

Nitrilotriacetic acid and its sodium salts

MAK value	–
Peak limitation	–
Absorption through the skin	–
Sensitization	–
Carcinogenicity (2007)	Category 3A
Prenatal toxicity	–
Germ cell mutagenicity	–
BAT value	–

Formula	$\text{N}(\text{CH}_2\text{COOH})_3$		
Substance	CAS number	Molecular formula	Molecular weight
Nitrilotriacetic acid	139-13-9	$\text{C}_6\text{H}_9\text{NO}_6$	191.14
Monosodium nitrilotriacetate	18994-66-6	$\text{C}_6\text{H}_8\text{NO}_6\text{Na}$	213.12
Disodium nitrilotriacetate	15467-20-6	$\text{C}_6\text{H}_7\text{NO}_6\text{Na}_2$	235.10
Disodium nitrilotriacetate, monohydrate	13566-03-5	$\text{C}_6\text{H}_7\text{NO}_6\text{Na}_2\text{H}_2\text{O}$	253.12
Trisodium nitrilotriacetate	5064-31-3	$\text{C}_6\text{H}_6\text{NO}_6\text{Na}_3$	257.09
Trisodium nitrilotriacetate, monohydrate	18662-53-8	$\text{C}_6\text{H}_6\text{NO}_6\text{Na}_3\text{H}_2\text{O}$	275.10
Synonyms	tri(carboxymethyl)amine aminotriethanoic acid triglycerine triglycollamic acid		
Chemical name	<i>N,N</i> -bis(carboxymethyl)glycine		

Substance	Solubility in water (25 °C)	Melting point (°C)	log K _{ow} [*]
Nitrilotriacetic acid	5.9 g/l (SRC 2007) 1.5 g/l (Anderson et al. 1985)	242, decomposition (SRC 2007)	–3.8 (SRC 2007)
Substance	Solubility in water (25 °C)	Melting point (°C)	log K _{ow}
Monosodium nitrilotriacetate	not specified	not specified	not specified
Disodium nitrilotriacetate	1000 g/l (SRC 2007)	not specified	–10.07 (SRC 2007)
Disodium nitrilotriacetate, monohydrate	not specified	not specified	not specified
Trisodium nitrilotriacetate	1000 g/l (SRC 2007)	not specified	–10.07 (SRC 2007)
Trisodium nitrilotriacetate, monohydrate	500 g/l (Anderson et al. 1985) 1000 g/l (SRC 2007)	not specified	–10.08 (SRC 2007)

This documentation is based mainly on published reviews of toxicological data (Anderson et al. 1985; IARC 1990, 1990), the classification proposed by the EU for trisodium nitrilotriacetate (EU 2002) and a selection of primary literature.

In this document, NTA is the abbreviation used for nitrilotriacetic acid, Na₂NTA for the disodium salt, Na₂NTA · H₂O for the disodium salt monohydrate, Na₃NTA for the trisodium salt, and Na₃NTA · H₂O for the corresponding monohydrate.

Nitrilotriacetic acid and its salts can form water-soluble chelate complexes with polyvalent metals such as calcium, magnesium, zinc or iron. These complexes are dealt with in this document only if they play a mechanistic role in the effects of nitrilotriacetic acid *in vivo*.

1 Toxic Effects and Mode of Action

Nitrilotriacetic acid and its monosodium and trisodium salts have been found to have nephrotoxic potential after oral administration with the diet or the drinking water. Na₃NTA causes carcinomas of the renal tubules and the transitional epithelium in

^{*} *n*-octanol/water partition coefficient

the kidneys and efferent urinary tract in rats after doses of 100 mg/kg body weight and day (corresponding to nitrilotriacetic acid doses of 74 mg/kg body weight and day) and above, as does nitrilotriacetic acid in mice after doses of 1125 mg/kg body weight and day and above. Tumours of the bladder occur only in female rats after nitrilotriacetic acid doses of 260 mg/kg body weight and day and above. Cytotoxic effects in the kidneys and an increased concentration of zinc in the urine are found after Na_3NTA doses of 38 mg/kg body weight and day (nitrilotriacetic acid doses of 27 mg/kg body weight and day) and above. In male rats, an increase in the incidence of bladder hyperplasia was observed after Na_3NTA doses of 10 mg/kg body weight and day (nitrilotriacetic acid doses of 7 mg/kg body weight and day) and above, but this was not significant.

After oral administration, about 50% of the nitrilotriacetic acid or its sodium salts is absorbed from the gastrointestinal tract and eliminated unchanged with the urine; the concentration of nitrilotriacetic acid is highest in the kidneys and the efferent urinary tract. The absorbed amount and thus the peak plasma concentration in humans, monkeys and rabbits is apparently somewhat lower than that in rats, mice and dogs. The LD_{50} values for Na_3NTA are somewhat lower than those for nitrilotriacetic acid, which is of low toxicity. Nitrilotriacetic acid and Na_3NTA are irritating to the conjunctiva of the eyes and slightly irritating to the skin. The available data and the chemical structure do not suggest there may be sensitizing effects on the skin. There are no data available for sensitization of the airways.

Nitrilotriacetic acid and its sodium salts do not induce gene mutations in vitro, with one exception (human epithelial cells, diphtheria toxin resistance), but are weakly clastogenic at concentrations of 1 mM and above. In vivo, DNA strand breaks and micronuclei are induced in the rat kidney, aneuploidy in somatic cells and germ cells of *Drosophila*, and aneuploidy in germ cells of the male mouse. All other in vivo genotoxicity tests yielded negative results. The positive results in vivo occurred at high doses. The mechanism of action has not been clarified, but it can probably be attributed to the high alkalinity of the trisodium salt or the acidity of nitrilotriacetic acid.

The available data show that fertility is not impaired up to the highest nitrilotriacetic acid dose tested of 335 mg/kg body weight and day in the rat. Prenatal development is not impaired after nitrilotriacetic acid doses of up to 335 mg/kg body weight and day in the rat, after doses of up to 250 mg/kg and day in the rabbit or of about 400 mg/kg body weight and day in the mouse. Maternal toxicity is not observed at these doses.

2 Mechanism of Action

Cytotoxicity

Under physiological conditions and at a neutral to alkaline pH, the complexation of nitrilotriacetic acid with zinc is greater than that with other metal ions which are

found in food in far higher quantities, such as calcium or iron. Nitrilotriacetic acid is only a moderately effective chelating agent, unlike EDTA for example; also unlike EDTA, it is absorbed by the intestinal tract very efficiently. Probably for this reason zinc deficiency does not arise; in fact, zinc is absorbed to an even greater extent and (mainly bound to nitrilotriacetic acid) is eliminated again (Anderson 1981).

The severity of the nephrotoxic effects of orally administered nitrilotriacetic acid can be amplified by a higher supply of zinc in the diet or by parenteral injection of zinc sulfate; the dose at which first effects occur is not affected. Intravenous infusion of 6 mmol/kg body weight and day as a sodium or potassium salt or a calcium or magnesium complex had weaker nephrotoxic effects after 6 days than oral administration of $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ with the diet for 3 days in concentrations of 73 mmol/kg (about 1000 mg/kg body weight corresponding to a systemically available nitrilotriacetic acid dose of 1.5 mmol/kg body weight, or about 290 mg/kg body weight) (Anderson et al. 1985). Evidently, the infused salts or complexes were eliminated before they could associate with the zinc. On the other hand, the intravenous infusion of ZnNaNTA doses of 3 mmol/kg body weight and day induced severe nephrotoxicity (coagulation necrosis of the renal tubules, anuria) and resulted in the animals' death within 48 hours; also other zinc salts had similar effects (Anderson et al. 1985).

Mechanisms of carcinogenesis

Kidney and ureter

Positive results in genotoxicity tests were obtained only after nitrilotriacetic acid concentrations of 1 mM and above. The physiological calcium and magnesium concentrations in serum (see Section 3.3, Table 2) and in the culture medium (Robbiano et al. 1999) are approximately 1 to 5 mM; the conditional complex formation constant of CaNTA and MgNTA at pH 7 is approximately 1000 (Table 2). This means that in the range of 1 to 2 mM nitrilotriacetic acid, the concentration of essential calcium in the culture medium can be reduced as a result of complex formation, which induces clastogenic effects. There are numerous things which indicate a predominantly cytotoxic and not primarily genotoxic mechanism of action: the mainly negative results in the studies of genotoxicity; the wide range of toxic effects of nitrilotriacetic acid or nitrilotriacetic acid metal complexes on the kidneys and transitional epithelia of the ureter; the hyperplastic effects; the non-linear concentration–effect relationships (described by the law of mass action) for the formation of chelate complexes from nitrilotriacetic acid and divalent metal ions; and the exclusive occurrence of tumours in nephrotoxic dose ranges.

Initially, vacuolization of the epithelial cells occurs in the renal tubules (Merski 1982), followed by the dose and time-dependent occurrence of regenerative hyperplasia and the increased incorporation of BrdU (detected only at the nitrilotriacetic acid dose of 688 mg/kg body weight) (Bahnmann et al. 1998; BASF AG 1997 c, 1998 a). In long-term studies with rats, the incidence of renal tubular cell hyperpla-

sia was significantly increased after Na_3NTA doses of about 100 mg/kg body weight and day and above (nitrilotriacetic acid doses of about 74 mg/kg body weight and day; Goyer et al. 1981), and age-related chronic nephropathy was more severe after Na_3NTA doses of 75 mg/kg body weight and day and above (nitrilotriacetic acid doses of about 56 mg/kg body weight) (Nixon et al. 1972).

The occurrence of numerous toxic effects in the kidneys is accompanied by an increase in zinc or ZnNTA in the primary urine and the urine. After the administration of nitrilotriacetic acid with the diet in concentrations of 7.3 mmol/kg (doses of about 100 mg/kg body weight and day) for 10 days, the levels of zinc in the urine of male and female rats were not increased; after doses which were 10 times higher, the zinc concentration in the urine was increased approximately 4-fold (Anderson 1981). A significant increase in the levels of zinc eliminated with the urine was detectable after nitrilotriacetic acid doses of 75 mg/kg body weight and day (Nixon et al. 1972), but not with Na_3NTA doses of and below 15 mg/kg body weight and day (nitrilotriacetic acid doses of about 11.2 mg/kg body weight) (Leibold et al. 2002; Nixon et al. 1972). Zinc in the urine is possibly an indicator of kidney damage. It is, however, difficult to specify the effective dose range as the data in Nixon et al. (1972) vary and calculating the absorbed doses from dietary concentrations of nitrilotriacetic acid depends, among other things, on the strain of rat (Anderson 1980). In addition, nitrilotriacetic acid was administered for only 10 days in the study of Anderson (1981). Nevertheless, it may be assumed that nitrilotriacetic acid doses of about 26 mg/kg body weight and day lead to hyperplasia in the renal pelvis (Hiasa et al. 1984).

Bladder

Hyperplasia of the bladder epithelium can be regarded as an indicator of cytotoxic effects and thus as a precursor of tumours. The incidence of hyperplasia in female rats is significantly increased after nitrilotriacetic acid doses of 70 mg/kg body weight and day and above (NCI 1977). Although hyperplasia of the bladder epithelium is observed in the males, no tumours were found.

In feeding studies with $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ in female rats, insoluble calcium–sodium–nitrilotriacetic acid crystals were found in the urine at urinary nitrilotriacetic acid concentrations of 3 mM and above (Na_3NTA doses of about 500 $\mu\text{mol/kg}$ body weight or nitrilotriacetic acid doses of about 100 mg/kg body weight and day); elimination of the crystals increased with the dose in linear fashion (Anderson 1980). At 1.4 mmol/kg body weight and day (267 mg/kg body weight and day) and above, the urine contained more nitrilotriacetic acid than the sum of the divalent metals, and free nitrilotriacetic acid could extract calcium from the transitional epithelium of the bladder (Anderson et al. 1985). In addition to possible irritation by the crystals formed, the formation of tumours in the bladder is attributed to a possible decrease in the calcium in bladder tissue, as a reduced calcium level stimulates the basal cell mitogenesis of bladder tissue *ex vivo*. The sex specificity was attributed to the increased urine volume in the male animals, but not in the female animals, after the intake of Na_3NTA (Anderson et al. 1985). As this

effect occurred only at nitrilotriacetic acid doses of about 1000 mg/kg body weight and day (Anderson et al. 1985), but the bladder tumours occurred at 260 mg/kg body weight and day and above, this does not explain the reason for the sex-specific occurrence of the bladder tumours.

Comparison of nitrilotriacetic acid with iron(III)nitrilotriacetic acid, copper nitrilotriacetic acid and aluminium nitrilotriacetic acid

As iron (III) nitrilotriacetic acid (Fe(III)-NTA) is carcinogenic, and the formation of Fe(III)-NTA via the complexation of iron from food or from the body is possible after the oral administration of nitrilotriacetic acid or its sodium salts, the following is an assessment of whether this complexation can occur to any significant extent. In addition, the effects of copper nitrilotriacetic acid and aluminium nitrilotriacetic acid are compared with those of nitrilotriacetic acid to allow a statement to be made on the involvement of these complexes.

Fe(III)-NTA was found to be genotoxic in a large number of test systems (Hartwig et al. 1993; Nakatsuka et al. 1990; Randerath et al. 1995; Toyokuni and Sagripanti 1993) and toxic in a large number of internal organs after intraperitoneal injection of doses in the range of 5 to 25 mg iron per kg body weight and day (up to total quantities of about 100 to 150 mg iron per rat), and caused tubular necrosis in the kidneys and cell damage and tumour promotion in the liver (Iqbal et al. 1995) and pancreas, the latter with diabetogenic effects (May et al. 1980). In these dose ranges, renal adenocarcinomas developed in the surviving rats after 6 to 12 months (Deguchi et al. 1995; Okada and Midorikawa 1982; Preece et al. 1989), for example after treatment with doses containing iron in the range of 5 to 7 mg/kg body weight and day for 3 months (Ebina et al. 1986). The intraperitoneal injection of Fe(III)-NTA was used in a model experimental testing to produce haemosiderosis in rats (Awai et al. 1979).

The mechanism of action of intraperitoneally injected Fe(III)-NTA is the result of the rapid penetration of iron into the abdominal organs with subsequent overloading of the cells by non-ferritin-bound iron. Intraperitoneal administration makes this possible before the Fe(III)-NTA complex hydrolyzes to form the thermodynamically favoured $\text{Fe}(\text{OH})_3$ (Leibold et al. 2002). In rats given single intraperitoneal injections of doses containing 15 mg iron per kg body weight, Fenton reactions take place, followed by the uninterrupted formation of OH radicals at an intracellular level and consequently also the accumulation of 8-hydroxy-2-deoxyguanosine in the renal DNA (Umemura et al. 1990; 1996).

There are two studies of the oral toxicity of Fe(III)-NTA available. In the first study with a very incomplete description of the methods and presentation of the results, 10000 mg Fe(III)-NTA was administered with the diet (iron doses of 200 mg/kg body weight and day) to Wistar rats for up to 30 weeks. Renal toxicity was apparently not evident. Lipid peroxidation and 8-hydroxy-2-deoxyguanosine were not investigated (Nakano et al. 1989). In a later, valid study in which 0, 50, 200 or 1000 mg/kg body weight and day of a Fe(III)-NTA colloid was administered by gavage to groups of 10 male Wistar rats for 28 days, however, renal toxicity and

increased lipid peroxidation were found in the high dose group and a (dose unrelated) increase in 8-hydroxy-2-deoxyguanosine in renal DNA in all three dose groups (BASF AG 1998 b). The limitations of the method used to determine 8-hydroxy-2-deoxyguanosine are discussed in Section 5.6.2. The increase in 8-hydroxy-2-deoxyguanosine confirms a certain bioavailability of colloidal Fe(III)-NTA also after oral administration, as nitrilotriacetic acid itself, which can be formed from Fe(III)-NTA, produced no increase in 8-hydroxy-2-deoxyguanosine in the same, likewise nephrotoxic, dose range (Leibold et al. 2002). No changes in the concentrations of iron and transferrin or the total iron binding capacity in serum were found with either nitrilotriacetic acid or Fe(III)-NTA after oral administration (BASF AG 1998 a 1998 b). In contrast, intraperitoneal injection of Fe(III)-NTA (total iron dose of 7 mg/kg body weight and day) increased serum iron and reduced the total iron binding capacity, which means that the relative transferrin load was increased (Leibold et al. 2002).

The effects of orally administered Fe(III)-NTA are relatively weak compared with those after the intraperitoneal route above all because the equilibrium between nitrilotriacetic acid, Fe(III)-NTA, $\text{Fe}(\text{OH})_3$ and other ligands is different after oral administration, and there is enough time for this equilibrium to take place:

For intraperitoneal injection of Fe(III)-NTA, a freshly prepared solution is made from nitrilotriacetic acid and FeCl_3 . At the moment of injection, the equilibrium of hydrolysis has not yet formed (recognizable by the change in colour from green to brown). Within a very brief space of time, the injected Fe(III)-NTA can, via the mesothelium, spread directly to the adjacent organs where it produces high concentrations of intracellular iron, cellular necrosis and genotoxic effects. Very high lung toxicity could therefore be expected after inhalation of FeNTA. After oral administration, however, following hydrolysis, equilibrium is reached; this is described by the system of the conditional complex formation constant (see Section 3.3), and, ultimately, favours the formation of insoluble $\text{Fe}(\text{OH})_3$ and free nitrilotriacetic acid; the latter can then interact with zinc and calcium.

In the case of oral administration, therefore, the formation of noticeable amounts of Fe(III)-NTA from nitrilotriacetic acid and nutritive or endogenous sources of iron *in vivo* can be excluded.

High acute and subacute liver toxicity and the development of liver cirrhosis were observed in Wistar rats after single or up to 141 intraperitoneal injections of copper nitrilotriacetic acid (corresponding to copper doses of 4 to 7 mg/kg body weight and day). No cirrhosis was observed when CuSO_4 was injected intraperitoneally; however, the animals died after just a few days. Intraperitoneal administration of nitrilotriacetic acid doses of 104 mg/kg body weight and day did not lead to cirrhosis (Toyokuni et al. 1989). This means that the chelate complex formed from copper and nitrilotriacetic acid causes toxic effects in a different target organ to that of nitrilotriacetic acid itself. This speaks against the involvement of a copper nitrilotriacetic acid complex in the effects of nitrilotriacetic acid or its sodium salts.

After rats were given repeated intraperitoneal injections of the aluminium com-

plex in doses corresponding to aluminium doses of 1.5 to 5 mg/kg body weight and day, damage to the liver and kidneys and, in the further course of the study, also to the brain (which is characteristic for aluminium) was observed (BUA 1987). After intraperitoneal injection of aluminium nitrilotriacetic acid for 3 months (aluminium doses of 1.5–2.0 mg/kg body weight and day), no tumours were found one year after the beginning of the treatment (Ebina et al. 1986). Also in the case of aluminium nitrilotriacetic acid, the liver as target organ speaks against the involvement of the aluminium nitrilotriacetic acid complex in the effects of nitrilotriacetic acid or its sodium salts.

3 Toxicokinetics and Metabolism

3.1 Absorption, distribution, elimination

According to the models of Guy and Potts (1993) and Wilschut et al. (1995), 0.002 or 0.1 mg nitrilotriacetic acid is absorbed from a saturated aqueous solution by a skin area of 2000 cm² in an hour. The model calculations are not suitable for the salts.

Rat

Fasting male Sprague Dawley rats were given oral doses of ¹⁴C-Na₂NTA of 12.3 mg (2 ml aqueous solution, pH 7.2; Na₂NTA doses of about 55 mg/kg body weight; labelling of carboxyl group). 1, 6, 12, 24, 48, 72 or 168 hours after administration, the radioactivity was determined in the urine and faeces (cumulative), the intestinal lumen, liver, heart, kidneys, intestinal tissue and remaining body tissue of 3 animals; the ¹⁴C concentration in the bones, muscle and blood were determined. Na₂NTA was absorbed well from the gastrointestinal tract. After one hour, only 29% of the radioactivity was found in the stomach and intestinal lumen (recovery: 95%), while 31% was found in the urine, 19% in the tissues of the stomach and intestine, 0.4% in the liver, 0.04% in the heart, 2% in the kidneys and 14% in the remaining body tissue. 24 hours after administration, 73% of the radioactivity was found in the urine and only 17% in the faeces and intestinal lumen, 0.3% in the other organs mentioned above, and 2% in the remaining body tissue (recovery: 92%). After 72 hours, 95% of the radioactivity was found in the urine and only 2% in the faeces, with traces in the other organs and 1% in the remaining body tissue (recovery: 98%). In the blood, bone and muscle tissue, the highest ¹⁴C concentration was found after one hour. Within 48 hours after the treatment, the ¹⁴C concentration in the blood and soft tissues had decreased to 0.04% to 0.06% of the administered dose. A half-life of about 3 hours was calculated for the soft tissues (Michael and Wakim 1971).

In addition, it was demonstrated using the same dose in rats that nitrilotriacetic acid is not eliminated via the gallbladder after intraperitoneal administration and is not subject to enterohepatic circulation after oral administration. Only about 4% of the ¹⁴C nitrilotriacetic acid is absorbed and transported via the lymphatic system,

and less than 1% of the administered radioactivity is exhaled in the form of CO_2 (Michael and Wakim 1971).

After oral administration of the low doses of 0.01, 0.1 or 1 mg per rat (^{14}C - Na_2NTA doses of 0.05, 0.5 or 5.0 mg/kg body weight, 2 animals per dose), cumulative absorption after 72 hours was lower (70%, 63% and 59% in the urine) compared with that after the high dose of 55 mg/kg body weight; 24%, 27% and 29% of the radioactivity remained in the faeces, and a maximum of 3% in the body (Michael and Wakim 1971).

A small quantity of the radioactivity of each single dose was incorporated into the bone and released only very slowly after repeated administration of 10 mg ^{14}C - Na_2NTA per rat and day (50 mg/kg body weight and day) for 5 days (Michael and Wakim 1971).

The toxicokinetics of Na_3NTA after repeated administration with the diet were investigated in female rats of the F344 and CD strains. Seven animals per group received unlabelled test substance in the form of $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ in concentrations of 0%, 0.05%, 0.1%, 0.3%, 0.5%, 0.75% or 2% in the diet for 32 days (F344; $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ doses of 0, 29, 56, 173, 286, 437 and 990 mg/kg body weight and day or nitrilotriacetic acid doses of 0, 20, 39, 120, 200, 304 and 688 mg/kg body weight) or 20 days (CD; $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ doses of 0, 39, 78, 235, 390, 586 and 1400 mg/kg body weight and day or nitrilotriacetic acid doses of 0, 27, 52, 163, 271, 440 and 970 mg/kg body weight and day), followed by administration with the diet of ^{14}C - $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ for 10 days at the same doses. The concentration of ^{14}C in plasma was directly proportional to the absorbed dose, and reached a ^{14}C nitrilotriacetic acid concentration of about 190 $\mu\text{mol/l}$ (corresponding to 36 mg/l, the value given in the publication is too high by a factor of 1000) in the high dose group in both strains; the dose–concentration relationship increased more steeply in the F344 rats. The following values were derived from graphs: up to a concentration of 0.75% in the diet, about 33% of the absorbed dose was eliminated with the urine in the F344 rats, and 27% in the CD rats. However, at a concentration of 2% in the diet, the amount eliminated increased to 33% in CD rats and 41% in F344 rats. The urine volume was also increased; to a greater extent in the CD rats than in the F344 rats. Up to a concentration of 0.75% in the diet, the urine volume of the test animals did not differ from that in the controls. The ratio of the specific activity of the investigated organs to that of the blood plasma was highest in the kidneys (about 6 to 9 times higher than in the plasma), followed by the liver (about 3 times higher), bladder (about 2 times higher) and heart (same as for the plasma; results similar in CD and F344 rats). In relation to the quantity of nitrilotriacetic acid absorbed, the concentration of nitrilotriacetic acid in the urine of F344 rats was up to 200 times higher than that in the plasma, whereas in CD rats the concentration in urine was only approximately 100 times higher than that in the plasma (Anderson 1980). However, as the absorption of nitrilotriacetic acid per kg body weight is higher by 50% in CD rats than in F344 rats, this difference is relativized; when the same concentrations are given in the diet, the nitrilotriacetic acid concentration in the urine is somewhat above 100 times higher than in the plasma.

The nitrilotriacetic acid concentration in the plasma ultrafiltrate and in the urine

increased in linear fashion in rats after administration with the diet for 10 days in concentrations of 0.73, 7.3 or 73 mmol/kg (as nitrilotriacetic acid in concentrations of 140, 1400 and 14 000 mg/kg or as $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ in concentrations of 200, 2000 and 20000 mg/kg). The zinc concentration in the urine was the same up to the middle concentration and four times higher at the high concentration. In another study, it was shown that nitrilotriacetic acid peak concentrations of more than 20 μM in the plasma ultrafiltrate increase the zinc concentration in the plasma ultrafiltrate (Anderson 1981).

In a 5-week feeding study 2% $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ was administered to young Sprague Dawley rats (doses of about 2000 mg/kg body weight and day corresponding to nitrilotriacetic acid doses of about 1390 mg/kg body weight) or 1.5% nitrilotriacetic acid (doses of about 1500 mg/kg body weight and day). Urine was collected for 3 days during the fourth week. The amount of nitrilotriacetic acid eliminated after the administration of nitrilotriacetic acid with the diet was found to be $2920 \pm 340 \mu\text{mol/kg}$ body weight and day, while that after the administration of $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ was $2840 \pm 620 \mu\text{mol/kg}$ body weight and day; this was about 40% of the ingested dose (food consumption was determined). This means that the amount of nitrilotriacetic acid eliminated with the urine is the same after equivalent doses of nitrilotriacetic acid and Na_3NTA (Anderson and Kanerva 1978 b).

The renal clearance of tritium-labelled nitrilotriacetic acid (C_{NTA}) at infusion rates of between 2.5 and 20 $\mu\text{mol/kg}$ body weight and hour was compared with that of inulin in rats: independent of the dose, the ratio was $0.89 C_{\text{NTA}}/C_{\text{inulin}}$. Simultaneous infusion of probenecid (Benuryl) and 4-aminohippuric acid showed that the elimination of systemically available nitrilotriacetic acid takes place via passive glomerular filtration only and no active secretion or tubular absorption takes place. Elimination is a zero-order process (Anderson et al. 1985).

In a later study using non-radioactive material, 4 male Wistar rats not fasting were given single gavage doses of Na_3NTA of 22 or 441 mg/kg body weight or doses of 453 mg/kg body weight and day (nitrilotriacetic acid doses of 336 mg/kg body weight) on 7 subsequent days. Up to 72 hours after administration, cumulative recovery in the urine was found to be 53%, 41% and 48%, and the elimination half-time 4.7, 6.2 and 5.4 hours, respectively (BASF AG 1997 b). The dose of 453 mg/kg body weight and day was found to be carcinogenic and highly nephrotoxic in feeding studies.

Mouse

Absorption of the substance was found to be rapid in groups of 5 male albino mice (strain not specified) not fasting which were given single oral ^{14}C nitrilotriacetic acid doses of 180 mg/kg body weight (labelling of the carboxyl group). Blood samples were taken 0.5, 1, 2, 3, 4, 5, 6, 7 and 8 hours after administration. The highest concentrations were found in the first two samples; only 20% of the peak concentration could still be detected after 3 hours. The radioactivity in blood, tissues, urine and faeces was determined by liquid scintillation, the radioactivity in urine and faeces was identified using thin-layer chromatography and gas chromatography/

mass spectrometry. The radioactivity was at its highest after one hour in the bladder, bones and kidneys. No radioactivity was found any more in any of the investigated organs, including the bones, after 8 hours. This suggests rapid elimination. 99% of the administered oral dose was eliminated within 24 hours; 96% with the urine, 3% with the faeces and less than 1% with the bile. Single ^{14}C -NTA doses of 45 mg/kg body weight were injected into the tail vein of 5 mice. Blood samples were taken at 2-minute intervals over the first 20 minutes, and 30 and 60 minutes after injection. The maximum was reached 4 minutes after administration. 60%, 40% and 20% of the maximum concentration was still detectable in the blood 20, 30 and 60 minutes after administration, respectively. The radioactivity was at its highest in the bladder, bones and kidneys after one hour. Also with this route of administration, no radioactivity was found any more in any of the investigated organs, including the bones, after 8 hours (Chu et al. 1978).

In contrast, in a study with autoradiographic examination of the whole body in which C57B₁ mice were given oral doses or NMRI mice intravenous injections of 0.93 mg ^{14}C labelled Na_3NTA (doses of about 46.5 mg/kg body weight assuming a body weight of 20 g; labelling in the carboxyl group), the radioactivity was found to accumulate in the skeleton; this still persisted after 48 hours (no other details; IARC 1990). Why the radioactivity was still detectable after 48 hours in this study remains unclear.

Dog

Seventy-two hours after a fasting male Beagle dog was given a single oral dose of ^{14}C - Na_2NTA of 50 mg/kg body weight, 69% of the radioactivity was detected in the urine (cumulative), 5% in the faeces, 0.07% in the intestinal lumen, 0.07% in the liver, 0.04% in the kidneys and 3% in the remaining body tissue (recovery rate 77%). The equivalent nitrilotriacetic acid concentrations in the blood, calculated from the ^{14}C concentrations determined, were 22.5 mg/l (one hour after administration), 0.35 mg/l (after 24 hours) and 0.22 mg/l (after 48 and 72 hours); after 72 hours, the concentration in the jawbone was 10.8 $\mu\text{g/g}$ and that in the teeth 3.0 $\mu\text{g/g}$ bone (Michael and Wakim 1971).

Seven fasting female Beagle dogs were given oral ^{14}C - Na_2NTA doses of 20 mg/kg body weight (labelling of the carboxyl group). In the first 4 hours after administration, blood and urine samples were collected by previously inserted catheters. After this, the catheters were removed and the animals were transferred to metabolic cages. Blood samples were taken at 15-minute intervals during the first 2 hours. The radioactivity was found above all in the serum and only small quantities in the blood cells. The highest concentrations of ^{14}C - Na_2NTA were determined in the serum 45 to 75 minutes after administration (15–16 $\mu\text{g/g}$, after conversion of the ^{14}C concentrations detected to the equivalent quantities of Na_2NTA); after 4 hours the concentration had decreased to about 4 $\mu\text{g/g}$, and after 24 hours only traces still remained. The peak plasma concentration was 15 mg/l. These values were taken from a graph and represent results obtained from one animal. From another graph, representing the cumulative elimination of radioactivity in one animal (determined

at intervals of 30 minutes), it can be seen that peak concentrations in the urine occurred about 1 to 2.5 hours after administration. After 4 hours about 75% of the administered radioactivity was found in the urine of this animal. After the treatment of 7 dogs, 3% of the administered radioactivity was found in the faeces (cumulative), 80% in the urine (cumulative), 0.04% in the intestinal lumen, 0.06% in the liver, 0.12% in the kidneys, 1.4% in vomit and 0.7% in the cage wash solution 72 hours after administration. The recovery was given as 89.3% (the radioactivity in the rest of the body was not specified). Small quantities of radioactively labelled test substance (2–3 µg/g bone substance) were found in three different bone samples from 8 treated animals. The author discusses whether this is the result of the formation of a calcium complex. 0.4 µg/g was determined in the kidneys, less than 0.3 µg/g in all other organs. 72 hours after the intravenous injection of 20 mg/kg body weight, only 0.7% of the radioactivity from ^{14}C - Na_2NTA was found in the faeces (cumulative), but 96% in the urine (recovery 97%) of 3 dogs. It may thus be concluded that practically no test substance enters the enterohepatic circulation (Budny 1972).

Rabbit

After one fasting male New Zealand rabbit was given a single oral dose of ^{14}C - Na_2NTA of 50 mg/kg body weight, 23% of the radioactivity was detected in the urine (cumulative), 33% in the faeces (cumulative), 26% in the intestinal lumen, 0.3% in the liver, 0.05% in the kidneys and 4% in the remaining body tissue (recovery 87%) after 72 hours. The test substance remained in the gastrointestinal tract for a longer period and was absorbed only slowly compared with in other species. The concentrations of ^{14}C nitrilotriacetic acid (after conversion of the ^{14}C concentrations determined to the equivalent amounts of nitrilotriacetic acid) in the blood were 1.7 mg/l (one hour after administration), 0.74 mg/l (after 24 hours), 0.76 mg/l (after 48 hours) and 0.80 mg/l (after 72 hours). The concentrations in different bone samples 72 hours after administration varied between 4.0 and 9.6 µg/g bone (Michael and Wakim 1971).

Rhesus monkey

Seventy-two hours after the administration of single doses of ^{14}C - Na_2NTA of 50 mg/kg body weight, 14% of the radioactivity was detected in the urine (cumulative), 65% in the faeces, 0.1% in the intestinal lumen, 0.06% in the liver, 0.01% in the kidneys and 1% in the remaining body tissue (recovery 81%) of a fasting female rhesus monkey. The concentrations of ^{14}C nitrilotriacetic acid (after conversion of the ^{14}C concentrations determined to the equivalent amounts of nitrilotriacetic acid) in the blood were 1.7 µg/ml (one hour after administration), 0.44 µg/ml (after 24 hours), 0.34 µg/ml (after 48 hours) and 0.41 µg/ml (after 72 hours); the concentrations in different bone samples 72 hours after administration varied between 0.2 µg/g (femur shaft) and 1.2 µg/g (femur epiphysis and jaw) (Michael and Wakim 1971).

Compared with the other species in this study, the monkey and rabbit were found

to absorb the lowest amounts after oral administration. It must, however, be taken into account that only one animal was used from each of these two species.

Humans

In a study, 10 mg ^{14}C nitrilotriacetic acid was administered to 8 fasting volunteers in gelatine capsule form (labelling of the carboxyl group; 0.11–0.17 mg/kg body weight). A peak plasma concentration of 6.5 $\mu\text{g/l}$ was determined around 2 hours after ingestion; in the blood cells, the maximum concentration was about 2 $\mu\text{g/kg}$ (data from one volunteer). The serum level was reduced by about half around 4 hours later. The concentration was below 0.1 $\mu\text{g/l}$ serum a mere 12 hours after administration. Over a period of 120 hours (89% recovery), only 12% of the radioactivity was eliminated with the urine and 77% with the faeces. 87% of the radioactivity eliminated with the urine was eliminated within the first 24 hours, only 8% on the second day, 2.5% on the third day and traces on the fourth and fifth days. Less than 0.1% of the radioactivity was exhaled within 72 hours (Budny and Arnold 1973).

In humans, about 12% of the ^{14}C nitrilotriacetic acid was absorbed after oral doses of 0.11 to 0.17 mg/kg body weight; the peak plasma concentration was 6.5 $\mu\text{g/l}$. Compared with the dog, in which a dose of 20 mg/kg body weight produced a peak plasma concentration of 15 mg/l, in humans a dose that was 130 times lower produced a peak plasma concentration which was 2300 times lower, which corresponds to a factor of about 17.

3.2 Metabolism

In the studies with rats already described in Section 3.1, nitrilotriacetic acid was eliminated unchanged with the urine after oral administration of ^{14}C - Na_2NTA doses of 55 mg/kg body weight (Michael and Wakim 1971). This was also the case in studies with mice after oral administration of ^{14}C nitrilotriacetic acid doses of 180 mg/kg body weight (Chu et al. 1978) and dogs after oral administration of ^{14}C - Na_2NTA doses of 20 mg/kg body weight (Budny 1972).

In the study with 8 volunteers described in Section 3.1, more than 96% of the radioactivity was present in the urine in the form of nitrilotriacetic acid, detected using thin-layer chromatography and reverse isotope dilution. The methods used yielded no evidence of the biotransformation of nitrilotriacetic acid (Budny and Arnold 1973).

Summary and species comparison

Depending on the dose, mode of administration and species, different amounts of nitrilotriacetic acid are absorbed by the gastrointestinal tract and eliminated with the urine in unchanged form after oral administration of the sodium salts. Relatively high amounts of more than 80% were absorbed after the administration

of high doses to fasting animals. With lower doses and animals that had been fed, the absorbed amounts decreased (rat, mouse and dog). Lower absorption levels were found also in pilot studies with rabbits, monkeys and volunteers. The following order was derived from the available data as regards absorption levels: mouse = Sprague Dawley rat > dog > Wistar rat > rabbit > humans = monkey; however, only one rabbit and one monkey were treated, the applied dose was very low in humans compared with in the other species, and the mice used were not fasting, unlike the other species.

As in humans, the peak plasma concentration in rats, mice and dogs was determined around one to two hours after ingestion. This was followed by a phase of decline with a half-time of about 3 hours; no considerable differences between the individual species were seen. Elimination took place exclusively by passive filtration in the glomeruli. The accumulation of nitrilotriacetic acid or the corresponding complexes with metals was demonstrated in the bones of rats and dogs. From long-term studies in rats, however, no adverse effects on bone substance and function are known (see Section 5.2).

The correlation between the external dose and the nitrilotriacetic acid concentration in the blood and urine of rats and dogs is shown in Table 1.

The lower level of absorption in humans compared with in mice, dogs and rats indicates the body burden may be lower and the peak plasma concentration is lower. It is not certain, however, whether this relation applies generally, as the doses administered to humans were far lower than those in the animal studies. It is also questionable whether such a difference also exists after inhalation exposure.

There are no data available as regards the toxicokinetics of the substance after

Table 1 Nitrilotriacetic acid concentrations in the blood and urine of rats and dogs

Species	Duration	Exposure	NTA concentration	References
rat, SD	short-term, gavage	Na ₂ NTA, 55 mg/kg body weight	9 mg/l (47 µM) (blood), peak concentration	Michael and Wakim 1971
rat, F344, CD	32 days, diet	Na ₃ NTA, 990/1400 mg/kg body weight and day (NTA doses of 688/970 mg/kg body weight and day) 1 mmol/kg body weight (Na ₃ NTA doses of 260 mg/kg body weight, NTA doses of 190 mg/kg body weight and day)	36 mg/l (190 µM) (plasma), 20 mM (urine) 40 µM (plasma), 4 mM (urine)	Anderson 1980
dog, beagle	short-term	Na ₂ NTA, 20 mg/kg body weight	15 mg/l (78 µM) (plasma), peak concentration	Budny 1972

dermal or inhalation exposure. Absorption of the respirable fraction after exposure to dust could be higher, and in turn the bioavailability of the substance. On the other hand, it can be assumed from the solubility of nitrilotriacetic acid in water and a half-time of 3 hours that accumulation in the lungs and in the kidneys does not occur.

3.3 Kinetics of metal ions in interaction with nitrilotriacetic acid

Nitrilotriacetic acid is available in physiological media and in low concentrations mainly in monoacetate and diacetate form. Because of its high negative charge density, the nitrilotriacetic acid ion is hardly able to penetrate into cells, and thus practically only reaches the extracellular space. Only here can it form chelate complexes with divalent metal ions, insofar as they are bioavailable.

Nitrilotriacetic acid is a chelating agent of only moderate potency (compared with other known chelating agents such as EDTA). The thermodynamics of chelation (complex formation) is described by the system of complex binding constants, based on the chemical equilibrium of the particular species participating in each individual reaction (complex, complex former, metal ion, OH^- and H^+ ions). According to the conditions actually present, therefore, the secondary reactions must also be taken into account, which in turn depend on the pH and the availability of individual ions.

The active complexing agent is the completely dissociated anion, NTA^{3-} . Although its concentration increases with the pH, the counter ion is generally not freely available at a high pH as a result of the formation of poorly soluble hydroxides. Thus, to form Fe(III)-NTA , for example, the solubility product for Fe(OH)_3 would need to be $\log L = -38.8$. The stability of the complex therefore depends on the pH. The true complex formation is thus described by a "conditional" complex binding constant ($\log C$) which varies in its constituents and level according to the pH. Each metal has a certain maximum value. Mathematically, this process consists of modifying the original concentration complex formation constants $\log K$ by the introduction of correction terms. These, designated as α coefficients, are indicators for the extent of hydrolysis or the actual availability of the ions participating in the equilibrium (Leibold et al. 2002). Thus, for example in the case of Fe(III)-NTA , for pH ranges of around 7, the original pH-independent concentration constant $\log K = 15.9$ is corrected by $\log \alpha_{\text{NTA}} = 2.8$ on account of the limited availability of NTA^{3-} at pH 7, and additionally by $\log \alpha_{\text{Me}} = 7.7$ because of the hydrolysis or precipitation of Fe(OH)_3 . Both α values are deducted from $\log K = 15.9$ in order to obtain the "conditional" constant $\log C = 5.4$ (BASF AG 2007 a). The maximum stability of the Fe(III)-NTA complex occurs at a pH of about 3. At this pH, the "conditional" constant is about 8.6. This decreases as the pH increases, amounting to only 5.4 at pH 7 and about 4.9 at pH 7.4. At pH 7, zinc salts do not yet undergo hydrolysis or precipitation in the form of Zn(OH)_2 . For this reason, the conditional

complexation constant for ZnNTA is within the neutral range of about 7.9, so that, at this pH, 300 times more zinc than iron is bound to nitrilotriacetic acid. At pH 7.4, copper is around 100 times more potent at binding than zinc, and zinc 500 times more potent at binding than iron, so that copper thus possesses a 50000 times more potent binding capacity than iron. These relations are shown in Table 2.

In mammalian cell cultures and in the kidneys and urinary tract, the complexation properties of nitrilotriacetic acid primarily cause disturbances in certain electrolyte balances, such as demonstrated for calcium and zinc. Their concentration–

Table 2 In vivo concentrations of some metal ions and their complex binding constants

	Calcium (Ca)	Magnesium (Mg)	Zinc (Zn)	Iron (Fe)	Copper (Cu)
total quantity in the organism (humans) (g)	>1000	20–30	1.5–2	4–5	0.1–0.15
concentration in rat feed (mg/kg)	about 1% (= 10 000)	about 0.1% (= 1000)	60	250	14
drinking water (mg/l)	about 80	about 10	< 0.05	0.02	< 0.001
serum, rat (μM)	2500	1000	18–25	10–90	25
serum, humans (μM)	2500–5000	1000–2000	15	30	20
rat urine ¹					
controls (μM)	2850 (\pm 710)	–	5.2 (\pm 4.8)	6.44 (\pm 3)	–
Na ₃ NTA doses of about 9 mg/kg (μM)	2790 (\pm 1150)	–	11.6 (\pm 6)	5.7 (\pm 1.5)	–
Na ₃ NTA doses of about 900 mg/kg (μM)	3440 (\pm 4600)	–	165.2 (\pm 125)	7.5 (\pm 2.9)	–
complex binding constants (without hydrolysis) log K ²	6.4	5.5	10.7	15.9	12.9
conditional complex binding constants with NTA (pH 7): log C ³	3.6	2.7	7.9	5.4	10.1
endogenous ligands in the plasma	about 1/3 bound to protein; freely available		freely exchangable ⁴	transferrin ⁵	initially albumin, then α -ceruloplasmin

¹ BASF AG 1998 a

² Anderson et al. (1985)

³ Calculation, see BASF AG (2007 a)

⁴ 50% bound to albumin only loosely, 70% to amino acids, 30% mainly to macroglobulins (Böhles 1991)

⁵ Binding capacity normally not fully utilized (Forth 1994)

effect relationships resemble titration or receptor binding curves, and thus increase disproportionately according to the mass action law. At correspondingly high nitrilotriacetic acid concentrations, essential metal ions are therefore removed from their endogenous ligands depending on the stability of their complex binding. As a consequence, in *in vitro* studies—for example in the context of genotoxicity—essential calcium first of all disappears from the culture medium and calcium nitrilotriacetic acid complexes precipitate or are able to penetrate the cell membrane as a result of their low charge density compared with that of nitrilotriacetic acid.

Under *in vivo* conditions it therefore depends on the concentrations at which a metal ion prevails in the extracellular space—as nitrilotriacetic acid only reaches the extracellular space—and how tightly this metal ion binds to its endogenous ligands. Thus, for example, calcium is freely available in large quantities. Zinc is also freely available in the plasma to a large extent. Although its concentration is 100 times lower, its complex binding constant is 10 000 times higher: this means that zinc is eliminated first. It also seems that ingested nitrilotriacetic acid is able to enhance zinc absorption from food or mobilization (see below).

In the extracellular space, iron is bound mainly to transferrin, which has a very much stronger affinity to iron than nitrilotriacetic acid (Bates et al. 1967), thus making the possible formation of Fe(III)-NTA even less probable than the relation of the conditional complexation constants for zinc and iron already suggest.

Compared with zinc, copper has a higher complex binding constant. However, it is bound more tightly by α -ceruloplasmin and is thus less freely available in food and the extracellular space. However, there is no evidence to suggest that binding to copper at corresponding nitrilotriacetic acid concentrations *in vivo* can be definitively excluded. The biological activity profile of CuNTA is, however, different to that of nitrilotriacetic acid (see Section 2).

Table 2 shows the *in vivo* concentrations of biologically important metal ions and their complex formation constants ($\log K$ and $\log C$).

The available studies with rats with increasing nitrilotriacetic acid doses therefore give the following picture:

After ingestion of low doses (15 and 6.7 mg/kg body weight and day; Anderson et al. 1985; Leibold et al. 2002), nitriloacetic acid still appears in the primary urine mainly unbound or as the sodium salt. At nitrilotriacetic acid doses of 56 and 186 mg/kg body weight and day, the simultaneous occurrence of nephrotoxic effects and a marked, dose-dependent increase in the level of zinc in the urine are observed (Nixon et al. 1972). CaNTA crystals could also be demonstrated in many experiments after nitrilotriacetic acid doses of more than 270 mg/kg body weight and day (Anderson 1980), though this was not a consistent finding. Zinc, however, remained at an increased level even after 4 weeks of high-dose treatment (nitrilotriacetic acid doses of about 688 mg/kg body weight and day) and was often the only ion to do so (Leibold et al. 2002). It is not clear where this zinc comes from. At least some of it could probably be mobilized or absorbed from the diet to an increased extent.

Even after prolonged administration of nephrotoxic doses of nitrilotriacetic acid, an increase in iron is found neither in the urine nor in the kidneys (Anderson and Kanerva 1979; Anderson et al. 1985; Leibold et al. 2002), at least as long as haematuria does not take place to any great extent. Other parameters of iron metabolism (plasma iron, transferrin and transferrin saturation, red blood cell count) are not affected by nitrilotriacetic acid. On the other hand, the intraperitoneal injection of Fe(III)-NTA preparations produces a great increase in iron in the kidneys and other peritoneal organs, and in plasma and transferrin (Anderson et al. 1985; Leibold et al. 2002).

4 Effects in Humans

In a repeated insult patch test with 66 volunteers, a 1% aqueous solution of a liquid detergent containing 20% Na_3NTA was used for the induction phase. 0.5 ml of the solution was applied to a cotton pad, which was attached to the upper arm using sticking plaster. In the induction phase, each volunteer received a total of 9 applications (3 times a week for 3 weeks). The volunteers removed the plaster after 24 hours, and the skin reaction was documented 48 to 96 hours later by trained staff. Two weeks after the induction phase, the challenge reaction was investigated both on the upper arm exposed during induction and on the untreated upper arm after a single application of the solution. The challenge treatment was carried out with the same strength of solution used for the induction treatment. No evidence of sensitization was found (Nixon 1971).

5 Animal Experiments and in vitro Studies

5.1 Acute toxicity

5.1.1 Inhalation

The exposure of groups of 10 male Sprague Dawley rats for 4 hours to Na_3NTA concentrations of 0, 3300, 3600 or 5000 mg/m^3 in the form of a non-micronized dust (mass median aerodynamic diameter (MMAD) 6.9 μm (70%)) or 5000 mg/m^3 of a micronized dust (MMAD 4.3 μm (90%); cyclon dust generator) did not have lethal effects. Salivation, breathing difficulties, wheezing, breathing through the mouth, partially closed eyes and reduced activity were observed in the exposed animals. All animals recovered after exposure and had no symptoms during the 14-day recovery phase. Body weights were normal at the end of the study after 15 days and no differences between the exposed animals and the controls were found at autopsy (Procter and Gamble Company 1971).

In an earlier inhalation hazard test, nitrilotriacetic acid and Na_3NTA in powder form (BASF AG 1968 a, 1968 b, 1978 a) and Na_3NTA as a 38% aqueous solution (BASF AG 1978 b) were not found to have lethal effects.

In a test for sensory irritation, mice were exposed for 5 minutes to four different aerosol concentrations of Na_3NTA of between 220 and 7600 mg/m^3 . None of the animals died. The respiratory parameters were determined with a polygraph and compared with the data from a control test in the same animals. Slight sensory irritation (parameters not defined) was observed at 220 mg/m^3 , moderate irritation at 1090 mg/m^3 and 1410 mg/m^3 and severe irritation at 7600 mg/m^3 (Procter and Gamble Company 1971). A NOAEC (no observed adverse effect concentration) was not obtained in this study.

In a more recent study of sensory irritation, rats were exposed several times for at least 30 minutes to an aqueous aerosol (MMAD: 2.5–3.0 μm) of $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ in concentrations up to 4300 mg/m^3 . This was tolerated without changes in lung weight. Disturbances in the respiration rate were found in 2 of 4 animals; this is typical of sensory irritation. This pattern was found also at 2900 mg/m^3 in 1 of 4 animals. After 900 mg/m^3 , intermittent irregular breathing was observed, but not the reflex to sensory irritation (CEFIC-EAC 2007).

5.1.2 Ingestion

The data available for the acute toxicity of nitrilotriacetic acid and its sodium salts after oral administration are shown in Table 3.

In a study with rats given $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ which meets present-day requirements, the LD_{50} was found to be 1300 mg/kg body weight for female animals and 1600 mg/kg body weight for male animals. No lethal effects were observed in the low dose group (625 mg/kg body weight). After the administration of 2500 mg/kg body weight, all animals died within 4 hours, and after doses of 1250 mg/kg body weight, 1 of 5 male animals and 2 of 5 female animals died within 24 hours. The symptoms observed were ataxia, tremor, reduced activity, hypothermia, a reduced respiration rate, lateral position, and oral and nasal discharge. In a number of animals of the two low dose groups, diarrhoea, reduced food intake (only after doses of 1250 mg/kg body weight), unkempt fur and discoloration of faeces or urine were observed up to 24 hours after administration. Symptoms no longer occurred after the second day after administration, and no unusual findings were found on autopsy after 14 days. The changes in the stomach and intestine (reddish liquid, discoloured mucous membranes) in animals found dead indicate irritation of the gastrointestinal tract. Similar results were reported in other studies with rats and rhesus monkeys. In dogs, doses up to 5000 mg/kg body weight produced vomiting shortly after oral administration (EU 2002).

Vacuolization in the cytoplasm of proximal tubules was observed in rats after single oral nitrilotriacetic acid doses of 300 mg/kg body weight and above (BASF AG 1997 a).

Table 3 Studies of acute toxicity after oral administration

Species	Substance	Dose (mg/kg body weight)	End point	References
rat	NTA	5340	LD ₅₀	Anderson et al. 1985
rat	NTA	> 6400	LD ₅₀	BASF AG 1968 a
rat	Na ₃ NTA	about 3900	LD ₅₀	BASF AG 1978 b
rat	Na ₃ NTA (77%)	3500	LD ₅₀	BASF AG 1968 b
rat	Na ₃ NTA	about 2100	LD ₅₀	BASF AG 1978 a
rat	Na ₃ NTA	1470 2220	LD ₅₀ ♀ LD ₅₀ ♂	EU 2002
rat	Na ₃ NTA	1680	LD ₅₀	EU 2002
rat	Na ₃ NTA · H ₂ O	1300 1600	LD ₅₀ ♀ LD ₅₀ ♂	EU 2002
rat	Na ₃ NTA · H ₂ O	3800 5300	LD ₅₀ ♀ LD ₅₀ ♂	EU 2002
rat	Na ₃ NTA · H ₂ O	3710	LD ₅₀	EU 2002
rhesus monkey	Na ₃ NTA	about 750	LD ₅₀	EU 2002
dog	Na ₃ NTA	> 5000	LD ₅₀	EU 2002

5.1.3 Dermal application

No lethal effects were observed after the occlusive application to the shaved skin of one male and one female rabbit of a 25% aqueous solution of Na₃NTA in doses up to 10000 mg/kg body weight. Motor activity was reduced (although without coordination problems) for two to three days and food intake was reduced in the higher dose groups; the animals were slightly weaker (no other details; EU 2002).

5.2 Subacute, subchronic and chronic toxicity

5.2.1 Inhalation

In an inhalation study with Na₃NTA, groups of 10 male Sprague Dawley rats were exposed to 0, 3, 40, 174 or 2300 mg/m³ (analysed concentrations; “micronized”, particle size: MMAD of the two high concentrations: 4.6–8.2 µm) for 6 hours a day for 4 days; the treatment was followed by a recovery period of 14 days. No clinical symptoms were observed at concentrations of 3 to 174 mg/m³. The highest concen-

tration produced irritation in the eyes and airways of all animals, which was reversible in the course of the 14-day recovery phase. The animals of this group lost weight between the third and fifth day (up to 4.3%), but had regained the loss in weight by the end of the recovery phase. Autopsy did not reveal any exposure-related changes in the trachea, kidneys, liver, small and large intestine, or the peribronchial lymph nodes (Anderson et al. 1985; Procter and Gamble Company 1971).

In another inhalation study by the same research group lasting 4 weeks, groups of 4 cynomolgus monkeys (2 animals per sex and group) and 12 Sprague Dawley rats and 12 Trueblood guinea pigs (6 animals per sex and group) were exposed to nitrilotriacetic acid concentrations of 0, 10, 213 or 342 mg/m³ (analysed concentrations; 83%–86% of the particles had a diameter of less than 10 µm, MMAD: 10 mg/m³: 6 µm, 213 and 342 mg/m³: 5.5 µm) for 6 hours a day on 5 days a week; the treatment was followed by a 2-week recovery phase. The low and medium concentration produced no clinical symptoms in rats and guinea pigs. At the high concentration, 2 rats and 4 guinea pigs had irregular breathing during the first two weeks of the study; monkeys had no irritation or other symptoms, with the exception of diarrhoea. Body weight gain was unaffected during the six weeks of exposure in all species and dose groups. Haematological investigations performed in rats and monkeys on days 28 and 42 revealed a concentration-dependent increase in the leukocyte count (significance not specified) in the male animals at both sampling times. The levels of aspartate aminotransferase and γ-globulin in the serum of the rats were increased at all concentrations on day 28. The aspartate aminotransferase activity had returned to within the control range on day 42, the concentration of γ-globulin remained increased. The toxicological relevance of these findings is not discussed. The sodium concentrations were increased in all groups in the monkeys. These increases were not concentration-dependent. In the rats, the elimination of zinc was increased in the high concentration group on day 28 compared with that in the controls (no statistical evaluation). As the inhaled concentration of 342 mg/m³ corresponds to a dose of about 80 mg/kg and day (assuming 100% absorption and a respiratory minute volume of 0.2 l/min in a rat weighing 300 g), a treatment-related effect can be assumed. Such a dose is within the range of the oral LOAEL (lowest observed adverse effect level).

The relative lung and kidney weights in rats and monkeys had a tendency to be increased (no statistical significance; however, only 4 to 6 rats and 2 monkeys per concentration were exposed). The absolute organ weights were not given. Red-brown discoloration of the surface of the lungs was found in the guinea pigs of the control and exposed groups (number of animals affected not specified). The organs were not examined histopathologically. Effects on lung function were investigated in monkeys and guinea pigs. With the exception of an increase in respiration rate at the lowest concentration and above, the lung function tests in monkeys revealed no consistent changes in the parameters, including the CO diffusion capacity, N₂ washout and total respiratory resistance on inhalation and exhalation. However, the small group size of 4 animals per concentration must be taken into consideration. The total respiratory resistance on inhalation and exhalation on day 41 was in-

creased in the guinea pigs of the high concentration group (Procter and Gamble Company 1971). As a result of methodological shortcomings, these lung function tests are not suitable for recognizing slight to moderate changes in lung function. As the lungs were not subjected to histopathological investigation, it is not possible to derive either a NOAEC or a valid LOAEC (lowest observed adverse effect concentration) from this study.

5.2.2 Ingestion

Table 4 shows the available studies with oral administration in the low dose range relevant for the derivation of a threshold limit value. As the increased renal elimination of zinc after exposure to nitrilotriacetic acid is thought to be responsible for the cytotoxic effects of the substance (Anderson et al. 1985), also the extent to which zinc is eliminated with the urine is given in this table.

A summary of the data available for the toxic effects of the substance after re-

Table 4 Effects of nitrilotriacetic acid in the low dose range after repeated oral administration

Species	Duration	Exposure to NTA [mg/kg body weight]	Effects	References
dog, beagle	7 months	1.7 (Na ₃ NTA doses of 2.5 mg/kg body weight), drinking water	bone formation decreased	Anderson and Danylchuk 1980
dog, beagle	90 days	5.6 (Na ₃ NTA doses of 8 mg/kg body weight; 300 mg/kg diet)	NTA concentration in bones increased, elimination of Zn in urine unchanged; NOAEL	Budny et al. 1973
rat, Wistar	4 weeks	6.7 (Na ₃ NTA doses of 9 mg/kg body weight; 150 mg/kg diet)	elimination of Zn in urine increased but not significantly; NOAEL	Bahnemann et al. 1998; BASF AG 1998 a
rat, SD	10 weeks	7.4 (Na ₃ NTA doses of 10 mg/kg body weight; 100 mg/l drinking water)	blood glucose increased (not reproducible in BASF AG 2006; Nixon et al. 1972)	Mahaffey and Goyer 1972
rat, F344	2 years	6.9 (Na ₃ NTA doses of 10 mg/kg body weight; 200 mg/kg diet)	hyperplasia of the bladder epithelium 3/23, only in ♂; LOAEL	NCI 1977
rat, CD	2 years	11.2 (Na ₃ NTA doses of 15 mg/kg body weight; 300 mg/kg diet)	Zn and NTA in the bones increased, Zn in urine not increased; NOAEL	Nixon et al. 1972

Table 4 (Continued)

Species	Duration	Exposure to NTA [mg/kg body weight]	Effects	References
rat, Wistar	30 weeks	26 (Na ₃ NTA doses of 37.5 mg/kg body weight; 500 mg/kg diet)	hyperplasia of the transitional epithelium in the renal pelvis	Hiasa et al. 1984
dog, beagle	90 days	26.4 (Na ₃ NTA doses of 38 mg/kg body weight; 1500 mg/kg diet)	♀: elimination of Zn in urine increased; NOAEL	Budny et al. 1973
rat, CD	2 years	56 (Na ₃ NTA doses of 75 mg/kg body weight; 1500 mg/kg diet)	Zn in urine significantly increased at various readings by differing amounts, incidence and severity of nephritis and nephrosis increased	Nixon et al. 1972
rat, F344	2 years	70 (Na ₃ NTA doses of 100 mg/kg body weight; 2000 mg/kg diet)	hyperplasia and dysplasia of the bladder, hyperplasia of the transitional epithelium of the renal pelvis; NOEL for tumours	NCI 1977
rat, SD	2 years	74 (Na ₃ NTA doses of 100 mg/kg body weight; 1000 mg/l drinking water)	incidence of tubular adenomas and hyperplasia increased	Goyer et al. 1981

NOAEL: no observed adverse effect level; LOAEL: lowest observed adverse effect level; NOEL: no observed effect level

peated oral exposure is shown in Table 5. Although most of the studies were not carried out in accordance with OECD test guidelines, an evaluation is still possible.

In feeding studies, Na₃NTA produced a marked increase in drinking water consumption and the volume of urine after doses of around 1000 mg/kg body weight (BASF AG 1998 a; 1998 b) as a result of the physiological regulation mechanisms which keep the sodium balance and the pH constant. When nitrilotriacetic acid is given with the diet, both the nitrilotriacetic acid and zinc are therefore diluted in the primary urine. This is not possible when Na₃NTA is administered with the drinking water. As Na₃NTA does not produce an increase in the urine volume up to concentrations of 7500 mg/kg diet in female animals (Na₃NTA doses of about 500 mg/kg body weight and day) (Anderson 1980), these considerations are not relevant to the low dose range.

Table 5 Effects of nitrilotriacetic acid and its sodium salts after repeated oral administration

Species, strain, number per group	Exposure	Findings	References
rat , Wistar, 5 ♂, 5 ♀	21 days , NTA doses of 0, 150, 500, 1500 mg/kg body weight and day; gavage	150 mg/kg body weight: NOAEL 500 mg/kg body weight: ♂: degeneration of straight renal tubules (1/5) 500 mg/kg body weight and above: ♀: relative kidney weights increased; ♂, ♀: vacuolization in cytoplasm of proximal renal tubules and increased regeneration of tubules 1500 mg/kg body weight: ♂, ♀: food consumption, body weights decreased, clinical symptoms of intoxication, serum urea increased, epithelial cells in renal tubules and transitional epithelium increased, CaNaNTA crystals in urine, degeneration of straight renal tubules; ♀: absolute kidney weights increased, increased elimination of renal granular desquamation; ♂: tubular hyperplasia (2/5), haematuria, relative kidney weights increased	BASF AG 1997 c
rat , Sprague-Dawley, 2 ♂	up to 31 days , (10, 14, 17, 21, 24, 28, 31 days), 0; 0.73, 7.3 mmol Na ₃ NTA/kg body weight and day (140, 1400 mg NTA/kg body weight and day); gavage	at 140 mg/kg body weight and above: vacuolization in cytoplasm of proximal renal tubules (from day 10; dose-dependent effect) 1400 mg/kg body weight: erosion and hyperplasia of transitional epithelium in renal pelvis, simple hyperplasia of renal tubules	Merski 1982
rat , Wistar, 5 ♂ per investigated parameter per dose	up to 4 weeks , Na ₃ NTA concentrations of 0, 150, 20000 mg/kg diet (Na ₃ NTA doses of 9 and 926 mg/kg body weight and day corresponding to NTA doses of 6.7 and 688 mg/kg body weight and day)	6.7 mg/kg body weight: elimination of Zn in urine increased 2-fold but not significantly; NOAEL: 688 mg/kg body weight: water consumption increased, food intake, body weights decreased; urine: volume increased, blood, epithelial cells, LDH, zinc increased 30-fold, opacity, specific gravity, creatinine, crystals decreased, elimination of Ca and Fe unchanged; kidneys: absolute and relative organ weights increased, lipid peroxidation increased, vacuoli-	Bahnemann et al. 1998 BASF AG 1998 a; Leibold et al. 2002

Table 5 (Continued)

Species, strain, number per group	Exposure	Findings	References
		zation in cytoplasm (RER and mitochondria), degeneration and hyperplasia in the proximal renal tubules (compensatory cell proliferation, slight lesions after 1 week), dilation of renal pelvis with focal hyperplasia, DNA synthesis in proximal and distal tubules of cortex and outer medulla increased	
rat , Sprague Dawley, 5–9 ♀ controls: 13 ♀, weaned	4 weeks , 0%, 0.5%, 0.75%, 1.5% NTA in the diet (NTA doses of about 375, 560, 1125 mg/kg body weight and day) ^{a)}	375 mg/kg body weight and above: urine volume decreased 560 mg/kg body weight and above: CaNaNTA crystals in urine 1125 mg/kg body weight: body weights, urinary pH decreased, elimination of Ca in urine increased	Anderson and Kanerva 1978 a
rat , Sprague Dawley, 5–9 ♀ controls: 13 ♀, weaned	4 weeks , 0%, 0.5%, 0.75%, 1.5%, 2% Na ₃ NTA · H ₂ O in the diet (NTA doses of about 260, 390, 780, 1040 mg/kg body weight and day) ^{a)}	260 mg/kg body weight: urine volume decreased 260 mg/kg body weight and above: body weights decreased, urinary pH increased 390 mg/kg body weight and above: CaNaNTA crystals in urine increased 780 mg/kg body weight and above: urine volume increased, elimination of Ca in urine increased, haemoglobin in urine increased	Anderson and Kanerva 1978 a
rat , Sprague Dawley, 5 ♂, 5 ♀ controls: 4 ♀, weaned	4 weeks , 0%, 1.5% NTA in the diet (NTA doses of about 1125 mg/kg body weight and day), 2% Na ₃ NTA · H ₂ O (NTA doses of about 1040 mg/kg body weight and day) ^{a)}	1125 mg/kg body weight (as NTA): ♂, ♀: CaNaNTA crystals in urine, Ca in urine increased, urinary pH decreased; ♂: haematuria, hydronephrosis 1040 mg/kg body weight (as Na ₃ NTA): ♂, ♀: urine volume, relative kidney weights increased, urinary pH increased, haematuria, CaNaNTA crystals in urine, hydronephrosis; ♂: body weights decreased; ♀: Ca in urine increased	Anderson and Kanerva 1979

Table 5 (Continued)

Species, strain, number per group	Exposure	Findings	References
rat, F344, 10 ♂, 10 ♀, 5–6 weeks old	4 weeks, 0%, 1.5% NTA in the diet (NTA doses of about 1125 mg/kg body weight und day), 2% Na ₃ NTA · H ₂ O (NTA doses of about 1040 mg/kg body weight and day) ^{a)}	1125 mg/kg body weight (as NTA): ♂, ♀: body weights, food efficiency decreased, relative kidney weights increased, urinary pH decreased, haematuria, CaNaNTA crystals in urine, Ca in urine increased; ♂: urine volume decreased 1040 mg/kg body weight (as Na ₃ NTA): ♂, ♀: relative kidney weights increased, urinary pH increased, haematuria, CaNaNTA crystals in urine, Ca in urine increased; ♂: body weights, food efficiency decreased; ♀: urine volume increased	Anderson and Kanerva 1979
rat, Sprague Dawley, 5 ♂, weaned	4 weeks, 0% or 2% Na ₃ NTA · H ₂ O in the diet (NTA doses of about 1040 mg/kg body weight and day) ^{a)}	1040 mg/kg body weight: body weight gains, body weights, food efficiency decreased, urine volume increased, urinary pH increased, CaNaNTA crystals in urine, relative kidney weights increased, hydronephrosis, mineralization and vacuolization in cytoplasm of epithelial cells of proximal renal tubules, degeneration and hyperplasia, erosion of transitional epithelium in renal pelvis with inflammatory reaction and hyperplasia	Alden et al. 1981
rat, CD or F344, 5 ♂, weaned	4 weeks, 0%, 2% (CD) or 3.5% (F344) Na ₃ NTA · H ₂ O in the diet (NTA doses of about 1040 and 1820 mg/kg body weight and day) ^{a)}	1040 mg/kg body weight (CD): body weights decreased, relative kidney weights increased, dilation of renal pelvis (hydronephrosis) and ureter, surface erosion and thickening of ureteral epithelium 1820 mg/kg body weight (F344): same as in the CD rats	Kanerva et al. 1984
rat, Sprague Dawley, 6 ♀, 4 ♂, controls: 10 ♀, 6 ♂, weaned	30 days, 0% or 2% Na ₃ NTA in the diet (NTA doses of about 1040 mg/kg body weight and day) ^{a)}	1040 mg/kg body weight: ♂, ♀: body weights decreased, food efficiency decreased, urinary pH increased, urinary elimination of Ca (♀/♂: 5.3-fold/5.0-fold), Zn (17.6-fold/16.6-fold), Na (1.7-fold/ 3.9-fold) increased, elimination of Ca and P with faeces decreased; ♀: elimination of Zn with faeces decreased (0.5-fold), Na increased, serum Zn increased	Michael and Wakim 1973

Table 5 (Continued)

Species, strain, number per group	Exposure	Findings	References
rat , Wistar, 5 ♂	35 days , Na ₃ NTA doses of 0 or 1 mmol/kg body weight and day with the drinking water (NTA doses of 0, 190 mg/kg body weight and day)	190 mg/kg body weight: Mg in blood, Cu in liver, S in kidneys decreased, Ca, Mg, Fe, P, Sr in duodenum decreased, Fe in liver increased, Zn in bones increased	Krari and Allain 1991
rat , Sprague Dawley, 4–10 ♂, weaned	5 weeks , 0%, 1.5% NTA (NTA doses of about 1125 mg/kg body weight and day), 2% Na ₃ NTA · H ₂ O in the diet (NTA doses of about 1040 mg/kg body weight and day) ^{a)}	1125 mg/kg body weight (as NTA): body weights decreased, relative kidney weights increased, urinary pH decreased, haemoglobinuria, Na in urine decreased, Zn, Ca in urine increased, water content of faeces increased, Na, K, Mg in faeces increased 1040 mg/kg body weight (as Na ₃ NTA): body weights decreased, relative kidney weights increased, urinary pH increased, haemoglobinuria, Na, Zn, Ca in urine increased, water content of faeces increased, Na, K, Mg in faeces increased	Anderson and Kanerva 1978 b
rat , Sprague Dawley, 6–8 ♂, weaned	7 weeks , or 7 weeks plus 5 weeks recovery period; 0%, 1.5% NTA (NTA doses of about 1125 mg/kg body weight and day), 2% Na ₃ NTA · H ₂ O in the diet (NTA doses of about 1040 mg/kg body weight and day) ^{a)}	1125 mg/kg body weight (as NTA): vacuolization in the cytoplasm of epithelial cells and hyperplasia of proximal renal tubules, nodular hyperplasia (basophilic) of the renal tubules, hyperplasia of the transitional epithelium of the renal pelvis (n = 1); all effects reversible 1040 mg/kg body weight (as Na ₃ NTA): as with NTA, effects in renal pelvis increased (erosion and inflammation; n = 4/7), hydronephrosis; all effects reversible except hydronephrosis	Myers et al. 1982
rat , Sprague Dawley, 9 ♂	10 weeks , 0%, 0.01%, 0.1% or 1% Na ₃ NTA in the drinking water (NTA doses of about 7.4, 74, 740 mg/kg body weight and day) ^{b)}	7.4 mg/kg body weight and above: blood glucose increased (significance not specified, histology of pancreas yielded no findings) 74 mg/kg body weight and above: relative kidney weights increased 740 mg/kg body weight: mortality increased, body weights decreased, vacuolization in cytoplasm of epithelial cells of renal tubules, glycosuria	Mahaffey and Goyer 1972

Table 5 (Continued)

Species, strain, number per group	Exposure	Findings	References
rat , CD, 21–25 ♂	10 weeks , 0%, 0.01%, 0.05%, 0.1% Na ₃ NTA in the drinking water (NTA doses of about 7.4, 37, 74 mg/kg body weight and day) ^{b)}	7.4 mg/kg body weight: NOAEL (study restricted to body weights, blood urea and blood glucose) 37 mg/kg body weight and above: blood glucose increased (effect slight, but significant)	Mahaffey and Goyer 1972
rat , not specified, 10 ♂, 10 ♀ (his- topathology of 5 ♂ and 5 ♀ per group)	90 days , Na ₃ NTA concentra- tions of 0, 2000 or 20 000 mg/kg diet (NTA doses of about 110 and 1115 mg/kg body weight and day) ^{a)}	110 mg/kg body weight: NOAEL 1115 mg/kg body weight: ♂, ♀: body weights decreased, relative liver and kidney weights increased, hydrone- phrosis, erythrocyte count decreased; ♂: haemoglobin decreased	Nixon 1971
rat , not specified, 40 ♂, 40 ♀ (his- topathology of 10 ♂ and 10 ♀ per group)	90 days , Na ₃ NTA concentra- tions of 0, 7500, 10 000 mg/kg diet (NTA doses of about 420 and 560 mg/kg body weight and day) ^{a)}	420 mg/kg body weight and above: ♂, ♀: relative kidney weights increased; ♂: vacuolization, degeneration of epithe- lial cells in renal tubules, tubular atro- phy and dilation	Nixon 1971
rat , Wistar, 15 ♂	30 weeks , 0%, 1% NTA (about 750 mg/kg body weight and day), 1% Na ₃ NTA · H ₂ O in the diet (NTA doses of about 520 mg/kg body weight) ^{a)}	750 mg/kg body weight (as NTA): bladder hyperplasia (1/15) 520 mg/kg body weight (as Na ₃ NTA · H ₂ O): bladder hyperpla- sia (11/15)	Kitahori et al. 1988
rat , Wistar, 19–20 ♂	28 weeks , Na ₃ NTA · H ₂ O con- centrations of 0 or 10 000 mg/kg diet (NTA doses of about 520 mg/kg body weight and day) ^{a)}	520 mg/kg body weight: body weights, absolute kidney weights de- creased, bladder hyperplasia (7/19, controls: 0/20)	Hiasa et al. 1985 a

Table 5 (Continued)

Species, strain, number per group	Exposure	Findings	References
rat , Wistar, 22–24 ♂	30 weeks , Na ₃ NTA · H ₂ O concentrations of 0, 3000, 10 000, 30 000 mg/kg diet (NTA doses of about 156, 520, 1560 mg/kg body weight and day) ^{a)}	156 mg/kg body weight and above: simple hyperplasia (no other details) of renal tubules (controls: 0/24, significance not specified), slight cystic dilation of renal tubules 1560 mg/kg body weight: hydronephrosis, simple and adenomatous tubular hyperplasia (incidences not evaluable because of pronounced diffuse hyperplasia between cystic lesions)	Hiasa et al. 1985 b
rat , Wistar, 24 ♂	30 weeks , Na ₃ NTA · H ₂ O concentrations of 0, 500, 10 000 mg/kg diet (NTA doses of about 26, 520 mg/kg body weight and day) ^{a)}	26 mg/kg body weight and above: hyperplasia of transitional epithelium in renal pelvis (dose-dependent effect, incidence not specified) 520 mg/kg body weight: body weight decreased, atypical cellular <i>foci</i> in renal tubules increased (not significant)	Hiasa et al. 1984
rat , Sprague Dawley, 193 ♂	704 days , 0% or 0.1% Na ₃ NTA in the drinking water (Na ₃ NTA doses of about 100 mg/kg body weight and day, NTA doses of 74 mg/kg body weight and day) ^{c)}	74 mg/kg body weight: mortality in first 550 days increased, increase in hyperplasia of greater severity in renal tubules, kidney tumours	Goyer et al. 1981
rat , F344, 24 ♂, 24 ♀	2 years , Na ₃ NTA · H ₂ O concentrations of 0, 200, 2000, 20 000 mg/kg diet (NTA doses of about 6.9; 70, 700 mg/kg body weight and day) ^{d)}	6.9 mg/kg body weight: ♂: hyperplasia of bladder epithelium (3/23) 70 mg/kg body weight: ♀: hyperplasia (13/24), dysplasia (3/24) of bladder epithelium; ♂: hyperplasia of transitional epithelium in ureter (4/24) and bladder (3/24) 700 mg/kg body weight: ♂, ♀: body weight gains decreased, hyperplasia of tubular epithelial cells and transitional epithelium in renal pelvis, increased tumour incidence in kidneys, ureter and bladder; ♂: mortality increased, age-related nephrosis increased, vacuolization in cytoplasm of tubular	Alden and Kanerva 1982 a; NCI 1977

Table 5 (Continued)

Species, strain, number per group	Exposure	Findings	References
		epithelial cells (100%, controls: 0%), nodular renal tubular hyperplasia; ♀: transitional epithelial hyperplasia and dysplasia in ureter	
rat , F344, 50 ♂, 50 ♀, controls: 20 ♂, 20 ♀	18 months, 6 months recovery period , Na ₃ NTA · H ₂ O concentrations of 0, 7500, 15 000 mg/kg diet (NTA doses of about 260 and 520 mg/kg body weight and day) ^{d)}	260 mg/kg body weight and above: ♂, ♀: body weight gains dose-dependently reduced, chronic inflammation of kidneys increased; ♀: hyperplasia of bladder epithelium and bladder tumours	NCI 1977
rat , F344, 50 ♂, 50 ♀, controls: 20 ♂, 20 ♀	18 months, 6 months recovery period , NTA concentrations of 0, 7500, 15 000 mg/kg diet (doses of about 375 and 750 mg/kg body weight and day) ^{d)}	375 mg/kg body weight and above: ♂, ♀: body weight gains dose-dependently reduced, chronic inflammation of kidneys increased 750 mg/kg body weight: ♂, ♀: tumour incidence increased; ♀: hyperplasia of bladder epithelium	Alden and Kanerva 1982 b; NCI 1977
rat , Charles River CD, 50 ♂, 50 ♀, controls: 100 ♂, 100 ♀	2 years , 5 animals per group killed after 6, 12, 19, or 24 months (metabolism study and autopsy); 0%, 0.03%, 0.15% or 0.5% Na ₃ NTA in the diet (NTA doses of about 11.2, 56, 186 mg/kg body weight and day) ^{d)}	11.2 mg/kg body weight and above: Zn and NTA in bones increased, Zn in urine not increased, NOAEL: 56 mg/kg body weight and above: Zn in urine increased twofold, incidence and severity of nephritis and nephrosis increased 186 mg/kg body weight: ♀: relative liver weight increased (12 months); ♂: mortality increased	Nixon et al. 1972
rat , Wistar, 50 ♂, 50 ♀	2 years , Na ₃ NTA concentrations of 0, 20 000 mg/kg diet, reduced to 15 000 mg/kg after 1 week (♂: Na ₃ NTA doses of 1170 mg/kg body weight and day	716 (♂) or 805 (♀) mg/kg body weight: BASF AG drinking water consumption increased, food consumption reduced, body weights decreased, body weight gains reduced; kidneys: absolute and relative weights increased, incidence and severity of chronic nephropathy increased, vacuolization in cytoplasm of tubular	BASF AG 2006

Table 5 (Continued)

Species, strain, number per group	Exposure	Findings	References
	or NTA doses of 805 mg/kg body weight and day; ♀: Na ₃ NTA doses of 1040 mg/kg body weight and day or NTA doses of 716 mg/kg body weight and day)	epithelial cells, pigment deposits, hyperplasia, cysts, pyelitis, mineralization of papillae; renal pelvis and ureter: dilation, diffuse hyperplasia; ♂: mortality increased, hydronephrosis, mesenterial and testicular arteritis, atrophy of testes, hypospermia, Leydig cell tumours, haemangiosarcomas in the kidneys, diffuse hyperplasia of parathyroid glands; ♀: focal ectasia of bile ducts or vessels in papillary region of kidneys	
mouse, C3H/He, 10 ♂	12 weeks, 0% or 1% NTA or Na ₃ NTA · H ₂ O in the diet (NTA doses of about 1000 mg/kg body weight and day) ^{e)}	1500 mg/kg body weight (as NTA): body weights increased, absolute kidney weights increased, renal hyperplasia (1/10), urea nitrogen, LDH, AST and ALT in blood increased 1000 mg/kg body weight (as Na ₃ NTA): urea nitrogen, LDH, AST, ALT in blood increased	Matsuki et al. 1992
mouse, B6C3F ₁ , 50 ♂, 50 ♀, controls: 20 ♂, 20 ♀	18 months, recovery period 3 months, NTA concentrations of 0, 7500, 15 000 mg/kg diet (NTA doses of about 780 and 1560 mg/kg body weight and day) ^{e)}	780 mg/kg body weight and above: ♀: body weight gains dose-dependently reduced; ♂: hydronephrosis 1560 mg/kg body weight: ♂, ♀: kidney tumours; ♂: body weight gains reduced; ♀: hydronephrosis	NCI 1977
mouse, B6C3F ₁ , 50 ♂, 50 ♀, controls: 20 ♂, 20 ♀	18 months, recovery period 3 months, Na ₃ NTA · H ₂ O concentrations of 0, 2500, 5000 mg/kg diet (NTA doses of about 260 or 520 mg/kg body weight and day) ^{e)}	260 mg/kg body weight and above: ♀, ♂: body weight gains dose-dependently reduced 520 mg/kg body weight: ♂, ♀: hydronephrosis	NCI 1977
dog, Beagle, 4 ♂, 4 ♀	90 days, 0%, 0.03%, 0.15% or 0.5 % Na ₃ NTA in the diet (Na ₃ NTA doses	5.6 mg/kg body weight: ♂, ♀: NTA concentration in bones increased; urinary Zn excretion not significantly changed	Budny et al. 1973

Table 5 (Continued)

Species, strain, number per group	Exposure	Findings	References
	of 8, 38 and 125 mg/kg body weight and day; estimated from graph; NTA doses of 5.6, 26.4 and 87 mg/kg body weight and day)	26.4 mg/kg body weight: ♂: urinary Zn excretion increased 1.75-fold ($p > 0.05$); ♀: urinary Zn excretion increased 6-fold ($p < 0.05$), no Zn deficiency 87 mg/kg body weight: ♂, ♀: clinical data, haematology, clinical chemistry, urinalysis, histopathology, analysis of bones without adverse findings; ♂: urinary Zn excretion increased 2.5-fold ($p < 0.05$); ♀: urinary Zn excretion increased 3.4-fold ($p < 0.05$), no Zn deficiency; NOAEL	
dog, Beagle, 2 ♂, controls: 22 ♂	7 months, Na ₃ NTA doses of 0 or 2.5 mg/kg body weight and day (NTA doses of 1.7 mg/kg body weight and day); drinking water	1.7 mg/kg body weight: effects on bones: bone formation rate decreased	Anderson and Danylchuk 1980

ALT: alanine aminotransferase; AST: aspartate aminotransferase; LDH: lactate dehydrogenase; RER: rough endoplasmatic reticulum

^{a)} whenever no other data were available from the authors, concentrations given in "mg/kg diet" have been converted into doses of "mg/kg body weight and day" using a conversion factor of 0.075. Calculation of the conversion factor: assumed body weight 200 g, assumed daily food intake 15 g (medium-term study, 4 to 30 weeks treatment);

^{b)} for an average daily drinking water intake of 20 ml and a body weight of 200 g;

^{c)} for an average daily drinking water intake of 10% of the body weight;

^{d)} conversion factor for the rat: 0.05 (long-term study) (assuming a body weight of 400 g and a daily food intake of 20 g);

^{e)} conversion factor for the mouse: 0.15 (medium and long-term study) (assuming a body weight of 20 g and a daily food intake of 3 g)

Rat

The main effects are the (cyto)toxic and proliferative effects on the tubular cells of the kidneys and on the transitional epithelium of the ureter and bladder. After Na₃NTA · H₂O doses of 10 mg/kg body weight and day (NTA doses of 6.9 mg/kg body weight and day) and above, a non-significant increase in the incidence of hyperplasia of the bladder epithelium was observed in male rats (Alden and Kanerva 1982 a; NCI 1977). The blood glucose concentration was increased in this dose range (Mahaffey and Goyer 1972). However, this finding is not considered relevant to the evaluation as it was not observed in other studies (BASF AG 2006; Nixon et al. 1972). After Na₃NTA doses of 9 and 15 mg/kg body weight and day (nitritotriacetic acid doses of

6.7 and 11.2 mg/kg body weight and day) urinary elimination of zinc was not increased, and no adverse effects occurred (Bahnmann et al. 1998; BASF AG 1998 a; Leibold et al. 2002; Nixon et al. 1972). After $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ doses of 37.5 mg/kg body weight and day (nitrilotriacetic acid doses of 26 mg/kg body weight and day) hyperplasia of the transitional epithelium of the renal pelvis was observed (Hiasa et al. 1984), and the incidences of nephritis and nephrosis were increased at and above 75 mg/kg body weight and day (nitrilotriacetic acid doses of 56 mg/kg body weight and day) (Nixon et al. 1972). At this dose, zinc was eliminated usually at a significantly increased level. After Na_3NTA doses of 100 mg/kg body weight and day (nitrilotriacetic acid doses of 70 mg/kg body weight and day), the frequency of epithelial hyperplasia in the bladder was significantly increased in females.

The design and results of the 2-year study of Nixon et al. (1972) are described below in greater detail: Na_3NTA was administered for 2 years to groups of 50 male and 50 female CD rats in the diet in concentrations of 0%, 0.03%, 0.15% or 0.5% (doses of about 15, 75 and 250 mg/kg body weight and day, corresponding to nitrilotriacetic acid doses of 11.2, 56 and 186 mg/kg body weight and day). The control group consisted of 100 male and 100 female animals. There was no recovery phase. Food consumption and body weights were determined once a week during the first eight weeks of the study and once a month from then onwards. The animals' health was monitored every day, and clinical symptoms were recorded in detail once a month. Intercurrent deaths and animals killed in moribund condition were subjected to gross pathological examination, and samples were taken for histopathological evaluation. Faeces and urine samples were collected from 5 male and 5 female animals per dose group after 6, 12, 19 and 24 months. Blood and bone samples were taken at the autopsy of these animals. Urinalysis comprised the volume and pH. The levels of Ca, Zn, Cu, Na, K, P and Mg in the urine and faeces were determined. The following blood parameters were determined: red and white blood cell counts, differential blood count, haemoglobin, haematocrit, cholesterol, calcium, inorganic phosphorus, total bilirubin, albumin, total protein, uric acid, urea nitrogen, glucose, lactate dehydrogenase, alkaline phosphatase and serum aspartate aminotransferase. Bone analysis included P, Cu, Mg, Ca and Zn as well as the dry and ash weight and the strength and the nitrilotriacetic acid content of the femur. The relative organ weights of the kidneys and liver were determined, and the spleen, liver, kidneys, lungs, heart, stomach, oesophagus, small intestine, adrenal gland, trachea, bladder, gonads, thyroid gland and bones were investigated histopathologically at the end of the study. After 12 months, but not after 6, 19 or 24 months, the relative liver weights of the female rats in the high dose group given doses of Na_3NTA of about 250 mg/kg body weight and day (nitrilotriacetic acid doses of 186 mg/kg body weight and day) were significantly increased. This dose significantly reduced survival in the male animals. At the low dose and above, zinc concentrations in the bones were increased. In all dose groups, apart from the lowest, increased zinc concentrations in the urine were found at various sampling times. The incidence and severity of nephritis and nephrosis was increased in the two high dose groups after two years. Other effects, including increased tumour incidences, were not observed.

The NOAEL (no observed adverse effect level) in this study is thus 15 mg/kg body weight and day for Na₃NTA (nitrilotriacetic acid doses of 11.2 mg/kg body weight and day). As no tumours of the bladder occurred up to the highest Na₃NTA dose of 250 mg/kg body weight and day (nitrilotriacetic acid doses of about 186 mg/kg body weight and day), the bladder was presumably not investigated as a target organ for tumour precursors. This must be taken into consideration when evaluating this study.

Other studies yielded the following results: nitrilotriacetic acid doses of 0.73 mmol/kg body weight and day (140 mg/kg body weight and day) produced vacuolization in the cytoplasm of the proximal tubules. Lower doses were not investigated (Merski 1982). Other effects were vacuolar and regenerative hyperplasia of the tubular cells, atrophy and dilation of the tubules, and dilation of the ureter and renal pelvis with papillary damage; the male animals apparently reacted more sensitively than the females. Erosion of the transitional epithelium in the renal pelvis with inflammatory reactions and hyperplasia, and hydronephrosis were found at the higher doses (nitrilotriacetic acid doses of ≥ 420 mg/kg body weight and day). LDH was released after doses of Na₃NTA of 926 mg/kg body weight and day (nitrilotriacetic acid doses of 688 mg/kg body weight and day) (Alden et al. 1981; BASF AG 1997 a, 1997 b; Goyer et al. 1981; Hiasa et al. 1985 b; Mahaffey and Goyer 1972; Merski 1982; Myers et al. 1982; NCI 1977; Nixon 1971; Leibold et al. 2002). One week after Wistar rats were given doses of Na₃NTA of 926 mg/kg body weight and day with the diet (nitrilotriacetic acid doses of 688 mg/kg body weight and day), cell-proliferative effects were observed in the distal tubules; within four weeks these had spread over the whole organ. In addition, increased lipid peroxidation was found, determined in malondialdehyde equivalents (Bahnmann et al. 1998; BASF AG 1998 a; Leibold et al. 2002). Interstitial nephritis developed in the Wistar rat (BASF AG 2006). In this strain, also arteritis and testicular damage was found after long-term administration of high doses (Na₃NTA doses of about 1040 mg/kg body weight and day or nitrilotriacetic acid doses of about 800 mg/kg body weight and day) (BASF AG 2006; see Section 5.7.2).

After the administration of Na₃NTA doses of 375 mg/kg body weight and day and above (nitrilotriacetic acid doses of about 260 mg/kg body weight and day) for 42 days, the pH of the urine was increased in female animals (Anderson and Kanerva 1978 a). Free nitrilotriacetic acid reduced the pH after doses of about 1125 mg/kg body weight and day and above (Anderson and Kanerva 1978 a; 1978 b; 1979). In a 90-day feeding study, the relative liver weights of male and female animals were increased after Na₃NTA concentrations of 20000 mg/kg diet (doses of about 2000 mg/kg body weight and day corresponding to nitrilotriacetic acid doses of 1115 mg/kg body weight and day). The histological investigation revealed no unusual findings (Nixon 1971). Increased relative liver weights without histopathological changes were also found in female animals after 0.5% Na₃NTA per kg diet (doses of about 250 mg/kg body weight and day corresponding to nitrilotriacetic acid doses of 186 mg/kg body weight and day) after 12 months, but not after 19 or 24 months (see above; Nixon et al. 1972).

Mouse

In the long-term carcinogenicity studies, body weight gains were reduced after $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ doses of 375 mg/kg body weight and day with the diet (nitrilotriacetic acid doses of 260 mg/kg body weight and day). After $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ doses of 750 mg/kg body weight and day (nitrilotriacetic acid doses of 520 mg/kg body weight and day) the incidence of hydronephrosis was increased. Similar effects were found also after very high doses of nitrilotriacetic acid (1125 and 2250 mg/kg body weight and day) (NCI 1977). The carcinogenic effects are described in Section 5.7.2. No NOAEL was determined for the mouse.

Dog

Unlike in the rat, in the dog no effects were found in the kidneys in a 90-day study, despite the fact that Na_3NTA doses of 38 mg/kg body weight and day and above (nitrilotriacetic acid doses of 26.4 mg/kg body weight and day) led to the increased elimination of zinc. Body weight gains were determined, the urine, faeces and blood (haematology and clinical chemistry) were analyzed and ophthalmological studies were carried out. Organ weights (organs not specified) were determined after autopsy and 28 tissue samples were taken for histopathological examination. The femoral bones were analyzed using a light microscope without previous decalcification. The nitrilotriacetic acid level in the bones increased in a dose-dependent manner to a maximum of 142 mg/kg bone. No treatment-related effects were found for any of the other parameters investigated in this study, including effects on bone tissue (Budny et al. 1973). The NOAEL for Na_3NTA was 125 mg/kg body weight and day (nitrilotriacetic acid doses of 87 mg/kg body weight and day).

In a drinking water study with Na_3NTA , 2 male Beagle dogs aged 15 months were given 2.5 mg/kg body weight and day (nitrilotriacetic acid doses of 1.7 mg/kg body weight and day) for 7 months. 22 animals from the same inbred colony aged between 10 and 26 months were used as controls. Bone samples were taken at the beginning and end of the study, and the effects on the Haversian lamellar system of the bones were investigated, but not the level of nitrilotriacetic acid. There was a statistically significant decrease in the bone formation rate of the osseous arches in the osteoid region. The zinc concentration in the bones was unchanged, as was the parathyroid hormone level in blood (Anderson and Danylchuk 1980). The relevance of these observations and whether they are adverse is unclear, as deviation from the control values was, according to the authors, only minimal. In other studies, even higher doses produced no effects on the bones of dogs (Budny et al. 1973) and rats (Nixon et al. 1972). In addition, the nitrilotriacetic acid level in the bones was not determined.

5.2.3 Dermal application

In 6 rabbits per treatment group (New Zealand; sex and number of animals in the solvent control not specified), 2 ml of a 2.5% aqueous solution of Na_3NTA per kg

body weight and day (corresponding to Na_3NTA doses of 50 mg/kg body weight and day) was applied to the scarified dorsal skin on 5 days a week for 28 days or to the intact dorsal skin for 91 days. Body weight gains and local irritation were recorded. Haematological and histopathological examinations were performed at the end of the study (investigated tissues not specified). No treatment-related effects were reported (Nixon 1971).

5.2.4 Intraperitoneal injection

Seven-week-old male Wistar rats were given 4 intraperitoneal injections with nitrilotriacetic acid doses of 75 mg/kg body weight within 6 days, or 5 injections of 75 mg/kg body weight, followed by 4 nitrilotriacetic acid injections of 150 mg/kg body weight within 13 days. Other groups of animals received FeNTA (no other details). While the surviving animals in the FeNTA groups were found to have tubular necrosis and large amounts of stainable iron in the kidneys and liver, no such effects were found in the animals treated with nitrilotriacetic acid. There were also no effects on body weight gains, histochemistry (activity of alkaline phosphatase and γ -glutamyl transpeptidase in the kidneys) or haematology; also liver function tests and urinalysis did not yield any unusual findings. However, a decrease in stainable iron of about 15% was found in the liver. The level of iron in the kidneys and the zinc and copper concentrations in the liver and kidneys were unchanged (Preece et al. 1989).

5.3 Local effects on skin and mucous membranes

In concentrated solutions, nitrilotriacetic acid and Na_3NTA cause slight irritation of the skin; in a study carried out according to OECD Test Guideline 404, a 38% solution of the trisodium salt did not have irritating effects. Nitrilotriacetic acid in crystalline form and Na_3NTA both in crystalline form and as powder were irritating to the eye. A 38% solution of the trisodium salt caused slight irritation of the eye. Na_3NTA dust concentrations of 220, 1090, 1410 and 7600 mg/m³ led to sensory irritation in the airways of increasing intensity.

5.3.1 Skin

An 80% preparation of nitrilotriacetic acid in the form of an aqueous paste caused slight irritation in the rabbit after exposure for 20 hours. 24 hours after the end of exposure, only questionable reddening of the dorsal skin was observed; the preparation had no effect on the ear. The effects were reversible after eight days (BASF AG 1968 a). Na_3NTA (purity about 77%) was also tested under the same experimental conditions. 24 hours after the end of exposure, slight reddening of the dorsal skin

was observed; there was no visible effect on the ear (BASF AG 1968 b). A repeat test (purity not specified) produced questionable reddening of the dorsal skin and slight reddening on the ear 24 hours after the end of exposure. Again, no effects were observed after eight days (BASF AG 1978 a).

Slight skin irritation, which was reversible after five days, was observed in a Draize test with a 25% aqueous solution of Na_3NTA . Occlusive application of an unspecified quantity for 24 hours produced no effects one hour after the end of exposure. Slight to marked reddening and slight oedema developed overnight in 1 of 3 animals. The reddening subsided almost completely within three days. All effects were reversible after five days (EU 2002).

No irritation was found with a 38% aqueous solution of Na_3NTA in the rabbit 24 hours or 8 days after a 20-hour exposure period (BASF AG 1978 b). In one study, 0.5 ml of an Na_3NTA solution (no details of solvent, pH or concentration) produced no irritation when applied to the skin of 3 rabbits (grade 0 for reddening and oedema, 0, 24, 48 and 72 hours after the end of exposure) (Rexolin Chemicals 1984 a).

5.3.2 Eyes

In an earlier study, nitrilotriacetic acid was tested as free acid in crystalline form. The instillation of 50 mm³ into the conjunctival sac of the rabbit eye produced slight reddening and severe swelling of the conjunctiva, and slight corneal opacity after one hour. Severe reddening and slight swelling of the conjunctiva and slight opacity of the cornea were reported 24 hours after the installation. Neither reddening nor oedema were evident eight days after the installation, but slight opacity of the cornea still persisted (BASF AG 1968 a).

In an earlier study, 50 mm³ of a 38% aqueous solution of Na_3NTA produced slight reddening and swelling one hour after installation. The slight reddening was still evident both 24 hours and eight days after the installation. The questionable swelling observed after 24 hours was reversible. Further effects observed were secretion one hour after the installation, and a slimy layer over the cornea 24 hours after the installation (BASF AG 1978 b). In a study carried out according to OECD Test Guideline 405, a 38% aqueous solution of Na_3NTA produced a primary irritation score of about 4 on a scale up to 110 and was regarded as not irritating. The slight irritation of the conjunctiva was reversible within eight days (no other details of the results) (BASF AG 1982).

After instillation of 0.1 ml of an Na_3NTA solution (no details of the solvent, pH or concentration) no effects were found in the cornea and iris. One hour after the instillation, conjunctival reddening (grade 2) was observed in all three rabbits. This persisted for 24 hours in only one animal. One hour after the instillation, grade 2 conjunctival swelling was diagnosed in 2 of 3 animals; within 24 hours this had, however, subsided (grade 1 or 0). No effects were visible four days after the instillation. Overall, the test substance caused only slight irritation (Rexolin Chemicals 1984 b).

On the other hand, more severe irritation was observed after the instillation of 100 mg $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ (in crystalline form, purity not specified) into the conjunctival sac of 3 rabbits. Conjunctival swelling with partial eversion of the eyelid, secretion copious discharge, moderate conjunctival reddening and congestion covering the iris were reported one hour after the instillation. The secretion and swelling (severity not specified) were reduced after 24 hours, and the eyes were rinsed with isotonic physiological saline. The swelling had subsided after five days, but slight conjunctival reddening and slight opacity of the cornea remained; these effects were observed also at the end of the study seven days after the instillation (EU 2002).

In an earlier study, Na_3NTA was tested in powder form (about 77% Na_3NTA). The instillation of 50 mm³ into the conjunctival sac produced slight conjunctival reddening and severe conjunctival swelling after one hour. Only slight conjunctival reddening was observed 24 hours after the instillation. Haemorrhage of the nictitating membrane was observed eight days after the instillation (no other details; BASF AG 1968 b). After a repeat test with Na_3NTA in powder form (no other details), severe reddening and slight swelling of the conjunctiva and slight opacity of the cornea were observed one hour after the instillation. These effects persisted for 24 hours. Slight conjunctival reddening was found at the reading after eight days. Further effects reported were secretion and haemorrhage (one hour after administration) and a slimy layer over the cornea (after 24 hours) (no other details; BASF AG 1978 a).

5.3.3 Respiratory tract

The studies of sensory irritation are described in Section 5.1.1 (CEFIC-EAC 2007; Procter and Gamble Company 1971). In the more recent of the two studies, a NOAEC of 900 mg/m³ was found for sensory irritation.

5.4 Allergenic effects

In a Buehler test, the skin of 20 guinea pigs was treated with 0.5 ml of a 50% Na_3NTA (purity 92.4%) formulation in water three times at 7-day intervals for induction and once for the challenge. The control group consisted of 10 animals. No skin reactions were observed either in the treated animals or in the controls. This study is of limited validity as the concentration used did not induce skin irritation during the induction phase (EU 2002).

5.5 Reproductive toxicity

An earlier feeding study in rats over two generations with Na_3NTA doses of up to 450 mg/kg body weight and day (NTA doses of 335 mg/kg body weight and day)

yielded no evidence of either impairment of fertility or embryotoxic effects. Earlier studies of developmental toxicity did not reveal adverse effects of nitrilotriacetic acid or Na_3NTA in the offspring of rats after Na_3NTA doses of up to about 450 mg/kg body weight and day (nitrilotriacetic acid doses of 335 mg/kg body weight and day), or of rabbits after nitrilotriacetic acid doses of up to 250 mg/kg body weight and day, or of mice after nitrilotriacetic acid doses of about 400 mg/kg body weight and day. No maternal toxicity occurred at the doses tested.

5.5.1 Fertility

In a long-term carcinogenicity study (BASF AG 2006) atrophic changes were found after 24 months in the testes of Wistar rats initially given 20 000 mg $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ in the diet followed after one week by 15 000 mg ($\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ doses of about 1170 mg/kg body weight and day or nitrilotriacetic acid doses of 800 mg/kg body weight and day). In addition, the animals had pronounced kidney damage and arteritis in the mesenteric, testicular and epididymal regions. In all other medium (Nixon 1971) and long-term studies (Nixon et al. 1972) with female and male rats in which the reproductive organs were investigated, nitrilotriacetic acid had no adverse effects in the gonads (see Table 5 and Section 5.2).

In an earlier two-generation study, groups of 20 male and 20 female CD rats were continuously given 0%, 0.1% or 0.5% Na_3NTA in the diet (about 0, 90 and 450 mg/kg body weight and day corresponding to NTA doses of 67 and 335 mg/kg body weight and day). It was not specified when the exposure period began, but this was presumably not until after mating. Animals of the F_0 generation were mated three times with each other: F_1 animals of the first litter (F_{1a}) were observed up to weaning, the offspring of the second litter (F_{1b}) were bred to become F_1 parents and the offspring from the third litter (F_{1c}) were used to study developmental toxicity. Animals from the F_1 generation were mated twice with each other: the first litter was observed up to weaning (F_{2a}) and the second litter again used to study developmental toxicity (F_{2b}). The food consumption and body weight gains of the F_0 and F_{1b} generations were recorded once a week for up to eight weeks after weaning. In each litter, the number of live pups at birth was determined, and four days later the number of live offspring was again recorded, together with the sex and body weight. Eight animals (even distribution of the sexes) were weighed again on day 21 after birth. Food consumption was slightly reduced in the high dose group in both sexes of the F_0 generation and in the male animals of the F_{1b} generation, but the reduction was not statistically significant. In the male animals of the F_0 generation the reduction in food consumption was statistically significant. In the male and female animals of both generations, body weight gains were slightly reduced at the high dose, but statistically significant only in the female F_0 animals and male F_{1b} animals. Relative to food consumption, the body weight gains were not impaired. Although the animals of the F_0 generation were given Na_3NTA with the diet after weaning for up to 10 months and those of the F_1 generation for up to 7 months, no adverse effects on the kidney tissue were observed.

No significant effects (data from F_{1a} and F_{1b} assessed together, and F_{2a}) were found on the number of pregnancies, stillbirths, live births per litter, live offspring on day 4 after birth or weaned young rats per litter, or on the lactation index (number of weaned pups/number of live pups on day 8 after birth). No details were given of the distribution of sexes. In the high dose group, the body weights of weaned pups in the F_{1a} generation were significantly ($p < 0.05$) reduced, but not in the F_{1b} and F_{2a} generations (Nolen et al. 1971). A NOAEL is not given by the authors. On the basis of the data provided, a NOAEL for systemic toxicity of 0.1% nitrilotriacetic acid in the diet (67 mg/kg body weight and day), and for fertility of 0.5% in the diet (335 mg/kg body weight and day) can be derived from this study.

5.5.2 Developmental toxicity

In the two-generation study with rats described in Section 5.5.1, 20 pregnant animals from the F_0 and F_{1b} generations were treated either throughout the entire study period or in satellite groups from days 6 to 15 of gestation only with concentrations of 0%, 0.1% and 0.5% Na_3NTA in the diet (about 0, 90 and 450 mg/kg body weight and day corresponding to nitrilotriacetic acid doses of 67 and 335 mg/kg body weight and day). The data for maternal toxicity are limited. Ten pregnant animals from each group were killed on days 13 and 21 of gestation. The dams killed on gestation day 13 did not differ from the dams investigated later with regard to the number of *corpora lutea*, implantations or resorptions (no other details). After exposure throughout the entire study period, the number of pregnant dams in the F_{1c} controls (60% in $n = 10$) and in the low dose group (50% in F_{1c}) was unusually low. No significant differences were found in the mean number of *corpora lutea* and dead foetuses and the mean body weights of male and female foetuses of the F_{1c} and F_{2b} generations compared with the values for the controls. The mean number of resorptions in the low dose group (but not in the high dose group) of the F_{2b} (but not in the F_{1c}) generation was significantly increased compared with that in concurrent controls, but was within the range of historical controls from this laboratory. No increase in the incidence of skeletal malformations was reported. The soft tissue primarily affected by malformations was the urinary tract system in the form of hydroureter and hydronephrosis. As the control groups of both generations were affected to the same extent, the authors regarded these effects as not being substance-related (Nolen et al. 1971). After exposure on days 6 to 15 of gestation, the number of pregnant rats varied between 50% and 70% in the treated groups (10 animals per group; controls see above). The body weights of the treated female animals were comparable to those of the controls. At the high dose, the number of live foetuses in the F_{1c} group was significantly increased compared with that in the corresponding controls. No other differences were found in the F_{1c} and F_{2b} generations compared with in the controls as regards the parameters investigated (see above) (Nolen et al. 1971). A NOAEL was not given by the authors. On the basis of the data, a NOAEL for developmental toxicity can be derived from this study of 0.5% nitrilotriacetic acid in the diet (335 mg/kg body weight and day).

In a study of the effects of Na_3NTA on the toxicity and teratogenicity of cadmium and methyl mercury in rats, in an additional series of experiments Na_3NTA was also tested alone. Groups of 20 pregnant Charles-River rats were given nominal doses of 0, 0.1 or 20 mg/kg body weight and day with the drinking water (actual Na_3NTA doses: 0, 0.11 and 25.3 mg/kg body weight corresponding to nitrilotriacetic acid doses of 0.08 and 18.8 mg/kg body weight and day) on days 6 to 14 of gestation. Caesarean section was performed on day 21 of gestation followed by gross pathological investigation for abnormalities, and the determination of the foetal weights and sex, the mean number of *corpora lutea* and the number of implantations, resorptions, and live and dead foetuses. Two thirds of the foetuses were investigated for visceral abnormalities, the rest for skeletal abnormalities. No exposure-related maternal toxicity was observed. In the groups treated with nitrilotriacetic acid alone, increased incidences of hydronephrosis, hydroureter and bladder defects (thin walls with incomplete development of the muscle layer) occurred. The incidence of hydronephrosis was not dose-dependent; the incidence of hydroureter and bladder defects was increased only in the high dose group. The authors regarded these effects as not treatment-related, as they were observed also in the historical controls of the laboratory with varying incidences (10%–40%). They assumed that these morphological variations correlate above all with the age of the foetuses (Nolen et al. 1972 b). In a similar study of the same research group, Na_3NTA was administered with the drinking water to groups of 20 rats in doses of 0 or 20 mg/kg body weight and day (nitrilotriacetic acid doses of 14.9 mg/kg body weight and day) on days 6 to 15 of gestation. Caesarean section was performed on day 21 of gestation. At the same dose of Na_3NTA as in the previous study, the incidence of hydroureter was the same as that in the control animals and the incidence of bladder defects even lower than that in the control group. Thus, the effects in the foetuses do not appear to be substance-related, although the target organs of nitrilotriacetic acid were involved. As in the previous study, no maternal or developmental toxicity was observed (Nolen et al. 1972 a). However, only a relatively low dose was tested in these two studies.

In a gavage study, groups of 20 pregnant New Zealand White rabbits were given doses of nitrilotriacetic acid in distilled water of 0, 2.5, 25, 100 or 250 mg/kg body weight and day on days 7 to 16 of gestation. A completely untreated control group was included in addition to the control group treated with the vehicle. The body weights of the treated dams were determined every three days during pregnancy, and the doses adjusted correspondingly. At the end of the study on gestation day 28, gross pathological examination was carried out for abnormalities. The foetal weights (sex not recorded), and the mean number of *corpora lutea*, implantations, resorptions, and live and dead foetuses were determined. A follow-up investigation of the foetuses was carried out for visceral and skeletal abnormalities. No significant, treatment-related differences between the controls and the treated groups were found for the investigated parameters. In this study, the toxic effects on the dams, such as effects on food intake, clinical symptoms and effects on body weight gains were not mentioned, merely that the gross pathological examination at the

end of the study yielded no abnormalities (Nolen et al. 1971). The authors do not give a NOAEL. On the basis of the data, a NOAEL for developmental toxicity of 250 mg/kg body weight and day can be derived from this study for nitrilotriacetic acid.

In a study with NMRI mice, groups of 10 pregnant animals were given 0% or 0.2% nitrilotriacetic acid in the drinking water (nitrilotriacetic acid doses of about 400 mg/kg body weight and day assuming drinking water consumption of 200 ml/kg body weight and day) on days 6 to 18 of gestation. Body weights were determined daily during pregnancy. The values did not differ from those of the controls. No significant effects on the number of live or dead foetuses, the number of resorptions, the mean number of live foetuses per litter or the foetal weights were observed at caesarean section on day 18 of gestation. The investigation for visceral and skeletal abnormalities revealed no treatment-related malformations. In additional investigations with radioactively labelled nitrilotriacetic acid it was demonstrated that nitrilotriacetic acid reaches the embryo or foetus after oral exposure of the dams and accumulates in the bone matrix (Tjälve 1972).

5.6 Genotoxicity

5.6.1 In vitro

Numerous studies are available, some of which are of only limited validity (Table 6). When evaluating the positive results, it must be borne in mind that certain metal complexes of nitrilotriacetic acid can have clastogenic effects. Thus, when the concentration of nitrilotriacetic acid in the culture medium exceeds the concentration of divalent metal ions (at and above around 2 mM), the test is more or less performed in the absence of essential ions (Anderson et al. 1985). This means that most of the test substance no longer consists of nitrilotriacetic acid but of a chelate complex with a metallic atom at its centre. When evaluating the tests, therefore, attention must also be paid to the presence of precipitations, as these can produce artefacts. In addition, when considering positive clastogenic findings with Na₃NTA, its alkalinity must also be taken into account, as NaOH *per se* can have clastogenic or aneuploidogenic effects, for example in CHO cells with metabolic activation (Morita et al. 1989) or in locusts (USEPA 1988). In the case of the positive results obtained with nitrilotriacetic acid, also acidity should be discussed as a cause, as a reduction in pH in the medium can induce clastogenic effects (Morita et al. 1992).

Summary

Nitrilotriacetic acid and its sodium salts yielded negative results in relevant indicator and mutagenicity tests with bacteria and yeast and fungus cells. The majority of the indicator tests with mammalian cells did not reveal any genotoxic effects. The studies of the induction of chromosomal damage (aberrations and micronuclei)

Table 6 Genotoxicity of nitrilotriacetic acid and its sodium salts in vitro

End point	Test system	Substance	Concentration range	Effective concentration	Cyto-toxicity*	Result*		References
						+ m.a.	- m.a.	
test for differential killing	<i>Escherichia coli</i> WP2	Na ₃ NTA	not specified	250 µg/ml (about 1 mM)	not specified	+	+	IARC 1999
SOS chromotest	<i>Escherichia coli</i> PQ37	NTA	0.01–1000 µg/test	–	not specified	–	–	EU 2002
gene mutation	<i>Salmonella typhimurium</i> TA97, TA98, TA100, TA1535	Na ₃ NTA · H ₂ O	100–10000 µg/plate	–	not specified	–	–	Zeiger et al. 1992
	<i>Salmonella typhimurium</i> TA98, TA100, TA1535, TA1537, TA1538, <i>Escherichia coli</i> WP2 uvrA	Na ₃ NTA · H ₂ O	3–3333 µg/plate	–	not specified	–	–	EU 2002
	<i>Salmonella typhimurium</i> TA98, TA100, TA1535, TA1537, TA1538	Na ₃ NTA	54–870 µg/plate	–	not specified	–	–	Loprieno et al. 1985
	<i>Salmonella typhimurium</i> TA97, TA98, TA100, TA1535, TA1537	NTA	33–2000 µg/plate	–	not specified	–	–	Zeiger et al. 1992
	<i>Salmonella typhimurium</i> TA92, TA100, TA103, TA104	NTA	10% in the medium (0.5 M)	–	not specified	not specified	–	Gava et al. 1989
	<i>Salmonella typhimurium</i> TA100	Na ₃ NTA	up to 870 µg/plate	–	not specified	–	–	IARC 1999
	<i>Salmonella typhimurium</i> (no other details)	Na ₃ NTA	1–600 µg/plate	–	not specified	not determined	–	EU 2002
	<i>Escherichia coli</i> WP2 uvrA	Na ₃ NTA	up to 100000 µg/ml (about 390 mM)	–	not specified	–	–	IARC 1999
	<i>Escherichia coli</i> WP2	NTA	up to 4000 µg/ml (21 mM)	–	not specified	not determined	–	IARC 1999

Table 6 (Continued)

End point	Test system	Substance	Concentration range	Effective concentration	Cytotoxicity*	Result*		References
						+ m.a.	- m.a.	
gene mutation	<i>Schizosaccharomyces pombe</i> PI	Na ₃ NTA	up to 40 µg/ml (about 0.16 mM)	-	not specified	-	-	Loprieno et al. 1985
	<i>Schizosaccharomyces pombe</i>	NTA	up to 4000 µg/ml (21 mM)	-	not specified	not determined	-	IARC 1999
	<i>Saccharomyces cerevisiae</i>	NTA	up to 4000 µg/ml (21 mM)	-	not specified	not determined	-	IARC 1999
	<i>Aspergillus nidulans</i>	Na ₃ NTA	1.8–72 mM	-	not specified	not specified	-	Crebelli et al. 1986
gene conversion	<i>Saccharomyces cerevisiae</i>	Na ₃ NTA	up to 40 µg/ml (about 0.16 mM)	-	not specified	-	-	Loprieno et al. 1985
	<i>Saccharomyces cerevisiae</i> D7	Na ₃ NTA	2, 10, 50 mM	-	not specified	not specified	-	Galli et al. 1988
crossing over	<i>Aspergillus nidulans</i>	Na ₃ NTA	10.6–42.5 mM	-	42.5 mM	not determined	-	Crebelli et al. 1986
	<i>Aspergillus nidulans</i>	Na ₃ NTA	10.6–42.5 mM	-	42.5 mM	not determined	-	Crebelli et al. 1986
aneuploidy	<i>Human lymphocytes</i>	Na ₃ NTA	0.001–1 mM	-	not specified	not determined	-	EU 2002; IARC 1999
	CHO cells	Na ₃ NTA	0.001–1 mM	-	not specified	not determined	-	EU 2002; IARC 1999
sister chromatid exchange	CHO cells	NTA	0.5–5 µg/ml (2.6–26.2 µM)	-	not specified	-	-	EU 2002; IARC 1999
	CHO cells	Na ₃ NTA	0.001–2 mM	-	not specified	not determined	-	Montaldi et al. 1985

Table 6 (Continued)

End point	Test system	Substance	Concentration range	Effective concentration	Cytotoxicity*	Result*		References
						+ m.a.	- m.a.	
un-scheduled DNA repair synthesis	mouse lymphocytes	Na ₃ NTA	0.001–1 mM	–	not specified	not determined	–	Montaldi et al. 1985
	V79 cells	Na ₂ NTA	0.5–1.5 mM	–	not specified	not determined	–	EU 2002
	primary rat hepatocytes	Na ₃ NTA · H ₂ O	1.6–3.2 mM	–	not specified	not determined	–	EU 2002, IARC 1999
					specified	determined	–	
DNA strand breaks	primary rat hepatocytes	Na ₃ NTA·H ₂ O	3.6 mM	–	not specified	not determined	–	EU 2002
	human lymphocytes	Na _x NTA	1–10 mM	1 mM	5 mM	not determined	±	Celotti et al. 1988
		Na ₂ NTA	1 mM	–	not toxic	not determined	–	Hartwig et al. 1993
	V79 cells	NTA	1.8–5.6 mM	1.8 mM	3.2 mM	not determined	+	Robbiano et al. 1999
chromosomal aberration	primary cultures of human kidney cells	NTA	1.8–5.6 mM	1.8 mM	5.6 mM	not determined	+	Robbiano et al. 1999
	primary cultures from rat kidney cells	Na ₃ NTA	2–6 mM	–	at 4 mM and above	not determined	–	Montaldi et al. 1987
	human lymphocytes	Na ₃ NTA	1–7.5 mM	–	toxic at high concentrations	not determined	–	EU 2002
	human lymphocytes	Na ₃ NTA	1–7.5 mM	–	toxic at high concentrations	not determined	–	EU 2002

Table 6 (Continued)

End point	Test system	Substance	Concentration range	Effective concentration	Cytotoxicity*	Result*		References
						+ m.a.	- m.a.	
	PT-K1 cells from kangaroo rat kidney	Na ₃ NTA · H ₂ O	2.5–10 mM	–	> 2.5 mM with 24 and 48-hour treatment	not determined	±	Kihlman and Sturelid 1970
	CHO cells	NTA	0.5–5 µg/ml (2.6–26.2 µM)	–	not specified	–	–	EU 2002
micronuclei	human lymphocytes	Na ₃ NTA	0.1–10 mM	–	not specified	not determined	–	EU 2002
	primary human kidney cell cultures	NTA	1.8–5.6 mM	5.6 mM (at 1.8 mM 2-fold increase, at 5.6 mM 3-fold increase, statistically significant at 5.6 mM)	3.2 mM	not determined	+	Robbiano et al. 1999
	primary cultures from rat kidney cells	NTA	1.8–5.6 mM	1.8 mM	5.6 mM	not determined	+	Robbiano et al. 1999
	hamster C1-1 cells	Na ₃ NTA	0.6–3 mM	2 mM	3 mM	not determined	(+)	Modesti et al. 1995

Table 6 (Continued)

End point	Test system	Substance	Concentration range	Effective concentration	Cytotoxicity*	Result*		References
						+ m.a.	- m.a.	
gene mutation HPRT TK ^{+/-}	V79 cells	Na ₃ NTA	0.1–15 mM	–	15 mM	not determined	–	EU 2002
	mouse lymphoma cells	Na ₃ NTA	2.4–9.1 mM (+ m.a.) 2.0–7.4 mM (– m.a.)	–	toxic at high concentrations	–	–	EU 2002
	mouse lymphoma cells	Na ₃ NTA	0.5–11.7 mM (+ m.a.) 0.5–4.7 mM (– m.a.)	–	toxic at high concentrations	–	–	EU 2002
	mouse lymphoma cells	Na ₂ NTA	up to 941 µg/ml (4 mM)	–	not specified	not determined	–	IARC 1999
resistance to diphtheria toxin	EUE cells (human epithelial cell line)	Na ₃ NTA · H ₂ O	0.002–11 mM	0.01 mM	11 mM	not determined	+	Grilli and Capucci 1985

m. a.: metabolic activation;
* (+): weakly positive; ±: ambiguous;
HPRT: hypoxanthine guanine phosphoribosyl transferase;

which produced negative results are of limited validity. One research group plausibly reported the induction of DNA strand breaks (comet assay) and micronuclei in primary cultures of rat and human kidney cells. Nitrilotriacetic acid concentrations were used in this case at which the concentration of divalent cations was minimized. This means that these findings are not relevant for the lower concentrations found under in vivo conditions (see above). In another study with C1-1 cells with positive results it was shown that the majority of the micronuclei were of clastogenic origin. In the recognized gene mutation tests with mammalian cells, Na₃NTA had no mutagenic effects. Only one study with human epithelial cells yielded positive results.

Bacteria

– Indicator tests

Nitrilotriacetic acid had no effect in a test for the induction of the SOS response in *Escherichia coli* (EU 2002). On the other hand, positive results were reported in a test for differential killing with *Escherichia coli* using 1 mM Na₃NTA (IARC 1999). This test is not included in the assessment of mutagenic effects, as it frequently responds also to substances that do not produce genotoxic effects in other test systems.

– Gene mutation tests

In a number of mutagenicity tests carried out in accordance with present-day requirements using different strains of *Salmonella typhimurium* or *Escherichia coli*, nitrilotriacetic acid and its sodium salts were not found to be mutagenic with or without metabolic activation (EU 2002; IARC 1999).

Yeast

In eukaryotic yeast cells such as *Schizosaccharomyces pombe* PI and *Saccharomyces cerevisiae* the induction of gene mutations was not observed with Na₃NTA or nitrilotriacetic acid (EU 2002; IARC 1999). Also in *Saccharomyces cerevisiae* D7, the induction of gene conversions was not found after treatment with Na₃NTA (Galli et al. 1988). Na₃NTA also did not induce gene mutation, mitotic crossing-over or aneuploidy in *Aspergillus nidulans* (Crebelli et al. 1986).

Mammalian cells

– Indicator tests

No increase in sister chromatid exchange was found in various established standard cell lines and mouse and human lymphocytes after incubation with nitrilotriacetic acid and its sodium salts (EU 2002). No increase in DNA repair synthesis was found with Na₃NTA · H₂O (EU 2002) in an UDS test with primary rat hepatocytes. A slight increase in the incorporation of ³H-thymidine was observed at non-cytotoxic concentrations of a nitrilotriacetic acid sodium salt in a poorly documented UDS test with phytohaemagglutinin-stimulated human lymphocytes. No information

about a statistical evaluation are available. It can, however, be assumed that the increase was not significant at this concentration range (Celotti et al. 1988). No increase in DNA strand breaks was observed in V79 cells (Hartwig et al. 1993).

On the other hand, an increase in DNA strand breaks was found in a comet assay in primary human and rat kidney cell cultures. Also in the non-cytotoxic concentration range, 1.8 to 5.6 mM nitrilotriacetic acid produced a dose-dependent increase in the tail length and tail movement parameters (Robbiano et al. 1999). As a result of complex formation, it is, however, probable that such nitrilotriacetic acid concentrations can reduce the concentration of essential divalent cations. In this case, valid test conditions can no longer be assumed.

Fe(III)-NTA, which has strong carcinogenic effects after intraperitoneal injection, induced 8-hydroxyguanine formation in the DNA of V79 cells (Hartwig and Schlegel 1995).

– Chromosomal aberration tests

No induction of chromosomal aberrations was observed in two tests with Na_3NTA in human lymphocytes and in one test with nitrilotriacetic acid in CHO cells. These studies have a number of methodological shortcomings, such as the absence of positive controls, no data for cytotoxicity, and high values in the negative controls (EU 2002; Montaldi et al. 1987).

A slight increase in aberrations was observed in PT-K1 cells (cell line from the kangaroo rat kidney) after treatment with $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ for 24 and 48 hours in a chromosomal aberration test. Treatment for 4 hours yielded negative results. Concentrations of over 2.5 mM were cytotoxic after 24 and 48 hours (Kihlman and Sturelid 1970). Positive controls were absent also here.

– Micronucleus tests

In a micronucleus test with primary kidney cell cultures from the rat, nitrilotriacetic acid caused a dose-dependent increase in the frequency of micronuclei which was significant at weakly cytotoxic concentrations (relative survival 90% at 1.8 mM). In similar experiments of this research group with human kidney cells, dose-dependent, positive results were likewise obtained. These were statistically significant at the highest concentration of 5.6 mM (with a relative survival of 80%) (Robbiano et al. 1999).

In a micronucleus test with C1-1 cells (a cell line from Chinese hamster cells), a concentration-dependent increase in the frequency of micronuclei was found after exposure to Na_3NTA for 24 and 48 hours; the effects were found to be more clastogenic than aneuploid (CREST staining). Concentrations of 2 mM with marginal effects on the mitotic frequency produced a significant doubling of the frequency of micronuclei (Modesti et al. 1995). The pH of the medium showed that the mitotic index at the highest concentration of 3 mM was 50% of the value in the controls. A significantly increased number of chromatid bridges and individual (lagging) chromosomes were observed in cells of the anaphase and telophase only at the highest concentration.

In another micronucleus test with human lymphocytes the frequency of micronuclei was not increased. Here too, there were no positive controls and no data regarding cytotoxicity were given (EU 2002).

In an abstract, zinc nitrilotriacetic acid concentrations of between 420 and 750 μM were said to have clastogenic effects in CHO cells although sodium nitrilotriacetic acid and calcium nitrilotriacetic acid had no clastogenic effects even at 12 000 to 21 000 μM (LeBoeuf et al. 1990). Similar effects were found in vitro also with other zinc salts (Thompson et al. 1989).

– Gene mutation tests

Four different studies with Na_3NTA and one with Na_2NTA are available for gene mutation in mammalian cells.

In HPRT gene mutation tests with V79 cells, Na_3NTA without metabolic activation was not found to be mutagenic up to cytotoxic concentrations (EU 2002).

No mutagenic effects were observed in two independent $\text{TK}^{+/-}$ mutation tests with L5178Y mouse lymphoma cells carried out in accordance with present-day requirements. The highest Na_3NTA concentration used in each test produced cytotoxic effects (EU 2002). Resistance to diphtheria toxin was found at concentrations of 0.01 mM and above; at 11 mM with cytotoxic effects in a test system which is not established with EUE cells (a cell line from human epithelial cells) and without metabolic activation (Grilli and Capucci 1985).

Na_2NTA was not found to have mutagenic activity in a $\text{TK}^{+/-}$ mutation test with L5178Y mouse lymphoma cells (IARC 1999).

5.6.2 In vivo

In *Drosophila*, nitrilotriacetic acid and its sodium salts produced mitotic recombinations and aneugenic effects, although the substance was not found to be mutagenic in SLRL and dominant lethal tests. Also dominant lethal tests in the mouse yielded negative results. Sister chromatid exchange and aneugenic or clastogenic effects were not induced in the bone marrow of rodents. However, toxicokinetic data show that nitrilotriacetic acid reaches the bone marrow. In the rat kidney, DNA damage was observed in the comet assay after high doses and there was an increase in the frequency of micronuclei. No increase in the formation of 8-hydroxy-2-deoxyguanosine in the kidneys was found at nephrotoxic doses. Aneugenic effects in the spermatocytes of mice after intraperitoneal injection of high (presumably alkaline) concentrations of Na_3NTA were confirmed when the study was repeated. Although the mechanism of action of the positive effects in vivo is unclear, the effects are believed to be attributable either to the high alkalinity of the trisodium salt or to the acidity of nitrilotriacetic acid. There are no studies available of gene mutations in mammals in vivo caused by nitrilotriacetic acid and its sodium salts.

The available studies of genotoxicity are shown in Table 7.

Table 7 In vivo studies of the genotoxicity of nitrilotriacetic acid and its sodium salts

Test system	Species, strain, number per group	Exposure; substance	Results	References
SMART test	<i>Drosophila melanogaster</i> , no other details	24 hours, 50 mM in the substrate; Na ₃ NTA	+	EU 2002
SMART test	<i>Drosophila melanogaster</i> , no other details	42 hours, 5–50 mM in the substrate; Na ₃ NTA	+	EU 2002
SLRL test	<i>Drosophila melanogaster</i> , no other details	3 days, 50 mM in the substrate; NTA	–	EU 2002
SLRL test	<i>Drosophila melanogaster</i> , no other details	single injections of 10 mM; NTA	–	EU 2002
SLRL test	<i>Drosophila melanogaster</i> , no other details	3 days, up to 4 mg/ml (21 mM) in the substrate; NTA	±	EU 2002; IARC 1999
SLRL test	<i>Drosophila melanogaster</i> , no other details	3 days, 4 mg/ml (21 mM) in the substrate; NTA	–	EU 2002; IARC 1999
SLRL test	<i>Drosophila melanogaster</i> , no other details	single injections of 1.1 mg/ml (5.8 mM); NTA	–	EU 2002
SLRL test	<i>Drosophila melanogaster</i> , no other details	not specified, 50 mM in the substrate; Na ₃ NTA	–	EU 2002
germ cell aneuploidy	<i>Drosophila melanogaster</i> , no other details	during larval stage, 4 mg/ml (21 mM) in the substrate; “NTA”	+	EU 2002
germ cell aneuploidy	<i>Drosophila melanogaster</i> , no other details	3 days, 50 mM in the substrate; Na ₃ NTA	+	EU 2002
DLM test	<i>Drosophila melanogaster</i> , no other details	3 days, 50 mM in the substrate; NTA	–	EU 2002
DLM test	<i>Drosophila melanogaster</i> , no other details	single injections of 10 mM; NTA	–	EU 2002
SCE test, bone marrow	mouse, 3–5 ♂	single intraperitoneal injections of 138, 275 mg/kg body weight; Na ₃ NTA	–	Russo et al. 1989
comet assay, kidneys	rat, Sprague Dawley, controls: 8 ♂; treated groups: 3 ♂	3 days at 490 mg/kg body weight and day or single doses of 735 mg/kg body weight, orally, after unilateral nephrectomy and intravenous administration of 250 mg folic acid/kg body weight; NTA	+	Robbiano et al. 1999

Table 7 (Continued)

Test system	Species, strain, number per group	Exposure; substance	Results	References
oxidative DNA damage, kidneys	rat, no other details	single intraperitoneal injections of 87 mg/kg body weight, sampling 3 and 24 hours after treatment; Na ₂ NTA	–	EU 2002
oxidative DNA damage, kidneys	rat, no other details	19 days, intraperitoneal doses of 29–58 mg/kg body weight and day; Na ₂ NTA	–	EU 2002
oxidative DNA damage, kidneys	rat, Wistar, 5 ♂	28 days, 9, 926 mg/kg body weight and day, diet; Na ₃ NTA · H ₂ O	–	Leibold et al. 2002
MN, kidneys	rat, Sprague Dawley, controls: 8 ♂; treated groups: 3 ♂	single oral doses of 735 mg/kg body weight, after unilateral nephrectomy and intravenous administration of 250 mg folic acid/kg body weight; NTA	–	Robbiano et al. 1999
MN, kidneys	rat, Sprague Dawley, controls: 8 ♂; treated groups: 3 ♂	3 days, 490 mg/kg body weight and day, orally, after unilateral nephrectomy and intravenous administration of 250 mg folic acid/kg body weight; NTA	+	Robbiano et al. 1999
MN, bone marrow	mouse, CD1 Swiss, 3 ♂	single intraperitoneal injections of 200, 400 mg/kg body weight; Na ₃ NTA	–	Montaldi et al. 1988
MN, bone marrow	mouse, NMRI, 5 ♂	2 days, 500, 1000, 2000 mg/kg body weight and day, orally; Na ₃ NTA	–	BASF AG 2004
aneuploidy, bone marrow	mouse, 3–5 ♂	single intraperitoneal injections of 138, 275 mg/kg body weight; Na ₃ NTA	–	Russo et al. 1989
DLT	mouse, ICR/Ha Swiss, 10 ♂	5 days, 1000 mg/kg body weight and day, orally, or single intraperitoneal injections of 125 mg/kg body weight; NTA	–	Epstein et al. 1972

Table 7 (Continued)

Test system	Species, strain, number per group	Exposure; substance	Results	References
aneuploidy, spermatocytes	mouse, C57BL/Cne×C57BL/Cne F ₁ , 2 investigations each with 3 ♂	single intraperitoneal injections of 138, 275 mg/kg body weight; Na ₃ NTA	+	Costa et al. 1988
aneuploidy, spermatocytes	mouse, Balb/c, 2 investigations with a total of 5 ♂	single intraperitoneal injections of 275 mg/kg body weight; Na ₃ NTA	+	Zordan et al. 1990
aneuploidy, spermatocytes	mouse, NMRI, 8–12 ♂	single oral doses of 0, 100, 330, 1000 mg/kg body weight; Na ₃ NTA	–	BASF AG 2000

±: ambiguous;

DLT: dominant lethal test;

SCE: sister chromatid exchange;

SLRL test: test for X-chromosomal recessive lethal mutations;

SMART: somatic mutation and recombination test

DLM: dominant lethal mutation;

MN: micronucleus;

Drosophila melanogaster

Studies with *Drosophila* are available for various end points.

In a SMART test, the administration of 5 to 50 mM Na₃NTA with the diet induced somatic mutations and recombinations. Two studies yielded positive results for chromosome loss or additional chromosomes in germ cells (EU 2002).

Injections of 10 mM and the administration of 50 mM with the diet yielded negative findings in dominant lethal tests; also numerous tests for X-chromosomal recessive lethal mutations (SLRL test) with dietary administration or by injection yielded negative results, with the exception of one ambiguous result (EU 2002).

Mammals

Somatic cells

– Indicator tests in mice and rats

In an SCE test with bone marrow cells of the male mouse, single intraperitoneal doses of Na₃NTA of 138 or 275 mg/kg body weight were not found to be genotoxic in 3 to 5 treated animals; with 25 metaphases per animal, only a few cells were evaluated. Samples were taken after 18 hours. No cytotoxic effects in the bone marrow were found (Russo et al. 1989).

In a study, a comet assay was combined with a micronucleus test in the rat kidney. For this purpose, male Sprague Dawley rats were subjected to unilateral ne-

phrectomy and given intravenous injections of folic acid of 250 mg/kg body weight (in 0.3 M sodium bicarbonate) 24 hours after the nephrectomy to stimulate mitosis. Thereafter, 3 animals were given either single gavage doses of nitrilotriacetic acid of 735 mg/kg body weight ($\frac{1}{2}$ LD₅₀) two days later or 490 mg/kg body weight ($\frac{1}{3}$ LD₅₀) on three successive days. The control group, consisting of 8 animals, was treated with the vehicle DMSO. Four days after the folic acid treatment, kidney cells were isolated for the comet assay. A significant increase ($p < 0.05$) in the parameter tail length was determined in both treated groups (Robbiano et al. 1999). The doses used were very high ($\frac{1}{2}$ and $\frac{1}{3}$ of the LD₅₀). In a study it was shown that doses of 440 and 990 mg/kg administered with the diet can lead to nitrilotriacetic acid concentrations in the urine of about 11 and 20 mM (Anderson 1980). Positive results were also found in a comet assay *in vitro* in concentration ranges of up to 5.6 mM (Robbiano et al. 1999) (see Section 5.6.1).

An increase in the amount of 8-hydroxy-2-deoxyguanosine in the DNA is a typical effect of Fe(III)NTA (see Section 2). This effect could not be demonstrated following the administration of nitrilotriacetic acid, for example in the rat kidney after single intraperitoneal injections of Na₂NTA doses of 87 mg/kg body weight or intraperitoneal injection of 29 to 58 mg/kg body weight and day for 19 days (EU 2002). Also after male Wistar rats were given Na₃NTA · H₂O doses of 9 or 926 mg/kg body weight and day (nitrilotriacetic acid doses of 6.7 or 688 mg/kg body weight and day) with the diet for 4 weeks, no increase in 8-hydroxy-2-deoxyguanosine was found compared with the levels in the controls, although the high dose produced marked nephrotoxic effects with corresponding compensatory cell proliferation. A non-nephrotoxic Fe(III)-NTA dose of 50 mg/kg body weight and day, likewise administered by gavage for 4 weeks, caused on the other hand a significant increase in 8-hydroxy-2-deoxyguanosine (BASF AG 1998 b). After Na₃NTA · H₂O doses of 9 mg/kg body weight and day (nitrilotriacetic acid doses of 6.7 mg/kg body weight and day), the spontaneous formation of 8-hydroxy-2-deoxyguanosine was significantly reduced compared with that in the controls (Leibold et al. 2002). The 8-hydroxy-2-deoxyguanosine was determined using HPLC; this method is accompanied by a relatively high range of scatter as a result of artefacts—with an already very high level of background interference—so that the level of statistical power, with just 5 animals in this case, must be regarded as low.

– Micronucleus tests

In the study described above, parallel to the comet assay also a micronucleus test was performed in rat kidney cells with nitrilotriacetic acid (see above for study design). A significant increase in the frequency of micronuclei was recorded after three doses, but not after single doses (Robbiano et al. 1999).

Na₃NTA was not found to be genotoxic in a micronucleus test in the bone marrow of mice. Groups of 3 male CD-1 mice were given intraperitoneal injections of 0, 200 or 400 mg/kg body weight and samples were taken 12, 24 or 48 hours later. The frequency of micronuclei at the high dose was merely double that of the concurrent controls (a maximum of 2.33‰ compared with 0.95‰, not statistically sig-

nificant). No cytotoxic effects were found in the bone marrow. The concurrent positive controls yielded significant effects (Montaldi et al. 1988). Overall, the results were negative, also when compared with historical controls for this test system (Mavournin et al. 1990). The results of this study are of limited validity because of the low number of animals per group.

In a valid micronucleus test carried out according to OECD Test Guideline 474, Na₃NTA was neither clastogenic nor aneugenic in the bone marrow of male NMRI mice. The test substance was administered twice with a 24 hour interval by gavage in doses of 0, 500, 1000 or 2000 mg/kg body weight. Preparation of the bone marrow was carried out 24 hours after the last administration. The treatment with Na₃NTA did not produce clinical findings, and no cytotoxic effects were found in the bone marrow. The results for the solvent control, the untreated control and the positive control were within the range of historical data (BASF AG 2004).

– Aneuploidy tests

In an aneuploidy test with bone marrow cells of male mice, single intraperitoneal doses of Na₃NTA of 138 or 275 mg/kg body weight did not produce aneuploid effects in 3 to 5 treated animals. However, with 300 to 555 second metaphases per group, only a few cells were evaluated. Samples were taken after 18 hours. No cytotoxic effects in the bone marrow were found (Russo et al. 1989).

Germ cells

– Chromosomal aberrations

In a dominant lethal test, nitrilotriacetic acid had no clastogenic effects in male germ cells. Groups of 10 male mice were given either single intraperitoneal injections of 125 mg/kg body weight, or oral doses of 1000 mg/kg body weight and day on five subsequent days. The treatment was carried out prior to mating with virgin animals. Three female animals were mated with one treated male per week for 8 weeks. There was no increase in pre-implantation and post-implantation losses in the pregnant animals (Epstein et al. 1972). However, the data are not described in detail, as the results of a total of 174 substances are reported in this paper.

– Aneuploidy

In a cytogenetic test system, the induction of aneuploid effects was investigated in the germ cells of male mice. Two experiments with 3 animals per group were carried out, and the results for all 6 animals per group were recorded in tabular form. The animals were given single intraperitoneal injections of Na₃NTA in bidistilled water of 0, 138 or 275 mg/kg body weight with an injection volume of 20 ml/kg body weight. Three hours after administration, 0.3 ml of 0.5% colchicin was injected to identify the spermatocytes in metaphase II. Samples were taken after a further three hours to determine the effects of the test substance during the first meiotic division. Cytogenetic analysis was carried out per animal in 100 cells in the second meiotic division. The number of hyperhaploid ($n = 21$ or 22 chromosomes)

metaphases relative to the total number of metaphases (euploid plus hyperhaploid) was given as the hyperhaploidy index. The treatment produced a significantly increased hyperhaploidy index after the high dose (3.5% compared with 0.5% in the controls) (Costa et al. 1988). There are no data available for the toxicity in general or in the investigated organ. The authors reported, however, that the same dose of nitrilotriacetic acid, although more highly concentrated and administered in doses of 10 ml/kg body weight or dissolved in physiological saline, had lethal effects. The authors suggest that the cause of the lethal effects was an imbalance in the water/salt equilibrium.

The study was repeated by the same research group to test EDTA under the same conditions. Nitrilotriacetic acid was administered as reference substance to 3 animals at the high dose of 275 mg/kg body weight. The controls were a total of 5 animals from two parallel experiments. This study was carried out using another strain of mouse (Balb/c). The positive results were confirmed, but EDTA was not found to have aneugenic effects. The hyperhaploidy index was 3.3%, compared with 0.4% in the controls; 500 metaphases were counted in the controls and 300 in the nitrilotriacetic acid group (Zordan et al. 1990).

To verify these results, the tests were repeated in a study with NMRI mice. Here, Na_3NTA was administered orally as test substance to avoid possible artefacts from the high pH after intraperitoneal injection. The doses were 0, 100, 330 or 1000 mg/kg body weight. The high dose was selected on the basis of preliminary tests to be close to the maximum tolerable dose. 100 spermatocytes in metaphase II per animal in the control group and 200 spermatocytes in metaphase II per animal in the treated groups were evaluated. All 12 animals in the control group were evaluated, but only between 8 and 9 of the total of 12 treated animals in the treated groups could be evaluated as the preparations could not be used or the effects of the treatment were lethal (no other details). The hyperhaploidy index was 0.13% to 0.5% in the treated groups and 0.5% in the concurrent solvent controls. No significant effects (hyperhaploidy index 1.0%) were found in the 7 evaluable animals of the positive control given intraperitoneal injections of diethylstilbestrol (BASF AG 2000). This means that the negative result for Na_3NTA is not meaningful and cannot be used to disprove the positive results described above.

5.7 Carcinogenicity

Nitrilotriacetic acid and its disodium or trisodium salts led, as a result of extensive tissue damage, to kidney tumours in rats and mice, and tumours of the efferent urinary passages (renal pelvis, ureter, bladder) in rats.

In a number of tumour promotion studies, nitrilotriacetic acid (after initiation with nitrosamines) was found to be a tumour promotor in the kidneys and bladder of rats and mice.

In three of a total of eight long-term studies, also the frequency of other types of tumour was increased (see Table 8). In these studies, nitrilotriacetic acid produced

phaeochromocytomas and hepatocellular adenomas in female F344 rats after doses of 750 mg/kg and adenocarcinomas of the preputial glands in the male animals (NCI 1977). It also produced pituitary tumours in male rats in a drinking water study (Goyer et al. 1981), and Na₃NTA doses of 1170 mg/kg body weight (NTA doses of 805 mg/kg body weight and day) produced Leydig cell tumours in Wistar rats (BASF AG 2006). Without exception only one sex was affected in each case and these types of tumour were usually not found in any of the other studies, even when they were performed at the same time using the same dose and the same strain of animal. The doses were already within the nephrotoxic range. In most cases, tumours were involved which occur spontaneously with a certain variability in the respective strain, and could not be assigned to the spectrum typical for nitrilotriacetic acid.

Reproducible tumours could be induced only in the kidneys and the epithelia of the lower urinary tract. These are the organs which were damaged also after short to medium-term administration of nitrilotriacetic acid.

5.7.1 Short-term studies

– Cell transformation studies

There are data available from three cell transformation tests with Na₃NTA performed without metabolic activation. With C3H/10T1/2 cells, negative results were obtained in a study by one laboratory and positive results by another. Na₃NTA did not induce cell transformation in BHK cells. Although positive results were obtained with rat embryo cells infected with Rauscher leukaemia virus, the criteria cited by the authors for a positive result were questionable. These studies are of limited validity (EU 2002); the results can therefore not be included in the evaluation.

– Initiation promotion studies

The ability of nitrilotriacetic acid and its sodium salts to promote tumour formation in the bladder and kidneys of rats after initiation with nitrosamines has been investigated in a number of studies (EU 2002; IARC 1990). The hyperplastic effects in the urogenital tract after treatment with nitrilotriacetic acid are presented in Section 5.2 and Table 5. The exposure duration in the promotion phase (no recovery phase) was 28 to 32 weeks in nearly all of the studies with rats described below. A conversion factor of 0.075 for the rat and of 0.15 for the mouse has been used whenever the administered dose given as “mg/kg diet” was not converted into “mg/kg body weight and day” by the authors themselves (see Table 5 for calculation).

The incidence of simple, papillary and nodular hyperplasia of the bladder after initiation by *N*-bis(2-hydroxypropyl)nitrosamine or *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine in the drinking water was increased by the subsequent administration of nitrilotriacetic acid or its sodium salts in the diet (3000–10 000 mg/kg diet corresponding to about 225–750 mg/kg body weight and day). After administration of the initiator, papillomas and carcinomas of the transitional epithelium were either

induced or their incidence was increased after $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ concentrations of 3000 mg/kg diet (225 mg/kg body weight and day corresponding to nitrilotriacetic acid doses of 156 mg/kg body weight and day) and above (EU 2002). In a study with Wistar rats, also a squamous cell carcinoma of the bladder developed after initiation and subsequent administration of $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ concentrations of 10000 mg/kg diet (doses of about 750 mg/kg body weight and day corresponding to nitrilotriacetic acid doses of 520 mg/kg body weight and day) (Hiasa et al. 1985 a). Less pronounced promoting effects were found in F344 rats in a study after initiation with *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine and the higher $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ concentration of 20000 mg/kg diet (doses of about 1500 mg/kg body weight and day corresponding to nitrilotriacetic acid doses of 1040 mg/kg body weight and day). No hyperplasia of the bladder epithelium or other effects (no other details) were observed after the administration of Na_3NTA alone (EU 2002). After initiation with *N*-ethyl-*N*-hydroxyethylnitrosamine, $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ concentrations of 0, 3000, 10⁴000 or 30⁴000 mg/kg diet (doses of about 0, 225, 750 and 2250 mg/kg body weight and day corresponding to nitrilotriacetic acid doses of 0, 156, 520 and 1560 mg/kg body weight and day) induced simple and adenomatous hyperplasia even at the lowest dose and above. A promoting effect with an increased incidence of kidney tumours was found at the medium dose and above. In the treated groups without initiation, simple hyperplasia was found at the low dose and above, and also adenomatous hyperplasia of the renal tubules in the high dose group. However, the incidence of kidney tumours was not increased (Hiasa et al. 1985 b).

In a similar study, $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ concentrations of 500 or 10 000 mg/kg diet (doses of about 38 and 750 mg/kg body weight and day corresponding to nitrilotriacetic acid doses of 26 and 520 mg/kg body weight and day) led to a dose-dependent increase in atypical cell foci. A promoting effect with an increased incidence of kidney tumours was found in the high dose group only. The tumours occurred mainly in the renal cortex, to a lesser extent in the renal medulla and not at all in the renal pelvis. After $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ doses of 38 mg/kg body weight and day (nitrilotriacetic acid doses of 26 mg/kg body weight and day) and above, the incidence of hyperplasia of the transitional epithelium in the renal pelvis increased in a dose-dependent manner even without an initiator. The incidences were not given, however (Hiasa et al. 1984).

In male Wistar rats (15 to 20 animals per group) the incidence of nephroblastomas, renal cell tumours (no other details) and tumours of the bladder (mainly papillomas) was significantly increased by Na_3NTA after two weeks initiation with *N*-nitroso-bis(2-hydroxypropyl)amine and subsequent exposure to 1% nitrilotriacetic acid or $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ in the diet (nitrilotriacetic acid doses of about 520 or 750 mg/kg body weight and day) for 30 weeks. The incidence of renal cell tumours was significantly increased also by nitrilotriacetic acid (IARC 1990). Neither Na_3NTA nor nitrilotriacetic acid were tested without an initiator.

Groups of 15 male Wistar rats were given *N*-ethyl-*N*-hydroxyethylnitrosamine with the diet in concentrations of 1000 mg/kg for 2 weeks. Unilateral nephrectomy was performed in the third week, and the animals were then given $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$

concentrations of 10⁴000 mg/kg diet (doses of about 750 mg/kg body weight and day corresponding to nitrilotriacetic acid doses of 520 mg/kg body weight and day) over the following 6, 10 or 18 weeks. Nitrilotriacetic acid led to increased incidences (which depended on the exposure duration) of simple and adenomatous hyperplasia in the kidneys, and of kidney tumours compared with those in the animals treated with the initiator only (Hiasa et al. 1991). Also in this study, Na₃NTA was not administered without an initiator to any group.

In an initiation promotion study, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine was administered to groups of 14 to 16 male C3H/He mice with the drinking water followed by 1% nitrilotriacetic acid or 1% Na₃NTA · H₂O in the diet for 12 weeks (nitrilotriacetic acid doses of about 1040 or 1500 mg/kg body weight and day). The incidence of dysplasia and carcinomas of the transitional epithelium of the bladder was increased compared with that in the untreated animals or animals treated with the initiator or promoter only. In addition, an increased incidence of hyperplasia and carcinomas of the transitional epithelium in the kidneys was observed in the animals treated with the initiator and nitrilotriacetic acid or Na₃NTA · H₂O (EU 2002).

– Cocarcinogenicity studies

The carcinogenic effects of dipentylnitrosamine (2000 mg/kg diet, for 8 weeks) on the liver of 15 male and 15 female F344 rats per group was significantly reduced by the simultaneous administration of Na₃NTA · H₂O in concentrations of 2430 or 7300 mg/kg diet (doses of about 180 and 550 mg/kg body weight and day corresponding to nitrilotriacetic acid doses of 127 and 380 mg/kg body weight and day) (Davis et al. 1981).

5.7.2 Long-term studies

The results of long-term carcinogenicity studies are shown in Table 8.

– Drinking water studies

In a drinking water study, 195 male Sprague Dawley rats in two separate groups were given 0.1% Na₃NTA in deionized water for 704 days; 193 controls were likewise given deionized water. This corresponds to a daily Na₃NTA dose of about 100 mg/kg body weight assuming a daily drinking water intake of 10% of the body weight. The dose cannot be verified as no data for drinking water consumption were given. The tissue prepared for the histopathological investigation of all animals comprised the thyroid gland, liver, lungs, kidneys, adrenal glands, heart, spleen, pancreas, brain, testes and skeletal muscle. The bones, eyes and gastrointestinal tract of 10 control animals and 10 treated rats without any gross pathological findings were investigated histopathologically. Where autopsy revealed changes in the bones, pituitary gland, ureter, bladder or other organs, also these organs were prepared for histopathology, although the ureter and bladder were not opened. An increase in hyperplasia of a higher degree of severity was found in the renal tubules.

Table 8 Studies of the carcinogenicity of nitrilotriacetic acid and its sodium salts

Author:	Lijinsky et al. 1973		
Test substance:	Na ₂ NTA (purity not specified)		
Species:	rat, MRC, 15 ♂, 15 ♀		
Administration route:	drinking water		
Concentration:	0%, 0.5%, 0.5% plus 0.2% sodium nitrite (Na ₂ NTA doses of about 400 mg/kg body weight and day ¹⁾ corresponding to nitrilotriacetic acid doses of 325 mg/kg body weight)		
Duration:	104 weeks, exposure duration 84 weeks, 5 days/week		
Toxicity:	no effects		
	Exposure concentration		
	0	5000 mg/l (NTA dose of 325 mg/kg body weight)	5000 mg/l plus sodium nitrite (NTA dose of 325 mg/kg body weight)
Survivors:	not specified not specified		not specified
Tumours:			
Kidneys:			
(not specified)	♂	0/15 (0%)	1/15 (7%)
	♀	0/15 (0%)	0/15 (0%)
		2/15 (13%)	0/15 (0%)
¹⁾ based on the dose of 100 mg/day and rat specified in the publication (Lijinski et al. 1973) and an assumed body weight of 250 g per rat			
Author:	Goyer et al. 1981		
Test substance:	Na ₃ NTA (purity not specified)		
Species:	rat, Sprague Dawley, only ♂ ¹⁾		
Administration route:	drinking water		
Concentration:	0%, 0.1%, (Na ₃ NTA doses of about 100 mg/kg body weight and day ²⁾ , corresponding to nitrilotriacetic acid doses of 74 mg/kg body weight)		
Duration:	704 days, 7 days/week		
Toxicity:	mortality increased during the first 550 days		
	Exposure concentration		
	0	1000 mg/l (NTA dose of 74 mg/kg body weight)	
Survivors:	80/193 (42%)		83/195 (43%)
Tumours/precursors:			

Table 8 (Continued)

Kidneys:		
hyperplasia of higher degree of severity	44/186 (24%)	67/183 (37%)**
	Exposure concentration	
	0	1000 mg/l (NTA dose of 74 mg/kg body weight)
tubular adenomas and adenocarcinomas	5/186 (3%)	29/183 (16%)**
Pituitary:	34/188 (18%)	43/183 (23%)
Total tumours	111/188 (59%)	131/183 (71%)**
total tumours without renal adenomas	109/188 (58%)	117/183 (64%)
total visceral tumours	39/186(21%)	59/183 (32%)**
visceral tumours without renal adenomas	35/186(19%)	37/183 (20%)
* p < 0.05; **p < 0.01;		
1) two control groups (102 and 91 animals) and two treated groups (102 and 93 animals) in each case, summarized report;		
2) with an average daily drinking water intake of 10% of the body weight		
Author:	Nixon et al. 1972	
Test substance:	Na ₃ NTA · H ₂ O or CaNaNTA · 2 H ₂ O	
Species:	rat, CD, 50 ♂, ♀, controls 100 ♂, ♀	
Administration route:	diet	
Concentration:	0%, 0.03%, 0.15%, 0.5% Na ₃ NTA, 0.5% CaNaNTA (Na ₃ NTA doses of about 0, 15, 75 or 250 mg/kg body weight and day corresponding to nitrilotriacetic acid doses of 11.2, 56, 186 mg/kg body weight and day)	
Duration:	24 months, no recovery phase	
Toxicity:	nitrilotriacetic acid doses of 11.5 mg/kg body weight and day: NOAEL; 56 mg/kg body weight and day and above: nephrosis increased, nephritis increased, zinc in urine increased; 186 mg/kg body weight and day: mortality increased (♂), relative liver weights increased (12 months; ♀)	
Tumours:	no increase in the incidence of tumours	
Author:	NCI 1977 (Stanford Research Institute)	
Test substance:	Na ₃ NTA · H ₂ O (purity 99.5%; analysed in the diet)	
Species:	rat, F344, 24 ♂, 24 ♀	
Administration route:	diet	

Table 8 (Continued)

Concentration:	0, 200, 2000, 20 000 mg/kg diet (Na ₃ NTA · H ₂ O doses of about 10, 100, 1000 mg/kg body weight and day corresponding to nitrilotriacetic acid doses of 6.9, 70 and 700 mg/kg body weight and day) ¹⁾				
Duration:	24 months, no recovery phase				
Toxicity:	NTA doses of 700 mg/kg body weight and day: ♂, ♀: body weight gains reduced, ♂: mortality increased (NCI 1977), age-related nephrosis increased, vacuolization in the cytoplasm of renal tubular epithelial cells (100% compared with 0% in the controls) (Alden and Kanerva 1982 a)				
<hr/>					
	Exposure concentration				
	0	200 mg/kg diet (NTA dose of 6.9 mg/kg body weight and day)	2000 mg/kg diet (NTA dose of 70 mg/kg body weight and day)	20 000 mg/kg diet (NTA dose of 700 mg/kg body weight and day)	
<hr/>					
Survivors:	♂	18/24(75%)	16/24 (67%)	24/24 (100%)	6/24 (25%)**
	♀	18/24(75%)	19/24 (79%)	16/24 (67%)	17/24 (71%)
<hr/>					
Tumours/precursors:					
Kidneys:					
hyperplasia of the tubular epithelial cells (cells with vacuolization in the cytoplasm) ²⁾	♂	0/0 ³⁾	not specified	not specified	96% ³⁾
hyperplasia of the tubular epithelial cells (basophilic and eosinophilic cell type) ²⁾	♂	5/10 (50%)	4/9 (44%)	5/10 (50%)	23/23 (100%)**
hyperplasia of the tubular epithelial cells ⁴⁾	♀	0/24 (0%)	0/24 (0%)	0/24 (0%)	11/24 (46%)**
nodular hyperplasia (vacuolized cell type) ²⁾	♂	0/0 ³⁾	not specified	not specified	57% ³⁾
nodular hyperplasia (basophilic and eosinophilic cell type) ²⁾	♂	0/0 ³⁾	not specified	not specified	74% ³⁾

Table 8 (Continued)

		Exposure concentration			
		0	200 mg/kg diet (NTA dose of 6.9 mg/kg body weight and day)	2000 mg/kg diet (NTA dose of 70 mg/kg body weight and day)	20 000 mg/kg diet (NTA dose of 700 mg/kg body weight and day)
adenoma-like hyperplasia ²⁾	♂	0% ³⁾	not specified	not specified	17% ³⁾
tubular adeno- mas and adeno- carcinomas	♂	0/24 (0%)	0/24 (0%)	0/24 (0%)	4/24 (17%)*
hyperplasia of the	♀	0/24 (0%)	0/24 (0%)	0/24 (0%)	4/24 (17%)*
transitional	♂	1/24 (4%)	0/23 (0%)	3/24 (13%)	7/24 (29%)*
epithelium in the renal pelvis ⁴⁾					
carcinomas of the	♀	1/24 (4%)	2/24 (8%)	0/24 (0%)	10/24 (42%)**
transitional	♂	0/24 (0%)	0/24 (0%)	0/24 (0%)	4/24 (17%)*
epithelium in the renal pelvis					
Ureter:					
hyperplasia of the	♂	0/24 (0%)	1/23 (4%)	4/24 (17%)	3/24 (13%)
transitional	♀	0/24 (0%)	0/24 (0%)	0/24 (0%)	5/24 (21%)*
epithelium ⁴⁾					
dysplasia	♀	0/24 (0%)	0/24 (0%)	0/24 (0%)	2/24 (8%)
carcinomas of the	♂	0/24 (0%)	0/24 (0%)	0/24 (0%)	8/24 (33%)**
transitional	♀	0/24 (0%)	0/24 (0%)	0/24 (0%)	6/24 (25%)**
epithelium					
Bladder:					
hyperplasia of the	♂	0/24 (0%)	3/23 (13%)	3/24 (13%)	8/24 (33%)**
transitional	♀	1/24 (4%)	1/24 (4%)	13/24 (54%)*	14/24 (58%)**
epithelium ⁴⁾					
dysplasia of the	♂	0/24 (0%)	1/23 (4%)	4/24 (17%)	0/24 (0%)
transitional	♀	0/24 (0%)	1/24 (4%)	3/24 (13%)	8/24 (33%)**
epithelium ⁴⁾					
carcinomas of the	♂	0/24 (0%)	0/24 (0%)	0/24 (0%)	1/24 (4%)
transitional	♀	0/24 (0%)	0/24 (0%)	0/24 (0%)	5/24 (21%)*
epithelium ⁴⁾					
papillomas	♀	0/24 (0%)	0/24 (0%)	1/24 (4%)	0/24 (0%)

Table 8 (Continued)

		Exposure concentration			
		0	200 mg/kg diet (NTA dose of 6.9 mg/kg body weight and day)	2000 mg/kg diet (NTA dose of 70 mg/kg body weight and day)	20 000 mg/kg diet (NTA dose of 700 mg/kg body weight and day)
Haematopoietic system⁴⁾:					
granulocytic leukaemia	♂	0/24 (0%)	6/24 (25%)	0/24 (0%)	0/24 (0%)
Thyroid gland:					
C-cell hyperplasia	♂	0/24 (0%)	1/23 (4%)	0/24 (0%)	0/24 (0%)
	♀	0/24 (0%)	0/24 (0%)	1/24 (0%)	5/24 (21%)*
C-cell adenomas	♂	0/24 (0%)	1/24 (4%)	4/24 (17%)	0/24 (0%)
	♀	0/24 (0%)	1/24 (4%)	0/24 (0%)	0/24 (0%)
C-cell carcinomas	♂	0/24 (0%)	3/24 (13%)	0/24 (0%)	0/24 (0%)
	♀	0/24 (0%)	2/24 (8%)	0/24 (0%)	0/24 (0%)
Total tumours⁵⁾					
benign	♂	8/24 (33%)	15/23 (65%)*	9/24 (38%)	18/24 (75%)**
	♀	13/24 (54%)	17/23 (74%)	15/24 (63%)	19/24 (79%)
malignant	♂	4/24 (17%)	8/24 (33%)	2/24 (8%)	11/24 (46%)*
	♀	9/24 (38%)	8/24 (33%)	10/24 (42%)	16/24 (67%)*
<p>* $p < 0.05$; ** $p < 0.01$; ¹⁾ conversion factor: 0.05; ²⁾ reevaluation of the data by Alden and Kanerva (1982 a, 1982 b); ³⁾ no incidences given; ⁴⁾ contradictory presentation of the incidences in the statistical analysis; ⁵⁾ without testicular tumours or without benign uterus tumours</p>					
Author:	NCI 1977 (Litton Bionetics)				
Test substance:	NTA (purity 99.5%; analysed in the diet)				
Species:	rat, F344, 50 ♂, 50 ♀; controls: 20 ♂, 20 ♀				
Administration route:	diet				
Concentration:	0, 7500, 15000 mg/kg diet (nitrilotriacetic acid doses of about 375 and 750 mg/kg body weight and day) ¹⁾				
Duration:	exposure duration 18 months, 6 months recovery period				
Toxicity:	7500 mg/kg and above: ♂, ♀: dose-dependent reduction in body weight gains; chronic inflammation of the kidneys increased				

Table 8 (Continued)

		Exposure concentration		
		0	7500 mg/kg diet (NTA dose of 375 mg/kg body weight and day)	15 000 mg/kg diet (NTA dose of 750 mg/kg body weight and day)
Survivors: (24 months)	♂	18/20 (90%)	39/50 (78%)	41/50 (82%)
	♀	6/20 (30%)	39/50 (78%)	39/50 (78%)
Tumours/precursors:				
Kidneys:				
tubular hyperplasia (basophilic and eosinophilic cell type) ²	♂	85% ³	not specified	100 % ^{3, 4}
hyperplasia	♂	0/20 (0%)	1/50 (2%)	1/49 (2%)
tubular adenomas and adeno- carcinomas	♂	0/20 (0%)	1/50 (2%)	5/49 (10%)
tubular adenomas	♀	0/20 (0%)	0/50 (0%)	1/50 (2%)
papillomas of the transitional epithelium	♀	0/20 (0%)	0/50 (0%)	1/50 (2%)
Ureter:				
hyperplasia of the transitional epithelium	♂	0/20 (0%)	0/50 (0%)	1/49 (2%)
papillomas of the transitional epithelium and papillary adenomas	♂	0/20 (0%)	0/50 (0%)	3/49 (6%)
Bladder:				
hyperplasia of the	♂	0/20 (0%)	0/50 (0%)	1/49 (2%)
transitional epithelium	♀	0/20 (0%)	2/50 (4%)	11/50 (22%)*
squamous cell carcinomas and carcinomas of the transitional epithelium	♀ ⁵	0/20 (0%)	2/50 (4%)	12/50 (24%)**
Liver:				
hepatocellular	♂	3/20 (15%)	2/50 (4%)	2/49 (4%)
adenomas/neoplastic nodules	♀ ⁵	2/20 (10%)	8/50 (16%)	22/50 (44%)**
hepatocellular carcinomas	♂	0/20 (0%)	3/50 (6%)	0/49 (0%)
Adrenal gland:				
phaeochromocytomas	♂	1/20 (5%)	9/50(18%)	5/49 (10%)
	♀ ⁵	1/20 (5%)	0/50 (0%)	14/50 (28%)*

Table 8 (Continued)

		Exposure concentration		
		0	7500 mg/kg diet (NTA dose of 375 mg/kg body weight and day)	15 000 mg/kg diet (NTA dose of 750 mg/kg body weight and day)
Lungs:				
alveolar/bronchiolar	♂	1/20 (5%)	5/50 (10%)	5/49 (10%)
adenomas and carcinomas	♀ ⁵	0/20 (0%)	3/50 (6%)	7/50 (14%)
Pituitary:				
adenomas	♂	1/20 (5%)	6/50 (12%)	2/50 (4%)
	♀	6/20 (30%)	10/50 (20%)	12/50 (24%)
Preputial gland:				
epithelial hyperplasia	♂	0/20 (0%)	0/50 (0%)	2/50 (4%)
adenocarcinomas	♂	0/20 (0%)	2/50 (4%)	4/50 (8%)
Total tumours				
benign	♂	18/20 (90%)	43/50 (86%)	46/50 (92%)
	♀	9/20 (45%)	18/50 (36%)	27/50 (54%)
malignant	♂	5/20 (25%)	13/50 (26%)	12/50 (24%)
	♀	7/20 (35%)	16/50 (32%)	25/50 (50%)
<p>*p < 0.05; **p < 0.01 ¹ conversion factor 0.05; ² reevaluation of the data by Alden and Kanerva (1982 b); ³ no incidences given; ⁴ no hyperplasia of the vacuolized cell type or nodular hyperplasia (compare with continuous exposure over 24 months); ⁵ contradictory presentation of incidences in the statistical analysis</p>				
Author:	NCI 1977 (Litton Bionetics)			
Test substance:	Na ₃ NTA · H ₂ O (purity 99.5%; analysed in the diet)			
Species:	rat, F344, 50 ♂, 50 ♀; controls: 20 ♂, 20 ♀			
Administration route:	diet			
Concentration:	0, 7500, 15 000 mg/kg diet (Na ₃ NTA · H ₂ O doses of about 375 or 750 mg/kg body weight and day corresponding to nitrilotriacetic acid doses of 260 and 520 mg/kg body weight and day) ¹			
Duration:	exposure duration 18 months, 6 months recovery period			
Toxicity:	nitrilotriacetic acid doses of 260 mg/kg and above: ♂, ♀: dose-dependent reduction in body weight gains; chronic inflammation of the kidneys increased			

Table 8 (Continued)

		Exposure concentration		
		0	7500 mg/kg diet (NTA dose of 260 mg/kg body weight and day)	15 000 mg/kg diet (NTA dose of 520 mg/kg body weight and day)
Survivors:	♂	18/20 (90%)	45/50 (90%)	36/50 (72%)
	♀	18/20 (90%)	46/50 (92%)	39/50 (78%)
Tumours/precursors:				
Kidneys:				
hyperplasia of the transitional epithelium	♂	0/20 (0%)	0/49 (0%)	3/50 (6%)
papillomas of the transitional epithelium	♂	0/20 (0%)	0/49 (0%)	1/50 (2%)
tubular adenocarcinomas	♂	0/20 (0%)	1/49 (2%)	1/50 (2%)
Ureter:				
papillomas	♂	0/20 (0%)	1/49 (2%)	0/50 (0%)
Bladder:				
hyperplasia of the transitional epithelium	♂	0/20 (0%)	0/49 (0%)	3/50 (6%)
	♀	0/20 (0%)	4/50 (8%)	5/49 (10%)
papillomas	♀	0/20 (0%)	0/50 (0%)	1/49 (2%)
squamous cell carcinomas and carcinomas of the transitional epithelium	♀	0/20 (0%)	4/50 (8%)	1/49 (2%)
Lungs:				
alveolar/bronchiolar adenomas and carcinomas	♂ ²	2/20 (10%)	10/49	3/50 (6%)
	♀	1/20 (5%)	5/50 (10%)	3/50 (6%)
Pituitary:				
adenomas	♂	2/20 (10%)	3/49 (6%)	1/50 (2%)
	♀	3/20 (15%)	16/50 (32%)	13/49 (27%)
Thyroid gland:				
C-cell hyperplasia	♂	1/20 (5%)	12/49 (25%)	4/50 (8%)
	♀	2/20 (10%)	5/50 (10%)	5/49 (10%)
Total tumours				
benign	♂	20/20 (100%)	47/49 (96%)	42/50 (84%)
	♀	10/20 (50%)	29/50 (58%)	17/49 (35%)
malignant	♂	5/20 (25%)	15/49 (31%)	13/50 (26%)
	♀	4/20 (20%)	11/50 (22%)	12/49 (25%)

in ♀ no tumours in kidneys and ureter, and in ♂ no tumours in the bladder;

¹ conversion factor 0.05;

² contradictory presentation of incidences in the statistical analysis

Table 8 (Continued)

Author:	BASF AG 2006		
Test substance:	Na ₃ NTA · H ₂ O; purity > 99.5%; analysed in the diet		
Species:	rat, Wistar, 50 ♂, 50 ♀		
Administration route:	diet		
Concentration:	0; initially 20000 mg/kg diet for 7 days, then 15 000 mg/kg diet (Na ₃ NTA · H ₂ O doses of about 1170 mg/kg body weight or nitrilotriacetic acid doses of 805 mg/kg body weight (♂) and Na ₃ NTA · H ₂ O doses of about 1040 mg/kg body weight or nitrilotriacetic acid doses of 716 mg/kg body weight and day (♀))		
Duration:	exposure duration 24 months		
Toxicity:	drinking water consumption increased, food consumption decreased, body weights decreased, mortality in ♂ increased; kidney weights (absolute and relative) increased, cysts, pyelitis, hydronephrosis, mesenterial and testicular arteritis; testicular atrophy		
<hr/>			
	Exposure concentration		
	0	15 000 mg/kg diet (NTA doses of 805 and 716 mg/kg body weight and day)	
<hr/>			
Survivors:	♂	38/50 (76%)	23/50 (46%)
	♀	36/50 (72%)	36/50 (72%)
<hr/>			
Tumours/precursors:			
Kidneys:			
Mesenchymal tumours:	♂	0/50 (0%)	6/50 (12%)*
– benign		0/50 (0%)	1/50 (2%)
– malignant		0/50 (0%)	5/50 (10%)*
– haemangiosarcomas		0/50 (0%)	3/50 (6%)
Testes:			
Leydig cell adenomas	♂	0/50 (0%)	7/50 (14%)**
<hr/>			
* p < 0.05; ** p < 0.01			
<hr/>			
Author:	NCI 1977 (Litton Bionetics)		
Test substance:	NTA (purity 99.5%; analysed in the diet)		
Species:	mouse, B6C3F ₁ , 50 ♂, 50 ♀, controls: 20 ♂, 20 ♀		
Administration route:	diet		
Concentration:	0, 7500 or 15000 mg/kg diet (nitrilotriacetic acid doses of about 1125 and 2250 mg/kg body weight and day) ¹		

Table 8 (Continued)

Duration:	exposure duration 18 months, recovery period 3 months			
Toxicity:	1125 mg/kg body weight and day and above: ♀: dose-dependent reduction in body weight gains, ♂: hydronephrosis 2250 mg/kg body weight and day: ♀: hydronephrosis, ♂: body weight gains reduced			
		Exposure concentration		
		0	7500 mg/kg diet (NTA dose of 1125 mg/kg body weight and day)	15 000 mg/kg diet (NTA dose of 2250 mg/kg body weight and day)
Survivors ² :	♂	19/20 (95%)	44/50 (88%)	39/50 (78%)
	♀	19/20 (95%)	35/50 (70%)	47/50 (94%)
Tumours/precursors:				
Kidneys:				
hyperplasia of the transitional epithelium	♀	0/20 (0%)	0/38 (0%)	1/49 (2%)
papillomas of the transitional epithelium	♂	0/20 (0%)	0/49 (0%)	1/44 (2%)
tubular adenocarcinomas	♂	0/20 (0%)	5/49 (10%)	22/44 (50%)**
	♀	0/20 (0%)	0/39 (0%)	4/50 (8%)
Haematopoietic system:				
malignant lymphomas	♀	1/20 (5%)	3/39 (8%)	5/50 (10%)
lymphoid hyperplasia of the spleen	♀	0/20 (0%)	5/38 (13%)	5/49 (10%)
Prostate:				
Cystic hyperplasia	♂	3/20 (15%)	9/48 (19%)	19/44 (43%)*
Total tumours				
benign	♂	5/20 (25%)	5/49 (10%)	5/44 (11%)
	♀	0/20 (0%)	4/39 (10%)	4/50 (8%)
malignant	♂	5/20 (25%)	11/49 (22%)	28/44 (64%)**
	♀	4/20 (20%)	10/39 (26%)	16/50 (32%)

*p < 0.05;

**p < 0.01;

¹ conversion factor 0.15;² one male animal in the low dose group, six male animals in the high dose group and eleven female animals in the low dose group escaped

Table 8 (Continued)

Author:	NCI 1977 (Litton Bionetics)			
Test substance:	Na ₃ NTA · H ₂ O (purity 99.5%; analysed in the diet)			
Species:	mouse, B6C3F ₁ , 50 ♂, 50 ♀, controls: 20 ♂, 20 ♀			
Administration route:	diet			
Concentration:	0, 2500 or 5000 mg/kg diet (Na ₃ NTA · H ₂ O doses of about 375 or 750 mg/kg body weight and day corresponding to nitrilotriacetic acid doses of 260 and 520 mg/kg body weight and day) ¹			
Duration:	exposure duration 18 months, recovery period 3 months			
Toxicity:	nitrilotriacetic acid doses of 260 mg/kg body weight and day and above: dose-dependent reduction in body weight gains ♂, ♀; 520 mg/kg body weight and day: ♂, ♀: hydronephrosis			
Tumours:	no treatment-related increase in tumour incidence (see text)			
<hr/>				
	Exposure concentration			
	0	2500 mg/kg diet (NTA dose of 260 mg/kg body weight and day)	5000 mg/kg diet (NTA dose of 520 mg/kg body weight and day)	
<hr/>				
Survivors:	♂ 20/20 (100%)	43/50 (86%)	46/50 (92%)	
	♀ 17/20 (85%)	43/50 (86%)	44/50 (88%)	
<hr/>				
Tumours:				
<hr/>				
leukaemia	♂ 0/20 (0%)	0/48 (0%)	4/50 (8%)	
	♀ 3/20 (15%)	7/48 (15%)	4/50 (8%)	
malignant tumours of the haematopoietic system	♂ 0/20 (0%)	4/47 (9%)	9/50 (18%)*	
alveolar/bronchiolar carcinomas	♂ 1/20 (5%)	6/48 (13%)	0/50 (0%)	
hepatocellular carcinomas	♂ 1/20 (5%)	5/48 (10%)	3/50 (6%)	
total malignant tumours	♂ 3/20 (15%)	15/48 (31%)	12/50 (24%)	
	♀ 6/18 (33%)	11/46 (24%)	13/47 (28%)	

* $p < 0.05$ ¹ conversion factor 0.15

There was no difference between the treated rats and controls with regard to simple hyperplasia or inflammation of the renal parenchyma. The frequency of renal tubular adenomas and adenocarcinomas was significantly increased ($p < 0.01$) compared with that in the controls. The increase in the incidence of pituitary tumours was not significant (Goyer et al. 1981). Whether the latter was a treatment-related effect or is within the range of the high biological variation is unclear. In addition, the

animals were under particular stress as regards the regulation of their water and electrolyte balance as a result of the high sodium intake and the presumably high pH of the drinking water. On reevaluation of the sections, the controls were found to have more pronounced age-related nephrosis than the exposed animals and the animals in the study of Nixon et al. (1972), in which no tumours occurred at a higher nitrilotriacetic acid dose (see below). This could be a reason for the different results of this drinking water study compared with those of feeding studies. Higher drinking water consumption by the older male animals is given as a further explanation, as this would result in a higher level of exposure from the drinking water, but lead to a higher dilution of nitrilotriacetic acid when administered with the diet (Anderson et al. 1985). Whether this effect late in the rats' lives can influence tumour incidence is unclear.

An earlier drinking water study with MRC rats is available in which a kidney tumour developed in one male animal after the administration of 0.5% Na_2NTA (doses of 400 mg/kg body weight and day corresponding to nitrilotriacetic acid doses of 325 mg/kg body weight and day). The number of animals used (15 males and 15 females per group) is, however, too low to obtain statistically valid findings. The exposure period lasted 84 weeks, and the recovery period 20 weeks (Lijinsky et al. 1973).

– Feeding studies

For the long-term studies, a conversion factor of 0.05 was used for the rat and of 0.15 for the mouse (calculation see Table 5) whenever the administered dose given as "mg/kg diet" was not converted into "mg/kg body weight and day" by the authors.

Charles-River-CD rats were used in the first 2-year feeding study with $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ already described in Section 5.2.2. Groups of 50 male and 50 female animals were given concentrations of 0%, 0.03%, 0.15% or 0.5% $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ or 0.5% calcium/sodium NTAH_2O ($\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ doses of about 0, 15, 75 and 250 mg/kg body weight and day corresponding to nitrilotriacetic acid doses of 11.2, 56 and 186 mg/kg body weight and day) in the diet. An increase in the incidence and severity of nephritis and nephrosis was found after two years in the two high dose groups. No other effects were observed. There was also no increase in tumour incidence (Nixon et al. 1972). The bladder was presumably not investigated for tumour precursors as no tumours of the bladder occurred up to the highest $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ dose of 250 mg/kg body weight and day (nitrilotriacetic acid doses of 186 mg/kg body weight and day).

The effects of nitrilotriacetic acid and $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ at higher dose levels were investigated in F344 rats and B6C3F₁ mice in a number of long-term studies conducted by the National Cancer Institute (NCI, USA). To a large extent these studies meet present-day requirements, with the exception of the small number of animals in the first study and a shortened exposure period in the subsequent studies. A total of 34 tissues/organs were examined histopathologically (see Table 8).

In a first study performed at the Stanford Research Institute (SRI), groups of

24 male and 24 female F344 rats were given $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ for 2 years in concentrations of 0, 200, 2000 or 20000 mg/kg diet (doses of about 10, 100, and 1000 mg/kg body weight and day corresponding to nitrilotriacetic acid doses of 6.9, 70 and 700 mg/kg body weight and day). The high dose led to significantly reduced body weight gains and an increase in hydronephrosis in both sexes, and significantly increased mortality in the males. In addition, an increase in hyperplasia in the tubules and transitional epithelia of the renal pelvis and ureter were observed in the male animals of the medium and high dose groups, and in the female animals of the high dose group. An increase in the incidence of hyperplasia in the bladder was found in the male animals of the low and medium dose groups, but this was neither dose-dependent nor statistically significant. The incidence was significantly increased in high-dose males and in medium and high-dose female animals. Dysplasia was found in the ureter and bladder. The incidences of the following tumours were increased in the high dose group: adenomas and adenocarcinomas of tubular origin and tumours of the transitional epithelium in the ureter of both sexes, tumours of the transitional epithelium in the renal pelvis of male animals and of the bladder in female animals (NCI 1977). On repeated investigation of the histopathological kidney sections from the males, vacuolized tubular epithelial cells and an increase in the incidence and severity of age-related nephropathy were found in the high dose group only (Alden and Kanerva 1982 a). In the authors' opinion, the incidences of the different lesions of the urinary tract system would possibly have been higher if more kidney and bladder sections had been investigated and the ureter prepared for histopathological investigation at autopsy on a routine basis. As a result of the methods used in this study, the ureteral tumours were discovered either during gross pathological examination or were dissected with the kidney by chance (NCI 1977). Higher incidences of these tumours must therefore be assumed.

In a second study, performed by Litton Bionetics (LBI), F344 rats were given $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ for 18 months with the diet in concentrations of 0, 7500 or 15000 mg/kg (doses of about 375 and 750 mg/kg body weight and day corresponding to nitrilotriacetic acid doses of 260 and 520 mg/kg body weight and day). This was followed by an exposure-free recovery period of six months. The control groups consisted of 20 male and 20 female animals and the exposed groups of 50 male and 50 female animals in each case. This treatment led to a dose-dependent reduction in body weights in male and female animals. The survival period of the treated animals was unchanged compared with that of the controls. The incidences of chronic inflammation in the kidneys were increased. A tubular cell adenocarcinoma was found in one male animal and a ureter papilloma in another male in the low dose group. One tubular cell adenocarcinoma and one papilloma of the transitional epithelium was found in the kidneys of one male in the high dose group in each case. In the female animals of the low dose group there were three transitional cell carcinomas and one squamous cell carcinoma of the bladder. In the high dose group, one carcinoma and one papilloma were found in the bladder of the female

animals. A dose-dependent, but not significant increase in pituitary adenomas was found in the female animals (NCI 1977).

Nitrilotriacetic acid was also tested using the same study design. The doses were about 375 and 750 mg/kg body weight and day. Marked carcinogenic effects in the urogenital tract were observed. The effects on body weights and mortality and the non-neoplastic lesions in the urogenital tract corresponded with those found in the study with $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$. In male rats, the incidence of tumours in the tubular epithelium of the kidneys and the transitional epithelium of the ureter were increased. Mainly tumours of the bladder were found in the females. Adenocarcinomas of the preputial gland were also observed, but these were not significantly increased. Also the incidence of neoplastic nodules, phaeochromocytomas and lung tumours was increased in female animals of the high dose group. With the exception of the lung tumours, this increase was statistically significant. It is unclear whether the terms liver adenomas and hepatocellular nodules used in the study denote foci, precursors of infiltrating carcinomas, or benign adenomas. The authors do not draw any conclusions from the increased incidences of tumours outside the urogenital region and draw attention to the wide range of variation and the fact that the results were not reproducible in two simultaneously performed studies with approximately the same dose in the same strain of rat (see above) (NCI 1977). Also in the other studies with nitrilotriacetic acid, these tumours were not found. There is no mechanistic explanation for the increased frequency of tumours outside the urogenital tract. Both doses were, however, in the nephrotoxic range and the nitrilotriacetic acid had led to a state acidosis. As regards the kidneys, a repeat investigation of the histopathological preparations from the male animals from the control and high dose groups revealed that simple hyperplasia of the eosinophilic and basophilic type in the tubular epithelium occurred also in the controls. Hyperplasia of the vacuolized cell type and nodular hyperplasia, however, were found only in the treated animals (Alden and Kanerva 1982 b).

In a feeding study, 50 male and 50 female Wistar rats were given $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ in doses of 1170 mg/kg body weight and day for the male and 1040 mg/kg body weight and day for the female animals (nitrilotriacetic acid doses of 805 and 716 mg/kg body weight and day). The drinking water consumption of the treated animals increased to approximately double that of the controls. Food consumption, body weights and particularly body weight gains were reduced. Mortality was increased in the males. The relative weights of the brain, heart, liver and spleen were significantly increased in the male and female animals and also the epididymis and testis weights in the male animals. The relative weights of the adrenal glands were significantly decreased in the males. After 24 months also the relative kidney weights were markedly increased and the incidence and severity of age-dependent nephropathy were increased. In addition, cysts, mineralization of the papillae, and fibrotic and in some cases necrotic pyelitis were found in the kidneys, and dilation of the renal pelvis and ureter and diffuse hyperplasia in the ureter were observed. In the tubular cells, vacuoles, pigment deposits and focal hyperplasia were found in the cytoplasm. Nodular or papillary hyperplasia of the transitional epithelium in the

renal pelvis was observed mainly in the males. Focal ectasis of the bile duct and vessels in the papillary region were found in the female animals. No tubular cell carcinomas occurred, although one benign and five malignant tumours of mesenchymal origin were found in the renal pelvis; three of the latter were haemangiosarcomas. Testicular atrophy was observed, and there was a significantly increased incidence of Leydig cell adenomas (14% compared with 0% in the current controls) (BASF AG 2006). Leydig cell tumours were found in 16 of 350 animals in five studies performed by the same laboratory between 1999 and 2006. The mean value is 4.6% with a range of 0% to 8% (BASF AG 2007 b); an exposure-related effect, therefore, cannot be excluded. In a review about Leydig cell tumours in rodents, the authors draw attention to species differences which suggest there to be a lower sensitivity in humans. The spontaneous formation of Leydig cell tumours is much more frequent in rodents than in humans. No increase in the incidence of Leydig cell tumours was found in epidemiological studies with both genotoxic and non-genotoxic substances that cause Leydig cell tumours in rats. The Leydig cells in humans are less sensitive to proliferative stimuli than those of the rat (Cook et al. 1999). In addition, the mortality of the male animals in the study with nitrilotriacetic acid was increased. As mesenchymal kidney tumours occurred in this study instead of tubular cell carcinomas, as found also in the tumour promotion study of Hiasa et al. (1984) with Wistar rats, it is possible that interstitial nephritis, with its greater tendency to produce tumours from the stroma, is more pronounced in Wistar rats.

Parallel to in the rat, nitrilotriacetic acid and $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ were investigated by Litton Bionetics (LBI) also in B6C3F₁ mice. The control groups consisted of 20 female and 20 male animals, and the treated groups of 50 male and 50 female animals.

Nitrilotriacetic acid was administered in concentrations of 0, 7500 or 15000 mg/kg diet (doses of about 1125 and 2250 mg/kg body weight and day). In the male animals of the high dose group, the incidence of tubular adenocarcinomas and malignant tumours of the prostate was significantly increased after exposure for 18 months and a three month recovery period. The body weights of the female animals were decreased (in a dose-dependent manner) in both groups, but in the males only in the high dose group. There was no increase in mortality. The incidence of hydronephrosis was increased (in a dose-dependent manner) in the male animals of both dose groups, but in the female animals only in the high dose group (NCI 1977).

B6C3F₁ mice were given $\text{Na}_3\text{NTA} \cdot \text{H}_2\text{O}$ concentrations of 0, 2500, or 5000 mg/kg diet (doses of about 0, 375 and 750 mg/kg body weight and day corresponding to nitrilotriacetic acid doses of 0, 260 and 520 mg/kg body weight and day) for 18 months. A dose-dependent reduction in body weights was observed, mortality was unaffected. Increased hydronephrosis was found in animals of both sexes in the high dose group after a three month recovery period. This was found only in one male and one female in the low dose group. The frequency of leukaemia and lymphomas was significantly increased in the male animals. This trend was not evident in the female animals, but the incidence in the female controls was already high at

15%. As there was no increase in the incidence of lymphomas in the parallel study with nitrilotriacetic acid, in which the dose was four times higher, the formation of these tumours is not considered to be treatment-related (NCI 1977).

– Parenteral studies

No treatment-related tumours were found in 30 mice after subcutaneous injection of 3.5 mg nitrilotriacetic acid in 0.05 ml distilled water once a week for 580 days (EU 2002). The study does not meet present-day requirements. Intraperitoneal injection of nitrilotriacetic acid doses of 6.1 to 9.2 mg/kg body weight and day once a day on 6 subsequent days for 12 weeks had caused no kidney damage or kidney tumours in 10 male and 10 female A/J mice by the end of a study for 420 days. Although the results do not exclude that there may be effects after high nitrilotriacetic acid doses and long exposure periods, they demonstrate the difference between nitriloacetic acid and Fe(III)-NTA, which is toxic and carcinogenic in equimolar quantities (Li et al. 1987). In two studies with rats, intraperitoneal administration of Fe(III)-NTA produced tubular necrosis, high mortality and tumours in the surviving animals (see Section 2; Ebina et al. 1986; Okada and Midorikawa 1982).

6 Manifesto

In long-term studies with rats and mice, tumours were found in the kidneys and the efferent urinary organs after oral administration of nitrilotriacetic acid and its sodium salts with the drinking water or the diet. Tumour-promoting effects on the kidneys and bladder were demonstrated in studies with rats and mice after initiation with nitrosamines. The lowest Na_3NTA dose at which kidney tumours occurred in rats was 100 mg/kg body weight and day (corresponding to a dose of nitrilotriacetic acid of 74 mg/kg body weight and day). Apart from possible genotoxicity (aneuploidy, clastogenicity), cytotoxic effects are to be considered as the cause of tumour formation.

The available data for genotoxicity do not indicate that nitrilotriacetic acid itself causes genotoxic effects. As a result of their complex-forming properties, Na_3NTA and nitrilotriacetic acid can, however, reduce the concentration of divalent essential ions in the medium and thus presumably have indirect genotoxic effects. Positive findings were obtained in vitro at 1 mM nitrilotriacetic acid and above. In addition, the alkalinity of Na_3NTA and the acidity of nitrilotriacetic acid may play a role in the positive in vivo findings observed. Consequently, no genotoxicity is to be expected in vivo at concentrations below 1 mM nitrilotriacetic acid. The cytotoxic effects are therefore predominant as regards tumour formation.

Ingested doses of nitrilotriacetic acid and their concentrations in urine and plasma correlate more or less linearly in rats up to Na_3NTA doses of 400 mg/kg body weight (Anderson 1980). Accordingly, an Na_3NTA dose of 15 mg/kg body weight corresponds in the rat to approximately 2 μM nitrilotriacetic acid in the plasma and

0.2 mM in the urine. Nitrilotriacetic acid concentrations at this level have no genotoxic effects *in vitro*.

In view of the mechanistically well documented dose range without genotoxic and cytotoxic effects, or increased cell proliferation in the kidneys or any noteworthy formation of nitrilotriacetic acid metal complexes, nitrilotriacetic acid and its sodium salts are candidates for Carcinogen Category 4. A NOAEL of 15 mg/kg body weight was identified for Na₃NTA (NTA doses of 11.2 mg/kg body weight and day) for renal toxicity based on a two-year feeding study in rats. However, the bladder seems to react more sensitively to Na₃NTA. This was demonstrated in a second long-term study by the increased incidence (not statistically significant) of bladder hyperplasia which occurred in male but not in female rats after doses of 10 mg/kg body weight (nitrilotriacetic acid doses of 6.9 mg/kg body weight and day). In the study with the NOAEL of 15 mg/kg body weight and day for Na₃NTA (nitrilotriacetic acid doses of 11.2 mg/kg body weight and day), the bladder as target organ was presumably not investigated for tumour precursors as no bladder tumours were found up to the highest tested Na₃NTA dose of 250 mg/kg body weight and day (nitrilotriacetic acid doses of 186 mg/kg body weight and day). There are no studies available which investigate the cytotoxic effects on the bladder. The mechanism of hyperplasia formation cannot, therefore, be completely explained. Thus, no systemic NOAEL can be derived.

Earlier 4-week inhalation studies with Na₃NTA in monkeys, rats and guinea pigs with lung function tests in monkeys and guinea pigs show, on the one hand, that concentrations of up to 213 mg/m³ are tolerated by rodents apparently without any adverse effects. Increased zinc concentrations in the urine after 342 mg/m³ (calculated as being equivalent to doses of about 80 mg/kg body weight and day in the rat), however, indicated incipient effects on the tubular and transitional epithelium. An increase in respiration rate was described in the monkeys at the lowest concentration of 10 mg/m³ and above, but the method described is inadequate and definitive conclusions concerning the effects of sensory irritation cannot be drawn. "Slight" irritation of the respiratory tract occurred at 220 mg/m³ in a test for sensory irritation in mice. However, this test can also not be included in the evaluation as a result of great methodological shortcomings. According to a more recent study of sensory irritation in the rat, the RD₅₀ value is about 4300 mg/m³ for Na₃NTA as test substance.

As there are no data for sensory irritation in humans or a NOAEL for effects in the bladder, no MAK value can be derived and nitrilotriacetic acid and its sodium salts are classified in Carcinogen Category 3A. Simultaneous inhalation exposure to iron compounds capable of being chelated by nitrilotriacetic acid should be avoided because of the possible formation and inhalation of FeNTA.

Both the dermal absorption of the trisodium salts after short-term and 90-day application *in vivo* and the model calculation with nitrilotriacetic acid indicate that absorption through the skin is of a low level. Nitrilotriacetic acid and its sodium salts are thus not designated with an "H".

A repeated insult patch test in volunteers at a very low nitrilotriacetic acid concentration and a Buehler test in guinea pigs provided no evidence of skin-sensitizing

effects, nor does the chemical structure of the substance suggest that it causes sensitization of the skin. There are no studies available of the sensitizing effects on the airways. Designation with "Sh" or "Sa" is therefore not possible.

Earlier studies of developmental toxicity provide no evidence of adverse effects of nitrilotriacetic acid or Na₃NTA in the offspring of rats up to the highest Na₃NTA dose tested of about 450 mg/kg body weight and day (nitrilotriacetic acid doses of about 335 mg/kg body weight and day), or in the offspring of rabbits up to nitrilotriacetic acid doses of 250 mg/kg body weight and day, or in the offspring of mice at the only nitrilotriacetic acid dose tested of about 400 mg/kg body weight and day. No maternal toxicity occurred at the tested doses. As no MAK value can be derived, nitrilotriacetic acid and its sodium salts cannot be classified in any Pregnancy Risk Group.

Nitrilotriacetic acid did not induce gene mutations *in vitro* with one exception (human epithelial cells, diphtheria toxin resistance), but was weakly clastogenic. *In vivo*, nitrilotriacetic acid induced DNA strand breaks and micronuclei in the rat kidney, aneuploidy in somatic cells and germ cells of *Drosophila* and aneuploidy in the germ cells of male mice. All other genotoxicity tests yielded negative results. In view of the unclarified mechanism of action and the assumption that the positive results are probably caused by the high alkalinity of the trisodium salts or the acidity of nitrilotriacetic acid, the substance is not classified in one of the categories for germ cell mutagens.

7 References

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completed 28.03.2007