adenomas and carcinomas in the trend test, but not in the incidence of carcinomas alone. In male B6C3F1 mice, a significant increase in thyroid gland adenomas occurred only at the highest concentration (CIIT 1993; Cattley et al. 1994).

DNA damage was found in vitro in the comet assay in thyroid gland cells of male rats (females were not tested); in the thyroid gland cells of male and female human donors, however, nitrobenzene did not induce DNA repair synthesis (UDS). In vivo, single oral doses at half the LD_{50} in male rats (females not tested) likewise produced DNA damage in the thyroid gland in the comet assay (Mattioli et al. 2006). The investigations by Mattioli et al. (2006) therefore confirm that the thyroid gland is the target organ for a possible overloading of reactive oxygen species; this could have produced the findings in the comet assay as well as the increase in the number of adenomas in mice and of adenomas and carcinomas in rats.

Another possible explanation for the mechanism by which the thyroid gland adenomas develop could be the induction of phase II enzymes in the liver. As a result of this induction, the degradation of the thyroid gland hormone thyroxine (T4) is increased by glucuronidation, and that of triiodothyronine (T3) by sulfation. This is followed by a compensatory increase in thyroid-stimulating hormone (TSH) with subsequent hyperplasia of the follicular cells (Hsu et al. 2007). It can be assumed that in female F344 rats nitrobenzene does not lead to a corresponding enzyme induction as, unlike in the males, no centrilobular hepatocytomegaly is found in the females. In addition, the fact that only F344 rats are found to have tumours in the thyroid gland, but not Sprague Dawley rats, corresponds to the findings in the liver, in which the male F344 rats were affected to a greater extent. Mice also were found

to have centrilobular hepatocytomegaly and to react in the same way with the induction of thyroid gland adenomas.

Hormonally induced tumour formation therefore seems to be the predominant mechanism in the thyroid gland after exposure to nitrobenzene; the increased formation of reactive oxygen species at high concentrations can have an additional impact on tumour formation.

Conclusions: The tumours in the thyroid gland are attributed to the induction of metabolizing enzymes, indicated by liver hypertrophy and centrilobular hepatocytomegaly, and the resultant disturbance in the metabolism of the thyroid gland hormones. Humans are much less sensitive to disturbances in this hormonal mechanism than rats (Meek et al. 2003); the tumours therefore have hardly any relevance for humans.

Lungs

In male B6C3F1 mice, the total incidence of adenomas and carcinomas in the lungs was increased in a statistically significant manner, but not that of carcinomas alone (CIIT 1993; Cattley et al. 1994). The incidences are in the range of historical controls, although the dose effect is clearly evident. There are no investigations available of the genotoxicity of nitrobenzene in the lungs.

In F344 and Sprague Dawley rats, no tumours occurred in the lungs (CIIT 1993; Cattley et al. 1994). The reason for this sex-specific and also species-specific occurrence of lung tumours in male mice treated with nitrobenzene is unclear (see also Section "Carcinogenicity"). In an NTP inhalation study, ethylbenzene was the only substance to induce lung tumours ("some evidence") in male mice exclusively, for which no explanation was given. In this review of the historical NTP data, also the different species-specificity in rats and mice remains unclear (Moore et al. 2013).

The activity of nitroreductase can be demonstrated in the lungs of rats (Ask et al. 2004). Also in mice, nitroreductase activity in the lungs, inducible by PAHs, can be demonstrated (Wang et al. 1992).

For 4-nitrophenol, a metabolite of nitrobenzene, enzymatic conversion to 4-nitrocatechol has been shown in rat liver microsomes. The presence of a highly active 4-nitrophenol hydroxylase, which is responsible for this step, can also be demonstrated in lung microsomes from sheep. The formation of catechol from benzene or nitrobenzene could be a reason for their possible carcinogenicity, as catechols are chemically reactive and are capable of binding to cellular macromolecules (WHO 2003).

With regard to lung tumour development, mice are presumably more sensitive than other species, as minimal bronchiolarization is frequently already present in untreated mice (Hedrich 2012). If bronchiolarization has taken place, cells of a cubic to high columnar type are found on the alveolar wall, with the differentiation of bronchiolar epithelial cells, such as cilia-bearing cells, Clara cells and mucosal cells. In mice, such findings are often obtained after exposure to irritants (Hedrich 2012).

The bronchiolarization of alveolar spaces is found also in humans suffering from idiopathic pulmonary fibrosis, in the form of the epithelialization of honeycomb cysts in areas of fibrotic restructuring (DePianto et al. 2014). It is considered to be a premalignant finding (Wang et al. 2009).

As pulmonary carcinomas result from fibrosis also in humans, these findings in mice per se are seen to be relevant for humans. In a study with rats, bronchiolarization was observed after the inhalation of ozone (Stockstill et al. 1995), which would support the hypothesis that this condition can be induced by reactive oxygen species.

After the inhalation of nitrobenzene, a clear effect on the number of animals with bronchiolarization is found, whereas this finding is not observed in controls. This finding is equally pronounced in females and in males; however, the tumours develop exclusively in the latter. Graduation of the severity of the effects was not carried out, however, so that no concentration–effect relationship can be derived.

A better agreement with the sex-specificity of lung tumours is found for bronchoalveolar hyperplasia. The latter frequently occurs in untreated mice, and its incidence increases with age and generally correlates with the bronchoalveolar tumours (Hedrich 2012).

The lung tumours found in mice are usually alveolar type 2 cell adenomas, which occur with a high spontaneous incidence (Hedrich 2012). This tumour type formerly occurred very rarely in humans, although the number of cases has been increasing over recent years, for example also in smokers of filter cigarettes. For this reason, the relevance for humans of the adenomas occurring in the lungs of mice is a matter of controversy at present. In the case of bronchiolarization, it is not the alveolar type 2 cells that proliferate, but the epithelial cells of the terminal bronchioles. These proliferate by penetrating into the adjacent pulmonary parenchyma (see above). Therefore, it would be important to know whether the increased incidences of bronchoalveolar tumours involve alveolar type 2 cell adenomas/adenocarcinomas (rarer in humans) or bronchiolar adenomas/adenocarcinomas in the terminal airway cells (frequent tumour-initiating cells in humans). Such details are, however, not available.

Conclusions: The lung tumours in male mice are in the range of historical controls, although a dose-dependent increase can be observed. Bronchiolarization is also found in mice. This can be seen as a reaction to the irritating effect of a substance and also as a pre-cancerous effect. The severity of this finding is equally pronounced in female animals, which do not develop tumours, and in males.

Bronchoalveolar hyperplasia agrees to a better extent with the tumours found exclusively in the males. The lung tumours occurring in mice are usually alveolar type 2 cell adenomas with a high spontaneous incidence; their relevance for humans is at present a subject of controversy.

The increased tumour induction can probably be attributed to oxidative stress (reactive oxygen species), combined with possible species and sex-specific toxicity. Oxidative stress can also lead to irritation. The relevance for humans of these processes is unclear.

Mammary gland

Adenocarcinomas of the mammary gland occur in mice with a high spontaneous incidence (Haseman et al. 1998), which is possibly increased by the toxicity of nitrobenzene at high concentrations. No other information is available concerning the mechanisms behind the development of these tumours.

Reproductive toxicity

The disturbance in the male reproductive organs resulting from exposure to nitrobenzene seems to originate from effects on Sertoli cells. Other studies showed that apoptosis is the reason for the loss of germ cells caused by nitrobenzene. It has been speculated that factors released from Sertoli cells might be responsible. In addition, the influence of reactive oxygen species cannot be excluded (US EPA 2009).

Toxicokinetics and Metabolism

Nitrobenzene is rapidly absorbed by humans and animals after inhalation, oral and dermal exposure. However, it is eliminated only slowly, mainly with the urine (documentation "Nitrobenzene" 2003).

Particularly after oral intake, also intestinal bacteria are involved in the reduction of nitrobenzene to nitrosobenzene. After inhalation, the nitroreductases found in the lungs, liver and kidneys play a role in the reduction of nitrosobenzene to phenylhydroxylamine.

There are great differences in the metabolism of the substance between the species and also between the individual strains of rat (see Figure 2). This was already described in the documentation of 1998 (documentation "Nitrobenzene" 2003).

*p-A*minophenol and its conjugates, for example, could be detected as the main metabolites in the urine of rabbits, mice and humans after oral exposure, though not in F344 or CD rats (US EPA 1985).

After the inhalation exposure of volunteers to nitrobenzene concentrations of 1 to 6 ml/m^3 for 6 hours, 6% to 37% was recovered from the urine in the form of 4-nitrophenol. 4-Aminophenol was not found. The initial half-life of the urinary elimination of 4-nitrophenol was 5 hours, the terminal half-life 60 hours. This indicates that the substance accumulates during the working week. After intravenous administration of ^{14}C-nitrobenzene to volunteers, 60% of the dose was excreted with the urine with a half-life of 20 hours (IARC 1996). The half-life of the urinary elimination of *p*-nitrophenol and *p*-aminophenol in rats that had inhaled 25 ml/m^3 for 8 hours is also within the range of 20 hours (as read from a graph) (Ikeda and Kita 1964).

Effects in Humans

There are no data available for humans concerning the effects of nitrobenzene on skin and mucous membranes, or its allergenic effects and reproductive toxicity.

Single exposures

The symptoms of nitrobenzene intoxication are a burning sensation in the mouth and throat, nausea, vomiting, dizziness, coordination disorders, cyanosis, a smell of bitter almonds in the exhaled air, restlessness, tachycardia, a drop in blood pressure, collapse, signs of paralysis, unconsciousness and coma. Nitrobenzene-induced

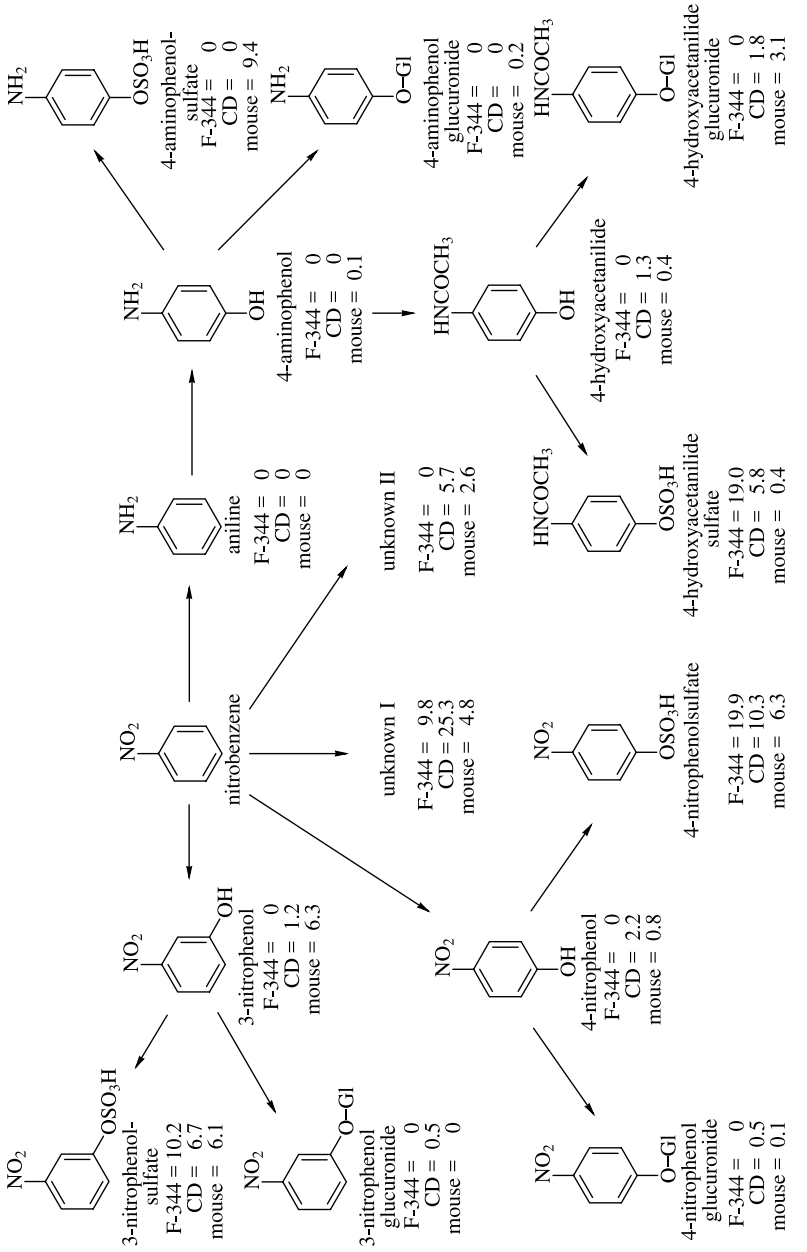

Figure 2 Metabolism of nitrobenzene in F344 rats, CD rats and mice. The figure shows the percentage of a dose of 225 mg/kg body weight recovered in the form of each metabolite in the 72-hour urine. Gl = glucuronic acid (documentation "Nitrobenzene" 2003).

methaemoglobin formation begins rapidly, within one hour, and can persist for days. It results in haemolytic anaemia, icterus and hypoxic damage in the internal organs. Deaths have been described in persons who had consumed nitrobenzene doses of between 5 and 16 ml and in persons exposed percutaneously (documentation "Nitrobenzene" 2003).

More recent cases not included in the documentation of 1998 (documentation "Nitrobenzene" 2003) with the typical symptoms of nitrobenzene intoxication have become available from India: a 5-year-old boy swallowed screen printing materials (no other details) containing nitrobenzene (no data for methaemoglobin level) (Gupta et al. 2000), a 32-year-old man developed cardiogenic pulmonary oedema, liver damage and a methaemoglobin level of 65% after the accidental intake of a dye (Agrawal et al. 2011) and a 24-year-old woman was found to have a methaemoglobin level of 56.5% after the intake of an unknown quantity of nitrobenzene (Chongtham et al. 1999). In an 82-year-old Spaniard, a methaemoglobin level of 70% and a nitrobenzene concentration in the blood of 3.2 µg/ml was found after the intake of a liquid containing nitrobenzene. The symptoms included coma, renal failure and ischaemic encephalopathy. He died three days after ingesting the nitrobenzene (Martínez et al. 2003).

Repeated exposure

The symptoms of chronic nitrobenzene poisoning are like those after acute intoxication. For the symptoms of intoxication in exposed persons, a LOAEL (lowest observed adverse effect level) of 40 ml/m^3 and a NOAEL (no observed adverse effect level) of 6 ml nitrobenzene/m^3 have been described (documentation "Nitrobenzene" 2003).

In a follow-up study with workers exposed to *o*-toluidine, aniline and nitrobenzene in a factory manufacturing rubber, the incidences of bladder cancer were still significantly increased. The incidence of bladder cancer correlated significantly with the calculated cumulative exposure. The authors attributed the tumour formation to exposure to *o*-toluidine (Carreón et al. 2014). As data for exposure concentrations are lacking and the workers were exposed to a mixture of substances, both this study and those preceding it cannot be used to assess the carcinogenicity of nitrobenzene in humans.

There are no suitable studies available for the assessment of the carcinogenicity of the substance in humans.

Animal Experiments and in vitro Studies

Subacute, subchronic and chronic toxicity

Inhalation

In rats and mice, particularly the spleen, liver, kidneys, testes, nose, brain and lungs were the target organs of nitrobenzene after repeated inhalation. These effects are

described in Table 1. No new studies have become available since the documentation of 1998 (documentation "Nitrobenzene" 2003).

While a NOAEC of 1 ml/m³ was obtained in the 2-year study with Sprague Dawley rats, pigmentation of the olfactory epithelium was observed in F344 rats at 1 ml/m³. At this concentration, pigmentation and extramedullary haematopoiesis are found also in the spleen of the F344 males. Both effects have a high spontaneous incidence which, however, is enhanced in a concentration dependent manner by exposure to nitrobenzene, at least in the male animals. Table 2 gives the incidences of effects in the different organs; it also shows a significant positive trend for these effects. In the liver of F344 and Sprague Dawley rats, eosinophilic foci, centrilobular hepatocytomegaly (males only) and spongiosis were observed as well as follicular cell hyperplasia in the thyroid gland of male F344 rats, and bilateral testicular atrophy and hypospermia in Sprague Dawley rats (Cattley et al. 1994; see also documentation "Nitrobenzene" 2003).

Nitrobenzene caused a concentration-dependent increase in methaemoglobin formation (see Table 4).

In the long-term study with mice, bronchiolarization in the lungs, centrilobular hepatocytomegaly and multinucleated hepatocytes, pigmentation of the olfactory epithelium and additionally in the females, degeneration of the olfactory epithelium occurred at the low concentration of 5 ml/m³ and above. Table 3 gives the incidences of effects in organs and tissues and shows the significant positive trend for follicular cell hyperplasia in the thyroid gland and degeneration of the olfactory epithelium in the males (Cattley et al. 1994, see also documentation "Nitrobenzene" 2003).

The critical effect for the derivation of a threshold limit value from the long-term studies in rodents is the bronchiolarization in mice. As the incidence of bronchiolarization was found to be very high (87%–92% compared with 0% in the controls) even at the lowest concentration, it is not possible to calculate a benchmark dose.

To summarize, a concentration without effects cannot be derived. The lowest LOAEC (lowest observed adverse effect concentration) of 1 ml/m³ was obtained from the long-term study with F344 rats with increased pigmentation of the olfactory epithelium as well as increased pigmentation and extramedullary haematopoiesis of the spleen in the males.

Oral administration

A toxicological assessment by the US EPA (2009) provides an overview of the studies with repeated oral administration which are suitable for the derivation of a threshold limit value. No new studies relevant to the evaluation have appeared since then. The studies of the toxicity of the substance after oral administration are summarized in Table 5.

No NOAEL could be derived from the 90-day studies with rats and mice carried out by the NTP (National Toxicology Program). For rats, the LOAEL was 9.38 mg/kg body weight and day, and for mice 18.8 mg/kg body weight and day. The systemic target organs—the liver, kidneys and haematological system—are the same as those found after inhalation exposure.

Table 1 Studies with repeated inhalation exposure to nitrobenzene relevant for the evaluation

Species, strain, number per group	Exposure	Findings	References
rats, F344, 10 ♂, 10 ♀	**90 days**, 0, 5, 16, 50 ml/m³, 6 hours/day, 5 days/week, whole-body exposure	**no NOAEC;** **5 ml/m³ and above**: ♂, ♀: spleen: acute sinusoidal congestion (all treated animals); increased haemosiderin pigmentation, ♂: blood: metHb and Ca^{2+} ↑, kidneys: minimal toxic nephrosis; **16 ml/m³ and above**: ♂: total bilirubin ↑, spleen: extramedullary haematopoiesis; focal accumulation of lymphocytes and macrophages and hyperplasia in the stroma, extending into spleen parenchyma in 1 ♂, bone marrow: hyperplasia, ♀: metHb ↑, liver and spleen weights ↑; **50 ml/m³**: ♂, ♀: spleen: size and weight ↑, spleen capsule: fibroblast hyperplasia (10 ♀, 10 ♂), on serosa surface mesenchymal cell proliferation enclosed by monocellular layer of differentiated mesothelial cells, focal accumulation of lymphocytes and macrophages and hyperplasia in the stroma extending into spleen parenchyma (6 ♀, 10 ♂), liver: weights ↑, light brown (necrosis) and red foci of about 1 mm² on surface, altered lobular structure, kidneys: moderate to severe toxic nephrosis in all animals, adrenal gland: basophilia in medulla cells, testes: size and weight ↓, moderate to severe degeneration of epithelial cells of the seminiferous tubules, cessation of maturation of primary and secondary spermatocytes, epididymis: Leydig cell hyperplasia, no mature sperms, proteinaceous material in the lumen of the spermatic duct, lymph nodes: proliferation of plasma and mast cells, accumulation of macrophages, multinucleated giant cells, ♀: spleen: extramedullary haematopoiesis	CIIT 1984

Table 1 (continued)

Species, strain, number per group	Exposure	Findings	References
rats, Sprague Dawley, 10 ♂, 10 ♀	**90 days**, 0, 5, 16, 50 ml/m³, 6 hours/day, 5 days/week, whole-body exposure	**no NOAEC;** **5 ml/m³ and above**: ♂, ♀: kidneys: toxic nephrosis (♂: 0/4/7/10, ♀: 0/0/2/5 at 0, 5, 16, 50 ml/m³), lungs: interstitial pneumonitis with emphysema without clear dose-dependency (♂: 1/6/5/5, ♀: 1/5/2/4 at 0, 5, 16, 50 ml/m³), infiltration of macrophages and lymphocytes in perivascular areas (♂: 2/5/4/7, ♀: 2/3/5/6 at 0, 5, 16, 50 ml/m³), nose: rhinitis (♂: 0/0/3/5, ♀: 0/0/0/1 at 0, 5, 16, 50 ml/m³), hyperplasia or metaplasia of epithelial cells of the anterior turbinates (♂: 0/0/3/6, ♀: 0/0/1/1 at 0, 5, 16, 50 ml/m³), goblet cell hyperplasia of the anterior septum (♂: 0/2/3/6, ♀: 0/0/0/5 at 0, 5, 16, 50 ml/m³), ♀: lungs: reactive lymphoid hyperplasia of the mesenteric lymph nodes: (♀: 2/6/9/9 at 0, 5, 16, 50 ml/m³); **16 ml/m³ and above**: ♂, ♀: weights of spleen and liver ↑, spleen: congestion in the small blood vessels, extramedullary haematopoiesis, liver: centrilobular hepatocytomegaly, enlarged cell nuclei, bone marrow: hyperplasia of the erythrocyte-forming cells, ♂: metHb ↑, spleen: haemosiderin pigmentation ↑, adrenal gland: basophilia of the medullary cells; **50 ml/m³**: ♂, ♀: spleen: number of lymphocytes and macrophages ↓, thickness of the splenic capsule ↑, liver: increased lobular pattern, pigmentation of Kupffer cells ↑, thyroid gland: hypertrophy of the colloid-filled follicles, ♂: testes: size ↓, atrophy, hyperplasia of interstitial cells, oedema, loss of seminal epithelium (starting at 5 ml/m³: 0/1/2/10 at 0, 5, 16, 50 ml/m³), epididymis: complete absence of mature sperms (starting at 5 ml/m³: 0/1/0/10 at 0, 5, 16, 50 ml/m³), ♀: metHb ↑, total bilirubin ↑, alanine aminotransferase ↑, Howell-Jolly bodies (nuclear fragments in erythrocytes), nose: hyperplastic changes (see above)	CIIT 1984

Table 1 (continued)

Species, strain, number per group	Exposure	Findings	References
rats, F344, 70 ♂, 70 ♀	**2 years**, 0, 1, 5, 25 ml/m³, 6 hours/day, 5 days/week, 10 ♂ and 10 ♀ per group examined already after 15 months, whole-body exposure	**no NOAEC;** **1 ml/m³ and above**: ♂, ♀: nose: pigmentation of olfactory epithelium, ♂: spleen: pigmentation and extramedullary haematopoiesis ↑; **5 ml/m³ and above**: ♂: liver: eosinophilic foci and centrilobular hepatocytomegaly; **25 ml/m³**: metHb ↑, erythrocyte count ↓, haematocrit and haemoglobin ↓, ♀: spleen: pigmentation, ♂: liver: spongiosis, kidneys: tubular epithelial hyperplasia; the incidences of organ changes are found in Table 2; chronic nephropathy (with accumulation of hyaline/eosinophilic droplets in the cytoplasm of the proximal tubular cells) in all animals (also in the controls), dose-dependent increase in severity	Cattley et al. 1994
rats, Sprague Dawley, 70 ♂	**2 years**, 0, 1, 5, 25 ml/m³, 6 hours/day, 5 days/week, 10 ♂ examined after 15 months, whole-body exposure	**1 ml/m³: ♂: NOAEC;** **5 ml/m³ and above**: ♂: liver: centrilobular hepatocytomegaly, positive trend for eosinophilic foci, nose: olfactory epithelium with pigmentation; **25 ml/m³**: ♂: metHb ↑, liver: spongiosis, testes: bilateral atrophy, hypospermia; the incidences of organ changes are found in Table 2; chronic nephropathy in all animals (also in the controls)	Cattley et al. 1994

Table 1 (continued)

Species, strain, number per group	Exposure	Findings	References
mice, B6C3F1, 10 ♂, 10 ♀	**90 days**, 0, 5, 16, 50 ml/m³, 6 hours/day, 5 days/week, whole-body exposure	**no NOAEC;** **5 ml/m³ and above**: ♂, ♀: spleen: haemosiderin pigmentation, extramedullary haematopoiesis ↑, ♂: spleen: lymphoid atrophy or degeneration, ♀: spleen: acute sinusoidal congestion, adrenal gland: cellular vacuolation in zona reticularis markedly ↑; **16 ml/m³ and above**: ♂: spleen: acute sinusoidal congestion; **50 ml/m³**: ♂, ♀: metHb ↑, spleen: hypertrophy, bone marrow: hyperplasia, lungs: hyperplasia of the bronchial mucosa (♂: 0/0/1/7; ♀ 0/2/2/6 at 0, 5, 16, 50 ml/m³), ♂: liver: basophilic hepatocytes with enlarged hypochromatic and partly multinucleated hepatocytes, ♀: liver: centrilobular hepatocytomegaly, spleen: lymphoid atrophy or degeneration	CIIT 1984
mice, B6C3F1, 70 ♂, 70 ♀	**2 years**, 0, 5, 25, 50 ml/m³, 6 hours/day, 5 days/week, 10 ♂ and 10 ♀ per group already examined after 15 months, whole-body exposure	**no NOAEC;** **5 ml/m³ and above**: ♂, ♀: lungs: bronchiolarization, nose: olfactory epithelium: pigmentation, ♂: liver: centrilobular hepatocytomegaly, multinucleated hepatocytes, ♀: nose: degeneration of the olfactory epithelium; **25 ml/m³**: ♂, ♀: lungs: bronchoalveolar hyperplasia, ♂: metHb ↑, haematocrit ↓, nose: degeneration of the olfactory epithelium, thyroid gland: follicular cell hyperplasia, testes: bilateral atrophy; hypospermia, ♀: nose: increased excretion from the respiratory epithelium; **50 ml/m³**: ♂, ♀: nose: glandularization of the respiratory epithelium, ♂: nose: increased excretion from the respiratory epithelium, testes: hypospermia, kidneys: cysts, ♀: thymus: involution, pancreas: mononuclear cell infiltration; the incidences of organ changes are shown in Table 3	Cattley et al. 1994

metHb: methaemoglobin

Table 2 Frequency of chronic toxic effects in rats (Cattley et al. 1994, see also documentation "Nitrobenzene" 2003)

			Exposure concentration (ml/m³)			
Findings	Strain / sex		0	1	5	25
liver	CD	♂	11/63 (17)*T)	3/67 (4)	8/70 (11)	19/65 (29)a)
eosinophilic foci	F344	♂	26/69 (42)*T)	25/69 (36)	44/70 (63)*	57/70 (81)*
		♀	6/70 (9)*T)	9/66 (14)	13/66 (20)	16/70 (23)*
centrilobular hepatocyto-	CD	♂	3/63 (5)*T)	1/67 (1)	14/70 (20)*	39/65 (60)*
megaly	F344	♂	0/69 (0)*T)	0/69 (0)	8/70 (11)*	57/70 (81)*
		♀	0/70 (0)	0/66 (0)	0/66 (0)	0/70 (0)*
spongiosis	CD	♂	25/63 (40)*T)	25/67 (37)	25/70 (36)	37/65 (57)*
	F344	♂	25/69 (36)*T)	24/69 (35)	33/70 (47)	58/70 (83)*
		♀	0/70 (0)*T)	0/66 (0)	0/66 (0)	6/70 (9)*
kidneys	CD	♂	54/63 (86)	60/67 (90)	63/70 (90)	59/65 (91)
chronic nephropathy	F344	♂	69/69 (100)	64/68 (94)	70/70 (100)	70/70 (100)
		♀	58/70 (83)	51/66 (77)	60/66 (91)	67/70 (96)
tubular epithelial	CD	♂	3/63 (5)	1/67 (1)	5/70 (7)	6/65 (9)
hyperplasia	F344	♂	2/69 (3)*T)	2/68 (3)	2/70 (3)	13/70 (19)*
		♀	0/70 (0)	0/66 (0)	2/66 (3)	2/70 (3)
thyroid gland	CD	♂	2/63 (3)	2/64 (3)	1/68 (1)	4/64 (6)
follicular cell hyperplasia	F344	♂	0/69 (0)*T)	1/69 (1)	2/70 (3)	4/70 (6)
		♀	1/69 (1)	–	–	0/68 (0)

Table 2 (continued)

			Exposure concentration (ml/m³)			
Findings	Strain / sex		0	1	5	25
nose	CD	♂	42/63 (67)*T)	49/64 (77)	60/66 (91)*	58/61 (95)*
pigmentation of olfactory	F344	♂	40/67 (60)*T)	53/67 (79)*	67/70 (96)*	68/69 (99)*
epithelium		♀	37/67 (55)*T)	54/65 (83)*	60/65 (92)*	66/66 (100)*
testes	CD	♂	11/62 (18)*T)	17/66 (26)	22/70 (31)	35/61 (57)*
bilateral atrophy	F344	♂	61/69 (88)	50/56 (89)	51/61 (97)	61/70 (87)
bilateral hypospermia	CD	♂	8/60 (13)*T)	12/65 (20)	15/67 (22)	32/59 (54)*
	F344	♂	15/69 (22)	21/54 (39)	12/59 (20)	12/70 (17)
spleen	CD	♂	58/63 (92)	56/67 (84)	61/69 (88)	60/65 (95)
extramedullary haemato-	F344	♂	53/69 (77)	62/69 (90)*	65/70 (93)*	61/70 (87)
poiesis		♀	60/69 (87)	62/66 (94)	60/66 (91)	65/69 (94)
pigment accumulation	CD	♂	59/63 (94)*T)	58/67 (87)	67/69 (97)	65/65 (100)
	F344	♂	55/69 (80)*T)	63/69 (91)*	64/70 (91)*	70/70 (100)*
		♀	62/69 (90)*T)	61/66 (92)	60/66 (91)	68/69 (99)*

*significant, $p < 0.05$; *T) significant positive trend, $p < 0.05$
a) numbers in brackets are percentages of the incidences

Table 3 Frequency of chronic toxic effects in B6C3F1 mice (Cattley et al. 1994, see also documentation "Nitrobenzene" 2003)

		Exposure concentration (ml/m³)			
Findings	Sex	0	5	25	50
lungs	♂	1/68 (1)*T)	2/67 (3)	8/65 (12)*	13/66 (20)*a)
bronchoalveolar hyperplasia	♀	9/53 (0)	2/60 (3)	5/64 (8)*	1/62 (2)
bronchiolarization	♂	0/68 (0)*T)	58/67 (87)*	58/65 (89)*	62/66 (94)*
	♀	0/53 (0)*T)	55/60 (92)*	63/64 (98)*	62/62 (100)*
thyroid gland	♂	1/65 (2)*T)	4/65 (6)	7/65 (11)*	12/64 (19)*
follicular cell hyperplasia	♀	2/49 (4)*T)	1/59 (2)	1/61 (2)	8/61 (13)
liver	♂	1/68 (1)*T)	15/65 (23)*	44/65 (68)*	57/64 (89)*
centrilobular hepatocytomegaly	♀	0/51 (0)*T)	0/61 (0)	0/64 (0)	7/62 (11)*
multinucleated hepatocytes	♂	2/68 (3)*T)	14/65 (22)*	45/65 (69)*	56/64 (88)*
	♀	0/51 (0)	0/61 (0)	0/64 (0)	2/62 (3)
nose	♂	10/67 (15)*T)	0/66 (0)	0/65 (0)	27/66 (41)*
glandularization of the respiratory epithelium	♀	0/52 (0)*T)	0/61 (0)	0/63 (0)	7/61 (11)*
increase in secretion from the respiratory epithelium	♂	0/67 (0)*T)	0/66 (0)	3/65 (5)	6/66 (9)*
	♀	2/52 (4)*T)	7/60 (12)	19/63 (30)*	32/61 (52)*
degeneration of olfactory epithelium	♂	1/67 (1)*T)	1/66 (2)	31/65 (49)*	41/66 (62)*
	♀	0/52 (0)*T)	19/60 (32)*	47/63 (75)*	42/61 (69)*
pigmentation of olfactory epithelium	♂	0/67 (0)*T)	7/66 (11)*	46/65 (71)*	49/66 (74)*
	♀	0/52 (0)*T)	6/60 (10)*	37/63 (59)*	29/61 (48)*
testes	♂	1/68 (1)	–	–	6/66 (9)
diffuse atrophy					
hypospermia					

Table 3 (continued)

		Exposure concentration (ml/m³)			
Findings	Sex	0	5	25	50
bone	♂	3/68 (4)*T)	10/67 (15)*	4/64 (6)	13/66 (20)*
hypercellular state	♀	4/52 (8)	–	–	9/62 (15)
thymus	♂	10/48 (21)	–	–	10/44 (23)
involution	♀	7/41 (17)	–	–	22/57 (39)*
kidneys	♂	2/68 (3)	–	–	12/65 (18)*
cysts	♀	0/51 (0)	–	–	0/62 (0)
pancreas	♂	3/65 (5)	–	–	3/64 (5)
mononuclear cell infiltration	♀	1/46 (2)	–	–	8/62 (13)*

*significant, $p < 0.05$; *T) significant positive trend, $p < 0.05$
a) numbers in brackets are percentages of incidences

Table 4 Methaemoglobin formation after inhalation of nitrobenzene over periods of different length

Dose [ml/m³]	Methaemoglobin [%]					
	F344 rats		Sprague Dawley rats		B6C3F1 mice	
	♂	♀	♂	♀	♂	♀
14 days	Medinsky and Irons (1985)					
0	0	3.6 ± 2.2	6.9 ± 1.3	4.8 ± 0.7	n. i.	n. i.
10	1.9 ± 0.7	4.8 ± 0.8	6.1 ± 0.5	6.3 ± 0.6	n. i.	n. i.
35	6.6 ± 0.2	6.6 ± 0.8	8.7 ± 1.0	7.3 ± 1.4	n. i.	n. i.
125	11.7 ± 1.2	13.4 ± 2.1	14.0 ± 1.0	31.3 ± 2.5	n. i.	n. i.
90 days	CIIT (1984)					
0	1.2 ± 0.8	1.6 ± 0.8	0.6 ± 0.2	2.1 ± 1.2	0.7 ± 0.6	1.3 ± 0.9
5	3.0 ± 1.0*	3.2 ± 0.9	0.9 ± 0.6	2.3 ± 0.6	1.6 ± 0.4	0.8 ± 0.5
16	4.4 ± 1.3*	3.9 ± 1.3*	3.2 ± 0.7*	3.7 ± 0.2	2.1 ± 1.3	2.0 ± 0.6
50	10.1 ± 1.2*	10.5 ± 1.5*	10.1 ± 2.0*	9.6 ± 2.5*	5.8 ± 1.7*	5.1 ± 0.8*
15 months	Cattley et al. (1994)					
0	2.9 ± 0.3	2.4 ± 0.4	1.2 ± 3.4	n. i.	n. i.	n. i.
1	3.2 ± 0.2	3.3 ± 0.4	4.1 ± 0.8**	n. i.	n. i.	n. i.
5	3.2 ± 0.4	3.2 ± 0.4	6.2 ± 1.6**	n. i.	n. i.	n. i.
25	4.7 ± 0.5**	5.9 ± 1.0**	5.9 ± 0.8**	n. i.	n. i.	n. i.

Table 4 (continued)

Dose [ml/m³]	Methaemoglobin [%]					
	F344 rats		Sprague Dawley rats		B6C3F1 mice	
	♂	♀	♂	♀	♂	♀
24 months	Cattley et al. (1994)					
0	3.9 ± 0.3	2.7 ± 0.4	2.8 ± 0.5	n. i.	2.0 ± 0.2	1.4 ± 0.2
1	3.3 ± 0.3	2.1 ± 0.2	2.9 ± 0.3	n. i.	n. i.	n. i.
5	4.2 ± 0.5	2.5 ± 0.3	2.4 ± 0.3	n. i.	1.9 ± 0.3	1.4 ± 0.2
25	5.3 ± 0.3**	5.0 ± 0.5**	4.6 ± 0.5*	n. i.	3.0 ± 0.4	2.2 ± 0.3
50	n. i.	n. i.	n. i.	n. i.	4.0 ± 0.5**	2.8 ± 0.2**

*$p < 0.05$; **$p < 0.01$; n. i. = not investigated

Allergenic effects

In a valid modified local lymph node assay (LLNA/IMDS (integrated model for the differentiation of skin reactions)) with female NMRI mice, nitrobenzene was tested at concentrations of 2%, 10% and 50% in acetone/olive oil (4:1; application volume 25 µl/ear). The investigated parameters were lymph node weights, the lymphocyte count in the lymph nodes, ear swelling and ear weights. There was no increased value for any of the parameters compared with those in the controls. For the three concentrations, the lymphocyte count indices were 0.96, 0.82 and 0.86, respectively; the test result is considered to be positive at a value of 1.4 and above (ECHA 2014).

An earlier skin sensitization study, in which 0.1 ml of a 10% nitrobenzene solution in dimethylformamide was applied to the outer sides of the ears of 6 guinea pigs daily for 3 days, did not yield any clear results. On day 7 after starting the test, 0.2 ml of the preparation in different concentrations (no further details) was applied to the shaved flanks of the animals. No skin reactions occurred 24 hours later (Stevens 1967).

The negative result obtained in another test with open epicutaneous application of a 3% preparation of the test substance in acetone for induction and a 0.3% preparation for the challenge (Sziza and Magos 1959) cannot be used in the evaluation because of incomplete documentation.

Reproductive and developmental toxicity

Fertility

Inhalation

In a 2-generation study with Sprague Dawley rats, inhaled nitrobenzene concentrations of 40 ml/m^3 and above led to a reduction in testis and epididymis weights, tubular atrophy in the testes, spermatocyte degeneration, and the presence of giant spermatocytes, with a resultant decrease in fertility. The NOAEL was 10 ml/m^3 (Sammelkapitel "MAK-Werte und Schwangerschaft" 1991, available in German only; documentation "Nitrobenzene" 2003).

Oral administration

Also after oral administration, nitrobenzene was found to be toxic to the reproductive organs. In a 90-day gavage study with F344 rats and B6C3F1 mice, atrophy of the testes was found (see Table 5). But also after single doses of 300 mg/kg body weight and day in F344 rats, nitrobenzene produced numerous histopathological findings such as necrosis of the germinal epithelium, multinucleated giant cells, a decrease in sperms and transient spermatogenic arrest (US EPA 2009).

Two fertility studies after oral administration are available.

In the more recent study, nitrobenzene doses of 60 mg/kg body weight and day were administered by gavage to groups of 10 male Sprague Dawley rats. These were mated with untreated females after 7, 14, 21, 28, 42, 56 or 70 days of treatment. One day after mating, the weights of the reproductive organs, sperm motility, progressive sperm motility, sperm count and sperm morphology were investigated. The testis and epididymis weights, sperm count, sperm motility and progressive motil-

Table 5 Studies with repeated oral administration of nitrobenzene relevant to the evaluation (US EPA 2009)*

Species, strain, number per group	Exposure	Findings
rat, F344, 6 ♂, 6 ♀	**28 days**, 0, 5, 25, 125 mg/kg body weight and day, gavage, 2-week recovery period	**5 mg/kg body weight: NOAEL;** **25 mg/kg body weight and above**: haemoglobin, haematocrit and erythrocytes ↓, MCV (haematocrit/erythrocyte count) and leukocytes ↑
rat, F344, 10 ♂, 10 ♀	**90 days**, 0, 9.38, 18.75, 37.5, 75, 150 mg/kg body weight and day, gavage	**9.38 mg/kg body weight: LOAEL;** **9.38 mg/kg body weight and above**: ♂, ♀: liver weights ↑, ♂: haemoglobin ↓, metHb ↑, kidney weights ↑, ♀: haemoglobin and haematocrit ↓, metHb and reticulocytes ↑; **18.75 mg/kg body weight and above**: ♂: haematocrit and erythrocytes ↓, reticulocytes ↑; **37.5 mg/kg body weight and above**: ♀: erythrocytes ↓, kidney weights ↑; **75 mg/kg body weight and above**: ♂: testis weights ↓; **150 mg/kg body weight:** lethal for 9 ♂ / 3 ♀
mouse, B6C3F1, 10 ♂, 10 ♀	**90 days**, 0, 18.75, 37.5, 75, 150, 300 mg/kg body weight and day, gavage	**18.8 mg/kg body weight: LOAEL;** **18.8 mg/kg body weight and above**: ♂, ♀: metHb ↑, ♀: liver weights ↑, reticulocytes ↑; **37.5 mg/kg body weight and above**: ♂: reticulocytes ↑; **75 mg/kg body weight and above**: ♂, ♀: haemoglobin ↓; **150 mg/kg body weight and above**: ♂: liver weights ↑; **300 mg/kg body weight:** ♂, ♀: hepatic cytomegaly, ♂: lethal for 3 animals, testis weights ↓

* Only end points with a clear dose–response relationship are included;
metHb: methaemoglobin; MCV: mean corpuscular volume (average volume of an erythrocyte)

ity were not significantly reduced until after 14 days of treatment, and the indices for sperm viability and fertility not until after 21 days of treatment. By day 21, the number of abnormal sperms was increased and in some cases there was no longer any motile sperm. The mating index was reduced only after 70 days. The authors concluded that sperm count and motility are the most sensitive parameters for the toxicity of nitrobenzene and that at least 14 days of exposure are necessary before effects on the fertility of the male rats are observed (Kawashima et al. 1995).

In a screening study carried out according to OECD Test Guideline 421, groups of 10 male and 10 female Sprague Dawley rats were given gavage doses of nitrobenzene of 0, 20, 60 or 100 mg/kg body weight and day for 14 days before mating, during mating and up to postnatal day 4, thus for 54 days in total. Significantly reduced testis and epididymis weights were found at dose levels of 60 mg/kg body weight and day and above. Histopathological examination revealed dose-dependent atrophy of the testes (0/10, 1/10, 10/10 and 10/10 at 0, 20, 60 and 100 mg/kg body weight and day), and, at 60 mg/kg body weight and day and above, Leydig cell hyperplasia and the loss of the intraluminal sperms in the epididymis. Effects on the offspring were seen on day 4 after birth at 20 mg/kg body weight and day and above in the form of reduced body weights in the male offspring; the survival index was reduced in the middle dose group in both sexes at birth and on day 4 after birth, and on day 4 after birth also in the high dose group. From day 19 of gestation, anaemia occurred in 6 dams of the middle dose group and neurological findings such as abnormal gait and torticollis were observed in one dam during lactation. Food consumption and body weight gains were reduced in the dams of the middle dose group during lactation. In the high dose group, the animals had unkempt fur, and salivation and anaemia were observed as from day 13 after treatment, and in some animals neurological abnormalities such as torticollis, circling movements and abnormal gait were found. Food consumption and body weight gains were reduced in the high dose group in both sexes. By the end of gestation 2 males and 7 females had died and 2 further females died during lactation (Mitsumori et al. 1994). The LOAEL obtained for foetotoxicity and toxicity was thus 20 mg/kg body weight and day in the parent animals.

In addition, the spermatotoxicity of nitrobenzene was shown in numerous other studies in which the substance was used as a positive control (US EPA 2009).

To summarize, a large number of studies show that nitrobenzene is toxic to the testes. The most sensitive end points are sperm count and sperm motility. In the end, exposure to nitrobenzene results in the complete loss of the germinal epithelium. Effects on fertility are observed only after exposure for more than 2 weeks, whereas damage to the reproductive organs has been demonstrated even after short-term exposure. The NOAEC for male reproductive toxicity in rats obtained from a 2-generation inhalation study is 10 ml/m^3.

Developmental toxicity

Inhalation

Two inhalation studies with rats of the embryotoxic potential of nitrobenzene, a 2-generation study and a teratogenicity study evaluated in the chapter "MAK-Werte und Schwangerschaft" (MAK values and pregnancy) 1991 are available (Sammelka-

pitel "MAK-Werte und Schwangerschaft" 1991, available in German only). In the teratogenicity study, no embryotoxic, foetotoxic or teratogenic effects were found even after inhalation of the clearly maternally toxic concentration of 40 ml/m³. In the 2-generation study, this high concentration produced reversible damage in the testes of the F0 males, although it was embryotoxic only insofar as the weight gains in the pups of the F1 generation were somewhat lower than those in the controls. For the rat, a NOAEC for developmental toxicity of 40 ml/m³ was obtained, with simultaneous, pronounced maternal toxicity, and a NOAEC for foetotoxicity of 10 ml/m³, without maternal toxicity.

Another, unpublished, company study dating from the 1980s has not yet been included in this assessment. In the respective range-finding study, 12 New Zealand White rabbits per group were exposed in whole animal chambers (whole-body exposure) to nitrobenzene concentrations of 0, 10, 40 or 80 ml/m³ from days 7 to 19 of gestation. The liver and kidney weights and the number of corpora lutea, live and dead foetuses, early and late resorptions and implantation sites were determined on day 20 of gestation. There were no substance-related findings. There were also no clinical signs or effects on the body weights of the dams. The only effect was increased methaemoglobin concentrations in the blood on days 13 and 19 of gestation (Biodynamics Inc 1984; US EPA 2009).

The main study comprised groups of 22 New Zealand White rabbits that were exposed whole-body to nitrobenzene concentrations of 0, 10, 40 or 100 ml/m³ for 6 hours daily, from days 7 to 19 of gestation. On gestation day 30, the number of corpora lutea, live and dead foetuses, early and late resorptions and implantation sites were determined and a complete examination of the foetuses for toxic effects on development was carried out. Apart from a slightly higher incidence of resorptions in dams of the 100 ml/m³ group (11 litters compared with 7 in the controls), there were no effects. The number of resorptions in the high concentration group was, in this test laboratory, close to that of historical control values. Maternal toxicity was evident in the increase in relative liver weights (up to 12%) and an increase in the methaemoglobin level by 40% and 60% in the two high concentration groups, respectively. The NOAEC for developmental toxicity obtained in rabbits was therefore 40 ml/m³ (Biodynamics Inc 1984; US EPA 2009).

Oral administration

In a screening study carried out according to OECD Test Guideline 421, groups of 10 male and 10 female Sprague Dawley rats were given gavage doses of nitrobenzene of 0, 20, 60 or 100 mg/kg body weight and day 14 days before mating, during mating and up to postnatal day 4 (for 53 days in total; see Section "Fertility"). On day 4, body weights were decreased in low dose male pups, in both sexes of the middle and high dose groups and at birth in the high dose group, in which the survival index was also reduced. Teratogenicity was not investigated. One dam died in the low and middle dose groups (Mitsumori et al. 1994). Thus, a LOAEL of 20 mg/kg body weight and day was obtained for foetotoxicity and maternal toxicity.

Genotoxicity

In vitro

Nitrobenzene is mutagenic only after metabolic activation and after the addition of Norharman in the Salmonella mutagenicity test (documentation "Nitrobenzene" 2003).

The new data for genotoxicity are described and presented in Table 6 below.

Investigations with cells from the target organs of nitrobenzene revealed DNA damage in renal and thyroid cells in the comet assay at concentrations of 7.6 µg/ml and above (Mattioli et al. 2006; Robbiano et al. 2004).

In the UDS test (DNA repair synthesis) with human thyroid cells or human and rat hepatocytes, nitrobenzene did not induce DNA repair synthesis (Butterworth et al. 1989; Mattioli et al. 2006).

In human blood lymphocytes, chromosomal aberrations were described. This study cannot be included in the assessment because of the inadequate and contradictory description of the results (Huang et al. 1995, 1996).

In human and rat primary kidney cells, nitrobenzene increased the frequency of micronuclei at high concentrations of 250 and 125 µM (31 or 15 µg/ml) and above, respectively (Robbiano et al. 2004).

In V79 cells, micronuclei were induced at concentrations of 0.1 µM (about 0.012 µg/ml) and above; about 72% of them were found to be CREST positive (Bonacker et al. 2004), which is indicative of an aneugenic effect.

In vivo

In rats, neither sister chromatid exchange nor chromosomal aberrations in the bone marrow were demonstrated after inhalation of concentrations of up to 50 ml/m^3 (Kligerman et al. 1983). No DNA repair synthesis in the rat liver (UDS test) was found after oral treatment with doses of up to 500 mg/kg body weight (Mirsalis et al. 1982).

After intraperitoneal injection of doses of up to 250 mg/kg body weight, no increase in the micronuclei frequency in the bone marrow of mice was found. The PCE/NCE ratio was not affected. Piloerection was observed at the low dose and above, breathing was irregular at the middle dose and above and apathy occurred at the high dose. In a range-finding study, mortality occurred at doses of 300 mg/kg body weight and above (BASF AG 1996) (see also documentation "Nitrobenzene" 2003).

Since then, studies with rats of the induction of DNA damage in the liver, kidneys and thyroid gland and of micronuclei in the kidneys, and a study of the binding of nitrobenzene to the DNA in the liver of mice have been published.

In mice, even at doses in the µg range, binding of nitrobenzene to the DNA in liver cells and to haemoglobin in the erythrocytes was demonstrated. ^{14}C-Labelled nitrobenzene was injected intraperitoneally and the DNA and the haemoglobin were investigated for ^{14}C-labelling after 2 hours. The dose of 0.1 µg/kg body weight served as a control, as it corresponded approximately to the nitrobenzene concentration in the ambient air (Li et al. 2003). The DNA binding was not specified in more detail. As a marker of exposure, this finding shows that binding to DNA takes

Table 6 Studies of the genotoxicity of nitrobenzene in vitro without metabolic activation systems in mammalian cells

End point	Test system	Conc.	Effective conc.	Cytotox.	Results	Remarks	References
DNA damage (comet assay)	primary kidney cells (human)	62–500 µM (7.6–62 µg/ml)	62 µM and above (7.6 µg/ml and above)	500 µM	+		Robbiano et al. 2004
	primary kidney cells (rat)	62–500 µM (7.6–62 µg/ml)	125 µM and above (15 µg/ml and above)	500 µM	+		Robbiano et al. 2004
DNA damage (comet assay)	primary thyroid follicular cells (rat)	1250–5000 µM (154–616 µg/ml)	1250 µM and above (154 µg/ml and above)	5000 µM	+		Mattioli et al. 2006
DNA repair synthesis (UDS)	primary thyroid cells (human)	1250–5000 µM (154–616 µg/ml)	–	5000 µM	–		Mattioli et al. 2006
	hepatocytes (human)	10–1000 µM (1.2–123 µg/ml)	–	–	–		Butterworth et al. 1989
	hepatocytes (rat)	10–1000 µM (1.2–123 µg/ml)	–	no data	–		Butterworth et al. 1989

Table 6 (continued)

End point	Test system	Conc.	Effective conc.	Cytotox.	Results	Remarks	References
chromosomal aberration	lymphocytes (human)	50–800 µM (6.2–98 µg/ml)	50 µM and above (6.2 µg/ml and above)	no data	+	results inadequately described, individual data lacking, contradictory concentrations reported	Huang et al. 1995
	lymphocytes (human)	50 mM (6200 µg/ml)	50 mM	no data	+	results inadequately described, individual data lacking, contradictory concentrations reported	Huang et al. 1996
micronucleus	V79	0.005–100 µM (0.0006–12 µg/ml)	0.1 µM and above (0.012 µg/ml)	–	+	72% CREST-positive	Bonacker et al. 2004
	primary kidney cells (human)	62–500 µM (7.6–62 µg/ml)	250 µM and above (31 µg/ml)	500 µM	+		Robbiano et al. 2004
	primary kidney cells (rat)	62–500 µM (7.6–62 µg/ml)	125 µM and above (15 µg/ml)	500 µM	+		Robbiano et al. 2004

conc. = concentration, cytotox. = cytotoxicity

place even at very low concentrations. However, whether this produces mutations remains unclarified.

In the comet assay with rats, single oral doses of half of the LD_{50} produced DNA damage in the target organs, the liver, kidneys and thyroid gland, and micronuclei in the kidneys (Mattioli et al. 2006; Robbiano et al. 2004). Lower doses were not investigated.

The in vivo studies of the genotoxicity of the substance are described in Table 7.

Genotoxicity of the metabolites

The nitrobenzene metabolite phenylhydroxylamine was mutagenic in the Salmonella typhimurium strain TA100 after metabolic activation and caused sister chromatid exchange in human fibroblasts (documentation "Nitrobenzene" 2003).

The metabolite nitrosobenzene produced DNA strand breaks in calf thymus DNA in the presence of NADH and copper(II) (Ohkuma and Kawanishi 1999).

To summarize, nitrobenzene was found to have clastogenic potential in vitro in mammalian cells at concentrations ≥ 7 µg/ml, and is aneugenic at markedly lower concentrations (approx. ≥ 0.012 µg/ml). In the mutagenicity test with Salmonella typhimurium, nitrobenzene alone was not mutagenic.

Especially at high toxic doses after oral administration, clastogenic effects were observed in the rat in vivo, whereas no clastogenic effects were induced in the bone marrow of rats and mice after intraperitoneal administration or inhalation at low doses.

Overall, the studies of the genotoxicity of nitrobenzene indicate a low genotoxic potential, similar to that of aniline, for which the formation of reactive oxygen species was confirmed to be the cause (see also Section "Mechanism of Action").

Carcinogenicity

Short-term studies

In human tumour cells from the lungs (WI-38) or the liver (Chang cells), no cell transformations were induced at nitrobenzene concentrations of up to 250 µg/ml (Styles 1978).

Long-term studies

Although the carcinogenicity studies with male and female Fischer 344 rats, male Sprague Dawley rats, and male and female B6C3F1 mice (Cattley et al. 1994; CIIT 1993) already included in the documentation of 1998 (documentation "Nitrobenzene" 2003) have shortcomings as regards the housing of the animals (loss of animals at the beginning of the study, wrongly assigned animals in an exposure group), these have no influence on the evaluation of the tumour incidences in the investigation (CIIT 1993). Despite these deficiencies, the study is therefore included in the assessment of the carcinogenicity of the substance.

Only the male rats were found to have tumours in the liver, kidneys and thyroid gland. For all three organs, the increase in the total incidences of adenomas and carcinomas was statistically significant, but not that of carcinomas alone. Positive

Table 7 Studies of the genotoxicity of nitrobenzene in vivo

Test system	Species, number of animals/group	Dose, exposure route, investigation time	Results	Remarks	References
in vivo/ex vivo					
SCE, lymphocytes from blood and spleen	rats, F344, 3 ♂	0, 5, 16, 50 ml/m³, 6 hours/day, 21 times in 29 days, inhalation	–	blood lymphocytes: mitotic index decreased in a dose-dependent manner	Kligerman et al. 1983
DNA repair synthesis (UDS), primary hepatocytes (^{3H}thymidine labelling)	rats, F344, 3 ♂	0, 200, 500 mg/kg body weight, gavage	–		Mirsalis et al. 1982
chromosomal aberrations, lymphocytes from blood	rats, F344, 3 ♂	0, 5, 16, 50 ml/m³, 6 hours/day, 21 times in 29 days, inhalation	–	mitotic index decreased in a dose-dependent manner and mitotic arrest	Kligerman et al. 1983
in vivo					
DNA binding, liver (accelerator mass spectrometry) detection limit: 1 binding/10^{11} DNA nucleotides	mice, Kunming, 2–3 ♂	0.1, 0.5, 2, 5, 20, 100 µg/kg body weight or 10 mg/kg body weight, ^{14}C-labelled nitrobenzene, intraperitoneal, examination after 2 hours	+	0.1 µg/kg body weight = control, as approximate nitrobenzene concentration in the air; DNA binding decreased in a dose-dependent manner, haemoglobin binding to erythrocytes increased in a dose-dependent manner, but increase very small	Li et al. 2003

Table 7 (continued)

Test system	Species, number of animals/group	Dose, exposure route, investigation time	Results	Remarks	References
DNA damage, kidneys (comet assay)	rats, Sprague Dawley, 3 ♂	0, 300 mg/kg body weight, oral, ½ LD_{50}, examination after 48 hours	+		Robbiano et al. 2004
DNA damage, kidneys, thyroid gland and liver (comet assay)	rats, Sprague Dawley, 3 ♂	0, 310 mg/kg body weight, oral, ½ LD_{50}, examination after 16 hours	+		Mattioli et al. 2006
micronucleus, kidneys	rats, Sprague Dawley, 3 ♂	0, 300 mg/kg body weight, oral, ½ LD_{50}, examination after 48 hours	+	no data for cytotoxicity	Robbiano et al. 2004
micronucleus, bone marrow	mice, B6C3F1, 5 ♂/5 ♀	I: 0, 62.5, 125, 250 mg/kg body weight, intraperitoneal, examination after 24 hours II: 0, 250 mg/kg body weight, intraperitoneal, examination after 48 hours	–	no cytotoxicity; **62.5 mg/kg body weight and above**: unkempt coat; **125 mg/kg body weight and above**: irregular breathing; **250 mg/kg body weight:** apathy and crouching posture; in pretest: ≥ **300 mg/kg body weight:** mortality	BASF AG 1996

results in the trend test were obtained for carcinomas of the liver and kidneys in F344 rats, but not in Sprague Dawley rats (see documentation "Nitrobenzene" 2003 and Table 8).

In the lungs of male B6C3F1 mice there was a statistically significant increase in the total incidence of adenomas and carcinomas, but not, however, in that of carcinomas alone. A significant increase in the incidence of thyroid gland adenomas was observed only at the high concentration. In this group, the incidence of adenocarcinomas of the mammary gland of female B6C3F1 mice was increased. The incidences in the other two exposed groups were not determined (see documentation "Nitrobenzene" 2003 and Table 8).

Summary and assessment of the tumours

The results of the investigations of genotoxicity indicate secondary genotoxic effects which are not of foremost importance for tumour formation.

The formation of tumours is considered to be mainly the result of chronic/cytotoxic damage, as, in the tissues which develop tumours, there are usually also chronic inflammatory, degenerative or proliferative changes present (documentation "Nitrobenzene" 2003 and see Table 1, Table 2 and Table 3 as well as Section "Mechanism of Action").

Liver

The liver tumours in male rats, in which the incidence of carcinomas was not significantly increased, occurred at the high concentration of 25 ml/m³; a significant positive trend was calculated. In addition, spongiosis hepatis, eosinophilic foci and centrilobular hepatocytomegaly were found.

As explained in the Section "Mechanism of Action", the liver tumours in the male rat are presumably the result of the formation of reactive oxygen species and centrilobular hepatocytomegaly. These two effects are both sex and species-specific, presumably because of the different metabolism. F344 and Sprague Dawley rats have both differences in metabolism and in their tumour incidences. As in both, humans and mice, *p*-aminophenol and its conjugates have been identified in the urine (US EPA 2009), the metabolism of the substance in humans could be similar to that in mice, where no increased incidences of liver tumours occurred. In the rat, *p*-aminophenol could not be demonstrated in the urine (see also Figure 2, Section "Toxicokinetics and Metabolism"). The relevance of the liver tumours for humans is not clear, although the liver is a target organ for the toxicity of nitrobenzene also in humans.

Kidneys

Renal adenomas and a non-significant increase in carcinomas were found only at the high concentration of 25 ml/m³ in the male F344 rats, but not in Sprague Dawley rats. Degenerative changes and thus chronic toxicity are assumed to be the cause also in this case (documentation "Nitrobenzene" 2003 and Section "Mechanism of Action"). Signs of renal toxicity (tubular epithelial hyperplasia) and adenomas were seen only in the 2-year study (Cattley et al. 1994), and only at the high concentration of 25 ml/m³, at which the animals of both sexes were found to have chronic nephropathy. However, in a 90-day study, minimal changes in the kidneys

Table 8 Studies of the carcinogenicity of nitrobenzene, see also documentation "Nitrobenzene" 2003

Author:	Cattley et al. 1994; CIIT 1993					
Substance:	nitrobenzene (purity > 99.8%)					
Species:	**rat**, F344/N, 70 ♂/70 ♀, **rat**, Sprague Dawley, 70 ♂					
Administration route:	inhalation					
Concentration:	0, 1, 5, 25 ml/m³					
Duration:	2 years, 5 days/week, 6 hours/day					
			Exposure concentration (ml/m³)			
			0	1	5	25
Liver, toxicity and preneoplasms						
centrilobular liver hepatocytomegaly	SD	♂	3/63 (5%)*T	1/67 (1%)	14/70 (20%)*	39/65 (60%)*
	F344	♂	0/69 (0%)*T	0/69 (0%)	8/70 (11%)*	57/70 (81%)*
		♀	0/70 (0%)	0/66 (0%)	0/66 (0%)	0/70 (0%)
spongiosis	SD	♂	25/63 (40%)*T	25/67 (37%)	25/70 (36%)	37/65 (57%)*
	F344	♂	25/69 (36%)*T	24/69 (35%)*T	33/70 (47%)	58/70 (83%)*
		♀	0/70 (0%)*T	0/66 (0%)	0/66 (0%)	6/70 (9%)*
eosinophilic foci	SD	♂	11/63 (17%)*T	3/67 (4%)	8/70 (11%)	19/65 (29%)
	F344	♂	26/69 (42%)*T	25/69 (36%)	44/70 (63%)*	57/70 (81%)*
		♀	6/70 (9%)*T	9/66 (14%)	13/66 (20%)	16/70 (23%)*

Table 8 (continued)

Liver, tumours (only in male animals)						
hepatocellular						
adenomas	SD	♂	1/63 (1.6%)	1/67 (1.5%)	2/70 (2.9%)	7/67 (10.4%)*
carcinomas		♂	2/63 (3.2%)	0/67 (0%)	2/70 (2.9%)	2/65 (3.1%)
adenomas and carcinomas		♂	2/63 (3.2%)	1/67 (1.5%)	4/70 (5.7%)	9/65 (13.8%)*
adenomas	F344	♂	1/69 (1.4%)	3/69 (4.3%)	3/70 (4.3%)	15/70 (21.4%)*
carcinomas		♂	0/69 (0%)*T	1/69 (1.4%)	2/70 (2.9%)	4/70 (5.7%)
adenomas and carcinomas		♂	1/69 (1.4%)*T	4/69 (6.0%)	5/70 (7.1%)	16/70 (22.9%)* a)
Thyroid gland, preneoplasms						
follicular cell hyperplasia	SD	♂	2/63 (3%)	2/64 (3%)	1/68 (1%)	4/64 (6%)
	F344	♂	0/69 (0%)*T	1/69 (1%)	2/70 (3%)	4/70 (6%)
Thyroid gland, tumours (only in male animals)						
follicular						
adenomas	F344	♂	0/69 (0%)	0/69 (0%)	2/70 (2.9%)	2/70 (2.9%)
adenocarcinomas		♂	2/69 (2.9%)	1/69 (1.4%)	3/70 (4.3%)	6/70 (8.7%)
adenomas and carcinomas		♂	2/69 (2.9%)*T	1/69 (1.4%)	5/70 (7.1%)	8/70 (11.4%)b)

Table 8 (continued)

Kidneys, toxicity and preneoplasms						
chronic nephropathy	SD	♂	54/63 (86%)	60/67 (90%)	63/70 (90%)	59/65 (91%)
	F344	♂	69/69 (100%)	64/68 (94%)	70/70 (100%)	70/70 (100%)
		♀	58/70 (83%)	51/66 (77%)	60/66 (91%)	67/70 (96%)
tubular epithelial hyperplasia	SD	♂	3/63 (5%)	1/67 (1%)	5/70 (7%)	6/65 (9%)
	F344	♂	2/69 (3%)*T	2/68 (3%)	2/70 (3%)	13/70 (19%)*
		♀	0/70 (0%)	0/66 (0%)	2/66 (3%)	2/70 (3%)
Kidneys, tumours (only in male animals)						
tubular adenomas	F344	♂	0/69 (0%)	0/68 (0%)	0/70 (0%)	5/70 (7.1%)*c)
carcinomas		♂	0/69 (0%)*T	0/68 (0%)	0/70 (0%)	1/70 (1.4%)d)
adenomas and carcinomas		♂	0/69 (0%)*T	0/68 (0%)	0/70 (0%)	6/70 (8.7%)*

* $p \leq 0.05$; *T significant positive trend, $p < 0.05$
historical controls: incidence, mean value, range (Haseman et al. 1998):
a) 35/903, 3.9%, 0–9%
b) 9/892, 1.0%, 0–4%
c) 9/902, 1.0%, 0–4%
d) 1/902, 0.1%, 0–2%

Table 8 (continued)

Author:	Cattley et al. 1994; CIIT 1993				
Substance:	nitrobenzene (purity > 99.8%)				
Species:	**mouse**, B6C3F1, 70 ♂/70 ♀				
Administration route:	inhalation				
Concentration:	0, 5, 25, 50 ml/m³				
Duration:	2 years, 5 days/week, 6 hours/day				
Toxicity:	50 ml/m³: body weights ↓				
		Exposure concentration (ml/m³)			
		0	5	25	50
Lungs, toxicity and preneoplasms					
bronchoalveolar hyperplasia	♂	1/68 (1%)*T	2/67 (3%)	8/65 (12%)*	13/66 (20%)*
	♀	0/53 (0%)	2/60 (3%)	5/64 (8%)*	1/62 (2%)
bronchiolarization	♂	0/68 (0%)*T	58/67 (87%)*	58/65 (89%)*	62/66 (94%)*
	♀	0/53 (0%)*T	55/60 (92%)*	63/64 (98%)*	62/62 (100%)*
Lungs, tumours					
bronchoalveolar					
adenomas	♂	7/68 (10.3%)*T	12/67 (17.9%)	15/65 (23.1%)	18/66 (27.3%)*
carcinomas	♂	4/68 (5.9%)	10/67 (15.0%)	8/65 (12.3%)	8/66 (12.1%)
adenomas and carcinomas	♂	9/68 (13.2%)*T	21/67 (31%)*	21/65 (32.3%)*	23/66 (34.4%)*a)

Table 8 (continued)

Thyroid gland, toxicity and preneoplasms									
follicular cell hyperplasia	♂	1/65	(2%)*T	4/65	(6%)	7/65	(11%)*	12/64	(19%)*
	♀	2/49	(4%)*T	1/59	(2%)	1/61	(2%)	8/61	(13%)
Thyroid gland, tumours									
adenomas	♂	0/65	(0%)*T	4/65	(6%)	1/65	(2%)	7/64	(11%)*
Mammary gland, tumours									
adenocarcinomas	♀	0/48	(0%)	n. i.		n. i.		5/60	(8.3%)*b)

* $p \leq 0.05$; *T significant positive trend, $p < 0.05$; n. i. = not investigated
historical controls: incidence, mean value, range (Haseman et al. 1998):
a) 251/1097, 22.9%, 10%–42% (Haseman et al. 1998)
b) 31/1902, 2.8%, 0%–8% (Haseman et al. 1998); up to 12% (CIIT 1993)

of F344 rats were described at concentrations of as little as 5 ml/m³ (CIIT 1984). The particular sensitivity of the F344 rat is presumably a result of chronic progressive nephropathy, which has no relevance for humans, and is probably based on the formation of reactive oxygen species or other toxic metabolites; these are formed in very different ways specific to the species, sex and also strain (see Section "Mechanism of Action").

Thyroid gland

In the thyroid gland of male F344 rats at the highest concentration, only adenomas together with carcinomas are not significantly increased (11.4%), the adenomas alone amounting to 2.9% and the carcinomas to 8.7%. In male B6C3F1 mice, a significant increase in thyroid gland adenomas (11%) occurred only at the highest concentration, but not in the case of carcinomas. Follicular cell hyperplasia is present in male but not in female rats, the same is true also for male and female mice; in this case, the higher incidence is found in the male animals (Cattley et al. 1994; CIIT 1993).

The tumours in the thyroid gland are attributed to the induction of xenobiotic-metabolizing enzymes in the liver and the resultant disturbances in the metabolism of the thyroid gland hormones (see Section "Mechanism of Action"). Regarding the disturbances in this hormonal mechanism, humans are far less sensitive than rats, so that the tumours are of hardly any relevance to humans.

Lungs

The incidence of lung tumours in male B6C3F1 mice, significant only when adenomas and carcinomas are combined, is within the range of historical controls (value of up to 34.4% compared with 10%–42% in controls, see Table 8). At concentrations of 25 ml/m³ and above, the animals were found to have increased incidences of bronchoalveolar hyperplasia and as from the low concentration a dose-dependent increase in bronchiolarization (87%–92% at 5 ml/m³ compared with 0% in the controls). In contrast to the tumour findings, this bronchiolarization, which can be seen as a pre-cancerous effect, occurred also in the females with incidences similar to those in the males. Bronchoalveolar hyperplasia agrees better with the tumour findings, which occur only in the male mice (Cattley et al. 1994; CIIT 1993).

There are no studies available for possible genotoxic effects in the lungs. In a review it has been shown that frequently only mice are affected by lung tumours, but not rats. Lung tumours are thus found only with 2 of 23 chemicals in both species (Haseman and Lockhart 1993). Sex-specific differences in lung tumour incidences in B6C3F1 mice were found in the NTP control groups, in which the male animals were found to have a somewhat higher spontaneous incidence than the females. However, compared with the incidences in controls, the increase in tumours induced by chemicals is higher in the females than in the males (Moore et al. 2013). In NTP inhalation studies, the only substance that likewise induced lung tumours ("some evidence") only in male mice but not in females, is ethylbenzene. In this case, however, no explanation is given for either the sex-specific effect or the different organ specificity in rats and mice.

The increased tumour induction in the lungs of mice caused by nitrobenzene is presumably due to oxidative stress (which presumably also induced the irritation)

combined with a—possibly species and sex-specific—toxic effect. As described also in the Section "Mechanism of Action", the relevance of these tumours for humans is not clear.

Mammary gland

The incidence of adenocarcinomas in the mammary gland of mice was determined only at the high concentration of 50 ml/m^3. At 8.3% it was within the range of that found in historical controls of up to 12% (see Table 8). As the mammary adenocarcinomas in mice occur with a high spontaneous incidence, which is possibly increased by the toxicity at high concentrations, and the incidence is nevertheless within the range of that in historical controls, these findings are not assumed relevant to humans.

Manifesto (MAK value/classification)

After inhalation exposure, nitrobenzene causes tumours in the liver, kidneys and thyroid gland of rats, and in the lungs and mammary gland of mice. Nitrobenzene has a low genotoxic potential. Effects relevant for a critical threshold limit are bronchiolarization in mice and pigmentation of the spleen (haemosiderosis) in rats.

Carcinogenicity.
After inhalation of high concentrations, nitrobenzene causes adenomas and in some cases also carcinomas. These occurred in the liver, kidneys and thyroid gland in rats and in the lungs and mammary gland of mice. The substance is genotoxic only at high doses. As discussed in detail in the Section "Carcinogenicity" and in the Section "Mechanism of Action", the tumours induced by nitrobenzene are caused primarily by chronic toxic mechanisms; also—presumably secondary—genotoxic or DNA damaging effects are found at high dose levels.

Nitrobenzene and aniline share the same metabolite, phenylhydroxylamine, which is responsible for the formation of reactive oxygen species. The induction of methaemoglobin (metHb) formation by nitrobenzene is markedly higher than by aniline, and also the corresponding effects are observed in the spleen. Unlike aniline, however, nitrobenzene does not induce tumours in the spleen, but in various other tissues. It remains difficult to establish a causal chain for metHb formation, from spleen toxicity up to tumour induction. The tumours are presumably formed in the sites with the highest cytotoxicity. This applies for all aromatic amines, in which the acute and chronic toxic effects play an important role in tumour promotion. Among other factors, proliferation is increased as a result of the toxicity in the typical target organs, which is accompanied by inflammation and increased proliferation. There is, however, no explanation as to which extent this is the result of non-specific cytotoxicity, caused for example by the increased degradation of erythrocytes, or whether specific, biochemical mechanisms, such as the inhibition of or an increase in apoptosis, play a role. The fact that tumour formation does not correlate with the extent of metHb formation (documentation "Monocyclic aromatic amino and nitro compounds" 2005) contradicts the concept of an "overloading of the spleen" as a result of erythrocyte breakdown.

As a member of the aromatic amines, and because it forms the same metabolites as aniline, which is classified in Carcinogen Category 4 (supplement "Aniline" 2010), nitrobenzene is considered to be a carcinogen, when the tumours are viewed overall.

Because of the non-linear dose–response relationship of the tumour incidences and as genotoxic effects play a minor role, nitrobenzene is classified in Carcinogen Category 4.

Germ cell mutagenicity.
In rats, after oral administration, especially at toxic doses, clastogenic effects were found in the liver, kidneys and thyroid gland. In mice and rats, after intraperitoneal administration and inhalation exposure, respectively, no clastogenic effects were induced in the bone marrow at low doses or concentrations. In vitro, aneugenic effects occurred at low concentrations, but such effects have not been investigated in vivo. The observed testicular atrophy confirms that nitrobenzene or its metabolites reach the germ cells. Investigations of the genotoxic effects in germ cells are not available. Overall, the studies of the genotoxicity of nitrobenzene indicate a low genotoxic potential similar to that of aniline, for which the formation of reactive oxygen species was confirmed to be the cause (see also Section "Mechanism of Action"). Therefore, nitrobenzene is not classified in one of the categories for germ cell mutagens.

MAK value.
The skin and eye irritation potential of nitrobenzene is low (see documentation "Nitrobenzene" 2003). No NOAEL could be derived from the oral 90-day studies with rats and mice carried out by the NTP. The LOAEL for the rat is 9.38 mg/kg body weight and day and for the mouse 18.75 mg/kg body weight and day. The systemic target organs, the liver, kidneys and blood, correspond to those affected after inhalation. The following toxikokinetic data are taken into consideration for the extrapolation of these LOAELs to a concentration in workplace air: the species-specific correction values for the rat and the mouse (1:4 and 1:7), the assumed oral absorption (100%), the body weight (70 kg) and the respiratory volume (10 m^3) of the person, and the assumed 100% absorption by inhalation. The corresponding calculated concentrations (lowest adverse effect concentrations (LAECs)) are 16.4 mg/m^3 and 18.8 mg/m^3 (3.2 and 3.7 ml/m^3), respectively.

In the long-term inhalation study with B6C3F1 mice, bronchiolarization in the lungs, hepatocytomegaly (centrilobular) and multinucleated hepatocytes, pigmentation of the olfactory epithelium and, in female animals, degeneration of the olfactory epithelium occurred at the low concentration of 5 ml/m^3 and above (Cattley et al. 1994). The critical effect for the derivation of a threshold limit is bronchiolarization in the mouse, which occurred with high incidences of 87% to 92% even at the low concentration of 5 ml/m^3 while in the controls an incidence of 0% was found. In mice, bronchiolarization often occurs after exposure to irritants (Hedrich 2012) and is relevant for humans (DePianto et al 2014; Wang et al. 2009). As bronchiolarization was found with a very high incidence even at the lowest concentration, it is not possible to calculate a benchmark value. The study cannot be used, therefore, for the derivation of a threshold limit.

From the long-term inhalation study with Sprague Dawley rats, a NOAEC of 1 ml/m^3 and a LOAEC of 5 ml/m^3, like in the mouse, was obtained.

In F344 rats, pigmentation of the olfactory epithelium and, in the males, pigmentation and extramedullary haematopoiesis in the spleen were found at the concentration of 1 ml/m³ (Cattley et al. 1994). All three effects occurred with a high spontaneous incidence and there was only a slight, concentration-dependent, increase in the effects. Since the authors did not provide any data regarding the severity of the findings, a clear concentration–effect relationship could nevertheless have been present. The increase in pigmentation of the olfactory epithelium could possibly be due to an increase in the age-related sensitivity of the animals; it is unclear whether this is an adverse effect.

Due to the formation of methaemoglobin caused by nitrobenzene, effects in the spleen are to be expected. These effects, which occur in male F344 rats at 1 ml/m³, are used as the starting point for the derivation of a MAK value. This value also protects against the critical effect, bronchiolarization in the mouse, which occurs at concentrations of 5 ml/m³ and above.

As, however, the effects in the spleen of male F344 rats are only of minimal severity and are to be regarded at best as an early stage, have a high spontaneous incidence, and in female F344 rats, male CD rats and mice the severity is not significantly increased by nitrobenzene up to at least 5 ml/m³, a NAEC (no adverse effect concentration) of 0.33 ml/m³ can be extrapolated. As effects on the spleen are more pronounced in rats than in humans, as found in studies with the metHb inducer aniline (supplement "Aniline" 2010), a NAEL (no adverse effect level) for systemic toxicity in humans at the same level is assumed. As this value is obtained from an animal study, the corresponding concentration for humans, according to the standard procedure of the DFG Commission, is established at half the NAEC. According to the formula by Buist et al. (2012), a blood:air partition coefficient of > 5 is obtained from the molar mass of 123.6 g/mol, the vapour pressure of 0.2 hPa and the $\log K_{OW}$ of 1.86 (ECHA 2014). It is therefore justified to use the increased respiratory volume at the workplace compared with that in the animal study in the extrapolation of the systemic effects on the spleen. Taking into account the preferred value approach, a MAK value of 0.1 ml/m³ has been established. This value is far below the LOAEC of 5 ml/m³ obtained from the long-term inhalation study with B6C3F1 mice.

Peak limitation.

The MAK value is derived from the LOAEC of 1 ml/m³ in F344 rats. The pigmentation of the olfactory epithelium in female animals has the highest percentage increase of the significant effects found in F344 rats at the concentration of 1 ml/m³. As an incidence of 55% was observed in the controls, this seems to be an increased age-related effect which was not yet seen in the 90-day inhalation study. Therefore, it is not an acute effect. The haemosiderin pigmentation of the spleen is a result of damaged erythrocytes and also not an acute effect caused by a peak concentration, as long as it does not result in the formation of methaemoglobin (metHb). Nitrobenzene is therefore classified in Peak Limitation Category II. The toxic effects start with the reductive metabolism. In humans, after inhalation and intravenous injection, elimination of the metabolites has been shown to take place at a somewhat slower rate. Because of the initial half-life of 5 hours, an excursion factor of 4 has been set (see supplement "Peak limitation" 2011). Concentrations of 1 to 6 ml/m³

were tolerated, apparently without irritation, by volunteers exposed for 6 hours (IARC 1996), so that irritation is not to be expected at 0.4 ml/m³. Methaemoglobin formation is not to be expected at the permissible peak concentration of 0.4 ml/m³, as in F344 rats the metHb level is only 3% at 5 ml/m³.

Prenatal toxicity.
In developmental toxicity studies with inhalation exposure, no effects on the offspring of rats were found up to the highest concentration tested of 40 ml/m³, which was toxic to the dams. In rabbits, an increased number of resorptions, which was close to that in historical controls, occurred at the highest concentration tested of 100 ml/m³. The NOAEC was 40 ml/m³. In a 2-generation inhalation study, the fertility index, the weights of the testes and epididymis and the number of sperms were reduced in the male F1 offspring at 40 ml/m³. The NOAEL for developmental toxicity on the male reproductive organs was 10 ml/m³.

In rats, after oral administration effects were found in the form of reduced body weights in the offspring at the low dose of 20 mg/kg body weight and day and above on day 4 after birth. The effect on the weights of the pups could also have been caused postnatally via the mother's milk. An effect resulting from bolus administration, compared with inhalation exposure, is also a possible explanation. No such findings were obtained in rats and rabbits after inhalation, so that the results after oral administration are not included in the assessment of developmental toxicity at the workplace.

As the 400 and 100-fold differences between the NOAECs for developmental toxicity for rats and rabbits and between the developmental toxicity to the male reproductive organs, respectively, and the MAK value of 0.1 ml/m³ are sufficiently large, no teratogenic effects occurred, and as it can be assumed that for nitrobenzene, as also found with aniline, the offspring do not react more sensitively as regards the formation of methaemoglobin than the adult animals, nitrobenzene is classified in Pregnancy Risk Group C.

Absorption through the skin.
After the exposure of volunteers at rest to nitrobenzene in exposure chambers, two thirds of the total uptake was absorbed from the gas phase via the lungs, and one third through the skin (documentation "Nitrobenzene" 2003). Therefore, the internal exposure to nitrobenzene via absorption through the skin may be increased by more than 25%, so that the designation of nitrobenzene with an "H" (for substances which can be absorbed through the skin in toxicologically relevant amounts) has been retained.

Sensitization.
As no findings on skin and respiratory sensitization are available for humans and a valid local lymph node assay with mice yielded negative results, the substance is not designated with "Sh" (for substances which cause sensitization of the skin) or with "Sa" (for substances which cause sensitization of the airways).

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