National Institute for Public Health
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Ministry of Health, Welfare and Sport

Overview of the available data on the mutagenicity and carcinogenicity of **hexachloroethane**

RIVM letter report 2024-0163
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Colophon

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Synopsis

Overview of the available data on the mutagenicity and carcinogenicity of hexachloroethane

Hexachloroethane is used to make products that release smoke, such as smoke pots and smoke bombs. These products are used in the military, among other places. Hexachloroethane is also found in many other products. The substance is used as a polymer additive, anti-moth agent, plasticizer and solvent for insect repellents. It is also used in metal processing to refine aluminium compounds.

The question is whether hexachloroethane can cause cancer (carcinogenic) and damage DNA (mutagenic). The Dutch Health Council has asked the RIVM to summarize scientific literature on these two properties. In total, the RIVM has summarized 22 studies in laboratory animals or humans on possible carcinogenic and mutagenic properties of the substance.

The Dutch Health Council uses the overview to assess whether hexachloroethane has the two properties. The Minister of Social Affairs and Employment (SZW) has asked the Dutch Health Council for this advice.

Keywords: hexachloroethane, mutagenicity, carcinogenicity, hazardous properties, hazard classification

Publiekssamenvatting

Overzicht van de beschikbare informatie over mutageniteit en carcinogeniteit van de stof hexachloorethaan

De chemische stof hexachloorethaan wordt gebruikt om producten te maken waarbij rook vrijkomt, zoals rookpotten en -bommen. Deze producten worden onder andere in het leger gebruikt. Daarnaast zit hexachloorethaan in veel verschillende producten. De stof wordt gebruikt als polymeeradditief, anti-mottenmiddel, weekmaker voor kunststoffen en oplosmiddel voor middelen tegen insecten. Het wordt ook bij de bewerking van metalen gebruikt om aluminiummengsels te raffineren.

De vraag is of hexachloorethaan kankerverwekkend is (carcinogeen) en schade aan het DNA kan veroorzaken (mutageen). De Gezondheidsraad heeft het RIVM gevraagd om wetenschappelijke literatuur over deze twee eigenschappen samen te vatten. In totaal heeft het RIVM 22 studies in proefdieren of mensen samengevat over mogelijke carcinogene en mutagene eigenschappen van de stof.

De Gezondheidsraad gebruikt het overzicht om te beoordelen of hexachloorethaan de twee eigenschappen heeft. De minister van Sociale Zaken en Werkgelegenheid (SZW) heeft de Gezondheidsraad om dit advies gevraagd.

Kernwoorden: hexachloorethaan, mutageniteit, carcinogeniteit, gevaarlijke eigenschappen, gevarenclassificatie

Contents

Summary

RIVM has summarised the available literature on the potential carcinogenicity and mutagenicity of hexachloroethane.
Hexachloroethane is primarily used in industrial settings as a solvent. Occupational exposure to hexachloroethane occurs in the defence sector where there is widespread use of hexachloroethane in explosives in the defence sector.
Workers are exposed during its production either by the chlorination of tetrachloroethylene in the presence of ferric chloride at 100-140°C or when hexachloroethane is a by-product in the industrial chlorination of saturated and unsaturated hydrocarbons.

In the current report, a total of 22 animal studies were summarised. The studies that are summarised here can be used to assess the potential mutagenicity and carcinogenicity of hexachloroethane. Such an assessment was beyond the scope of the current report.

1 Introduction

The aim of current research is to summarize the available data from studies with laboratory models, test animals and humans on the substance hexachloroethane. The focus of the current literature overview will be on the mutagenic and carcinogenic properties of this substance.
At the request of the Dutch Minister of Social Affairs and Employment, the Health Council of the Netherlands will use the summaries to assess the mutagenic and carcinogenic properties and to provide a recommendation for its classification.

The current RIVM-report does not include an assessment of the reported mutagenic and carcinogenic effects of hexachloroethane, nor does it provide a recommendation for classification of the substance based on the CLP-criteria (Regulation EC No 1272/2008[1]).

The literature search strategy as applied by the Health Council of the Netherlands which forms the basis of current literature overview is presented in chapter 2. In chapter 3 the substance identity of hexachloroethane is provided. Chapter 4 presents information on international classifications of hexachloroethane. Available information on manufacture and use, monitoring (i.e. environmental and biological exposure monitoring), and exposure is presented in chapters 5, 6 and 7, respectively. A summary of the (toxico)kinetics of hexachloroethane is described in chapter 8. Chapter 9 describes an overview of the data on mutagenicity. Finally, the data on carcinogenicity and mutagenicity are presented in chapter 10.

[1] https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=celex%3A32008R1272

2 Literature search strategy

The Health Council of the Netherlands has performed a literature search using PubMed and Scopus. Relevant *in vitro* and *in vivo* studies were selected by the Health Council for an extensive summary. There were no relevant human studies available. Studies were exclusively on hexachloroethane. This resulted in 22 studies.

For the current report, RIVM summarized the data of the selected studies. RIVM also consulted the REACH registration dossier of hexachloroethane (publicly available on ECHA website[2]) and secondary sources, which included the ATSDR (Agency for Toxic Substances and Disease Registry) Toxicological profile for hexachloroethane (1997) [1], the IARC (International agency for research on cancer) monograph (1999) [2], the information on the IRIS (Integrated Risk Information System) toxicological review (2011) [3], and the NTP (National Toxicology Program) monograph on hexachloroethane (1989) [4]. These were used to retrieve information on substance identification, classification, manufacture, monitoring and toxicokinetics.

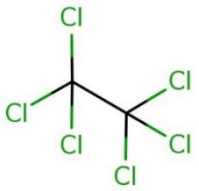
[2] https://chem.echa.europa.eu/100.000.606/overview?searchText=hexachloroethane

3 Substance identification

3.1 Name and other identifiers of the substance

The identity of hexachloroethane is presented in Table 1 below.

Table 1 Substance identity and information related to the molecular and structural formula of hexachloroethane

Name(s) in the IUPAC nomenclature or other international chemical name(s)	Hexachloroethane 1,1,1,2,2,2-hexachloroethane
Other names (usual name, trade name, abbreviation)	Avlothane Distokal Distopan Distopin Egitol Ethane hexachloride Ethane, 1,1,1,2,2,2-hexachloro- Ethane, hexachloro- Ethylene hexachloride Falkitol Fasciolin Hexachlor-aethan Hexachloroethylene Mottenhexe Perchloroethane Phenohep
ISO common name (if available and appropriate)	N/A
EC/EINECS number (if available and appropriate)	200-666-4
EC name (if available and appropriate)	Hexachloroethane
CAS number	67-72-1
Other identity code (if available)	N/A
Molecular formula	C_2Cl_6
Structural formula	
SMILES notation (if available)	ClC(Cl)(Cl)C(Cl)(Cl)Cl
Molecular weight or molecular weight range	236.74 g/mol
Information on optical activity and typical ratio of (stereo) isomers (if applicable and appropriate)	N/A
Description of the manufacturing process and identity of the source (for UVBC substances only)	N/A
Degree of purity (%) (if relevant for the entry in Annex VI)	N/A

N/A: Not applicable

3.2 Physico-chemical properties of hexachloroethane

The physico-chemical properties of hexachloroethane were obtained from the REACH registration dossier and Pubchem [5, 6] is presented in Table 2.

Table 2 Summary of physico-chemical properties of hexachloroethane

Properties	**Value**
State of the substance at normal temperature and pressure	Solid
Melting/freezing point	186.8°C
Boiling point	No result
Relative density	2.091 at 20°C
Vapour pressure	27.998-106.658 Pa at 20-30°C
Surface tension	4.37-4.39 at 25°C and pH 5-7
Water solubility	Insoluble in water, with a solubility of only 50 mg/L @ 22.3°C
Partition coefficient n-octanol/water	LogP = 4.14
Flash point	N/A
Flammability	Not flammable
Explosive properties	Slight
Self-ignition temperature	No information
Oxidising properties	Can react
Granulometry	D[4,3] =269.343 µm D<50%=208.25 µm D<3.6% = <45 µm
Stability in organic solvents and identity of relevant degradation products	No information
Dissociation constant (pKa)	No information
Viscosity	No information

4 International classifications

4.1 European Commission

Hexachloroethane has three active registrations under Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) [5].

It has currently no harmonized classification in Annex VI of the CLP-Regulation (EC) 1272/2008. It is self-classified as carc. 2 (H351) by 18 notifiers.

It is also restricted by Annex XVII of the REACH Regulation. 'The chemical shall not be placed on the market, or used as substance, or in mixtures, where the substance or mixture is intended for the manufacturing or processing of non-ferrous metals' [5].

Additionally, this chemical is restricted under the European Union (EU) Cosmetics Regulation 1223/2009 Annex II—List of substances prohibited in cosmetic products (number 197) [7].

4.2 The Health Council

No previous reports were found on the website of the Health council.

4.3 IARC

In 1999, IARC classified hexachloroethane as "possibly carcinogenic to humans" (Group 2B), because there is inadequate evidence in humans for the carcinogenicity and sufficient evidence in experimental animals for the carcinogenicity [2].

4.4 Other countries

Restrictions are also in place in other countries.

In the United States of America, the US-EPA has classified hexachloroethane as a Group C, possible human carcinogen [3, 8] and the State of California has determined under Proposition 65 that hexachloroethane is a carcinogen [9].

In Germany, hexachloroethane is classified by the Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area as a category 3 carcinogen. This means that "the substance causes concern that it could be carcinogenic for man but cannot be assessed conclusively because of lack of data. A MAK or BAT value can be established, provided no genotoxic effects have been detected for the substance or its metabolites or the genotoxic effect is not the main effect" [10].

In Australia, according to the Hazardous Chemical Information System (HCIS), hexachloroethane is classified as "H351 (Suspected of causing cancer)" [11].

5 Manufacture and uses

5.1 Manufacture

Hexachlorethane is registered under the REACH Regulation and is manufactured in and/or imported to the European Economic Area, at a total tonnage band of ≥ 10 to < 100 tonnes per year [5].

Hexachloroethane is usually produced by the chlorination of tetrachloroethylene in the presence of ferric chloride at 100-140°C. Hexachloroethane can also be a by-product in the industrial chlorination of saturated and unsaturated hydrocarbons. The product may be used captively in-house or recycled in feedstock to produce tetrachloroethylene or carbon tetrachloride [3].

5.2 Uses

5.2.1 European Union

There are no consumer uses registered in the REACH dossier, but among workers there is widespread use of hexachloroethane in explosives in the defence sector [5]. REACH does not provide publicly available information for the current situation in The Netherlands.

Hexachloroethane has been used for the manufacture of degassing pellets to force air bubbles out of molten ore in aluminium foundries, but this use has been phased out in the EU since 1997 [12, 13].

5.2.2 United States of America

Hexachloroethane is primarily used by the military for smoke pots, smoke grenades, and pyrotechnic devices. Hexachloroethane has also been used as a polymer additive, a moth repellent, a plasticizer for cellulose esters, an insecticide solvent, and in metallurgy for refining aluminium alloys [3].

Hexachloroethane is also an industrial chemical used in the United States in military smoke and pyrotechnic devices and as an intermediate in the organic chemicals industry. It is released to the environment from these uses, primarily to the atmosphere.

5.2.3 Australia

The Australian National Industrial Chemicals Notification and Assessment Scheme (NICNAS) reports the following international uses of hexachloroethane [14]:

- camphor substitute in nitrocellulose explosives, pyrotechnics and smoke devices
- lubricating oil additive
- in fluxes
- in fire extinguishing fluids
- plasticiser for cellulose esters
- solvent in organic synthesis
- polymerisation catalyst
- vulcanising agent

- in metallurgy for refining aluminium alloys, recovering metal from ores, or smelting products.
- as a retarding agent in fermentation
- in insecticides
- in veterinary medicine.

6 Monitoring

Gas chromatography analysis (GC analysis) is usually used to measure hexachloroethane in environmental media and in biological fluids and tissues [1]. Electron capture detector or flame ionization detector may be used for identification of hexachloroethane. However, the most sensitive method for identification is a mass-spectrometer (MS) coupled to a gas-chromatography (GC) column [1].
Analytical methods for hexachloroethane in different environmental and biological matrices were collated by the Agency for Toxic Substances and Disease Registry US (ASTDR) in 1997 as part of their toxicological profile for hexachloroethane (Tables 3 and 4). A literature search did not reveal significant updates to the methods listed in ASTDR. These methods are sufficiently sensitive for determining hexachloroethane concentrations at very low levels. The detection limit for hexachloroethane in biological tissue is 0.001 µg/g with recoveries ranging from 50 to 130%. Recoveries from environmental media ranges from 40 to 90% (Table 3).

6.1 Environmental exposure monitoring

Hexachloroethane is mainly used for the manufacture of explosives in the defence sector. Releases to the environment are therefore linked to this activity and may occur when it is used in smoke and pyrotechnic devices. In smoke devices it is about 44.5-46% wt/wt of the solid material. It makes up about 0.3% to 5% of the reagents in the device that are released to the air at a burn efficiency of 70%.
Hexachloroethane may also be released to air during combustion and incineration of chlorinated wastes, from hazardous waste sites, and in small amounts during chlorination of sewage effluent prior to discharge and chlorination of raw water during drinking water treatment [1].

Hexachloroethane has been measured in air, water, soil, waste and food using GC analysis. Representative methods for quantifying hexachloroethane in these media that have been approved by US regulatory bodies NIOSH and US EPA are listed in Table 3. An organic solvent such as methylene chloride is usually used to separate it from the environmental sample matrix [1].

Table 3 Overview of methods for the analysis of hexachloroethane in environmental samples. All references were quoted in ASTDR (1997) [1]

Sample matrix	Preparation method	Analytical method	Sample detection limit	Percent recovery	Reference
Air	Collect on activated coconut shell charcoal in glass tube; desorb with carbon disulfide	GC/FID	0.01 mg/sample	98	NIOSH 1994

Sample matrix	Preparation method	Analytical method	Sample detection limit	Percent recovery	Reference
Water/waste water	Extract with methylene; exchange to hexane, Florisil cleanup, if required	GC/ECD	0.03 µg/L	99	EPA 1982
Waste water	Extract continuously with methylene chloride under alkaline and then acidic conditions	Isotope dilution, capillary column GC/MS	10 µg/L	No data	EPA 1990c
Water/waste water	Extract with methylene; chloride under alkaline then acidic conditions	Packed column GC/MS	1/6 µg/L	40-113	APHA 1992
Water/soil/ wastes	Extract with methylene; exchange to hexane, Florisil or GPC cleanup, if required	Capillary column GC/ECD	1.6 µg/L[a]	83-96	EPA 1990a
Water/soil/ wastes	Extract with methylene; exchange to hexane, Florisil or GPC cleanup, if required	Packed column GC/ECD	0.03 µg/L[a]	~74	EPA 1990b
Food (fish milk, butter, corn oil)	Extract with acetonitrile; cleanup with Florisil; elute with petroleum ether and ethyl ether/petroleum ether	GC/ECD	No data	80	Yurawecz and Puma 1986

[a] Method detection limit (MDL) in reagent water. Estimated quantitation limits for other matrices are: 10 MDL in groundwater, 670-10,000 MDL in soil, and 100,000 MDL in nonaqueous wastes.
ECO= electron capture detector; FID = flame ionization detector; GC = gas chromatography; GPC = gel permeation chromatography; MS = mass spectrometry

6.2 Biological exposure monitoring

Hexachloroethane concentrations have be determined in biological fluids and tissues including blood, urine, faeces, liver, kidney, adipose tissue, and breath using a range of different analytical methods. Table 4 contains a representative list of the different analytical methods. Gas chromatography (GC) is the most common method used. A mass spectrometer (MS) coupled with a GC is very sensitive method and can be used to detect levels in tissues as low as 0.001 µg/g. Recoveries range from 50 to 130%. Separation from the sample matrix is achieved by purging with an inert gas, followed by trapping on an absorbent cartridge or by extraction with hexane [1].

Table 4 Overview of methods for determining hexachloroethane in biological materials. This list was obtained from ASTDR (1997) [1]. The references quoted are from the original ASTDR report.

Sample matrix	Preparation method	Analytical method	Sample detection limit	Percent recovery	Reference
Blood	Purge and trap on Tenax GC cartridge; desorb thermally	GC/MS	~3 ng/mL[a]	~80[a]	Pellizzari et al. 1985a
Blood	Purge and trap on Tenax GC cartridge; desorb	GC/MS	0.028 ppb	93-41	Ashley et al. 1992
Adipose tissue	Macerate tissue in water; tap on Tenax GC cartridge; desorb thermally	GC/MS	~ 6 ng/g[a]	~50[a]	Pellizzari et al. 1985a
Breath	Collect on Tenax GC cartridge; dry over calcium sulfate; desorb thermally	Capillary column GC/MS	No data	~70-130	Pellizzari et al. 1985b

Sample matrix	Preparation method	Analytical method	Sample detection limit	Percent recovery	Reference
Urine	Extract with hexane; successively wash with water, sodium hydroxide, hydrochloric acid, and water	GLC/ECD	No data	>90	Fowler 1969b
Faeces	Macerate under warm hexane; successively wash with water, sodium hydroxide, hydrochloric acid, and water	GLC/ECD	No data	>90	Fowler 1969b
Blood, liver, kidney, fat	Extract with hexane	GC/ECD	0.001 µg/g	No data	Nolan and Karbowski 1978

[a] Typical or expected values for halocarbons by this method. Data were not reported for hexachloroethane;
ECD = electron capture detector; GC = gas chromatography; GLC = gas liquid chromatography; MS = mass spectrometry

7 Exposure

Hexachloroethane is relatively persistent in the environment. The routes of potential human exposure to hexachloroethane are inhalation, dermal contact, and ingestion [1, 15]. However, air is the medium of most concern for human exposure.

7.1 General population exposure

Release of hexachloroethane into the environment has reduced following the decision in Europe to phase it out in the non-ferrous metal industry (98/241/EC) [16] and its subsequent restriction under REACH (Directive (76/769/EEC, Entry 41) [17]. It is still imported and used in the EU and there is potential for general population exposure. However, this is expected to be minimal and limited to populations living close to military sites that use pyrotechnic devices that contain hexachloroethane or, to populations living in the vicinity of hazardous waste sites or industrial sources [1]. Other sources of exposure for the general population include real estate, paper and allied products, lumber and wood products and amusement and recreation services [18].

7.2 Occupational exposure

Occupational exposure to hexachloroethane may occur during its manufacture, transportation, or use. The National Occupational Exposure Survey conducted from 1981 to 1983 in the US estimated workers were potentially exposed to hexachloroethane in seven industries (Business Services; Machinery, Except Electrical; Chemicals and Allied Products; Primary Metal; Electric and Electronic Equipment; Transportation by Air; and Printing and Publishing) (NIOSH 1990 as quoted in NTP 2021). However, the most common occupational exposure occurs while working with smoke or pyrotechnic devices that contain hexachloroethane. Most of the hexachloroethane in a smoke pot or grenade is used up by the smoke-producing reaction, but small amounts (5% or less) remain after the smoke has formed and could result in further exposure. One study reported hexachloroethane concentrations in smoke ranging from 0.64 to 1.26 mg/m^3. Plasma concentrations of hexachloroethane in workers exposed to hexachloroethane in loading and packing operations for smoke munitions production rose from 0.08 ± 0.14 µg/L to 7.3 ± 6.0 µg/L after more than five weeks of work in those areas, despite the use of protective equipment, including disposable overalls and compressed-air-fed visors or full-facepiece masks with filters [1]. Exposed groups are most likely to be military personnel or civilians working with these devices.

Other occupations with potential exposure to hexachloroethane include cleaners and charwomen, millwrights, miscellaneous machine operatives, plumbers and pipefitters, and electricians [18].

8 (Toxico)kinetics

The information below is a summary of The Toxicological Review of Hexachloroethane performed by the US EPA in 2011 [3].

8.1 Human data

8.1.1 Absorption

No studies have evaluated hexachloroethane absorption in humans by oral or inhalation exposure. The dermal absorption rate has been described as limited and the absorption of a saturated hexachloroethane solution across human skin was estimated to be 0.023 mg/cm^2/hour .

8.1.2 Distribution

There are limited data on the distribution of hexachloroethane in humans. Hexachloroethane was identified in >50% of follicular fluid samples of women undergoing *in vitro* fertilisation, at average concentrations of 232 ±27 pg/mL. This suggests post-absorptive distribution to reproductive organs.

8.1.3 Metabolism

There are no data available on hexachloroethane metabolism in humans.

8.1.4 Excretion

No available studies evaluated the hexachloroethane elimination in humans.

8.2 Animal data

8.2.1 Absorption

Oral exposure studies in animals have demonstrated that hexachloroethane is absorbed and primarily distributed to fat.

In sheep, hexachloroethane is absorbed slowly after oral exposure with maximal venous blood concentrations of 10-28 µg/mL reached at 24h after oral administration of 500 mg/kg hexachloroethane. In rabbits fed ^{14}C-radiolabeled hexachloroethane absorption was estimated to be 19-29% based on the amount of radioactivity in urine and expired air. Studies in rats and mice using ^{14}C-radiolabeled hexachloroethane (500 mg/kg for rats; 1,000 mg/kg for mice) administered orally in corn oil indicated that the amounts absorbed were 65–71% and 72–88%, respectively, based on the amount of radiolabel detected in expired air and total excreta (urine and faeces).

8.2.2 Distribution

Animal studies have consistently demonstrated that hexachloroethane is distributed to fat, kidney, liver, and blood.

Fowler (1969) evaluated the tissue distribution of hexachloroethane 8.5h after injection with 500 mg/kg into the rumen and lower duodenum of two Scottish Blackface sheep. The substance was widely distributed and the highest tissue levels were found in fat of sheep number 1 (1.1 µg/g). Sheep number 2 had only trace amounts of hexachloroethane in

tissue. Hexachloroethane was also detected in bile after 15 minutes, while after 27 minutes in blood.

Tissue clearance was studied in male F344 rats that were on a diet with 62 mg/kg/day hexachloroethane for 57 days. The highest tissue concentrations of hexachloroethane were in fat (303 ± 50 µg/g tissue), which were 3-fold greater than the concentration in the kidney and over 100-fold greater than blood and liver concentrations. Liver concentrations increased in the first 3 days post exposure but began to decrease by day 6. Blood, fat, kidney, and liver half-life were all approximately 2-3 days after peak concentrations were reached. In another study by the same authors, hexachloroethane levels in tissues were compared between male and female rats exposed to 1, 15, and 62 mg/kg/day hexachloroethane in the diet. Both sexes exhibited comparable levels (although levels in males were slightly greater) of hexachloroethane in blood, liver, and fat; concentrations in fat were the highest for both sexes. Kidney concentrations of hexachloroethane were higher in male rats compared with female rats, particularly at the highest dose (47-fold greater). Blood levels of hexachloroethane did not correlate well to either the exposure dose or the concentration in the kidney.

8.2.3 Metabolism

Data from *in vivo* and *in vitro* studies support a conclusion that metabolism of hexachloroethane is incomplete, with excretion of unmetabolized hexachloroethane in exhaled air and possibly in urine. *In vivo* metabolism data for hexachloroethane are limited to three studies suggesting limited metabolism for hexachloroethane in rats, mice, rabbits and sheep.

Following oral administration of 500 mg/kg hexachloroethane (in six sheep), blood measurements were 10–28 µg/mL for hexachloroethane, 0.6–1.1 µg/mL for tetrachloroethylene , and 0.06–0.5 µg/mL for pentachloroethane. *In vitro* experiments confirmed the presence of the metabolites tetrachloroethylene and pentachloroethane in liver slices.

Hexachloroethane metabolism was evaluated in rat and mice by making a mass balance based on radioactivity measurements in tissues and excreta after oral administration of 125 or 500 mg/kg and 250 or 1000 mg/kg hexachloroethane to rats and mice respectively, for four weeks. Four animals per dose orally received unlabeled hexachloroethane, followed by a single dose of ^{14}C-radiolabeled hexachloroethane. Both rats and mice metabolized 30% of the parent compound, based on the mass balance between dose and the estimated sum of metabolites. This could be an underestimation since exhaled air was likely to include volatile metabolites. The major urinary metabolites, determined qualitatively by high performance liquid chromatography, were trichloroethanol and trichloroacetic acid (TCA) for both rats and mice.

Rabbits that were fed ^{14}C-radiolabeled hexachloroethane at 500 mg/kg excreted 5% of the applied radioactivity in urine over 72h, indicating slow metabolism. Reported urinary metabolites include trichloroethanol (1.3%), dichloroethanol (0.4%), TCA (1.3%), dichloroacetic acid (0.8%), monochloroacetic acid (0.7%), and oxalic acid (0.1%). The

expired air contained hexachloroethane, carbon dioxide, tetrachloroethylene, and 1,1,2,2-tetrachloroethane (TCE was not observed in expired air). Quantitative data on the volatile metabolites in exhaled air were not reported.
In vitro studies using liver microsomes indicated that hexachloroethane metabolism involves phenobarbital-inducible cytochrome P450 (CYP450) enzymes. However, no specific enzymes have been identified. The CYP450 enzymes induced by phenobarbital include those from the 2A, 2B, 2C, and 3A subfamilies.

The proposed metabolic pathway in Figure 1 is based on limited information; therefore, it is likely that intermediate chemical reactions are not captured in the figure.

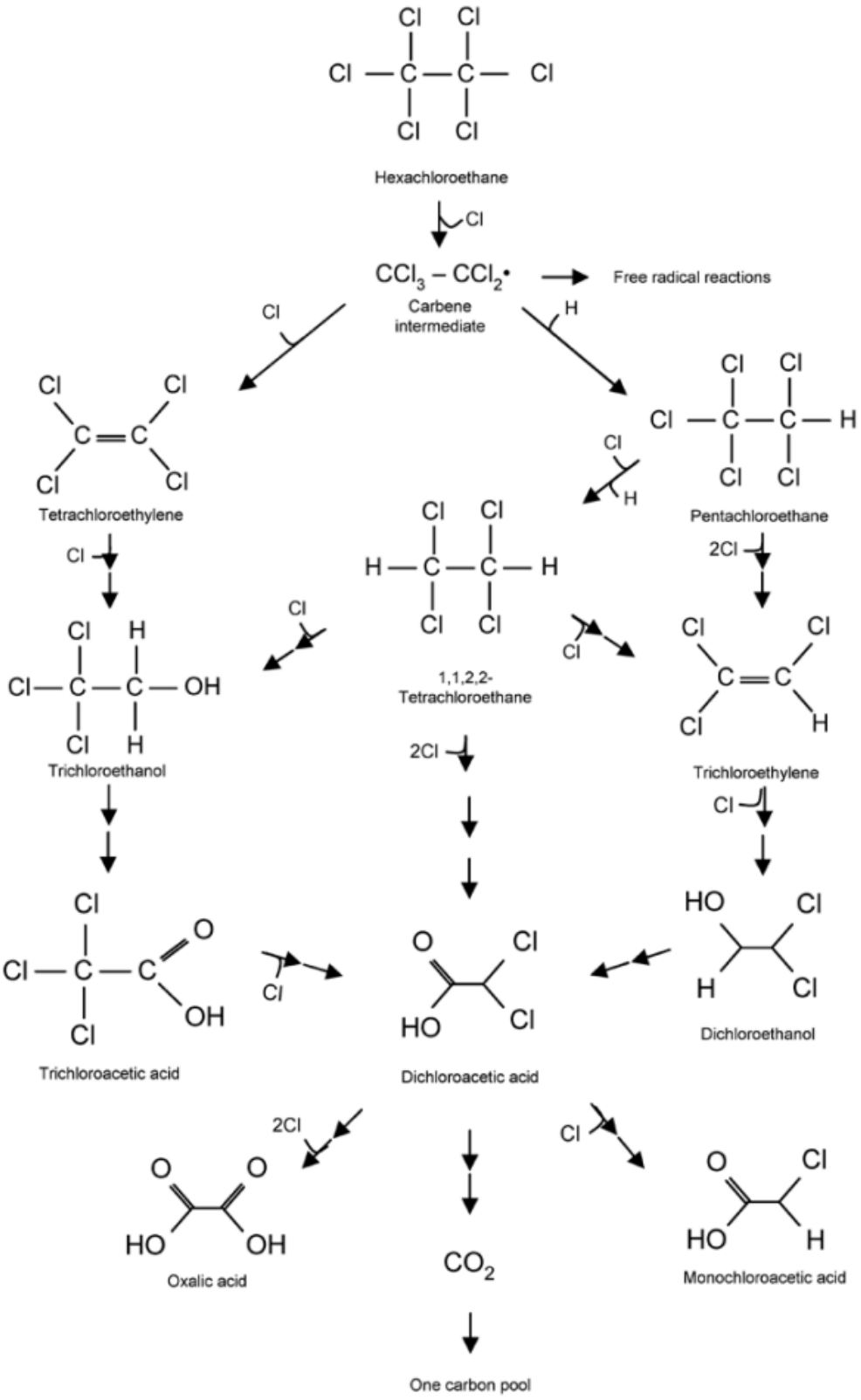


Figure 1 Plausible metabolic pathway of hexachloroethane, as presented in US EPA, 2011 [3].

8.2.4 Excretion

Animal studies indicated that the major routes of hexachloroethane elimination are either by faecal matter or by expired air. Sheep studies indicated that orally administered hexachloroethane is eliminated by the faecal route without absorption and metabolism, while rodent studies provided evidence that hexachloroethane is absorbed and eliminated by exhalation. It is unknown why there is a difference in elimination between sheep and rodents.

Rabbits fed [14C]-radiolabelled hexachloroethane at 0.5 g/kg eliminated 14–24% of the radioactivity in expired air during a 3-day period following exposure. Only 5% of the radiolabel was detected in urine. Faecal measurements were not conducted.

A single 500 mg/kg dose of hexachloroethane was orally administered to two sheep. Urine and faeces were collected over four days for analysis. More than 80% of the total faecal excretion of hexachloroethane occurred in the first 24 hours and only small amounts were detected in the urine.

Excretion of radiolabelled hexachloroethane was studied in rats and mice following four weeks of oral administration of 500 mg/kg/day in rats and 1000 mg/kg/day in mice. In both rats and mice, most of the radiolabel was detected in expired air, indicating this to be a major route of elimination. Less than 2.5% of the exhaled radioactivity was found in CO_2, with rats exhaling slightly more than mice. On the other hand, the amount of radioactivity in the excreta was lower in rats than in mice.

9 Mutagenicity

9.1 Summary of *in vitro* mutagenicity tests

Data on *in vitro* mutagenicity testing of hexachloroethane are summarised in Table 5, followed by a summary in text. In general, only statistically significant results are reported.

Table 5 Summary table of in vitro mutagenicity tests with hexachloroethane

<table>
<tr><th>Reference</th><th>Assay: microorganism or cell type</th><th>Exposure conditions</th><th>Results</th><th>Remarks</th></tr>
<tr><td colspan="5">Cellular assays</td></tr>
<tr><td>Tu et al. (1985) [19]</td><td>BALB/c-3T3 cell transformation assay
Counts of Type III foci

30 days exposure

N=2</td><td>HCE (97-99% purity)

Concentrations: 0, 0.16, 0.8, 4.0, 20.0, 100.0 µg/mL

Positive control: 3-methylcholanthrene (MCA)

No statistical analysis performed</td><td>Cytotoxicity<table><tr><th>Concentration (µg/mL)</th><th>Relative surviving fraction</th></tr><tr><td>0</td><td>1.00</td></tr><tr><td>0.16</td><td>1.00</td></tr><tr><td>0.8</td><td>0.78</td></tr><tr><td>4.0</td><td>0.85</td></tr><tr><td>20.0</td><td>1.00</td></tr><tr><td>100.0</td><td>0.16</td></tr></table>BALB/c-3T3 cell transformation assay: negative</td><td>Non-guideline study

Positive cytotoxicity assay and negative in transformation assay</td></tr>
<tr><td>Doherty et al. (1996) [20]</td><td>Cytochalasin B-blocked micronucleus assay

Human lymphoblastoid cell line AHH-1, with negative cytochrome CYP1A1 activity

MCL-5 cell line

h2El cell line, containing a cDNA for CYP2E1

N=2</td><td>HCE (purity unknown)

Concentrations: 0, 0.01, 0.05, 0.1 mM

Exposure: one cell cycle (18h for AHH-1, 24h for MCL-5 and h2El) at 37°C

Vehicle: growth medium

Statistical analysis: Chi-test</td><td>No significant induction of micronuclei in any cell line.

Toxicity was observed from 0.05 to 0.1 mM</td><td>Non-guideline study

There is a type-o in the table in the original paper where the last concentration says “0.01 mM” instead of “0.1mM”</td></tr>
<tr><td>Hendriks et al. (2012) [21]</td><td>Toxtracker assay</td><td>HCE (purity unknown)</td><td>Genotoxicity profile:</td><td>Assay validation study</td></tr>
</table>

Reference	Assay: microorganism or cell type	Exposure conditions	Results	Remarks
	C57/B16 B4418 wild-type mES cells	Concentrations: based on European Center for Validation of Alternative Methods (ECVAM) suggested list of chemicals for validation of in vitro genotoxicity test assay. Compounds used based on cytotoxicity, where the highest concentration induced significant cell death (10–25% viable cells after 24h treatment) Exposure time: 3h A maximum concentration of 10mM was used Compounds that required metabolic activation, cells were exposed for 3h in the presence of 1% S9 rat liver extract No statistical analysis performed	Ames test: negative *In vivo* genotoxicity: negative *In vitro* genotoxicity: negative mES DsRed reporters: negative GFP induction: negative	The actual concentrations used for HCE were not described
Thougaard et al. (2014) [22]	MN assay TK6 cells N=2	HCE (purity unknown) Concentrations:	MN and EMA assays: Negative	Assay validation study Compounds were tested up to a concentration of 1 mM

Reference	Assay: microorganism or cell type	Exposure conditions	Results	Remarks
		+S9: 0, 92, 104, 138, 156, 207, 233 µM -S9: 0, 89, 117, 133, 176, 200, 264 µM Exposure time: 24h Positive control: 15 µg/mL methyl methanesulfonate (MMS) for testing without S9, 15 µg/mL cyclophosphamide (CPA) for testing with S9 No statistical analysis performed	Cytotoxicity >90% at all concentrations	or, if precipitation was observed, up to the solubility limit The concentrations used showed no cytotoxicity
Micro-organisms				
Weeks et al. (1979) [23]	Ames test *S. typhimurium* (TA98, TA100, TA1535, TA1537, TA1538) and *S. cerevisiae* strain (D4) (+/- S9)	HCE (99.8% purity) Concentrations: 0.1, 1.0, 10, 100, and 500 µg/plate Tested with and without S9 Vehicle: DMSO Positive and solvent controls using both directly active positive chemicals and those that require metabolic	No result details were available. According to the authors, the test material did not induce any significant increase in revertant colonies with or without metabolic activation, neither in *S. typhimurium* nor in *S. cerevisiae* strains	Non-guideline study Test performed prior to guideline availability but with procedures similar to standard methods and with acceptable restrictions

<table>
<tr><th>Reference</th><th>Assay: microorganism or cell type</th><th>Exposure conditions</th><th>Results</th><th>Remarks</th></tr>
<tr><td></td><td></td><td>activation were run with each assay. No further details described

No statistical analysis performed</td><td></td><td></td></tr>
<tr><td>Nakamura et al.
(1987)
[24]</td><td>Umu test system

S. typhimurium TA1535/pSK1002</td><td>HCE (purity unknown)

Concentrations: up to 42 µg/mL

Tested with and without S9</td><td>Negative</td><td>Non-guideline study

Limited study information</td></tr>
<tr><td>Crebelli et al. (1988) [25]</td><td>Mitotic segregation assay

Aspergillus nidulans diploid strain P1</td><td>HCE (98% purity)

Concentrations: 0, 0.0025, 0.005, 0.01, 0.02, 0.04 %v/v

Exposure time: 3h

Statistical analysis: factor analysis and Bray-Curtis Ordination</td><td>Cytotoxicity:<table><tr><td>Concentration (%v/v)</td><td>Survival (%)</td></tr><tr><td>0</td><td>100</td></tr><tr><td>0.0025</td><td>100</td></tr><tr><td>0.005</td><td>82</td></tr><tr><td>0.01</td><td>81</td></tr><tr><td>0.02</td><td>65</td></tr><tr><td>0.04</td><td>48</td></tr></table>In italics the lowest concentration arresting conidial germination.

No effects observed in yellow sectors in abnormal and normal colonies.</td><td>Non-guideline study

Positive results in cytotoxicity test and negative results in mitotic segregation</td></tr>
<tr><td>Milman et al. (1988) [26]</td><td>Ames test and BALB/c-3T3 neoplastic transformation assay</td><td>HCE (97-99% purity)</td><td>Ames test: negative</td><td>Non-guideline study</td></tr>
</table>

Reference	Assay: microorganism or cell type	Exposure conditions	Results	Remarks
	Reverse mutation assay with *S. typhimurium* strains TA98, TA100, TA1535, TA1537 BALB/c-3T3 neoplastic transformation assay (only type III foci scored)	Concentrations: tested concentrations for the Ames test and BALB/c-3T3 neoplastic transformation assay not described Tested with and without S9 No statistical analysis performed	BALB/c-3T3 neoplastic transformation assay: negative	For the Ames test and BALB/c-3T3 neoplastic transformation assay, the tested concentrations are not described
Bronzetti et al. (1989) [27]	Induction of mitotic gene conversion and reverse point mutation Yeast D7 strain of *Saccharomyces cerevisiae*	HCE (purity unknown) Concentrations: 5, 7.5, 10, 12.5 mM With and without S9 Exposure time: 2h Positive control: 2 mM methyl methanesulfonate (MMS) for testing without S9, 300 mM dimethylnitrosamine (DMNA) for testing with S9 Statistical analysis: Wilcoxon's rank method and student t-test	HCE induced a significant increase ($p \leq 0.01$) of gene conversion only in logarithmic growth phase cells Reverse point mutation: negative	Non-guideline study The presence of a negative control is not mentioned

Reference	Assay: microorganism or cell type	Exposure conditions	Results	Remarks
Mersch-Sundermann (1989) [28]	Ames test Bacterial reverse mutation test: *S. typhimurium* strain with TA97, TA98, TA100 and TA102	HCE (98% purity) Ames test: 10 µg/L, 100 µg/L, 1 mg/L, 10 mg/L, 100 mg/L, 1g/L, 10 g/L. Tested with and without S9 fraction Exposure time: 48h Positive control: applied, but not specified Spot-testing (substance added in central puncture of agar plate): 100 µL undiluted substance Preincubation method: 5, 10, 25, 50 or 100 µL undiluted substance per plate (=3000 µL) with S9 fraction for 30 min at 37°C. No statistical analysis performed	Classical Ames test: positive in TA98 without S9. Positive in TA97 and TA98 with S9 Spot test: positive in TA98 without S9. Negative with S9, but toxic to bacteria Preincubation method: negative with and without S9	Article in German, abstract in English Positive control was included, no details mentioned Negative control was not included
NTP (1989)	Ames test and cytogenic test <u>Ames test:</u>	HCE (>99%) <u>Ames test:</u>	<u>Ames test:</u> negative, with and without S9	Non-guideline study

Reference	Assay: microorganism or cell type	Exposure conditions	Results	Remarks
	S. typhimurium with strains TA98, TA100, TA1535 and TA1537 (N=2/laboratory) <u>Cytogenic tests (sister chromatid exchanges (SCEs) test and chromosome aberration test):</u> Cultured Chinese hamster ovary (CHO) cells	Concentrations: 0, 10, 33, 100, 333, 1000 µg/plate (Case Western Reserve University) 0, 100, 333.3, 1000, 3333.3, 10000 µg/plate (SRI international) Positive controls: 2-aminoanthrancene in all strains with S9. 4-nitro-o-phenylenediamine (TA98), sodium azide (TA100 and TA1535), and 9-aminoacridine (TA1537) in all strains without S9 <u>SCE test:</u> Concentrations: Without S9: 10, 33 100, 330 µg/mL With S9 (Trial 1): 100, 330, 1000 µg/mL With S9 (Trial 2): 400, 600, 800, 1000 µg/mL Positive controls: -S9 Mitomycin C (0.005 µg/mL), +S9 Cyclophosphamide (1.5 µg/mL) Negative control: DMSO	<u>SCE test:</u> No induction of SCEs in the absence of S9. With S9, exposure to ≥330 µg/L resulted in a significant increase in SCEs as well as cell cycle delay Precipitation of HCE at ≥330 µg/L <u>Chromosome aberration test:</u> no induction of chromosomal aberrations up to 500µg/mL without S9 or 1000 µg/mL with S9, but a considerable delay in cell cycle time	

Reference	Assay: microorganism or cell type	Exposure conditions	Results	Remarks
		Exposure time: 26h without S9 and HCE (+2h with BrdU and colcemid without HCE), or 2h with S9 (+26h with BrdU and the final 2h with colcemid) Statistical analysis: trend test (P<0.003) <u>Chromosome aberration test:</u> Concentrations: high dose was limited by toxicity or solubility but not exceed 5 mg/mL Exposure time: 8h without S9 (+2h with colcemid) or 2h with S9 (+10h with fresh medium and final 2h with colcemid)		
Roldán-Arjona et al. (1991) [29]	Ara mutagenicity assay *S. typhimurium* strain BA13 and BAL13 N=2	HCE (98% purity) Concentrations: 0, 1.5, 3, 6, 12, 15, 22.5, and 30 µM Tested with and without S9 Exposure time: 20 min	Ara test: negative	Non-guideline study Survival rate of cells at 100% for all concentrations

Reference	Assay: microorganism or cell type	Exposure conditions	Results	Remarks
		No statistical analysis performed		
Tafazoli et al. (1998) [30]	Micronucleus (MN) and comet assays <u>MN assay:</u> Lymphocytes cultured from two donors <u>Comet assay:</u> Lymphocytes cultured from donors	HCE (purity >99%) <u>MN assay:</u> Donor A: 0, 0.05, 0.10, 0.50, 1.00 (+S9, -S9 not tested) Donor D: 0, 1, 2, 4, 8, 16 mM (+S9 and -S9) Positive controls: 0.1 mM cyclophosphamide and 0.44 µM mitomycin C Exposure time: 3h with S9, or 48h without S9. Statistical analysis: Chi-test and one-tailed *t*-test <u>Comet assay:</u> Concentrations: 0, 1, 4, 16 mM Positive control: 2 mM ethylmethane sulfonate (EMS)	<u>MN assay:</u> Statistically significant increase in MN frequency was found only in donor D and the repeated experiment in the presence and absence of S9. <u>Comet assay:</u> Negative results either for the tail length or for tail moment at the concentrations tested, with or without S9.	<u>MN assay:</u> different concentration used depending on the donor <u>Comet assay:</u> lower concentrations used than with the MN assay donor D

Reference	Assay: microorganism or cell type	Exposure conditions	Results	Remarks
		Exposure time: 3h with S9, or 48h without S9. Statistical analysis: *t*-test		
Smart et al. (2011) [31]	Measurement of serine139-phosphorylated histone H2AX (ɣH2AX), a biomarker of DNA double-strand breaks (DSBs) in mouse lymphoma L5178Y cells (tk+/−)	HCE ('highest purity available') Concentrations: not specified, between 0.1 and 2.5 mM Exposure time: 3h without S9 or 24h with S9 No statistical analysis performed	Exposure for 3h to 2.5 mM with S9 resulted in a 2-fold increase in γH2AX as well as high levels of cytotoxicity (RCC 1%)	Non-guideline study The authors argue that the increase in γH2AX was associated with almost complete cell lethality (RCC 1%), suggesting that the observed DNA damage was likely a consequence of cytotoxicity rather than direct genotoxicity

CHO = Cultured Chinese hamster ovary cells; HCE = hexachloroethane; MN = micronucleus; SCE = sister chromatid exchanges; EMA = ethylmethane sulfonate

Cellular assays

Tu et al. (1985)

In a non-guideline study conducted by Tu et al. (1985) [19], a BALB/c-3T3 cell transformation assay was performed to count type III foci. Cells were exposed for 30 days to hexachloroethane (N=2) to concentrations of 0, 0.16, 0.8, 4.0, 20.0, or 100.0 µg/mL. Results show that cytotoxicity was positive at a concentration of 100.0 µg/mL (16% survival). The results of the transformation assay were negative.

Doherty et al. (1996)

In a non-guideline study conducted by Doherty et al. (1996) [20], a cytochalasin B-blocked micronucleus assay was performed with human lymphoblastoid cell line AHH-1, MCL-5 cell line, and h2El cell line. Cells were exposed for 30 days to hexachloroethane (N=2) to concentrations of 0, 0.01, 0.05, or 0.1 mM. The exposure was performed during one cell cycle (18h for AHH-1, 24h for MCL-5 and h2El) at 37°C. The vehicle used was growth medium. Results show no significant increase but toxicity in micronucleated cells in all of the cell lines was observed from 0.05 to 0.1 mM.

Hendriks et al. (2012)

In a non-guideline study conducted by Hendriks et al. (2012) [21], a Toxtracker assay was performed with C57/Bl6 B4418 wild-type mES cells. The toxtracker assay uses C-terminal green fluorescent protein (GFP)-tagged fusion proteins. GFP reporter genes were located on bacterial artificial chromosomes, thereby enabling transcriptional regulation of the reporters by their own physiological promoter. The Bscl2-GFP reporter is selectively activated after exposure to genotoxic agents and its induction is associated with inhibition of DNA replication and activation of the ataxia telangiectasia and Rad3-related protein signalling pathway.
Cells were exposed to hexachloroethane for 3h to a nominal concentration which was not specified. Compound concentrations that were used for the validation were based on cytotoxicity, where the highest concentration induced significant cell death (10–25% viable cells after 24h treatment). A maximum concentration of 10mM was used. The results of the Toxtracker test were negative.

Thougaard et al. (2014)

In a validation study conducted by Thougaard (2014) [22], a micronucleus assay (MN) was performed in TK6 cells. Cells were exposed to hexachloroethane for 24h with or without S9 mix. The concentrations used were different in presence or absence of S9 mix. With S9, the concentrations were 0, 92, 104, 138, 156, 207, and 233 µM and without S9 mix, the concentrations were 0, 89, 117, 133, 176, 200, and 264 µM. Results showed that the MN assay was negative. Cytotoxicity was performed at the same time and results show that it was above 90% at all concentrations. Methods mentioned that compounds were tested up to a concentration of 1 mM or, if precipitation was observed, up to the solubility limit.

Micro-organisms

Weeks et al. (1979)

In a non-guideline study conducted by Weeks et al. (1979) [5, 23], the mutagenicity potential of hexachloroethane was assessed by performing a bacterial reverse mutation assay in *S. typhimurium* (TA-98, TA-100, TA-1535, TA-1537, TA-1538) and *S. cerevisiae* (D4) strains (+/- S9). Hexachloroethane concentrations of 0.1, 1.0, 10, 100 and 500 µg/plate (+/- S9) were prepared in DMSO. Positive controls were included in this assay.
According to the authors, the test material did not induce any significant increase in revertant colonies with or without metabolic activation, neither in *S. typhimurium* nor in *S. cerevisiae* strains. No result details were available.

Nakamura et al. (1987)

In a non-guideline study conducted by Nakamura et al. (1987) [24], the mutagenicity potential of hexachloroethane was assessed by performing a *umu* gene expression test in *S. typhimurium* TA1535/pSK1002. The strain was exposed to hexachloroethane with or without S9 fraction at different concentrations. The highest tested concentration was 42 µg/mL and showed negative results. No more details were available.

Crebelli et al. (1988)

In a non-guideline study conducted by Crebelli et al. (1988) [25], *Aspergillus nidulans* diploid strain P1 was exposed to hexachloroethane for 3h. Induction of mitotic segregation was evaluated. The concentrations tested were 0, 0.0025, 0.005, 0.01, 0.02, and 0.04 %v/v. Cytotoxicity results show a survival rate of 100% at concentrations 0 and 0.0025 %v/v. It decreased to 82% at 0.005, 81% at 0.01, 65% at 0.02, and 48% at 0.04 %v/v. No effects were observed in yellow sectors in abnormal and normal colonies. To conclude, positive results in cytotoxicity test and negative results in mitotic segregation were observed.

Milman et al. (1988)

In a non-guideline study conducted by Milman et al. (1988) [26], a reverse mutation assay with *S. typhimurium* strains TA98, TA100, TA1535 and TA1537, with and without S9 fraction was performed. Furthermore, a BALB/c-3T3 neoplastic transformation assay (only type III foci scored) was also performed with hexachloroethane.
The concentrations and the time of exposure for the Ames test and BALB/c-3T3 neoplastic transformation assay were not described. Results of the Ames test as well as the BALB/c-3T3 neoplastic transformation assay were negative.

Bronzetti et al. (1989)

In a non-guideline study conducted by Bronzetti et al. (1989) [27], yeast D7 strain of *Saccharomyces cerevisiae* was used to evaluate the induction of mitotic gene conversion and reverse point mutation after exposure to hexachloroethane for 2h. The concentrations used were 5, 7.5, 10, and 12.5 mM, with or without mammalian metabolic activation (S9). Results show that hexachloroethane induced a significant increase ($p \leq 0.01$) of gene conversion only in logarithmic growth phase cells. The

reverse point mutation showed a negative result, both with and without metabolic activation.

NTP (1989)
Hexachloroethane was tested in an Ames test, a sister chromatid exchange (SCE) test, and a chromosomal aberration test.
Hexachloroethane was incubated with the Salmonella typhimurium tester strains TA98, TA100, TA1535, and TA1537, either with or without S9-mix for 20 minutes at 37 °C before the addition of soft agar supplemented with L-histidine and D-biotin and subsequent plating on minimal glucose agar plates. Incubation was continued for an additional 48 hours. Hexachloroethane was tested in duplicate in all four strains at multiple laboratories. Each test consisted of triplicate plates of concurrent positive and negative controls and of at least five doses of hexachloroethane. The high dose was limited by toxicity or solubility but did not exceed 10 mg/plate.
Hexachloroethane, tested at concentrations up to 10 mg/plate, was not mutagenic in Salmonella typhimurium strains TA98, TA100, TA1535, or TA1537, both with or without S9.

Hexachloroethane was tested in cultured Chinese hamster ovary (CHO) cells for induction of sister chromatid exchanges (SCEs) both in the presence and absence of S9 and cofactor mix. Cultures were handled under gold lights to prevent photolysis of bromodeoxyuridine (BrdU)-substituted DNA. Each test consisted of concurrent solvent and positive controls and of at least three doses of the study chemical; the high dose was limited by toxicity or solubility but did not exceed 5 mg/ml. In the SCE test without S9, CHO cells were incubated for 26 hours. BrdU was added 2 hours after culture initiation. After 26 hours, the medium containing the study chemical was removed and replaced with fresh medium plus BrdU and colcemid, and incubation was continued for 2 more hours. In the SCE test with S9, cells were incubated with hexachloroethane, serum-free medium, and S9 for 2 hours. The medium was then removed and replaced with medium containing BrdU and no study chemical; incubation proceeded for an additional 26 hours, with colcemid present for the final 2 hours.
Hexachloroethane did not induce SCEs in CHO cells in the absence of S9; with S9, doses of 330 µg/ml hexachloroethane and above resulted in a significant increase in SCEs as well as toxicity, as evidenced by cell cycle delay; precipitation of hexachloroethane occurred at all doses of 330 µg/ ml and above.

In the chromosomal aberration test without S9, cells were incubated in McCoy's 5A medium with the study chemical for 8 hours; colcemid was added, and incubation was continued for 2 hours. In the test with S9, cells were treated with hexachloroethane and S9 for 2 hours, after which the treatment medium was removed and the cells were incubated for 10 hours in fresh medium, with colcemid present for the final 2 hours.
No induction of chromosomal aberrations was observed after treatment with up to 500 µg/ml hexachloroethane in the absence of S9 or 1,000 µg/ml in the presence of S9. In these tests, treatment with hexachloroethane caused a considerable delay in cell cycle time, indicating toxicity.

Mersch-Sundermann (1989)
In a non-guideline study conducted by Mersch-Sundermann (1988) [28], a reverse mutation assay with *S. typhimurium* strains TA97, TA98, TA100 and TA102 with and without S9 fraction was performed. The microorganisms were exposed to hexachloroethane for 48h. Three test strategies were performed: a classical Ames test (decadic dilution from 10g/L to 10 µg/L), a spot test (undiluted substance added to a central punctured hole on the agar plate), and an Ames test with a preincubation of S9 fraction for 30 min (5, 10, 25, 50 or 100 µL of undiluted substance per plate). The classical Ames test and spot test were weakly positive in TA98 without S9 fraction. The Ames test was also weakly positive in TA97 and TA98 with S9 fraction.

Roldán-Arjona et al. (1991)
In a non-guideline study conducted by Roldán-Arjona et al. (1991) [29], an Ara test was performed with *S. typhimurium* strain BA13 and BAL13. The micro-organism was exposed in duplicate to hexachloroethane for 20 min. The concentrations used were 0, 1.5, 3, 6, 12, 15, 22.5, and 30 µM. Pre-incubation was performed with or without S9 mix. The results of the Ara test were negative. The survival rate stays at 100% at all concentrations.

Tafazoli et al. (1998)
In a non-guideline study conducted by Tafazoli et al. (1998) [30], a micronucleus (MN) test and comet assays were performed on lymphocytes cultured from two donors. Lymphocytes were exposed to hexachloroethane for 3h with S9 or for 48h without S9 mix. Concentrations tested in the MN assay for the first donor were 0, 0.05, 0.10, 0.50, and 1.00 mM (+/-S9 not tested). For the second donor the concentrations were 0, 1, 2, 4, 8, and 16 mM (+/-S9). 0.1 mM cyclophosphamide and 0.44 µM mitomycin C were used as positive controls. Concentrations tested in the comet assay were: 0, 1, 4, and 16 mM. 2mM ethylmethane sulfonate (EMS) was used as a positive control. Results showed a statistically significant increase in MN frequency only in one donor in the presence and absence of S9 mix. The comet assay showed negative results for the measured DNA damage parameters (tail length, tail moment, and fraction of total cellular DNA in the tail) at the concentrations tested, with or without S9 mix. It should be noted that the concentrations in the comet assay were lower than with the MN assay with one donor.

Smart et al. (2011)
In a non-guideline study conducted by Smart et al. (2011) [31], measurement of serine139-phosphorylated histone H2AX (ɣH2AX), a biomarker of DNA double-strand breaks was performed on mouse lymphoma L5178Y cells (tk+/−) to evaluate a flow cytometry assay for ɣH2AX detection. Cells were exposed for 3h (+S9) or 24h (+S9) to hexachloroethane. The concentrations hexachloroethane used were not specified, but between 0.1 and 2.5mM. Results showed that after an exposure for 3h with S9 at 2.5 mM, a 2-fold increase in γH2AX over controls was observed. The authors concluded that the increase in γH2AX was associated with almost complete cell lethality (relative cell count 1%), suggesting that the observed DNA damage was likely a consequence of cytotoxicity rather than direct genotoxicity.

9.2 Summary of *in vivo* animal mutagenicity tests

Data on *in vivo* mutagenicity testing of hexachloroethane are summarised in Table 6, followed by a summary in text. In general, only statistically significant results are reported.

Table 6 Summary table of in vivo animal mutagenicity studies with hexachloroethane

Reference	Test system	Dose and route	Observations and results	Remark
Intraperitoneal (i.p.) injection				
Lattanzi et al. (1988) [32] Non-guideline	Male Wistar rats (N=6) and male BALB/c mice (N=12) Extra mice and rats used for organ testing Statistics: student t-test	[^{14}C]-HCE (98% purity) Concentration: 127 µCi/kg bw Euthanasia 22h after dosing Organ testing: microsomal and cytosolic fractions obtained from livers, kidneys, lungs, and stomachs of rats and mice. Some animals pretreated i.p. with phenobarbital (100 mg/kg bw two days prior to sacrifice).	HCE binding to proteins and especially of RNA from organs of both species was in general higher than binding to DNA. HCE binding to DNA from kidney, lung, and stomach was lower than to liver DNA Liver microsomes: rat and mouse catalysed HCE binding to DNA at similar levels. Significant difference from control in both species ($p<0.01$) Kidney microsomes: in rats HCE binding to DNA was more efficient than mouse DNA binding. Significant difference from control in both species ($0.05>p>0.01$) Lung and stomach microsomes: no binding in both species. Cytosolic fractions: HCE binding to DNA from all organs was higher than in	

Reference	Test system	Dose and route	Observations and results	Remark
			microsomal fractions, except for lung cystol. In mouse liver cytosols greater binding to DNA than in rat liver cytosols.	
Bronzetti et al. (1989) [27] Non-guideline	Swiss albino mice (CD1), hepatic microsomes Effects on Cytochrome P-450 (Cyt P450), Pentoxyresorulin O-dealkvlase (PROD), and Ethoxvresorufin O-dealkvlase (EROD) Sacrifice after 24h N=not described Statistics: Wilcoxon's rank method	HCE (98% purity) Route of exposure: i.p. injection Concentrations: 70% of LD_{50} (3150 mg/kg bw) and 35% of LD_{50} (1575 mg/kg bw) Control: corn oil	Cyt P450 content: -1575 mg/kg bw; (-25.93% ($p \leq 0.02$) -3150 mg/kg bw: 41.67% ($p \leq 0.01$) EROD content: -1575 mg/kg bw; (-36.46% ($p \leq 0.01$) -3150 mg/kg bw: 55.85% ($p \leq 0.01$) PROD content: -1575 mg/kg bw; (-23.52% ($p \leq 0.02$) -3150 mg/kg bw: 52.40% ($p \leq 0.01$)	
Crebelli et al. (1999) [33] Non-guideline	Mouse bone marrow MN assay CD-1 mice N=5 animals/sex	HCE (purity unknown) Concentrations: 0 (olive oil), 2000, 4000 mg/kg (LD_{50} > 4000 mg/kg) Positive controls: 1.0 mg/kg bw colchicine (COL) and 2.0 mg/kg bw mitomycin C (MMC)	Mouse bone marrow MN assay results: negative, no significant increase in frequency of micronucleated polychromatic erythrocytes The two positive controls showed a significant induction of micronuclei as compared to the control ($p<0.001$)	Authors argue that "The comparison of the results with findings provided by other authors *in vitro*, indicate that mouse bone marrow is weakly sensitive to the genotoxic effects induced by halogenated hydrocarbons in other test systems."

Reference	Test system	Dose and route	Observations and results	Remark
		Sacrifice after 24h and 48h exposure Statistics: -incidence of micronuclei: Chi- test -PCE/NCE ratio; *t*-test -variability: two-way ANOVA.		
Oral gavage				
Story et al. (1986) [34] Non-guideline	Rat liver Foci test Male Osborne Mendel rats N=10 animals/group Statistics: student t-test one sided and Bartlett's chi-square test	HCE (98% purity) <u>Initiation protocol:</u> rats had 2/3 partial hepatectomies and 24h later received 500 mg/kg bw (MTD) HCE by gavage. Six days later, rats received phenobarbital (0.05 %; w/w) via the diet for 7 weeks, after which they received control diet for 7 more days Control: 2 ml/kg bw corn oil via gavage or 30 mg in 5 ml/kg bw diethylnitrosamine (DEN) (30 mg/kg bw) via gavage, followed by control diet or	No initiating activity, but statistically significant promoting capability Increased mean absolute and relative liver weights Number of Enzyme-Altered Foci in Rat Liver: positive in presence of DEN ($p<0.05$)	

Reference	Test system	Dose and route	Observations and results	Remark
		diet containing 0.05% (w/w) phenobarbital <u>Promotion protocol:</u> rats were initiated with 30 mg in 5 ml water/kg bw DEN via i.p. injection or 5 ml/kg bw water, 24h after partial hepatectomies. Six days later, rats received 500 mg/kg bw HCE (MTD) in corn oil by gavage, 5 days/week for 7 weeks. Control: corn oil		
Milman et al. (1988) [26] Non-guideline	Rat liver Foci test Male Osborne Mendel rats N=10 rats/group Statistics: not specified	HCE (97-99% purity) Initiation protocol: rats had 2/3 partial hepatectomies and 24h later received 500 mg/kg bw (MTD) HCE by gavage. Six days later, rats received phenobarbital (0.05 %; w/w) via the diet for 7 weeks, after which they received control diet for 7 more days Control: 2 ml/kg bw corn oil via gavage or 30 mg in 5	No initiating activity, but statistically significant promoting capability Increased mean absolute and relative liver weights Number of Enzyme-Altered Foci in Rat Liver: positive in presence of DEN ($p<0.05$)	

Reference	Test system	Dose and route	Observations and results	Remark
		ml/kg bw diethylnitrosamine (DEN) (30 mg/kg bw) via gavage, followed by control diet or diet containing 0.05% (w/w) phenobarbital <u>Promotion protocol:</u> rats were initiated with 30 mg in 5 ml water/kg bw DEN via i.p. injection or 5 ml/kg bw water, 24h after partial hepatectomies. Six days later, rats received 500 mg/kg bw HCE (MTD) in corn oil by gavage, 5 days/week for 7 weeks. Control: corn oil		
Dietary study				
Gorzinki et al. (1985) [35] Non-guideline	Sub-chronic oral toxicity test Fischer 344 rats N=10 animals/sex/group 16 weeks Clearance test: N=20 males 8 weeks	HCE (99.4% purity) <u>Subchronic:</u> Concentrations: 0, 1, 15, 62 mg/kg bw/day Target dose levels: 3, 30, 100 mg/kg bw/day <u>Clearance:</u> Concentration: 62 HCE mg/kg bw/day	<u>Sub-chronic:</u> Increased kidneys weight with males being more sensitive (dose response) than females (small increase) 62 mg/kg bw/day: Liver: increased liver weight in males ($p<0.05$), increased relative liver	

Reference	Test system	Dose and route	Observations and results	Remark
		Euthanasia: 3, 6, 13, 22, or 31 days (3 to 4 rats) Statistical analysis: Dunnett's test and ANOVA	weight ratio in females ($p<0.05$) Kidneys: Slight hypertrophy and/or dilation of proximal convoluted tubules. Atrophy and degeneration of renal tubules (with a degree of peritubular fibrosis in high dose males) - 62 mg/kg bw/day: 10/10 males and 6/10 females - 15 mg/kg bw/day: 7/10 males and 2/10 females - 1 mg/kg bw/day: 2/10 males and 1/10 females - 0 mg/kg bw/day: 1/10 males and 1/10 females Liver: Slight swelling of hepatocytes: - 62 mg/kg bw/day: 8/10 males and 0/10 females - 15 mg/kg bw/day: 6/10 males and 0/10 females - 1 mg/kg bw/day: 3/10 males and 0/10 females - 0 mg/kg bw/day: 4/10 males and 0/10 females	

Reference	Test system	Dose and route	Observations and results	Remark
			Concentration HCE higher in male than female kidneys Clearance: extraction efficiencies for HCE from blood, liver, kidney and fat into hexane were 98.6%, 82.9%, 100% and 71%, respectively $T_{1/2}$ = 2.5 days NOAEL: 1 mg/kg/day	

MTD = maximum tolerated dose; HCE = hexachloroethane

Lattanzi et al. (1988)
Lattanzi et al. (1988) [32] conducted a non-guideline study to investigate the capacities of [^{14}C]-hexachloroethane to bind to DNA. For this, 6 male Wistar rats and 12 male BALB/c mice received an intraperitoneal (i.p.) injection of 127 µCi/kg hexachloroethane. Euthanasia was performed 22h after the injection. Regarding the test specifics on organs: microsomal and cytosolic fractions were obtained from livers, kidneys, lungs, and stomachs of rats and mice. Some animals (number not described) were pretreated i.p. with 100 mg/kg bw phenobarbital (PB) two days prior to sacrifice.
Results showed that hexachloroethane binding to proteins and especially of RNA from organs of both species was in general higher than binding to DNA. Binding values to DNA from kidney, lung, and stomach were lower than DNA binding values from the liver. In the liver microsomes, rat and mice catalysed HCE binding to DNA at similar levels. Significant difference from control in both species ($p<0.01$). In the kidney microsomes, hexachloroethane binding to DNA was more efficient than in mice. Significant difference from control in both species ($p<0.01$). For the lung and stomach, microsomes, the binding of DNA, RNA and proteins was inactive in both species.
Hexachloroethane binding to DNA in cytosolic fractions from all organs was higher than those in microsomal fractions, except for lung cystol. Mouse liver cytosols produced greater hexachloroethane binding to DNA than rat liver cytosols.
Additionally, the results from the polynucleotides mediated by microsomal fractions mouse liver showed significant increase on Poly(A), Poly(G), Poly(C), and Poly(U). The authors describe that hexachloroethane has a high oncogenic potency index (OPI), DNA binding ability in vivo (CBI), and in vitro (microsome cytosol-mediated binding index, MCMBI) in mouse liver.

Bronzetti et al. (1989)
Bronzetti et al. (1989) [27] conducted a non-guideline study to investigate the *in vivo* effects on Cytochrome P-450 (cyt. P450), Pentoxyresorufin *O*-dealkylase (PROD), and Ethoxvresorufin *O*-deethylase (EROD) activities. Swiss albino mice (CD1) received an intraperitoneal (i.p.) injection of hexachloroethane. Concentrations used correspond to 35% (1575 mg/kg bw) and 70% of the LD_{50} (3150 mg/kg bw). Corn oil is used as a control. Euthanasia was performed 24h after the injection and the activity of Cyt. P450, PROD, and EROD were measured in hepatic microsomes. The number of animals used for the study is not described. Results show a significant decrease ($p\leq0.01$) in cyt P450 levels for both the low and high dose (-25.93% and -41.67%, respectively), a significant reduction ($p\leq0.01$) of EROD activity for both the low and high dose (-36.46% and -55.85%, respectively), and a significant ($p\leq0.01$) decrease of PROD activity at the low and high dose (-23.52% and 52.40%, respectively).

Crebelli et al. (1999)
In a non-guideline study conducted by Crebelli et al. (1999) [33], CD-1 mice (5 animals/sex) received an i.p. injection of hexachloroethane to perform a bone marrow MN assay. Concentrations tested were 2000 or 4000 mg/kg, representing approximately 40 and 70-80% of the LD_{50}. Positive controls were 1.0 mg/kg body weight colchicine (COL) and 2.0

mg/kg body weight mitomycin C (MMC). Euthanasia was performed 24h and 48h after exposure. The incidences of micronuclei were compared with the Chi- test and the ratio polychromatic/normochromatic erythorcytes (PCE/NCE) were compared by the t-test. Variability between readers was tested by ANOVA.
Results of the bone marrow MN assay were negative. There was no significant increase in frequency of micronucleated polychromatic erythrocytes. The two positive controls showed a significant induction of micronuclei as compared to the control ($p<0.001$).

Oral gavage
Story et al. (1986)
In a non-guideline study, Story et al. (1986) [34] conducted a rat liver foci assay. The study was divided into two separate experiments because of the large number of rats to be given partial (2/3) hepatectomies. In the initiation protocol, Osborne Mendel rats (10 males/group) received hexachloroethane (98%) in corn oil by gavage at concentrations of the MTD (500 mg/kg bw) 24h after the partial hepatectomies. Six days after, the rats received 0.05% (w/w) phenobarbital by dietary exposure for 7 weeks. Control animals received corn oil by gavage or diethylnitrosamine (DEN) (30 mg/kg bw) via i.p. injection.
In the promotion protocol, rats (10 male animals/group) were administered 30 mg DEN by i.p. injection of 5.0 ml/kg bw water as a negative control 24h after the partial hepatectomy. Six days later the rats received 500 mg/kg bw hexachloroethane in corn oil by gavage, 5 days per week for 7 weeks. Control animals received the same volume of corn oil (2.0 ml/kg bw).
Results show an increased mean absolute and relative liver weights as well as an increase of the number of enzyme-altered foci in rat liver in presence of DEN ($p<0.05$). So hexachloroethane did not induce initiating activity, but has statistically significant promoting capability.

Milman et al. (1988)
In a non-guideline study, Milman et al. (1988) [26] conducted a rat liver foci assay. In the initiation study, Osborne Mendel rats (10 male rats/group) first underwent 2/3 partial hepatectomies and received 500 mg/kg (MTD) hexachloroethane by gavage 24 hours later. Six days after, the rats received phenobarbital (0.05 %; w/w) in the diet for 7 weeks, then control diet for 7 more days, after which they were euthanised. Control rats received 2 ml/kg bw corn oil by gavage or 30 mg DEN followed by a control diet or phenobarbital-containing diet for 7 weeks.
During the promotion study, rats (10 males/group) received 30 mg in 5 ml water/kg bw DEN or 5 ml/kg bw water 24h after the partial hepatectomies. Six days after, the rats received hexachloroethane (MTD) in corn oil by gavage, for 5 days/week for 7 weeks. Control rats received corn oil.
An enzyme-altered Foci test was performed on the livers. Results were positive and showed a significantly increased number of total enzyme-altered foci (type I plus type II) when administered in the promotion phase after treatment with DEN in the initiation phase ($p<0.05$).
The experiment done on the same rats as the BALB/c-3T3 neoplastic transformation assay (see 8.1) that had 2/3 of their liver removed.

Gorzinski et al. (1985)
In a 16-week feeding study performed by Gorzinski et al. (1985), F344 rats (N=10/sex/dose) were exposed to 0, 1, 15, and 62 mg/kg bw/day hexachloroethane for 57 days. After exposure, rats received a hexachloroethane-free diet.
No lethality was observed. Males were slightly more sensitive than females to nephrotoxic properties of hexachloroethane. Slight hypertrophy and/or dilation of proximal convoluted tubules. Atrophy and degeneration of renal tubules (with a degree of peritubular fibrosis in high dose males). It was observed at 62 mg/kg bw/day in 10/10 males and 6/10 females, at 15 mg/kg bw/day in 7/10 males and 2/10 females, at 1 mg/kg bw/day in 2/10 males and 1/10 females, at 0 mg/kg bw/day in 1/10 males and 1/10 females. Statistically significant ($p<0.05$) increased kidney and liver weights were observed at the highest dose in males. At 15 and 62 mg/kg bw/day, hypertrophy and/or dilation, atrophy, and degeneration of the renal tubules was observed. Renal toxicity in females manifested as very slight renal tubular atrophy and degeneration at the highest dose. Slight swelling of the hepatocytes in males was observed at 62 mg/kg bw/day in 8/10 males and 0/10 females, at 15 mg/kg bw/day in 6/10 males and 0/10 females, at 1 mg/kg bw/day in 3/10 males and 0/10 females, 0 mg/kg bw/day in 4/10 males and 0/10 females. Additionally, significant increase in liver weight in males ($p<0.05$) and significant increase in relative liver weight ratio in females ($p<0.05$) was noted. In females, increased relative liver weight ratio was observed in the highest dose, but no microscopic alterations were observed. Half-life of hexachloroethane was 2.5 days. Clearance extraction efficiencies from blood, liver, kidney and fat into hexane were 98.6%, 82.9%, 100% and 71%, respectively. Kidney concentration of hexachloroethane was higher in males than females.

10 Carcinogenicity

10.1 Summary of animal experiments

The carcinogenicity studies of hexachloroethane in experimental animals are summarized in Table 7, followed by a summary in text. In general, only statistically significant results are reported. In studies where statistical significance of the results was not reported, the listed tumour incidences in the table were limited to the control group and groups where actual lesions occurred.

Table 7 Summary table of in vivo animal experiments with hexachloroethane (only significant results, unless stated otherwise)

Reference	Study design and animal species	Data on exposure and effect endpoints	Observations and results	Remarks
Gavage				
NIH (1978)/ Weisburger (1977) [15, 36] Non-guideline study	Repeated dose toxicity/carcinogenicity study Osborne-Mendel rats (N=50/sex/dose) B6C3F1 mice (N=50/sex/dose) Gavage, 5d/week 78 weeks exposure + 12 weeks recovery for the mice and a total of 110 weeks for rats A sub-chronic study performed to determine dose. Rats and mice (N=2/species), of which the lowest doses causing death, were selected as the highest level of the sub-chronic study. The sub-chronic study was performed to	HCE (98% purity) Concentrations: Rats: 250 and 500 mg/kg bw/day → TWA doses were 113 and 227 mg/kg bw/day Mice: 500 and 1000 mg/kg bw/day (week 1-8), 600 and 1200 mg/kg bw/day (week 9-78) → TWA doses were 360 and 722 mg/kg bw/day Statistical analysis: Cox method, one-tailed Fisher exact test, Cochran-Armitage test Control: corn oil	Increased mortality (dose-dependent and statistically significant) in rats, but not in mice Tubular nephropathy was observed in all treated animal groups <u>Rats</u>: -increased incidence of follicular-cell adenomas or carcinomas in thyroid in male rats: 0 mg/kg bw/day: 2/18 (11%, n.s. P<0.05) 250 mg/kg bw/day: 3/36 (8%, n.s. P<0.05) 500 mg/kg bw/day 5/28 (18%, n.s. P<0.05) -increased incidence of mammary gland fibroadenomas in females: 0 mg/kg bw/day: 6/20 (30%, n.s. P<0.05) 113 mg/kg bw/day: 13/50 (26%, n.s. P<0.05) 227 mg/kg bw/day: 9/50 (18%, n.s. P<0.05) <u>Mice</u>: -increased incidence of hepatocellular carcinomas:	

Reference	Study design and animal species	Data on exposure and effect endpoints	Observations and results	Remarks
	determine the maximum tolerated dose (MTD) for the chronic test. If there was no effect on weight gain, doses were increased		Pooled control: 6/60 (M; 10%, n.s. P<0.05) and 2/60 (F; 3%, n.s.) matched control: 3/20 (M; 15%, n.s. P<0.05) and 2/20 (F; 10%, n.s P<0.05) 600 mg/kg bw/day: 15/50 (M; 30%, sign. P=0.008) and 20/50 (F; 40%, sign. p<0.001) 1200 mg/kg bw/day: 31/49 (M; 63%, sign. p<0.001) and 15/49 (F; 31%, sign. p<0.001)	
NTP (1989) Non-guideline	Repeated dose toxicity/carcinogenicity study F344/N rats N=50 animals/sex/group Gavage, two years, 5 days/week	HCE (>99%) Concentrations: Males: 0, 10, 20 mg/kg/day → TWA doses were 0, 7 and 14 mg/kg bw/day Females: 0, 80, 180 mg/kg/day → TWA doses were 0, 57 or 114 mg/kg bw/day Control: corn oil	No significant difference in survival rate Mean body weight: 20 mg/kg/day: 5-6% lower than control after week 81 180 mg/kg/day: 5-9% lower than control between week 41 and 101. Increased total incidence and overall severity (mean±S.D.) of nephropathy: <u>Males</u>: 20 mg/kg bw/day: 47/50 (n.s.), 2.68±0.16 (sign. P<0.05) <u>Females</u>: 80 mg/kg bw/day: 42/50 (sign. P<0.01), 1.38±0.11 (sign. P<0.01) 180 mg/kg bw/day: 44/49 (sign. P<0.01), 1.69±0.12 (sign. P<0.01)	Limited information For (non)-neoplastic lesions, percentages were not calculated with only the survival animals but with the total number animals.

Reference	Study design and animal species	Data on exposure and effect endpoints	Observations and results	Remarks
			Increased linear mineralization of renal papillae: Males: 10 mg/kg bw/day: 15/50 (30%, sign. P<0.05) 20 mg/kg bw/day: 32/50 (64%, sign p<0.05) Increased hyperplasia of pelvic transitional epithelium (only observed in males): 0 mg/kg bw/day: 0/50 (0%, n.s.) 10 mg/kg bw/day: 7/50 (14%, sign. P<0.05) 20 mg/kg bw/day: 7/50 (14%, sign. p<0.05) Increased renal tubular hyperplasia in male rats: 20 mg/kg bw/day: 11/50 (22%, sign. P<0.01) Increased total incidences of renal adenomas or carcinomas in male rats: 20 mg/kg bw/day: 7/50 (14%, sign. P<0.01) Increased adrenal pheochromocytoma in males: 10 mg/kg bw/day: 26/45 (58%, sign. P<0.01)	

Reference	Study design and animal species	Data on exposure and effect endpoints	Observations and results	Remarks
			20 mg/kg bw/day: 19/49 (39%, n.s.) Increased combined adrenal pheochromocytoma: 10 mg/kg bw/day: 28/45 (62%, sign. P<0.01)	

TWA= time weighted average; M=male; F=female; n.s.=not significant; sign.=significant

NIH (1978)/Weisburger (1977)
In a non-guideline study conducted by NIH (1978)/Weisburger (1977) [15, 36], a single dose range finding study was performed in two male rats and two female mice. Animals received up to 10 dose levels in corn oil by gavage. The lowest doses causing death were selected as the highest level for the 8-week sub-chronic study. The main objective for the sub-chronic study was to determine the maximum tolerated dose (MTD) for the chronic study. In the sub-chronic study, six groups of rats and mice (N=5/sex/species) were used (of which one control group receiving corn oil only). Rats receive 0, 178, 316, 562, 1000 and 1789 mg/kg bw/day hexachloroethane by gavage, for 5 days/week for 6 weeks. Mice received 0, 316, 562, 1000, 1780 and 3160 mg/kg bw/day by gavage, during the same exposure time. Animals were weighted weekly, which served as a guide for the dosage during the next week. After the dosing period, the animals were observed an additional 2 weeks. Animals were then euthanized and gross necropsy was performed.
At 562 mg/kg bw/day all rats survived, at 1000 mg/kg bw/day some rats survived, and at 1789 mg/kg bw/day all rats died. Mean body weight gain was -38% for male rats and -18% for female rats at 1000 mg/kg bw/day. All mice survived exposure to ≤1000 mg/kg bw/day hexachloroethane and all female mice survived exposure to 1780 mg/kg bw/day. At 3160 mg/kg bw/day, 1/5 male mice and 2/5 female mice survived. Mean body weight gain was substantially less in mice receiving 3160 mg/kg bw/day.
A MTD of 500 mg/kg bw/day was determined for both rats and mice. A 'low' and 'high' dose were determined for the chronic study. These were for rats 250 and 500 mg/kg bw/day and for mice 500 and 1000 mg/kg bw/day hexachloroethane.

Carcinogenicity of hexachloroethane was tested in Osborne-Mendel rats and B6C3F1 mice of (N=50/sex/dose/species). From week 1 to 78 rats received 250 and 500 mg/kg bw/day hexachloroethane in corn oil by gavage. From week 23, a pattern started with 4 weeks exposure and 1 week of rest. From week 1 to 8 mice received 500 and 1000 mg/kg bw/day in corn oil by gavage. From week 9 to 78, mice received 600 and 1200 mg/kg/bw/day.
Results showed a statistically significant dose-response increase in mortality in male and female rats, but not in mice. Tubular nephropathy was observed in all treated animal groups. There were effects on neoplastic and non-neoplastic lesions as well as on tumour development. An increased incidence of follicular-cell adenomas or carcinomas in the thyroid in male rats was noted (high: 5/28, low:3/36, control: 2/18) with an earlier onset: 60, 92, and 111 weeks respectively (Table 8). In females, higher incidence of mammary gland fibroadenomas was seen at low (13/50) and high dose (9/50) as compared to control (6/20), with an earlier onset: 94, 57, 106 weeks respectively. In the mice, significant increase of hepatocellular carcinoma in males and females was observed (control:3/20 and 2/20; low:15/50 and 20/50; high:31/49 and 15/49 respectively).

Table 8 Summary table of occurrence of tumours from hexachloroethane

	Males			Females		
Tumour type	Control	Low	High	Control	Low	High
Rat						
Pituitary adenoma and carcinoma	2	4	0	7	15	7
Adrenal tumours	1	2	0	2	1	1
Thyroid adenomas and carcinomas	2	3	6	2	5	4
Mammary fibromas and fibroadenomas	0	0	0	7	13	10
Mammary carcinoma	0	1	0	0	3	1
Kidney tumours	0	5	0	0	1	3
Metastases	4	3	0	1	1	1
Total tumours	13	22	12	22	50	27
Number of animals examined	20	49	50	20	50	49
Animals with tumours	9	17	11	14	33	20
Mouse						
Lung adenomas and carcinomas	0	2	3	1	1	4
Malignant histiocytic lymphoma	0	0	0	4	9	7
Liver-hepatocellular carcinoma	3	15	29	2	20	15
Metastases	0	1	0	0	1	0
Total tumours	4	17	37	9	40	30
Number of animals examined	20	50	49	20	50	49
Animals with tumours	4	17	32	8	32	26

NTP (1989)
In 3 non-guideline studies conducted by NTP [4], F344/N rats were exposed for 16 days, 13 weeks, or 2 years to hexachloroethane.

In the 16-days study, 5 animals/sex/group were exposed to 0, 187, 375, 750, 1500, and 3000 mg/kg bw of hexachloroethane in corn oil by gavage.
Results showed a full mortality in the groups of 1500 or 3000 mg/kg, and 1/5 males and 2/5 females died in the 750 mg/kg group, respectively. Regarding the body weight changes, in the 750 mg/kg group, males had 25% lower mean body weights and females 37% lower mean body weights than controls. Clinical signs observed in the 750 mg/kg group were dyspnoea, ataxia, prostration, and excessive lacrimation.
Pathology results showed that all dosed males had hyaline droplet formation in the cytoplasm of the renal tubular epithelial cells. In the 187 and 375 mg/kg groups, tubular cell regeneration and eosinophilic granular casts in the tubule lumina at the corticomedullary junction in the kidney was observed.

In the 13-weeks study, 10 animals/sex/group were exposed to 0, 47, 94, 188, 375, and 750 mg/kg bw of hexachloroethane.
Results showed mortality at 750 mg/kg bw of 5/10 males and 2/10 females. A mean body weight decrease was observed in males (-19%) and females (-4 %) compared to controls in the 750 mg/kg group. Clinical signs observed at doses of 94 mg/kg and higher were post-gavage hyperactivity for both sexes and convulsions at doses of 375 and

750 mg/kg bw (the week of first appearance decreased with increased dose).
Additionally, pathology results showed that all dosed males had hyaline droplet formation in the cytoplasm of the renal tubular epithelium. In the 750 mg/kg bw groups, all animals had an increased relative organ weight of the liver, heart, kidney, and brain. At the same dose, hepatocellular necrosis was observed in 2/5 males and 8/10 females, and all males had haemorrhagic necrosis of the urinary bladder. All five males who died before schedule had renal papillary necrosis, and degeneration and necrosis of the renal tubular epithelium. In the 375 mg/kg bw group, hepatocellular necrosis was noted in 1/10 males and 4/10 females, and 2/10 females in the 188 mg/kg bw group. Dose response was also observed regarding the kidney changes.

In a 2-years study conducted by the NTP, 50 males/group were exposed to 0, 10, and 20 mg/kg bw/day hexachloroethane in corn oil by gavage, and 50 females/group were exposed to 0, 80, and 180 mg/kg bw/day hexachloroethane in corn oil by gavage, all for 5 days/week. Dosing was based on the lesions of the kidneys in males and livers of females observed in the 13-week study.
Results showed no significant differences in mortality rate in any group. The mean body weight in the 20 mg/kg bw/day group was 5-6% lower compared to the control animals after week 81, and in the 180 mg/kg bw/day group a decrease of 5-9% was observed between week 41 and 101.
Pathology results showed (non)-neoplastic lesions in males and females with a dose response severity of nephropathy. In males, renal hyperplasia was significantly increased (+22%; 11/50 animals) in the high dosed group. The combined incidence of renal adenomas or carcinomas was significantly increased (+14%; 7/50 animals) in males in the high dose group. Adrenal pheochromocytoma and combined pheochromocytoma were significantly increased in males at the mid dose group (+58%; 26/45 animals and +62%; 28/45 animals, respectively), but not in the high dose group.

11 Additional information

Vogel (1993)

Vogel and Nivard (1993) [37] conducted an eye mosaic assay, an *in vivo* short-term test measuring genetic damage in somatic cells of Drosophila after treatment of larvae (non-guideline). Between 12 and 15 pairs of flies were permitted to lay eggs in bottles for 3 days on food supplemented with hexachloroethane dissolved in a solvent (4—4.5 ml/100 ml food) before mixing it into standard food. Response was weakly positive ($+^{w}$). This means that the clone frequency was significantly enhanced compared with both concomitant and pooled controls, but no more than about a 'doubling' of the spontaneous spot frequency is associated with signs of toxicity meaning that a dose—response relationship could not be established.

Mitoma et al. (1985)

In a non-guideline metabolism study conducted by Mitoma et al. (1985) [38], male Osborne Mendel rats and male B6C3F1 mice (4-6 weeks old) were exposed to hexachloroethane (98%) 5 days per week for 4 weeks followed by a single radiolabelled dose for a metabolism test. Animals were kept for an extra 48h in metabolic cages after the radioactive dose was given. Concentration used were 125 and 500 mg/kg bw/day (¼ MTD and MTD) for the rats and 250 and 1000 mg/kg bw/day (¼ MTD and MTD) for the mice. For metabolism results see section 8.2.3. Mice metabolize chlorinated hydrocarbons at a higher rate than rats.

12 References

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