Committee for Risk Assessment

RAC

Annex 1

Background document

to the Opinion proposing harmonised classification and labelling at EU level of

acetaldehyde; ethanal

EC Number: 200-836-8
CAS Number: 75-07-0

CLH-O-0000001412-86-120/F

The background document is a compilation of information considered relevant by the dossier submitter or by RAC for the proposed classification. It includes the proposal of the dossier submitter and the conclusion of RAC. It is based on the official CLH report submitted to public consultation. RAC has not changed the text of this CLH report but inserted text which is specifically marked as 'RAC evaluation'. Only the RAC text reflects the view of RAC.

Adopted

16 September 2016

CLH report

Proposal for Harmonised Classification and Labelling

**Based on Regulation (EC) No 1272/2008 (CLP Regulation),
Annex VI, Part 2**

Acetaldehyde

EC Number: **200-836-8**

CAS Number: **75-07-0**

Index Number: **605-003-00-6**

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Version number: 2.0 **Date: June 2015**

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Part A.

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Part A.

1 PROPOSAL FOR HARMONISED CLASSIFICATION AND LABELLING

1.1 Substance

Table 1: Substance identity

Substance name:	***acetaldehyde, ethanal***
EC number:	***200-836-8***
CAS number:	***75-07-0***
Annex VI Index number:	***605-003-00-6***
Degree of purity:	***confidential***
Impurities:	***confidential***

1.2 Harmonised classification and labelling proposal

Table 2: The current Annex VI entry and the proposed harmonised classification

	CLP Regulation
Current entry in Annex VI, CLP Regulation	Flam. Liq. 1, H224 Eye Irrit. 2, H319 STOT SE 3, H335 Carc. 2, H351
Current proposal for consideration by RAC	Carc. 1B, H350 Muta. 1B, H340
Resulting harmonised classification (future entry in Annex VI, CLP Regulation)	Flam. Liq. 1, H224 Eye Irrit. 2, H319 STOT SE 3, H335 Carc. 1B, H350 Muta. 1B, H340

1.3 Proposed harmonised classification and labelling based on CLP Regulation

Table 3: Proposed classification according to the CLP Regulation

CLP Annex I ref	Hazard class	Proposed classification	Proposed SCLs and/or M-factors	Current classification [1)]	Reason for no classification [2)]
2.1.	Explosives	None		None	Not evaluated
2.2.	Flammable gases	None		None	Not evaluated
2.3.	Flammable aerosols	None		None	Not evaluated
2.4.	Oxidising gases	None		None	Not evaluated
2.5.	Gases under pressure	None		None	Not evaluated
2.6.	Flammable liquids			Flam. Liq. 1	
2.7.	Flammable solids	None		None	Not evaluated
2.8.	Self-reactive substances and mixtures	None		None	Not evaluated
2.9.	Pyrophoric liquids	None		None	Not evaluated
2.10.	Pyrophoric solids	None		None	Not evaluated
2.11.	Self-heating substances and mixtures	None		None	Not evaluated
2.12.	Substances and mixtures which in contact with water emit flammable gases	None		None	Not evaluated
2.13.	Oxidising liquids	None		None	Not evaluated
2.14.	Oxidising solids	None		None	Not evaluated
2.15.	Organic peroxides	None		None	Not evaluated
2.16.	Substance and mixtures corrosive to metals	None		None	Not evaluated
3.1.	Acute toxicity - oral	None		None	Not evaluated
	Acute toxicity - dermal	None		None	Not evaluated
	Acute toxicity - inhalation	None		None	Not evaluated
3.2.	Skin corrosion / irritation	None		None	Not evaluated
3.3.	Serious eye damage / eye irritation			Eye Irrit. 2	
3.4.	Respiratory sensitisation	None		None	Not evaluated
3.4.	Skin sensitisation	None		None	Not evaluated
3.5.	Germ cell mutagenicity	Muta. 1B			
3.6.	Carcinogenicity	Carc. 1B		Carc. 2	
3.7.	Reproductive toxicity	None		None	Not evaluated
3.8.	Specific target organ toxicity –single exposure			STOT SE 3	
3.9.	Specific target organ toxicity – repeated exposure	None		None	Not evaluated
3.10.	Aspiration hazard	None		None	Not evaluated
4.1.	Hazardous to the	None		None	Not evaluated

	aquaticenvironment				
5.1.	Hazardous to the ozone layer	None		None	Not evaluated

[1)] Including specific concentration limits (SCLs) and M-factors
[2)] Data lacking, inconclusive, or conclusive but not sufficient for classification

Labelling: Signal word: Danger
Hazard statements: H224, H319, H335, H350, H340
Precautionary statements: not harmonized

Proposed notes assigned to an entry:

: none

2 BACKGROUND TO THE CLH PROPOSAL

2.1 History of the previous classification and labelling

Acetaldehyde is classified for carcinogenicity in Annex VI of regulation (EC) No 1272/2008 as follows: Carc 2 (suspected human carcinogen; H351: suspected of causing cancer). The substance is not classified for mutagenic activity. The classification by the European Commission dates from 1991. The existing classification with Carc. Cat 2 is based on the same carcinogenicity studies as in this proposal. However, there is new information regarding mutagenicity. This proposal for changing the harmonised classification is based on the report of the Health Council of the Netherlands.(1)

2.2 Short summary of the scientific justification for the CLH proposal

In 1999, IARC concluded that there was inadequate evidence in humans for the carcinogenicity of acetaldehyde, and that there was sufficient evidence in experimental animals.(2) Therefore, IARC classified the substance in Group 2B ('possibly carcinogenic to humans').

In 2010, IARC evaluated the risk of cancer due to alcohol consumption, including acetaldehyde. It confirmed that there was sufficient evidence in animal experiments for the carcinogenicity of acetaldehyde.(3) Moreover, in 2012 IARC concluded that 'acetaldehyde associated with alcohol consumption' is carcinogenic to humans (Group 1).(4)

Acetaldehyde is an intermediate substance in the metabolism of ethanol, and it has been suggested that acetaldehyde accounts for a great part of the toxic effects of ethanol. However, this proposal focuses on acetaldehyde alone and does not consider combined exposure with ethanol and ethanol-related adverse health effects.

On mutagenicity, sufficient evidence has been found for *in vivo* mutagenicity testing in somatic cells of mammals. There is limited evidence that acetaldehyde is genotoxic (sister chromatid exchanges) in germ cells of mice (Madrigal-Bujaidar et al. 2002), when the substance was given by intraperitoneal injection.(5) These findings indicate that acetaldehyde is able to reach the germ cells, and interacts with the genetic material, which would be in line with the findings on absorption and distribution kinetics. However, in another animal study no abnormal sperm cells, and no meiotic micronuclei in spermatids were observed at dose levels inducing acute toxicity (Lähdetie et al. 1988).(6) Overall, it is considered that some evidence exists that acetaldehyde has potential to cause mutations in germ cells. Therefore, it is recommended to classify the substance in category 1B.

On carcinogenicity, there is little or no epidemiological data to support statements concerning an association between exposure to acetaldehyde and cancer. Therefore, human data are considered insufficient to make a final conclusion on the carcinogenic potential of acetaldehyde in humans. For animal data, there is sufficient evidence of carcinogenicity, since a causal relationship was established between malignant tumours in animals and chronic inhalation to acetaldehyde in two studies (Woutersen et al. 1986, Feron et al. 1982), the main route of exposure in an occupational environment.(7, 8) According to the CLP classification criteria, acetaldehyde should, therefore, be classified as "presumed to have carcinogenic potential for humans", which corresponds to classification in category 1B. Supporting evidence for its carcinogenic potential is that the substance has mutagenic properties.

2.3 Current harmonised classification and labelling

2.3.1 Current classification and labelling in Annex VI, Table 3.1 in the CLP Regulation

The classification of acetaldehyde is harmonised in Annex VI of CLP under the index number 605-003-00-6 as follows:

Table 3.1 CLP Regulation
Flam. Liq. 1 - H224
Eye Irrit. 2 - H319
STOT SE 3 - H335
Carc. 2 - H351

2.3.2 Current classification and labelling in Annex VI, Table 3.2 in the CLP Regulation

This paragraph is considered irrelevant seen the repeal of Directive 67/548/EEC with effect from 1 June 2015.

2.4 Current self-classification and labelling

2.4.1 Current self-classification and labelling based on the CLP Regulation criteria

The registrants and most notifiers use the harmonised classification:

Flam. Liq. 1 - H224

Eye Irrit. 2 - H319

STOT SE 3 - H335

Carc. 2 - H351

However, the following additional classifications were applied by some of the other notifiers:

Acute Tox. 4 – H302

Acute Tox. 3 – H311

Eye Dam. 1 - H318

Skin Sens. 1 – H317

Muta 2 – H341

STOT SE 2 – H371

Aquatic Chronic 2 – H411

2.4.2 Current self-classification and labelling based on DSD criteria

This paragraph is considered irrelevant seen the repeal of Directive 67/548/EEC with effect from 1 June 2015.

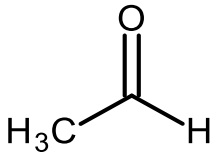
RAC general comment

Note about the public consultation

Two separate consultations were conducted. Additional to the standard public consultation, targeted views were sought from stakeholders on the genetic polymorphism of aldehyde dehydrogenase 2 (ALDH 2) and its releveance to the harmonised classification and labelling of acetaldehyde.

Introductory observations

Acetaldehyde (ethanal) is an organic substance, which occurs in various food and industrial products. It appears as an intermediate metabolic product in plants and animals.



Absorption and distribution

In human volunteers, a significant uptake (45-70%) by the respiratory tract of inhaled acetaldehyde (100 to 800 mg/m^3) was observed after a very short exposure duration of 45 to 75 seconds.

In an inhalation study in rats, acetaldehyde was distributed to the blood, liver, kidney, spleen, heart, myocardium and skeletal muscle. The levels in the blood were reduced quickly. There is no direct evidence that acetaldehyde reaches the germ cells, testes or ovaries after exposure via physiological routes. In the public version of the REACH registration dossier for acetaldehyde, a 4 week repeated dose study (consistent with OECD 407) by the oral route is available. No effects were reported on the weights of the testes or ovaries in this study.

Metabolism and reactivity towards DNA

In humans, acetaldehyde is primarily produced by oxidation of ethanol through alcohol dehydrogenase (ADH) in the liver.

Acetaldehyde is further oxidized to acetic acid in a NAD-dependent reaction by ALDH. ALDH exists in the cells of most tissues, including liver and mucosal tissue of the respiratory tract. In addition, data indicate that ALDH is expressed in the testes of mice. Further enzymes are involved in the metabolism of acetaldehyde but to a negligible extent.

There is a mitochondrial and a cytosolic form of ALDH. In human liver, only the mitochondrial form oxidizes acetaldehyde. However, in rodents, both forms of ALDH contribute to the metabolism of acetaldehyde.

ADH and ALDH exhibit human genetic polymorphisms and ethnic variations. At least 19 ALDH genes have been identified in humans and similar numbers of ALDH genes appear to be present in other mammalian species. A toxicologically relevant polymorphism involves the mitochondrial ALDH2, where the ALDH2*2 shows little or no catalytic activity. This inactive form is found in up to approximately 50% of the Asian population but is absent in Caucasians. No reliable data are available on the half-life of acetaldehyde in humans with different genotypes of ALDH; the consequence of this mutation on the systemic bioavailability of acetaldehyde is unclear.

However, there is one *in silico* study (provided during the second public consultation) on the effects of different ALDH2 genotypes on the concentration of acetaldehyde in human nasal tissue after inhalation exposure (Teeguarden *et al*., 2008). In this study, a physiologically based pharmacokinetic model was used to assess whether ALDH2 polymorphism has an impact on acetaldehyde concentrations and acidification in nasal tissues. Although low exposure levels led to high acetaldehyde concentrations in nasal epithelium, in this model the modelled concentrations did not differ significantly between different genotypes (full activity, intermediate activity and zero activity). The authors concluded that ALDH2, as a high-affinity but low-capacity enzyme, does not contribute significantly to acetaldehyde metabolism in the nasal tissue. Metabolism through ALDH2 seems to be saturated even at low concentrations of inhaled acetaldehyde (50 ppm). Therefore, in human nasal tissues, acetaldehyde metabolism is more likely to occur through the activity of isoenzymes of the ALDH1 subfamily: low-affinity, high-capacity enzymes with no known polymorphisms in humans.

In general, data indicate a highly effective metabolism. In laboratory studies, half-time values in the blood for acetaldehyde were found to be three minutes in rats (after repeated exposure by inhalation) and mice (following a single intraperitoneal injection).

Acetaldehyde is a highly reactive electrophile which reacts with nucleophilic groups of cellular macromolecules, such as proteins and DNA, to form adducts. It has been shown that acetaldehyde that is incubated with ribonucleosides and deoxyribonucleosides forms adducts with cytosine or purine nucleosides, and one of acetaldehyde guanosine adducts is N2-ethylguanosine.

Excretion

Data on elimination are limited. In rabbits and rats, metabolites (but not the parent compound) were found in urine after intravenous administration of acetaldehyde. In dogs, minor amounts of acetaldehyde were found in the urine following a single administration of acetaldehyde via a stomach tube, although in most dogs no acetaldehyde was detected in the urine at all. In general, it appears that systemic levels of acetaldehyde following exposure will be low and will decrease quickly after the end of exposure.

Data on Alcohol Consumption and Cigarette Smoking

Acetaldehyde is the major metabolite of ethanol. Ethanol is oxidised to acetaldehyde by ADH and acetaldehyde is then converted to acetate by ALDH2. Acetaldehyde is also a component of cigarette smoke. In the CLH dossier, Germ Cell Mutagenicity section, a summary of studies on acetaldehyde-DNA adduct formation in alcoholics and smokers was provided. Furthermore, the Carcinogenicity section contained a number of studies which look at the link between the genetic polymorphism of ALDH2 and cancer development in humans. RAC considered these data on the effects of smoking and alcohol consumption in humans not relevant for the assessment of the classification of acetaldehyde and therefore, these studies are not taken into account in this opinion.

3 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

A change in the harmonised classification of acetaldehyde is proposed because there is new data especially on mutagenicity, which warrants a more severe classification for germ cell mutagenicity and carcinogenicity compared to the current harmonised classification.

Part B.

SCIENTIFIC EVALUATION OF THE DATA

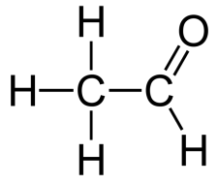
1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substance

Table 4: Substance identity

EC number:	200-836-8
EC name:	acetaldehyde, ethanal
CAS number (EC inventory):	75-07-0
CAS number:	75-07-0
CAS name:	acetaldehyde
IUPAC name:	acetaldehyde
CLP Annex VI Index number:	605-003-00-6
Molecular formula:	C_2H_4O
Molecular weight range:	44.05256 g/mol

Structural formula:



1.2 Composition of the substance

Table 5: Constituents (non-confidential information)

Constituent	Typical concentration	Concentration range	Remarks
Acetaldehyde	confidential	confidential	mono constituent substance

Current Annex VI entry:

Table 6: Impurities (non-confidential information)

Impurity	Typical concentration	Concentration range	Remarks
confidential			The known impurities are not expected to affect the classification.

Current Annex VI entry:

Table 7: Additives (non-confidential information)

Additive	Function	Typical concentration	Concentration range	Remarks
confidential				

Current Annex VI entry:

1.2.1 Composition of test material

Relevant information on the purity is given in the respective study summaries when available.

1.3 Physico-chemical properties

Table 8: Summary of physico - chemical properties

Property	Value	Reference	Comment (e.g. measured or estimated)
State of the substance at 20°C and 101,3 kPa	Liquid	IUCLID 2000	
Melting/freezing point	-123.5 °C	SCCNFP 2004[2]	
Boiling point	20.4 °C	SCCNFP 2004[2]	
Relative density	0.78 g/cm3 at 20 °C	IUCLID 2000	
Vapour pressure	98 kPa at 20 °C	SCCNFP 2004[2]	
Surface tension	-	IUCLID 2000	
Water solubility	Miscible at 20 °C	IUCLID 2000	
Partition coefficient n-octanol/water	log P, 0.43	IARC 1999[3]	
Flash point	-40 °C (open cup), -38 °C (closed cup)	IARC 1999[3]	
Flammability	Extremely flammable	IUCLID 2000	
Explosive properties	-	IUCLID 2000	
Self-ignition temperature	-		
Oxidising properties	-		
Granulometry	-		
Stability in organic solvents and identity of relevant degradation products	-		
Dissociation constant	13.6 at 25 °C	NTP 2010	
Viscosity	0.2456 mPa x sec at 15 °C	SCCS 2012	

2 MANUFACTURE AND USES

2.1 Manufacture

Not relevant for classification.

2.2 Identified uses

Acetaldehyde is an aldehyde, occurring widely in nature. For instance, it occurs naturally in coffee, bread, and ripe fruit, and is produced by plants as part of their normal metabolism. Acetaldehyde is also formed endogenously in humans in small amounts, for instance during the breakdown of ethanol in the body. It is, furthermore, present in tobacco smoke.

Acetaldehyde is produced on a large industrial scale for many purposes and uses.(9) For instance, it is used as an intermediate in the production of acetic acid; in the production of cellulose acetate, pyridine derivates, perfumes, paints (aniline dyes), plastics and synthetic rubber; in leather tanning

and silvering mirrors; as a denaturant for alcohol; in fuel mixtures; as a hardener for gelatine fibres; in glue and casein products; as a preservative for fish and fruit; in the paper industry; and as a flavouring agent.

Acetaldehyde has a full registration. However, no use information is publicly available from the registration.

3 CLASSIFICATION FOR PHYSICO-CHEMICAL PROPERTIES

Not evaluated in this dossier.

4 HUMAN HEALTH HAZARD ASSESSMENT

4.1 Toxicokinetics (absorption, metabolism, distribution and elimination)

The data presented below is a summary from evaluations and reviews by others, such as IARC,(2-4) IPCS,(10) DFG,(11), CERI (12), and SCCNFP.(13)

Absorption, distribution and elimination
In human volunteers, a significant uptake (45-70%) by the respiratory tract of inhaled acetaldehyde (100 to 800 mg/m3) was observed after a very short exposure duration of 45 to 75 seconds.

In an inhalation study (1 litre/minute for 1-hr, between 1-20 mM) in 3 male SD rats, acetaldehyde was distributed in the blood, liver, kidney, spleen, heart, myocardium and skeletal muscle. Levels of acetaldehyde in the blood were reduced quickly, with a half-life of 3.1 minutes. Following acetaldehyde inhalation, peripheral blood acetaldehyde levels were highest; other tissue levels were similar except for the liver, which had a much lower level (Table 9). The concentration in the liver was relatively low due to the rapid metabolism of acetaldehyde. In the same study, acetaldehyde was also measured after a single intragastric ethanol administration (3 gr/kg bw). Acetaldehyde was found in the same tissues compared to inhalation exposure, but the liver levels were higher instead of lower, due to the formation of acetaldehyde in the metabolism of ethanol (Table 9) (14).

Table 9: The tissue distribution of acetaldehyde following acetaldehyde inhalation and intragastric ethanol administration (14)

Tissue	Acetaldehyde inhalation (nmol/g)	Ethanol administration (nmol/g)
Blood*	1210	4.2
Liver	55	9.4
Kidney	213	2.1
Spleen	183	2.1
Heart muscle	277	2.3
Skeleton-muscle	345	1.7

*Blood levels were expressed as nmol/ml. Rats were exposed to acetaldehyde gas for 1 hour (1-20 mM). The acetaldehyde levels were determined immediately after discontinuation of inhalation and 3 hours after the intragastric administration of ethanol (3 g/kg body weight).

Limited data obtained from animal experiments suggest that acetaldehyde (administered by intraperitoneal injection) may be partially transferred from maternal to foetal blood. It is also found in foetal liver.
In a few studies acetaldehyde was detected in the blood and brain of animals, which were given the substance by intragastric administration or intraperitoneal injections. After an oral administration of ethanol at a dose of 4,500 mg/kg in male and female Wistar rats, it was confirmed that produced acetaldehyde was distributed in the blood and brain interstitial fluid.
No data are available on dermal or percutaneous absorption.

Data on elimination are very limited. In one study using dogs, a single administration of acetaldehyde via a stomach tube revealed the presence of the substance in urine in minor quantities, but in most dogs no urinary acetaldehyde could be detected at all. Most likely this is due to the rapid metabolism of the substance in the liver.

This was supported by studies in rabbits and rats, where metabolites were found in urine after intravenous administration of acetaldehyde.

Metabolism
Acetaldehyde is metabolized to acetic acid by nicotinamide adenine dinucleotide (NAD)-dependent aldehyde dehydrogenase (ALDH), which exists in the cells of most tissues, including the liver, mucosal tissue of the respiratory tract, and the testes of mice. Eventually it is degraded to carbon dioxide and water by the citric acid cycle. A minor part of the substance is probably oxidized by cytochrome P450 2E1, and by different aldehyde oxidases.
There are two types of ALDH, a mitochondrial and a cytosolic form. The kinetic characteristics of the enzymatic reaction of liver mitochondrial ALDH are similar among human, rat and Syrian hamster. The Km value of human cytosolic ALDH1 was approximately 180 ìM, but those of rat and Syrian hamster were 15 and 12 ìM, respectively. In human liver, mitochondrial ALDH alone oxidizes acetaldehyde at physiological concentrations, but in rodent liver, both mitochondrial and cytosolic ALDHs have a role in acetaldehyde metabolism.

Acetaldehyde dehydrogenases show genetic polymorphism that gives rise to differences in vulnerability in humans concerning toxicity. Approximately 40% of Oriental population is inactive in mitochondrial ALDH2, which is associated with alcohol intolerance.

In general, data indicate a highly effective metabolism, in that half-time values in the blood for acetaldehyde were found to be three minutes in rats (after repeated exposure by inhalation) and mice (single intraperitoneal injection). For humans, no reliable data on half-times are available.

Acetaldehyde is a highly reactive electrophile, which reacts with nucleophilic groups of cellular macromolecules, such as proteins and DNA, to form adducts. It is shown that acetaldehyde (purity: 99%) that is incubated with ribonucleosides and deoxyribonucleosides forms adducts with cytosine or purine nucleoside, and one of acetaldehyde guanosine adducts is N2-ethylguanosine.

Conclusion
The available information from laboratory animals and humans indicate that acetaldehyde becomes systemically available after oral and inhalation exposure. However, the data also show that due to the rapid metabolism as indicated by the half-time values in blood of 3 minutes the systemic exposure can be expected to be low and to decrease quickly after the end of exposure. There is no direct evidence that acetaldehyde reaches the germ cells or the testes and ovaries after exposure via physiological routes of exposure. However, as acetaldehyde reaches the systemic circulation and several organs it is considered likely that acetaldehyde will also reach the testes and ovaries.

4.2 Acute toxicity

Not evaluated in this dossier.

4.3 Specific target organ toxicity – single exposure (STOT SE)

Not evaluated in this dossier.

4.4 Irritation

Not evaluated in this dossier.

4.4.1 Skin irritation

Not evaluated in this dossier.

4.4.2 Eye irritation

Not evaluated in this dossier.

4.4.3 Respiratory tract irritation

This paragraph is considered irrelevant seen the repeal of Directive 67/548/EEC with effect from 1 June 2015.

4.5 Corrosivity

Not evaluated in this dossier.

4.6 Sensitisation

Not evaluated in this dossier.

4.6.1 Skin sensititsation

Not evaluated in this dossier.

4.6.2 Respiratory sensitisation

Not evaluated in this dossier.

4.7 Repeated dose toxicity

Not evaluated in this dossier.

4.8 Specific target organ toxicity (CLP Regulation) – repeated exposure (STOT RE)

Not evaluated in this dossier.

4.9 Germ cell mutagenicity (Mutagenicity)

4.9.1 Non-human information

4.9.1.1 In vitro data

Data on in vitro mutagenicity testing are presented in Table 10.

Table 10 Summary of in vitro mutagenicity studies

Method	Cell type	Concentration Range*	Results - negative + positive	Klimisch(15) Score**	References
Micro-organisms					
Reverse mutation; multi-substance study	*S. typhimurium* TA98, TA100, TA1535, TA1537	0 – 10,000 µg/plate	- (tested in two laboratories)	2	Mortelmans et al. 1986(16)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	0.005, 0.01, 0.1, 1.0, 5.0, and 10 µg/plate: + and – S9	-	2	ECHA registration data, in vitro.001, study report 1979 (echa.europe.eu;)
Reverse mutation	*S. typhimurium* TA100, TA102, TA104	0.1 – 1.0 ml/chamber, vapour; - and + S9	-	2	Dillon et al. 1998(17)
Reverse mutation	*S. typhimurium*	Max. non-toxic	-	3; only one	Marnett et al.

	TA104	dose: 2,515 µg/ml; -S9		strain tested	1985(18)
Reverse mutation	*S. typhimurium* TA102	0 – 3 µg/plate; cytotoxic over 5,000 µg/plate	-	3; only one strain tested, no positive control	Chang et al. 1997(19)
Reverse mutation	*S. typhimurium* TA1535, TA1537	10 µg/plate (exact dose not given)	-	3; one dose tested only	Rosenkranz 1977(20)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537	0.5% in air (highest dose; - and + S9)	-	4; from secondary source	JETOC 1997(21)
Reverse mutation	*S. typhimurium* TA98 and TA100	No exposure concentration given; +/– S9	-	4; abstract only	Sasaki and Endo 1978(22)
Reverse mutation	*E. coli WP2 uvrA*	Six different concentrations in the range of 0.02 to 10 mM for 18 hours (- S9)	- (also alkylation rate did not increase)	2	Hemminki et al. 1980(23)
Reverse mutation	*E. coli WP2 uvrA*	0.5% in air (highest dose; - and + S9)	-	4; from secondary source	JETOC 1997(21)
Reverse mutation	*E. coli WP2 uvrA*	0.1%	+	4; abstract only; no data on controls; no data on viability	Igali and Gaszó 1980(24)
Chromosomal aberration	*Aspergillus nidulans*	Up to 300 µg/ml; -S9	+ (chromosomal malsegregation); percentage survivors decreases from 100 µg/ml onwards	3	Crebelli et al. 1989(25)
Forward mutation	*Yeast*	23400 µg/ml	(+)	4	Bandas, 1982 (26)
Mammalian cells					
Gene mutation	Human TK6 cells; mutants determ-ined at the *hprt* and *tk* locus	0.001, 0.005, 0.01, 0.05, 0.25, 0.5, 1.0, 2 and 4 mM for 24 hours	- *hprt* locus; + *tk* locus (dose-dependent increase, starting at 0.05 mM)	1	Budinsky et al. 2013(27)
Gene mutation	Human lympho-cytes, *hprt* locus	0 – 2.4 mM (24 hr-treatment, 0-0.6 mM (48-hr treatment); doses selected were based on low-cytotoxicity);	+ (dose-related increase in number of mutants)	2	He and Lambert 1990(28)

		-S9			
Gene mutation spectrum	Human lymphocytes, *hprt* locus	2.4 mM for 22 hours; cloning efficiency was 50% at 1.2 mM compared to control	+ (mutation spectrum of acetaldehyde induced mutations was different from control)	2	Noori and Hou 2001(29)
Gene mutation	Human lymphocytes from donors, *hprt* locus	1.2 to 2.4 mM for 24 hours; 0.2 to 0.6 mM for 48 hours	+ (dose-dependent increase in number of mutants); large genomic deletions; most lesions are likely point mutations	3; no positive control; no data on cytotoxicity	Lambert et al. 1994(30)
Gene mutation; multi-substance study	Mouse lymphoma L5178T cells, *tk* locus	176 – 352 µg/ml; -S9	+; growth reduces with increasing exposure	2	Wangenheim and Bolcsfoldi 1988(31)
Gene mutation	Human fibroblast cell line with shuttle vector plasmid containing *supF* suppressor tRNA gene	0, 0.25, 0.5, 1.0 and 2.0 M	+ (after replication). Mutations were specified as tandem based substitutions (GG→TT); single-strand and double strand DNA mutations increased with increasing dose	2	Matsuda et al. 1998(32)
Gene mutation (6-TG resistant mutations)	Normal human fibroblasts	Concentrations up to 10 mM for 5 hours; positive and negative control included; cell viability tests performed	+ (bell-shaped dose-response relationship); survival at 5 mM was 50%; cells treated with 8 and 10 mM showed delayed recovery of the growth rate.	2	Grafström et al. 1994(33)
Chromosome aberrations	Different DNA-repair deficient Chinese hamster ovary cells	0.3, 0.6, 1.0, 1.8, 2.5 and 3.6 mM for 2 hours; 100 metaphases scored/group	CA: + (concentration-related increase)	2; no positive control	Mechilli et al. 2008(34)
Chromosome aberration	Primary rat skin fibroblasts	0.1 - 10 mM for 12 and 24 hours; 50 metaphases analysed/dose	12 hours: - 24 hours: + ($p<0.05$), except lowest dose, concentration-	3; no positive controls; no data on cytotoxicity	Bird et al. 1982(35)

			related increase in aneuploidy		
Chromosome aberration	Chinese hamster embryonic diploid fibroblasts	0, 20, 40 and 60 µg/ml; -S9	+	3; no data on cytotoxicity; no positive control	Dulout and Furnus 1988(36)
Chromosome aberration	Human peripheral lymphocytes (from 3 healthy volunteers)	0, 0.001 and 0.002 % (v/v); 100 or 200 mitoses scored/sample	-	3; no positive control; no data on cytotoxicity	Obe et al. 1979(37)
Chromosome aberration	Human peripheral blood lymphocytes	0.02 and 0.04 mg/mL culture medium; no positive control	+	4; abstract only	Badr and Hussain 1977(38)
Chromosome aberration	Human lymphocytes	7.8 µg/ml	+	4	Obe et al. 1978 (39)
Chromosome aberration	Human lymphocytes	15.6 µg/ml	+	4	Obe et al. 1979 (40)
Chromosome aberration	Human lymphocytes	15.9 µg/ml	+	2	Bohlke et al. 1983 (41)
Chromosome aberration	Human lymphocytes	7.8-15 µg/ml	+ (dose dependent)	4	Obe et al. 1979 (37)
Chromosome aberration (nondisjunction)	*Aspergillus nidulans*	200 µg/ml	+	4	Crebelli et al. 1989 (25)
Micronuclei	Human lymphoblastoid TK6 cells	0.005, 0.01, 0.05, 0.25, 0.5, 1.0, and 2 mM; plates sealed due to volatility substances	+ (dose-related increase, starting at 0.25 mM); with increasing exposure also the number of apoptotic cells increased	1	Budinsky et al. 2013(27)
Micronuclei	Human lymphoblastoid TK6 cells	8 different concentrations tested, between 0.005 and 4 mM; negative and positive controls included; only data analysed when cytotoxicity was below 55%	+ (0.25, 0.5 and 1.0 mM)	2	ECHA registration data, in vitro.002, study report 2010 (echa.europe.eu)
Micronuclei; multi-substance study	Human lympho-cytes isolated from peripheral blood from one healthy non-	0, 0.6, 0.8 and 1.0 mM	+ (dose-related increase, $p<0.05$); - (after hybridization with a centromeric DNA probe)	2; optimal doses were assessed determining degree of	Migliore et al. 1996(42)

	smoking donor			decrease in bi-/mononucleated ratio	
Micronuclei; multi-substance study	HepG2 and Hep3B cells	0, 0.9 and 9 mM for 24 hours; per experimental point 1,500 cells evaluated.	+ (concentrations-related increase)	2; no data on cytotoxicity	Majer et al. 2004(43)
Micronuclei	MCL-5 human lymphoblastoid cell line	0 – 2 % (v/v; a range of 6 differ-rent concentra-tions) for 22 hours; > 4,000 cells per dose examined	+ (from 0.4 % onwards, *p<0.05*), dose-dependent increase -: aneuploidy	2; no positive control included	Kayani and Parry 2010(44)
Micronuclei	Primary rat skin fibroblasts	0.1 - 10 mM for 12, 24 or 48 hours; > 1,000 cells analysed/ dose	+ (*p<0.05*; except lowest dose tested)	3; no positive controls; no data on cytotoxicity	Bird et al. 1982(35)
Micronuclei	V79 Chinese hamster cells	0.5 – 10 mM (MN);	+ (dose-dependent increase)	2; No positive control	Speit et al. 2008(45)

* + or - S9, with or without metabolic activation system.

** Klimisch score is expressed in reliability levels (cited from original publication):

- Reliability 1 (reliably without restriction). For example, guideline study (OECD, etc.); comparable to guideline study; test procedure according to national standards (DIN, etc.).
- Reliability 2 (reliable with restrictions). For example, acceptable, well-documented publication/study report which meets basic scientific principles; basic data given: comparable to guidelines/standards; comparable to guideline study with acceptable restrictions.
- Reliability 3 (not reliable). For example, method not validated; documentation insufficient for assessment; does not meet important criteria of today standard methods; relevant methodological deficiencies; unsuitable test system.
- Reliability 4 (not assignable). For example, only short abstract available; only secondary literature (review, tables, books, etc.).

Micro-organisms

Acetaldehyde was not mutagenic to *Salmonella typhimurium* or *E. coli WP2 uvrA*, with or without metabolic activation. It induced chromosome malsegregation in *Aspergillus nidulans* and forward mutations in yeast.

Mammalian cells

Overview of key studies

Budinsky et al. (2013) found formation of micronuclei (MN) and thymidine kinase (tk) mutants in a TK6 cell culture after 4 and 24 hours exposure to acetaldehyde. The lowest concentration that consistently induced the formation of MN was 0.25 mM. There was a close dose-response linkage between MN formation and cytotoxicity, with 80-90% survival at 0.25 mM (Figure 1). An increase in TK mutants was observed from 0.05 mM (Figure 2). There was no significant increase in mutation frequency at the HPRT locus (27).

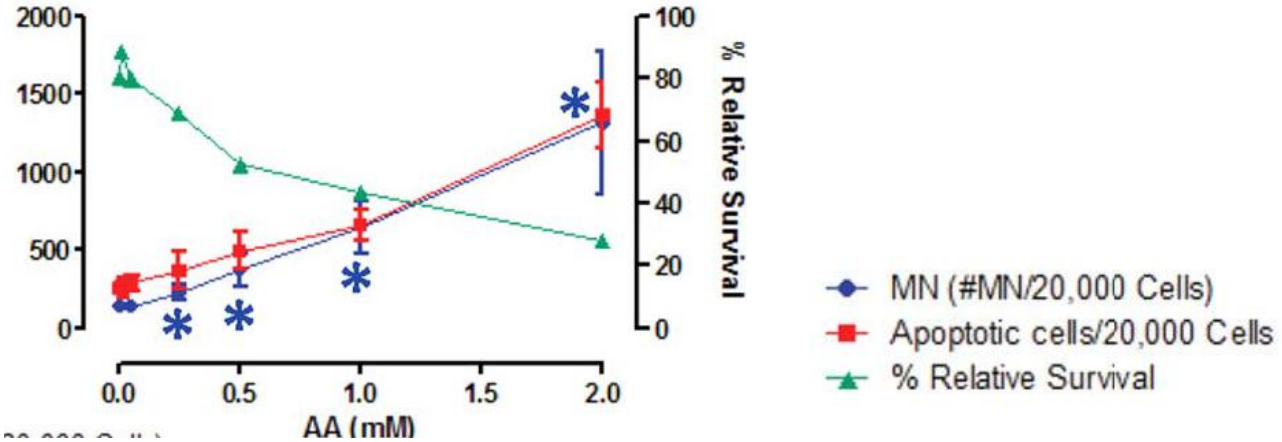


Figure 1: Micronucleus formation after exposure to acetaldehyde (4 hr). After 4 hr, the AA exposures were discontinued and the cells were processed for flow cytometry measurements of MN, apoptosis, and percent relative survival. The data represent the average of four replicates/concentration and standard deviation from two separate studies that were combined. The asterisks represent MN responses that were statistically different from the VC ($P < 0.05$) using Dunnett's Test. The EMS positive control group results: MN: 1591 ± 329; relative survival: 44.4%; apoptosis: 1208 ± 292).

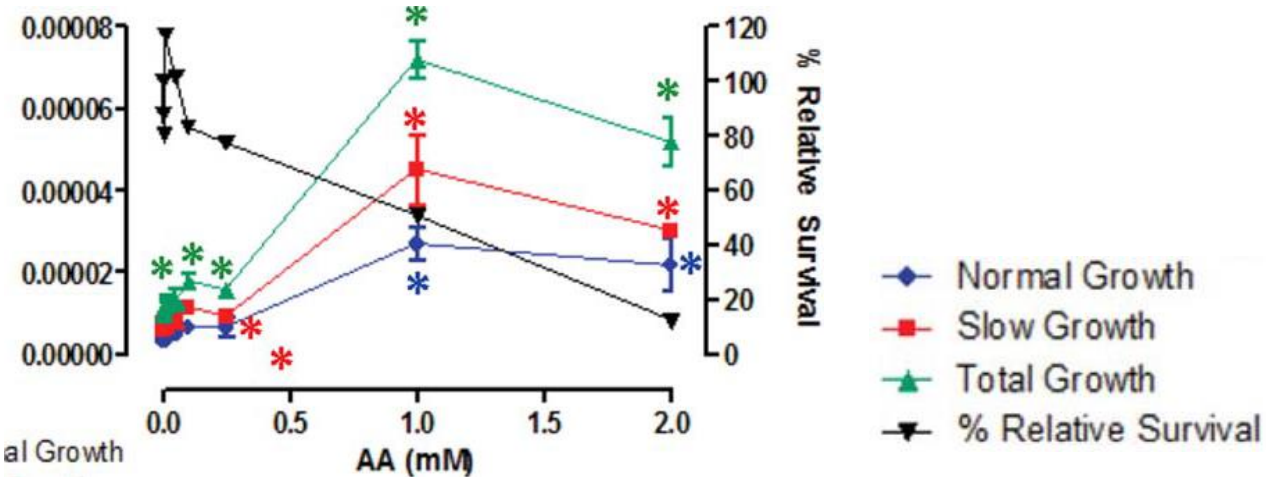


Figure 2: The mutation frequency at the TK locus, following 24 hours incubation with acetaldehyde. Points represent the average ± standard deviation of 5 replicates. Dunnett's test ($P < 0.05$) indicated by the asterisks identify the mutation frequency response that was statistically different from the controls. The normal growth, slow growth, and total growth results are

represented by the blue circles, red squares, and green triangles, respectively. Total growth represents the combined results for normal and slow growth mutants. The inverted black triangles represent the % relative survival.
Separate positive controls, using EMS at 20 and 200 3M, were conducted. The 20 and 200 3M EMS positive controls in the AA study showed a normal growth MF of 1.87E - 05 and 2.44E - 04, respectively; a slow growth MF of 1.68E - 05 and 6.46E - 05, and a total MF of 3.55E - 05 and 3.09E - 04.

In a study by Mechilli et al (2008), induction of chromosomal aberrations (CAs) and sister chromatid exchanges (SCEs) by acetaldehyde (AA) was evaluated in parental and different DNA repair-deficient Chinese hamster ovary (CHO) cell lines to elucidate the mechanisms involved in the protection against AA-induced chromosome damage. Cell lines employed included the parental (AA8), nucleotide excision repair (UV4, UV5, UV61), base excision repair (EM9), homologous recombination repair (HRR) (irs1SF, 51D1)-deficient and Fanconilike (KO40) ones. Concentration dependent increases in both CAs and SCEs were observed. The ranking of different cell lines for sensitivity to induction of CAs by AA was 51D1 > irs1SF > KO40 > UV4 > V33-EM9-AA8 > UV61-UV5 in a descending order (Table 11). Cells deficient in HRR were most sensitive followed by Fanconi anaemia like (KO40) suggesting these pathways, especially HRR is very important for the repair of AA-induced lesions. These observations also suggest that interstrand cross links are primary biologically relevant DNA lesions induced by AA for induction of CAs. Only marginal differences were found between the cell lines for induction of SCEs (34).

Table 11. Relative sensitivity values for induction of CAs; relative sensitivity values for induction of abnormal cell and SCEs (34)

Cell line	CAs		Abnormal cells or SCEs	
	1 mM	1.8 mM	F_{ab}, 0.6 mM	F_{SCE} 0.6 mM
AA8	1	1	1	1
EM9	1.43	2.50	1	1.25
V3-3	1.78	0	1.29	1.29
KO40	2.96	6.70	2.36	1.21
51D1	31.9	67.1	27.28	0.93
irs-1SF	9.52	0	3.50	0.70
UV61	0.42	0.94	0.36	1.68
UV4	2.6	4.40	2.36	0.68
UV5	0.27	0.63	0.21	1.20

A recent micronucleus test (OECD 487) was provided in the substance registration dossier, in which eight concentrations acetaldehyde were tested (0.005 - 4.0 mM). Acetaldehyde induced an increase in micronuclei at levels of 0.25 mM in *in vitro* incubations for 4 hours with human TK6 cells. At levels ≤ 0.05 mM Acetaldehyde did not induce chromosomal damage in human cells. Levels above 1 mM showed marked cytotoxicity (>55% cytotoxicity, based on relative survival compared to unexposed controls) (ECHA registration data, in vitro.002, study report 2010).

Majer et al (2004) investigated the sensitivity of two human derived hepatoma (HepG2, Hep3B) to dietary and lifestyle related carcinogens, including acetaldehyde. Acetaldehyde induced a dose dependent increase in micronuclei in both cell lines (Figure 3). A two-fold increase over the background was found at 11.2 mM (43).

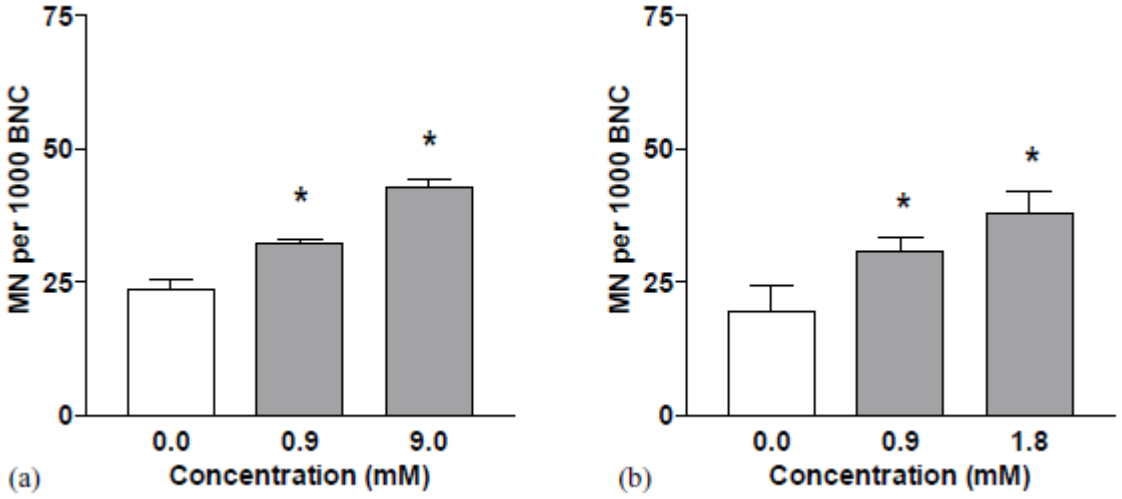


Figure 3: Induction of micronuclei (MN) in HepG2 cells (a) and in Hep3B cells (b) by acetaldehyde. The cells were exposed for 24 h to acetaldehyde. Subsequently, they were incubated with cytochalasin B (final concentration 3 g/ml) for another 26 h. Each bar represents the means ±S.D. of three parallel cultures. Per experimental point 1500 cells were evaluated. * significantly different from control (Dunnett'stest, $P < 0.05$) (43).

Kayani & Parry (2010) looked at the ability of ethanol and acetaldehyde to induce chromosomal changes using in vitro CBMN assay (Cytokinesis Blocked Micronucleus assay) in conjunction with immunofluorescent labeling of kinetochores. Kinetochore staining was used with a view to differentiate, between the genotoxic effects of both chemicals, and ascertain the mechanisms of genotoxicity induction. Both ethanol and acetaldehyde produced statistically significant ($P < 0.05$) dose dependent increase in MN induction as compared with the controls over the dose range tested (Table 12). In the case of acetaldehyde most of the MN had originated by a clastogenic mechanism (44).

Table 12: The effect of acetaldehyde on MN formation in human lymphoblastoid cell line MCL-5 (44)

Dose (% v/v)	Number of cells scored	CBPI	% Cytostasis	BN cells with micronuclei (MNBn) (%)	Apoptosis (%)	Necrosis (%)	Relative proportions of kinetochore positive	
							K^+	K–
00	4036	1.55	0	0.85	0.37	7.84	0.47	0.53
0.005	5097	1.22	60	1.86*	3.53**	8.74	nt	nt
0.010	5044	1.21	61.81	2.08*	2.60**	13.29**	0.46	0.54
0.015	5043	1.21	61.81	2.28*	2.47**	10.82**	0.33	0.67
0.020	4906	1.19	65.45	2.60*	1.85**	11.69**	0.34	0.66
0.025	4919	1.19	65.45	3.73*	1.70**	17.78**	0.32	0.68

CBPI – Cytokinesis Blocked Proliferation Index.
nt: not tested.
MN = micronuclei, K^+ = kinetochore positive, K– = kinetochore negative.
* Significant increase $P < 0.05$ compared with control cultures.
** Significant increase $P < 0.01$ compared with control cultures.

Because the comet assay is increasingly used for the detection of cross-linking agents, Speit et al (2008) characterized the effects of acetaldehyde in the comet assay in relation to cytotoxicity and other genetic endpoints such as the induction of sister chromatid exchange (SCE) and micronuclei (MN).
The standard alkaline comet assay did not indicate induction of DNA strand-breaks by AA in a range of concentrations from 0.2 to 20 mM. AA at a concentration of 20 mM was clearly cytotoxic and reduced cell growth and population doubling to less than 50% of the control. Using the comet assay modification with proteinase K, slightly enhanced DNA migration was measured in comparison to treatment with AA only. No significant induction of cross-links by AA (measured as reduction of gamma ray-induced DNA migration) was determined by the comet assay. A small and reproducible but statistically not significant effect was measured for the AA concentration 20 mM. A clear and concentration-related increase in the frequency of sister chromatid exchange (SCE) and micronuclei (MN) was already measured at lower concentrations (0.2 and 0.5mM, respectively) (Figure 4). These results suggest that the comet assay has a low sensitivity for the detection of AA-induced DNA lesions leading to the induction of SCE and MN. These findings were further supported by results found in literature (45).

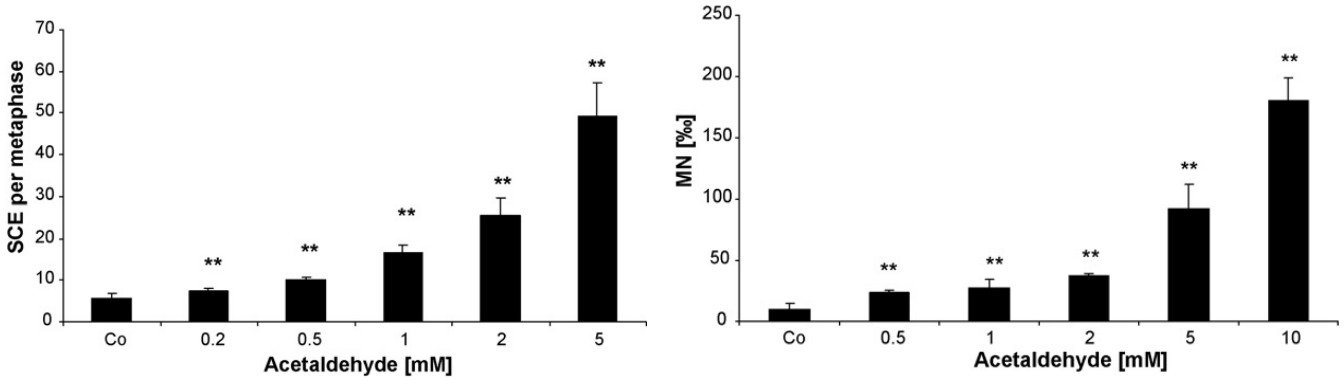


Figure 4: Induction of SCE and MN by acetaldehyde in V79 cells. Results are given as the mean±S.D. of three independent tests. (**) Significance at the 1% level for Dunnett test; Co, untreated control culture (45).

Summary and conclusions

Acetaldehyde showed positive responses in various *in vitro* mammalian mutagenicity assays. Acetaldehyde without metabolic activation induced gene mutation in mouse lymphoma L5178Y cells, chromosomal aberrations and micronuclei in SD rat primary skin fibroblasts. The induction of these gene mutations and chromosomal aberrations was dose-dependent. Acetaldehyde also induced chromosome aberrations in embryonic diploid fibroblasts of Chinese hamster and micronuclei in V79 Chinese hamster cells.
In human lymphocytes, dose-dependent gene mutations, chromosomal aberrations, and micronuclei were induced.
The results were generally consistent over the different studies. However, a particular observation was the absence of a significant increase in gene mutations at the *hprt* locus in the study by Budinsky et al. (2013), as mutations were observed at the *tk* locus in this study. No explanation was offered for this difference. Gene mutations at the *hprt* locus were reported in other studies. This might be related to the concentrations tested, as the highest concentration used by Budinsky for this endpoint was 2.0 mM, while up to 2.4 mM was used in other studies.
Overall, acetaldehyde is considered to induce mutagenicity in mammalian cells *in vitro*.

4.9.1.2 In vivo data

A summary on the in vivo mutagenicity of acetaldehyde is shown in Table 13.

Table 13 Summary of in vivo mutagenicity studies (animal studies)

Method	Animal	Exposure conditions	Results	Klimisch(15) score*	References
Somatic cell mutagencicity					
Gene mutation and micronuclei	Wildtype and knock-out mice with inactive ALDH2[1] gene; micro-nuclei deter-mined in reti-culocytes; mutations were deter-mined by T-cell receptor (TCR) gene mutation assay	Inhalation, 125 and 500 ppm vapour, continuously for two weeks; negative control was inhalation of clean air	*Micronuclei*: + in knock-out mice (*p<0.05*); - in wild-type mice. *Mutation* (TCR mutant frequency): + in knock-out mice (*p<0.05*); - in wild-type mice.	2	Kunugita et al. 2008(46)
Gene mutation and micronuclei	Wildtype and knock-out mice with inactive ALDH2 gene; micronuclei determined in reticulocytes; mutations were deter-mined by TCR gene mutation assay	Oral administration, 0 and 100 mg/kg bw, daily, once a day for two weeks; 5 – 10 animals/group	*Micronuclei*: + in knock-out mice (*p<0.05*); - in wild-type mice. *Mutation* (TCR mutant frequency): + in knock-out mice (*p<0.05*); - in wild-type mice	2	Kunugita et al. 2008(46)
Micronuclei; multi-substance study	Male SD and F344 rats, bone marrow erythrocytes	250 mg/kg bw, intraperitoneal injection. Highest dose tested was	+ (both cell types)	2; only highest dose tested	Wakata et al. 1998(47)

[1] ALDH2, aldehyde dehydrogenase 2 family (mitochondrial), converts acetaldehyde into acetate.

	and peripheral blood erythrocytes	maximum tolerated dose; at least four animals/group			
Micronuclei	5 male CD-1 mice	0 – 400 mg/kg bw, Intraperitoneal injection, three dose levels; tests on acute toxicity performed	+ (dose-related increase)	2	Morita et al. 1997(48)
Micronuclei	Male Han rats, 5 animals/group	Single intraperitoneal injection of 125 or 250 mg/kg bw; blood samples collected after 0, 24, 48 and 72 hours	+ (at 24 and 48 hours), dose-related increase; no data at 72 hours due to toxicity	2	Hynes et al. 2002(49)
Chromosomal aberrations	Rat embryos	Single intra-amniotic injection of 7,800 mg/kg bw	+	4; original publication available in Russian only	Bariliak and Kozachuk 1983(50)
Germ cell mutagenicity					
Meiotic micronuclei in spermatids	C57BL/6J x C3H/He mouse early spermatids	125, 250, 375 and 500 mg/kg bw per day, single dose, intraperitoneal injection; 4 animals/group	- ; survival rate was significantly decreased in highest exposure group	2	Lähdetie 1988(6)
Sex-linked recessive lethal mutations; multi-substance study	*Drosophila melanogaster*	1) Single injection of 22,500 ppm; 2) 25,000 ppm in feed; data presented on mortality and sterility	+ (injection) - (feed)	2	Woodruff et al. 1985(51)

* See footnote in Table 10 for explanation of the Klimisch-scores.

Germ cells

Lähdetie (1988) studied the induction of meiotic micronuclei in spermatids of mice.(6) Mice (4 animals per group) were given a single intraperitoneal injection of acetaldehyde at a concentration of 0 (control vehicle), 125, 250, 375 and 500 mg/kg bw. A group of mice served as positive control (cyclophosphamide injection). Thirteen days after treatment the mice were killed to examine the presence of meiotic micronuclei in early spermatids (1,000 spermatids scored per mouse). Compared to the vehicle control, the number of spermatids with micronuclei did not increase after acetaldehyde treatment, whereas in the positive control it did. The author reported that at a dose of

500 mg/kg bw all animals died due to acute toxicity, whereas all survived at lower doses. In a separate experiment, the author also investigated the sperm morphology in mice treated with acetaldehyde for a short period (up to 250 mg/kg bw; 5-day exposure regimen). However, acetaldehyde did not decrease sperm count, testis weight or seminal vesicle weight, nor did it induce abnormal sperm at the doses. The highest administered dose was lethal to half of the animals in the group.

In a sex-linked recessive lethal mutation assay, acetaldehyde was positive after injection (Woodruff et al. 1985).(51) This shows that the substance induces mutations in germ lines of the insect.

Somatic cells

Kunugita et al. (2008) studied the induction of gene mutations and micronuclei in knock-out mice having an inactive acetaldehyde dehydrogenase (Aldh2, converts acetaldehyde into acetate) gene.(46) Both wildtype and the knockout mice inhaled acetaldehyde at concentrations of 0, 225 or 900 mg/m^3, continuously for two weeks. In addition, groups of mice (5-10 animals per group) were given acetaldehyde orally at doses of 0 or 100 mg/kg bw, once a day for two weeks. Two weeks after the last exposure, all animals were killed and the number of reticulocytes with micronuclei was determined. Also the mutations in the *TCR* gene of T-lymphocytes was measured. Irrespective the route of exposure, in knockout mice, the number of micronuclei positive cells, and the frequency of *TCR* gene mutations in lymphocytes was statistically significantly increased compared to the respective controls. In wildtype animals, acetaldehyde did not cause any effects on these endpoints. See Table 14 for a summary of the results.

In a well-performed study, Wakata et al. (1998) showed that in bone marrow polychromatic and peripheral blood erythrocytes of SD and F344 rats, micronuclei were induced after exposure to acetaldehyde by a single intraperitoneal injection of 250 mg/kg bw.(47) Bone marrow and blood cells were harvested 24 hours after the treatment. The maximal micronucleated polychromatic erythrocyte frequency in bone marrow was 0.43%; the mean for the negative control (saline) was 0.15 ± 0.13%, the mean positive control (cyclophosphamide, 20 mg/kg) was 2.9 ± 1.5%. The highest frequency of micronucleated reticulocytes in peripheral blood was 0.33; the negative control had a mean of 0.07 ±0.08%, the positive control a mean of 0.67 ±0.46%.

In addition, Morita et al. (1997) reported on acetaldehyde-induced micronuclei in bone marrow polychromatic erythrocytes of male CD-1 mice.(48) Five/six mice received the substance by a single intraperitoneal injection. Dose levels were based on acute toxicity test results. Two different lots were used, because the experiment was performed in two different laboratories. Twenty four hours after injections, bone marrow cells were harvested for the micronucleus assay. In Table 15 a summary of the results is shown.

Hynes et al. (2002) exposed male Wistar Han rats (5 animals per group) to acetaldehyde by a single intraperitoneal injection of 125 or 250 mg/kg bw.(49) For micronuclei testing, peripheral blood cells were harvested 0, 24, 48 and 72 hours after the injection. Micronuclei were scored by flow cytometric analysis. The study included negative (vehicle) and positive (cyclophosphamide) controls. Acetaldehyde at a dose of 250 mg/kg bw induced micronuclei, with maximum increases at 48 hours (see Table 16).

Table 14 Induction factors of micronuclei and TCR gene mutations in knockout mice (Kunugita et al 2008).(46)

Exposure route	Exposure level	Micronuclei in reticulocytes	Mutant frequency in T-cell receptor gene
Knock-out mice (Aldh2 -/-)			
Inhalation	0 (control)	-	-
	225 mg/m^3	1.8 *	Not determined
	900 mg/m^3	1.9/unspecified **/***	1.7**
Oral administration	0 (control)	-	-
	100 mg/kg bw	2/1.7 **/***	2.4/1.6 **/***
Wildtype mice (Aldh2 +/+)			
Inhalation	0 (control)	-	-
	225 mg/m^3	-	-
	900 mg/m^3	-	-
Oral administration	0 (control)	-	-
	100 mg/kg bw	-	-

* compared to Aldh2 +/+ control mice (p<0.05); ** compared to Aldh2 +/+ control mice (p<0.01); *** compared to Aldh2 -/- control mice (p<0.05).

Table 15 Induction of micronuclei in male CD mice (Morita et al. 1997).(48)

Manufact. lot	LD_{50}	Dose	Percentage of micronuclei in bone marrow cells		
	mg/kg bw	mg/kg bw	mean	SD	p-value*
Wako	470	0	0.12	0.08	-
		95	0.22	0.15	0.132
		190	0.33	0.10	0.010
		380	0.85	0.21	0.000
Merck	338	0	0.12	0.08	-
		100	0.10	0.07	0.726
		200	0.44	0.11	0.002
		300	0.62	0.16	0.000
		400	1.10	0.25	0.000

* P-value of pairwise comparisons.

Table 16 Induction of micronuclei in blood cells of rats treated with acetaldehyde (Hynes et al. 2002).(49)

Dose (mg/kg bw)	**Time (h)**	**Laboratory***	**Mean RET** ± SD**	**Mean MNRET** per 20,000 RET ± SD**	**Mean MNNCE** ± SD**
0	0	GW	1.29 ± 0.29	0.13 ± 0.06	0.01 ± 0.00
		LL	1.47	0.14	0.01
125	24	GW	0.80 ± 0.12	0.21 ± 0.07	0.01 ± 0.00
		LL	0.91	0.19	0.01
	48	GW	1.32 ± 0.21	0.30 ± 0.09	0.01 ± 0.00
		LL	1.37	0.19	0.01
	72	GW	1.82 ± 0.18	0.14 ± 0.05	0.01 ± 0.00
		LL	1.65	0.18	0.01
250	24	GW	1.00 ± 0.42	0.28 ± 0.07	0.02 ± 0.01
		LL	0.99	0.32	0.01

48	GW	1.31 ± 0.25	0.33 ± 0.11	0.02 ± 0.01
	LL	1.14	0.39	0.01
72	GW	1.90 ± 0.42	0.14 ± 0.05	0.01 ± 0.01
	LL	1.42	0.16	0.01

* GW, GlaxoWellcome; LL, Litron Laboratories. ** RET, reticulocytes; MNRET, micronucleated reticulocytes; MNNCE, micronucleated monochromatic erythrocytes. No data on statistical significance presented.

These studies show that acetaldehyde is inducing mutation in the bone marrow after intraperitoneal injection or in ALDH2 knock-out mice after inhalation but not in wild-type mice after inhalation, suggesting metabolism is an important factor in the ability of acetaldehyde to reach distant sites. No mutations were found in spermatids of mice, although this was endpoint was investigated in only one study.

4.9.2 Human information

Table 17 summarizes a few studies performed on humans, in which effects were related to acetaldehyde. Acetaldehyde exposure in these studies was due to alcohol abuse and/or smoking.

Table 17 Summary of human studies

Method	Population	Cells	Results and remarks	Quality and/or reliability of study	References
DNA-adducts (^{32}P-postlabelling)	Alcohol abusers (n=24) and controls (n=12)	Peripheral white blood cells (granulo-cytes and lymphocytes)	+ in alcohol abusers compared to controls (p<0.001). Average adduct levels in abusers (adducts /10^7 nucleotides): - granulocytes: 3.4 ± 3.8 - lymphocytes: 2.1 ± 0.8 Levels in controls were below LOD	Reliability low in that subjects in the alcoholic group were heavy smokers; in control group one moderate smoker.	Fang and Vaca 1997(52)
DNA-adducts	Cancer-free male Japanese alcoholic patients with different acetaldehyde dehydrogenase (ALDH) genotypes	Peripheral white blood cells	+, adduct level was significantly higher in alcoholics with ALDH2*1*2 genotype compared to alcoholics with ALDH2*1*1 genotype.	Past exposure to ethanol; no non-alcoholic healthy controls included	Matsuda et al. 2006(53)
Acetaldehyde specific DNA-adducts (N^2-	Smokers, before and after smoking	Leucocytes	Decrease in number of N2-ethylidene-dGuo adducts after	Reliability low, because of smoking	Chen et al. 2007(54)

ethylidene-deoxiguanosine)	cessation		cessation (28%). Note: cigarette smoke contains acetalde-hyde, but also other potential carcinogens.	history participants and co-exposure

Acetaldehyde–DNA adducts have been observed in granulocytes and lymphocytes of human alcohol abusers (52, 53) and leucocytes of smokers (54).
In comparison with controls, Fang and Vaca (1997) (52) found 13- and 7-fold higher adduct levels in respectively granulocytes and lymphocytes of alcohol abusers. However, the alcohol abusers were also heavy smokers, and the values of the controls were all below the limit of detection, limiting the reliability of these percentages.
Matsuda et al. (2006) enrolled 19 alcoholic patients with the ALDH2*1/2*1 genotype and 25 alcoholic patients with the ALDH2*1/2*2 genotype. The averages of age, daily ethanol consumption, duration of drinking, and daily cigarette consumption were not significantly different between the two groups. The average levels of three acetaldehyde-derived adducts were significantly higher in ALDH2*1/2*2 alcoholics. The average level of blood N^2-Et-dG adducts in ALDH2*1/2*2 and ALDH2*1/2*1 alcoholics were 28.3 and 3.9 adducts per 10^9 bases, respectively.

Chen et al. (2008) (54) found a decrease in DNA-adducts of 28% in leucocytes of volunteers after 4 weeks of smoking cessation. Levels of acetaldehyde in mainstream cigarette smoke typically range from 500 – 1000 μg/cigarette. The most important confounder was alcohol consumption, for this reason, subjects were eligible only if they consumed less than six alcoholic beverages per month and abstained during the study. Nevertheless, occasional drinking might have been undetected and could potentially contribute to acetaldehyde DNA adducts. The only modifier in this study was the race of the participants. When the data were stratified by race, there was no change in adduct levels in whites, but a significant 57% decrease was observed in the black plus other group (consisting of 7 blacks, 1 American Indian, and one person of mixed racial background).

The data indicate the intrinsic property of acetaldehyde to react in vivo in humans with DNA.

4.9.3 Other relevant information

In the Tables 18 and 19 data are shown on the DNA damaging and genotoxic (other than mutagenicity) properties of acetaldehyde.

Table 18 Summary of other information on DNA damage

Method	Cell type	Concentration	Results	Klimisch(15) score**	References
In vivo studies					
DNA-protein crosslinks	Male Fischer-344 rats; DNA-protein cross-links studied in nasal respiratory mucosa and olfactory cells	1) Inhalation; 100, 300, 1,000 and 3,000 ppm; single 6-hour exposure 2) inhalation; 1,000 ppm; 6-hours/day, daily, 5-days	1) + (respiratory mucosa; dose-dependent increase, *p<0.05*); - (olfactory mucosa) 2) + (respiratory mucosa); + (olfactory	2	Lam et al. 1986(55)

		samples of three rats were combined	mucosa, *p<0.05*)		
In vitro tests using human cells					
DNA single and double strand breaks	Human lymphocytes from two healthy donors	0, 1.56, 6.25, 25 and 100 mM for one hour; for each dose 50 cells were analysed from each subject	+ (single strand breaks at all exposures) + (double strand breaks at 100mM only) Authors reported that > 80% of cells were not viable after exposure to 100 mM for 2 hours	2; no positive control	Singh and Khan 1995(56)
Comet assay*	Human peripheral blood lymphocytes	3, 10, 30 and 100 mM for one hour; doses were based on cytotoxicity data	+ (dose-dependent)	2	Blasiak et al. 1999(57)
Comet assay*	Human lymphocytes, gastric and colonic mucosa cells	3 mM (lympho-cytes), 100 mM (gastric and colonic mucosa cells)	+ No differences were noted among the different cell types; viability was over 70% at the tested doses	2; one dose tested only	Blasiak et al. 2000(58)
Comet assay*	Human bronchial epithelial cells	Exposure to 3, 10, 30 and 100 mM for 1 hour in thiol free medium	+, dose-dependent effects - for single strand breaks	2	Grafström et al. 1994(33)
DNA-adducts	DNA form primary human liver cells, samples from normal liver	Incubation of cells with 5.7 mM [$^{13}C_2$]acetaldehyde; 12 liver samples analysed	+ (N^2-ethyl-deoxiguanosine adducts)	3	Wang et al. 2006(59)
Alkaline elution assay*	Human lymphocytes	10 – 20 mM for 4 hours	+, DNA cross-links - ,DNA strand-breaks	3; No data on cytotoxicity; no positive controls	Lambert et al. 1985(60)
Alkaline elution assay*; multi-substance study	Normal human bronchial epithelial cells and humane leucocytes	1 mM for 1 hour	- (without metabolic activation); at 1 mM no significant growth reduction noted	3; only one concentration used	Saladino et al. 1985(61)
Alkaline elution assay*	Human bronchial epithelial cells	10 mM for 1 hour	-	3; only one dose tested; no data on	Grafström et al. 1986(62)

				controls; 10 mM acetaldehyde induced 50% cytotoxicity	
DNA-protein crosslinks	EBV-transformed human Burkitt's lymphoma cells (EBV, Epstein Barr virus)	0.035, 0.175, 0.875, 3.5 and 17.5 mM for 2 hours; Maximum tolerated dose was 17.5 mM	+ (> 5 mM, *p<0.05*)	2	Costa et al. 1997(63)
DNA-adducts	normal epithelial cells, and SV40T antigen-immor-talized human buccal epithelial cells	1-100 mM for one hour; ^{32}P-postlabeling assay	+ (N^2-ethyl-3'-dG-monophosphate adducts, dose-dependent	2	Vaca et al. 1998(64)
In vitro tests using rodent cells					
Comet assay*	V79 Chinese hamster cells	0.2 – 20 mM	-; authors reported more than 50% reduction of cell viability at 20 mM	2; no positive control	Speit et al. 2008(45)
Cell transformation	Mouse C3H 10T1/2 cells	10-100 µg/ml	-	4	Abernathy et al. 1982 (65)
Cell transformation	Mammalian cells	0.44 µg/ml (3 hours)	-	4	Eker & Sanner 1986 (66)
Alkaline elution assay*	Chinese hamster ovary cells (K1 cells)	0.5, 1.5 and 4.5 mM for 90 minutes	- (strand breaks); + (crosslinks); cell viability > 80%	2; no positive control	Marinari et al. 1984(67)
Alkaline elution assay*; multi-substance study	Primary rat hepatocytes	0.03, 0.3 and 3 mM for 3 hours; cytotoxicity < 55%	-	3	Sina et al. 1983(68)
Other test systems					
DNA-adducts	Calf thymus DNA	1 M for 30 minutes at 37 °C; negative control included	+ (without metabolic activation)	3; only one concentration tested	Ristow and Obe 1978(69)
DNA-adducts	Calf thymus DNA	0.01-40 mM for 20 to 96 hours	+ (mainly N^2-ethylidene-deoxi-guano-sine DNA-adducts, but also (< 10%) 1,N-propano-deoxi-guanosine, N^2-dimethyldioxane-deoxiguanosine, and a cross-link adduct detected).	2	Wang et al. 2000(70)
DNA-adducts	Calf thymus DNA	1.8 mM for 92 hours; ^{32}P-	+ (N^2-ethyl-3'-dG-monophosphate	3	Fang and Vaca 1995(71)

		postlabeling assay	adducts)		
DNA-adducts	Calf thymus DNA in 2'-deoxy-guanosine-3'-monophosphate	Up to 79,000 µg/ml;	+	3	Fang and Vaca 1997(52)
DNA-protein crosslinks	Calf thymus DNA in 2'-deoxy-guanosine-3'-monophosphate	100, 300 and 1,000 mM for one hour	+	3	Lam et al. 1986(55)
Alkaline elution assay*	*Saccharomyces cerevisiae* (yeast)	0.85 M for 2 or 4 hours	+	3; no positive control; no data on statistical analysis	Ristow et al. 1995(72)
DNA damage	*E. coli polA*	7800 µg/ml	-	3	Rosenkranz, 1977 (20)
DNA repair host-mediated assay, in vivo; multi-substance study	repair-deficient *E.coli* K-12 *uvrB/recA*; tests performed in mice	Highest tested concentration 370 mM/L; - and + S9	- (- and + S9)	3; method not validated	Hellmer and Bolcsfoldi 1992(73)

* Comet assay and alkaline elution assay: DNA single and double strand breaks, DNA cross-links.

** See footnote in Table 10 for explanation of the Klimisch-scores.

Table 19 Summary of genotoxicity studies

Method	Cell type	Concentration	Results and remarks	Klimisch(15) Score*	References
In vitro tests using rodent cells					
Sister chromatid exchange	Different DNA-repair deficient Chinese hamster ovary cells	0.3, 0.6, 1.0, 1.8, 2.5 and 3.6 mM for 2 hours; 250 metaphases scored/group	+	2; no positive control	Mechilli et al. 2008(34)
Sister chromatid exchange	Chinese hamster ovary cells	0, 30, 100 and 300 µM; - S9	+ (dose-dependent increase	2	Brambilla et al. 1986(74)
Sister chromatid exchange	V79 Chinese hamster cells	0.2 – 5 mM	+ (dose-dependent increase)	2; No positive control	Speit et al. 2008(45)
Sister chromatid exchange	Chinese hamster ovary cells	0, 0.8, 2, 4, 7.8, 39.4 and 78 µg/ml; + and – S9; 20 metaphases/sample scored	+, dose-related response	3; no data on cytotoxicity; no positive control	de Raat et al. 1983(75)
Sister chromatid exchange	Chinese hamster ovary cells	$0.25x10^{-3}$, $0.5x10^{-3}$, $1x10^{-3}$, and $1.5x10^{-3}$ % (v/v); - S9; 100	+	3; no positive controls, no data on	Obe et al. 1979(40)

		mitoses scored/ sample		cytotoxicity	
In vitro tests using human cells					
Sister chromatid exchange	Human peripheral lymphocytes	0 – 1,080 µM; -S9; reduction of cell growth noted above 720 µM	+, dose-related response	2; no positive controls	Böhlke et al. 1983(76)
Sister chromatid exchange	Human peripheral lymphocytes	1 – 100 µM	+	2; no positive controls	Knadle 1985(77)
Sister chromatid exchange	Human lymphocytes and fibroblast of normal subjects	40, 400 and 800 µM;	+	3; limited information on test protocol	Véghelyi and Osztovics 1978(78)
Sister chromatid exchange	Human lymphocytes	0, 63, 125, 250 500 and 2,000 µM; -S9	+ (dose-dependent increase)	3; no positive controls; no data on cytotoxicity	Norppa et al. 1985(79)
Sister chromatid exchange	Human lymphocytes	0, 0.0005, 0.001, and 0.002 % (v/v); -S9	+, dose-related response	3; no positive controls; no data on cytotoxicity	Ristow and Obe 1978(69)
Sister chromatid exchange	Human lymphocytes	0 – 500 µM; - S9	+, dose-related response	3; no data on cytotoxicity; no positive controls	Sipi et al. 1992(80)
Sister chromatid exchange	Human peripheral lymphocytes	100 – 400 µM; - S9; exposure performed in capped bottles	+ (dose-dependent increase)	3; no positive controls; no data on cytotoxicity	Helander and Lindahl-Kiessling 1991(81)
Sister chromatid exchange	Human peripheral lymphocytes	$2x10^{-3}$ % (v/v); + or – acetaldehyde metabolizing enzyme ALDH	+	3; no positive controls, no data on cytotoxicity	Obe et al. 1986(82)
Sister chromatid exchange	Human lymphocytes	100 – 2,400 µM; - S9	+ (dose-dependent increase	3; no positive controls used, no data on cytotoxicity	He and Lambert 1985(83)
Sister chromatid exchange	Human peripheral lymphocytes	0 – 0.001% (v/v); - S9	+ (dose-dependent increase)	3; limited information on test protocol	Jansson 1982(84)
Rodents (in vivo somatic cell tests)					
Sister chromatid exchange	Bone-marrow cells of Chinese hamsters (strain not specified)	Single intra-peritoneal injection of 0.01, 0.1 and 0.5 mg/kg bw; 6-7 animals/ dose;	+ at the highest exposure level only; at this level signs of intoxica-tion were noted;	2	Korte et al. 1981(85)

		negative and positive control included	no signs of intoxication at 0.1 and 0.01 mg/kg bw		
Sister chromatid exchange	Male mouse (NIH) bone marrow cells	0.4, 4.0, 40 and 400 mg/kg bw, single intraperitoneal injection	+ (40 and 400 mg/kg bw, *p<0.05*) Mitotic index and average generation time did not differ from control	3; number of mice per group not given; no positive control	Torres-Bezauri et al. 2002(86)
Sister chromatid exchange	Male CBA mouse	Single intraperi-toneal injection of 1 or 0.5 mL of a 10^{-4} % (v/v) solu-tion; one animal/ dose	+	3; low number of animals in study, no positive controls	Obe et al. 1979(37)
Rodents (in vivo germ cell tests)					
Sister chromatid exchange	Mouse spermatogonial cells	Single intraperitoneal injection; 0.4, 4.0, 40 and 400 mg/kg bw; 4 – 5 animals/ concentration; cells were isolated, 53 h after injection.	+ (all doses applied, *p<0.05*); no clear exposure-response relationship observed	2; authors did test for intoxication; concentrations used were considered non-toxic/-lethal	Madrigal-Bujaidar et al. 2002(5)

* See footnote in Table 10 for explanation of the Klimisch-scores.

In vitro studies: DNA damage and genotoxicity

Acetaldehyde caused DNA strand breaks and cross-links in human lymphocytes *in vitro* without metabolic activation, but not in human bronchial epithelial cells and in human leukocytes, or in rodent cells. Acetaldehyde–DNA adducts have been found in vitro in calf thymus DNA and in 2′-deoxyguanosine-3′-monophosphate. It induced dose-dependent sister chromatid exchanges in Chinese hamster ovary cells and human lymphocytes in a wide range of studies. Overall, these studies show the intrinsic property of acetaldehyde to react with DNA in vitro.

In vivo studies

Germ cells

Madrigal-Bujaidar et al. (2002) injected NIH mice (4-5 mice per group) with acetaldehyde at concentrations of 0 (vehicle control), 0.4, 4, 40 and 400 mg/kg bw (single treatment), or cyclophosphamide (positive control).(5) Fifty-three hours later, the animals were killed, and the tunica albuginea was removed from each testes to obtain spermatogonial cells in the seminiferous tubules. A statistically significant increase in the number of cells with sister chromatid exchange

and a clear dose response relationship was reported (30 metaphases per mouse scored; see Table 20). The authors determined a LD_{50}-dose of 560 mg/kg bw.

Somatic cells

Lam et al. (1986) reported on the formation of DNA-protein crosslinks in the nose tissue of male Fischer-344 rats after inhalation exposure.(55) The animals were exposed to acetaldehyde at concentrations of 0,180, 540, 1,800 and 5,400 mg/m^3 for a single six hours, or to 5,400 mg/m^3, 6 hours a day for 5 consecutive days. Immediately after the final exposure the animals were killed, and nasal respiratory mucosa was obtained for further examination. After a single inhalation, a dose dependent increase in DNA-protein crosslinks was observed in the respiratory mucosa, but not in the olfactory mucosa. Short-term repeated inhalation induced DNA-protein crosslinks in the respiratory and the olfactory mucosa.

In bone marrow cells of Chinese hamsters (6-7 animals per group), a single intraperitoneal injection of acetaldehyde increased the number of sister chromatid exchanges at the two highest doses applied (0.1 and 0.5 mg/kg bw; Korte et al., 1981).(85) The authors reported that exposure to concentrations of 0.6 mg/kg bw and higher was lethal.

Table 20 Sister chromatid exchanges in spermatogonial cells of mice treated with acetaldehyde (Madrigal-Bujaidar et al. 2002).(5)

Dose (mg/kg bw)	SCE/cell ± SD	SCE increase
0	1.9 ± 0.16	
0.4	2.9 ± 0.33*	1.1
4	4.1 ± 0.34*	2.2
40	4.6 ± 0.51*	2.7
400	5.1 ± 0.8*	3.2
50 (cyclophosphamide)	6.0 ± 0.1*	4.1

SCE, sister chromatid exchange. * Statistically significant different compared to control, $p < 0.05$.

4.9.4 Summary and discussion of mutagenicity

Below, only data are summarized of reliable (with or without restrictions) experimental design (according to the Klimisch criteria (1997)).(15)

In vitro studies

Numerous data have been presented on the mutagenic and genotoxic properties of acetaldehyde in bacteria and mammalian cells. Overall, negative outcomes were found in bacteria using the reverse mutation assay, whereas most in vitro assays with mammalian cells gave positive outcomes. These included gene mutations, chromosome aberrations, micronuclei, DNA-strand breaks, DNA-adducts, DNA-protein crosslinks, and sister chromatid exchanges in both rodent and human cells (the latter were mainly lymphocytes). In some of these positive studies, also a dose-related response was found.
The only mammalian in vitro assay that gave mainly negative outcomes was the alkaline elution assay. However, two these studies had low reliability, as they tested only one concentration and two

studies reported positive results for DNA cross-links, together with negative results for DNA strand-breaks. The presence of DNA or DNA-protein crosslinks may affect the outcomes of an alkaline elution test.

Taken together, the data show that acetaldehyde can damage DNA directly and induce mutations *in vitro*.

In vivo studies in somatic cells

After inhalation of acetaldehyde, a dose-dependent increase of DNA-crosslinks was found in the respiratory and olfactory mucosa of rats.
Acetaldehyde also induced micronuclei in bone marrow and blood cells in mice and rats, and sister chromatid exchange in the bone marrow of mice and hamsters after intraperitoneal injection.
Gene-mutations and micronuclei were induced in reticulocytes of ALDH2 knock-out mice, after inhalatory or oral administration, but not in wild-type mice.

According to Buddinsky et al. (2013), the key event after acetaldehyde exposure involves Schiff's base formation with DNA and proteins to elicit genotoxicity and/or cytotoxicity. DNA repair, apoptosis and other stress-related adaptive responses, and replacement of proteins or redundancy in protein function all act in opposition of these adducts. This is followed by metabolic deactivation of acetaldehyde via ALDH2. If the action of ALDH2 is sufficient, and when it is combined with DNA repair, apoptosis, and other stress-related responses, no increase in genotoxic outcomes will occur. In vivo, tissue acidification occurs, caused by the production of acetic acid, which adds to the cytotoxicity of DNA and protein adducts. Because of the constant presence of acetaldehyde in cells, the dose-response for mutagenicity will depend on the capacity of cells to maintain homeostatic levels of the agent.

These data suggest that acetaldehyde is a direct acting mutagen in vivo, of which the potential to induce mutations at distant sites depends strongly on the activity of ALDH2.

Data on humans are limited, but show the formation of DNA adducts in white blood cells related to acetaldehyde exposure through alcohol (ab)use and smoking (see Table 17). The available studies also showed that variation in the ALDH2 genotype indeed influenced the occurrence of DNA-adducts (Matsuda et al. 2006 and indirectly via race Chen et al. 2007).

The available kinetic data shows that acetaldehyde can reach the systemic circulation and several organs. The intraperitoneal studies show that when sufficient acetaldehyde reaches the systemic circulation it induces genotoxicity and mutagenicity in vivo. This is confirmed by the inhalation studies by Kunugita (2008) which showed that in animals without ALDH2, which most likely have higher systemic acetaldehyde levels, were positive whereas wild type animals were negative for the induction of micronuclei.

Germ cell genotoxicity

Two animal studies were found on germ cell genotoxicity by acetaldehyde, both in mice. The first is the study by Lähdetie et al. (1988), in which a single intraperitoneal injection of acetaldehyde did not induce meiotic micronuclei in early spermatids nor sperm abnormalities.(6) The second study is published by Mardigal-Bujaidar et al. (2002), and considers the induction of sister chromatid exchanges in mouse spermatogonial cells.(5) Although no clear dose-response relationship could be

assessed, the authors reported that acetaldehyde induced sister chromatid exchanges (see Table 13). This difference in results might be related to a difference in sensitivity between the two assays. In relation to this, degradation of acetaldehyde could be of influence, as Maredigal-Bujaidar showed that blockage of aldehyde dehydrogenase resulted in an increase in SCEs at normally non-genotoxic doses (0.004 and 0.04 mg/kg bw). However, considering these uncertainties and the non-physiological route of exposure, it cannot be concluded that acetaldehyde is genotoxic in germ cells on these studies alone.

4.9.5 Comparison with criteria

Annex VI of CLP states for the hazard class germ cell mutagenicity that "the classification in Category 2 is based on positive evidence obtained from experiments in mammals and/or in some cases from in vitro experiments, obtained from:
- Somatic cell mutagenicity tests in vivo, in mammals; or
- Other in vivo somatic genotoxicity tests which are supported by positive results from in vitro mutagenicity assay"

In vivo in somatic cells, the following effects were observed:
- increases of DNA-crosslinks at local sites after inhalation
- micronuclei and sister chromatid exchanges in bone marrow and blood cells after intraperitoneal injection
- gene-mutations and micronuclei in reticulocytes of ALDH2 knock-out mice, after inhalatory or oral administration
- DNA adducts in humans after exposure through alcohol and/or smoking

These findings are supported by in vitro studies in mammalian cells, which showed gene mutations, chromosome aberrations, micronuclei, DNA-strand breaks, DNA-adducts, DNA-protein crosslinks, and sister chromatid exchanges in both rodent and human cells.
Also the available kinetic information shows that acetaldehyde is systemically available after exposure via relevant routes.

Thus the genotoxic and mutagenic effect of acetaldehyde warrants at least classification in category 2.

According to the criteria in Annex VI of the European regulation No. 1272/2008, classification as a mutagen in category 1 is warranted when positive evidence for *in vivo heritable germ cell* mutagenicity in humans (1A) or mammals (1B) has been reported. No data have been presented on human germ cell mutagenicity, and the only animal germ cell mutagenicity study did not show mutagenic activity (Lähdetie et al., 1988).(6) Overall, due to a lack of data it is concluded that there is no positive direct evidence for in vivo heritable germ cell mutagenicity of acetaldehyde.
In addition, substances may be categorized in 1B if there are "positive results from in vivo somatic cell mutagenicity tests in mammals, in combination with some evidence that the substance has potential to cause mutations to germ cells". The latter may be based on a) "supporting evidence from mutagenicity/genotoxicity tests in germ cells in vivo", or b) "by demonstrating the ability of the substance or its metabolites to interact with the genetic material of germ cells". Sufficient evidence has been found for in vivo mutagenicity testing in somatic cells of mammals. Regarding the second part of the criterion, there is limited evidence that acetaldehyde is genotoxic (sister chromatid exchanges) in germ cells of mice (Madrigal-Bujaidar et al. 2002), when the substance was given by intraperitoneal injection.(5) These findings indicate that acetaldehyde is able to reach

the germ cells, and interacts with the genetic material, which would be in line with the findings on absorption and distribution kinetics. As described in 4.1, acetaldehyde is rapidly taken up after inhalation and oral exposure. In rats, acetaldehyde was distributed in the blood, liver, kidney, spleen, heart, myocardium and skeletal muscle. However, in another animal study no abnormal sperm cells, and no meiotic micronuclei in spermatids were observed at dose levels inducing acute toxicity (Lähdetie et al. 1988).(6)

An important factor for the distribution of acetaldehyde in the body is the activity of the enzyme acetaldehyde dehydrogenase (ALDH2). It is known that this enzyme has a high degree of genetic polymorphism in humans, which influences the occurrence DNA adducts in white blood cells due to exposure to acetaldehyde through alcohol (ab)use and smoking. Thus it cannot be excluded that acetaldehyde may reach the germ cells, especially in humans with a mutated form of ALDH2.

Overall, it is considered that some evidence exists that acetaldehyde has potential to cause mutations in germ cells. Therefore, it is recommended to classify the substance in category 1B.

4.9.6 Conclusions on classification and labelling

Based on the available data, it is recommended to classify acetaldehyde as a germ cell mutagen in category 1B, "substance to be regarded as if they induce heritable mutations in the germ cells of humans".

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

In vitro studies

Data have been presented on the mutagenic and genotoxic properties of acetaldehyde in bacteria and mammalian cells. Overall, negative outcomes were found in bacterial mutagenicity assays, whereas most *in vitro* assays with mammalian cells gave positive outcomes. These included tests for gene mutations, chromosome aberrations, micronuclei, sister chromatid exchanges and DNA-strand breaks. Additionally, acetaldehyde formed DNA-adducts and DNA-protein crosslinks in both rodent and human cells (the latter were mainly lymphocytes). In some of these positive studies, a dose-related response was found.

The only mammalian *in vitro* assay that gave mainly negative outcomes was the alkaline elution assay. However, two of these studies had low reliability (as they tested only one concentration) and two studies reported positive results for DNA cross-links, together with negative results for DNA strand-breaks. The presence of DNA or DNA-protein crosslinks may affect the outcomes of an alkaline elution test.

Overall, the data show that acetaldehyde can damage DNA directly and induce mutations *in vitro*.

In vivo studies in somatic cells

After inhalation of acetaldehyde, a dose-dependent increase in DNA-crosslinks was found in the respiratory and olfactory mucosa of rats. Acetaldehyde also induced micronuclei in bone marrow and blood cells in mice and rats and sister chromatid exchanges (SCE) in the bone marrow of mice and hamsters after intraperitoneal injection. Gene-mutations and micronuclei were induced in reticulocytes of knock-out mice, which had an inactive ALDH2 gene, after inhalatory or oral administration. In the same experiment, mutations and micronuclei were not induced in wild-type mice.

The key event after acetaldehyde exposure involves Schiff's base formation with DNA and proteins to elicit genotoxicity and/or cytotoxicity. DNA repair, apoptosis and other stress-related adaptive responses, and replacement of proteins or redundancy in protein function all act in conjunction to to reduce the impact of the formation of these adducts. This is followed by metabolic deactivation of acetaldehyde via ALDH2. If the action of ALDH2 is sufficient, and when it is combined with DNA repair, apoptosis, and other stress-related responses, no increase in genotoxic outcomes will occur.

In vivo, tissue acidification occurs, caused by the production of acetic acid, which adds to the cytotoxicity of DNA and protein adducts. Because of the constant presence of (endogenous) acetaldehyde in cells, the dose-response for mutagenicity will depend on the capacity of cells to maintain the intracellular acetaldehyde concentration at sufficiently low levels.

These data suggest that acetaldehyde is a direct acting mutagen *in vivo*, of which the potential to induce mutations at distant sites depends strongly on the activity of ALDH2.

Data from humans show the formation of DNA adducts in white blood cells related to acetaldehyde exposure through alcohol abuse and smoking. The available studies also show that variation in the ALDH2 genotype influences the occurrence of DNA adducts.

The available kinetic data shows that acetaldehyde can reach the systemic circulation and several organs. Laboratory studies involving intraperitoneal injection of acetaldehyde to animals show that when sufficient acetaldehyde reaches the systemic circulation it induces genotoxic and mutagenic lesions. This is confirmed by inhalation studies which showed that micronuclei were induced in mice lacking ALDH2, which most likely had higher systemic acetaldehyde levels, but not in wild type mice.

Germ cell genotoxicity

Two animal studies were found on germ cell genotoxicity by acetaldehyde, both in mice. In the study by Lähdetie *et al.* (1988), a single intraperitoneal injection of acetaldehyde did not induce meiotic micronuclei in early spermatids nor sperm abnormalities. The second study, by Mardigal-Bujaidar *et al.* (2002), addressed the induction of SCE in mouse spermatogonial cells. Although no clear dose-response relationship could be established, the authors reported that acetaldehyde induced SCE.

This difference in results might be related to a difference in sensitivity between the two assays. In relation to this, degradation of acetaldehyde could be of influence, as Maredigal-Bujaidar *et al.* (2002) showed that inhibition of aldehyde dehydrogenase activity resulted in an increase in SCEs at normally non-genotoxic doses (0.004 and 0.04

mg/kg bw). However, considering these uncertainties and the nonphysiological route of exposure, it cannot be concluded that acetaldehyde is genotoxic in germ cells based on these studies alone.

Comparison with criteria

According to Annex VI of CLP, classification in germ cell mutagenicity Category 2 is based on positive evidence obtained from experiments in mammals and/or in some cases from *in vitro* experiments, obtained from:

- Somatic cell mutagenicity tests *in vivo*, in mammals; or
- Other *in vivo* somatic genotoxicity tests which are supported by positive results from *in vitro* mutagenicity assay"

In vivo in somatic cells, the following effects were observed:

- increases of DNA-crosslinks at local sites after inhalation
- micronuclei and SCE in bone marrow and blood cells after intraperitoneal injection
- gene-mutations and micronuclei in reticulocytes of ALDH2 "knock-out" mice, after inhalation exposure or oral administration
- DNA adducts in humans after exposure through alcohol ingestion and/or smoking

These findings are supported by *in vitro* studies in mammalian cells, which showed gene mutations, chromosome aberrations, micronuclei, DNA-strand breaks, DNA-adducts, DNA-protein crosslinks, and SCE in both rodent and human cells.

Also, the available kinetic information shows that acetaldehyde is systemically available after exposure via relevant routes. Thus the genotoxic and mutagenic effect of acetaldehyde warrants at least classification in category 2.

According to the criteria, classification in category 1 is warranted when positive evidence for *in vivo* heritable germ cell mutagenicity in humans (1A) or mammals (1B) has been reported. No data have been presented on human germ cell mutagenicity, and the only animal germ cell mutagenicity study did not show mutagenic activity (Lähdetie *et al.,* 1988). Overall, due to a lack of data it is concluded that there is no direct positive evidence for *in vivo* heritable germ cell mutagenicity of acetaldehyde.

In addition, substances may be categorised in 1B if there are "positive results from *in vivo* somatic cell mutagenicity tests in mammals, in combination with some evidence that the substance has potential to cause mutations to germ cells". The latter may be based on a) "supporting evidence from mutagenicity/genotoxicity tests in germ cells *in vivo*", or b) "by demonstrating the ability of the substance or its metabolites to interact with the genetic material of germ cells".

With acetaldehyde, positive results have been found in *in vivo* mutagenicity tests in somatic cells of mammals. Regarding the second part of the criterion, there is limited evidence that acetaldehyde is genotoxic (SCE) in germ cells of mice (Madrigal-Bujaidar *et al*., 2002), when the substance was given by intraperitoneal injection. These findings indicate that acetaldehyde is able to reach the germ cells, and interacts with the genetic material, which would be in line with the findings on absorption and distribution kinetics. Acetaldehyde is rapidly taken up after inhalation and oral exposure. In rats, acetaldehyde was distributed in the blood, liver, kidney, spleen, heart, myocardium and skeletal

muscle. However, in another animal study no abnormal sperm cells and no meiotic micronuclei in spermatids were observed at dose levels inducing acute toxicity.

An important factor for the distribution of acetaldehyde in the body is the activity of the enzyme ALDH2. It is known that this enzyme has a high degree of genetic polymorphism in humans, which influences the occurrence of DNA adducts in white blood cells due to exposure to acetaldehyde through alcohol abuse and smoking. Thus it cannot be excluded that acetaldehyde may reach the germ cells, especially in humans with a mutated form of ALDH2.

Overall, the DS considered that some evidence exists that acetaldehyde has potential to cause mutations in germ cells and proposed classification for germ cell mutagenicity in Category 1B.

Comments received during public consultation

Comments were received during the first public consultation from three MSCAs, two industry trade associations, a US-based industry expert working group on acetaldehyde and a private individual.

Two MSCAs agreed with the proposal to classify in Category 1B. One of them highlighted the *in vivo* germ cell SCE study indicating that acetaldehyde can reach the germ cells and interact with the genetic material. They agreed with the DS that the negative result of the *in vivo* germ micronucleus assay might be related to a lower sensitivity of this study compared to the SCE study.

The remaining MSCA and the private individual agreed that the substance should be classified for mutagenicity, but in Category 2 rather than Category 1B. The two industry associations disagreed with the proposal to classify in Category 1B, but acknowledged that classification in Category 2 may be appropriate. The expert working group proposed that acetaldehyde should not be classified for this end point.

The MSCA that proposed Category 2 presented an independent analysis of the data. They noted that the positive *in vivo* SCE study indicates that acetaldehyde can reach the germ cells and interact with the genetic material. However, the induction of indicator effects (e.g., SCEs) does not necessarily lead to mutations (e.g. due to repair mechanisms). Indeed, acetaldehyde was negative in the *in vivo* mutagenicity test (micronuclei in spermatids, i.p. route of exposure), in which it can be assumed that acetaldehyde reached the germ cells (due to comparable test performance between the two studies). The MSCA also commented that the failure to exclude the possibility that acetaldehyde may reach germ cells, especially in humans with a mutated form of ALDH2, is alone not sufficient to classify in Category 1B. The MSCA concluded that the criteria for Category 1B are not met, as there is no evidence that acetaldehyde has the potential to cause mutations in germ cells, but that Category 2 is appropriate based on positive results in the *in vitro* studies and *in vivo* studies in somatic cells.

Both industry associations conducted an independent assessment of the data and both made similar comments. The *in vivo* SCE study suggested positive effects in germ cells, but it did not show a dose-dependent effect. Furthermore, i.p. injection is not an

appropriate route of exposure and does not reflect normal intake in humans. They also suggested that the biological relevance of this study type (SCE) has been called into question, which led to the deletion of the respective OECD guideline for the *in vitro* SCE assay in 2014. Finally, they stated that the DS's assumption that acetaldehyde will reach the germ cells in humans is not based on robust evidence. However, both industry associations acknowledged the positive *in vitro* studies and the findings in somatic cells. To account for these findings, they suggested that classification in Category 2 may be appropriate.

The US-based working group provided detailed comments. They disagreed with the DS; acetaldehyde should not be viewed as a stochastic genotoxic substance. Most significantly, the expert group commented that the *in vivo* SCE study in germ cells did not provide evidence that acetaldehyde has the potential to cause mutations in the germ cells. They argued that SCE are not mutational end-points and there is a general lack of understanding regarding the mechanism associated with this test. The International Workshop on Genotoxicity Testing (IWGT) has recently identified several experimental protocols for evaluating germ cell mutagenicity; none of these recognised SCE as a legitimate end point for establishing germ cell mutagenicity (Yauk *et al*., 2015). Furthermore, the negative *in vivo spermatid* micronucleus study showed that acetaldehyde does not have mutagenic potential in male germ cells.

The working group commented that according to the CLP criteria and associated guidance, the likely route of exposure should be taken into account when deciding on classification; i.p. injection is an irrelevant route of exposure in humans. They argued that i.p. injection could cause the normal homeostatic mechanisms that protect against mutations from this endogenous agent to be overwhelmed and it is notable that all of the positive *in vivo* mutagenicity studies have employed this exposure route.

The working group also noted that acetaldehyde is a ubiquitous substance in food and beverages (either naturally occurring or intentionally added), and is a product of normal cellular metabolism. Cellular sensitivity to acetaldehyde is determined by intracellular ALDH activity, which varies among cell types. An additional mutational load would only be manifested when physiological concentrations are exceeded. Several papers were cited which provided evidence of a threshold for mutagenicity induced by acetaldehyde, including in an *in vitro* test with human TK cells in which micronuclei were not increased at concentrations below 50 µM acetaldehyde. In contrast, blood acetaldehyde concentrations in wild type mice exposed to 125 µM or 500 ppm acetaldehyde by inhalation 24 hours per day for 14 days were only 1.65 µM or 1.72 µM; i.e., well below this threshold found for micronucleus formation.

Taking all of the above into consideration, the working group concluded that no change in the classification of acetaldehyde is warranted (i.e., the substance should remain 'not classified' for mutagenicity).

According to the private individual who commented, acetaldehyde occurs widely as a trace component in foodstuffs and is also formed endogenously in humans. As such, humans have evolved multiple detoxification mechanisms and are capable of breaking it down quickly once formed. Whilst it may be theoretically possible that the substance could reach more distant organs (e.g., testes and ovaries), there is no experimental

evidence to support this.

This individual discussed the weight that should be applied to the different study types (i.e., greater weight to *in vivo* studies, studies conducted according to standard protocols, and studies that use a physiologically relevant route of exposure, i.e., oral or inhalation). Studies conducted by 'artificial' routes (e.g. i.p. injection) should be interpreted with caution as they bypass important detoxification mechanisms, and this route of exposure is not relevant for humans. Of the somatic cell studies, the *in vivo* micronucleus test in mice reticulocytes should be given the highest weighting. This produced negative results by the inhalation and oral routes in wild type animals. The ALDH "knockout" mouse results should be disregarded for classification purposes; this provides useful information on mode of action but is not a 'natural' situation and not part of standard testing protocol procedures.

The individual considered that the 2 *in vivo* studies in germ cells should be given heavy weighting, although genotoxicity to germ cells cannot be concluded on the basis of these studies alone (one study was negative, and the other showed effects but without a clear dose-response relationship). The individual concluded that based on inconclusive or negative results *in vivo* in germ cells, and negative results *in vivo* in somatic cells by relevant routes of exposure (inhalation and oral), at most the substance should be classified in Category 2.

The second public consultation did not provide any significant specific further information relating to this endpoint.

Assessment and comparison with the classification criteria

In vitro data

Acetaldehyde was not mutagenic to *S. typhimurium* or *E. coli WP2 uvrA*, with or without metabolic activation. However, in the absence of metabolic activation, acetaldehyde induced gene mutations (in human TK6 cells, human lymphocytes, human fibroblasts and mouse lymphoma L5178T cells), chromosome aberrations (in human lymphocytes, Chinese hamster ovary cells, primary rat skin fibroblasts and Chinese hamster embryonic diploid fibroblasts), and micronuclei (in human lymphocytes, HepG2 and Hep3B cells, primary rat skin fibroblasts and Chinese hamster V79 lung cells). Results were generally consistent across studies.

Among an extensive database of additional genotoxicity studies with acetaldehyde, positive results have also been found in the comet assay and tests for SCEs in a variety of mammalian cell cultures. Also, acetaldehyde has induced DNA adducts in mammalian cells. In contrast, negative results have been reported in alkaline elution assays with human bronchial epithelial cells and primary rat hepatocytes.

Although the DS considers that a large number of these studies are 'not reliable', or the reliability 'not assignable' according to the Klimisch categories, RAC concludes there is sufficient information available to conclude that acetaldehyde has mutagenic potential in cultured mammalian cells.

In vivo data – somatic cells

In a well conducted study, Wakata *et al.* (1998), micronuclei were detected in bone marrow erythrocytes and peripheral blood erythrocytes in male Sprague-Dawley and F344 rats given a single i.p. injection of (250 mg/kg bw/d) acetaldehyde. Supporting this study, dose-related increases in micronuclei were found in the bone marrow of male CD-1 mice (0-400 mg/kg bw/d) (Morita *et al.* 1997) and peripheral blood cells of male Han rats (125 or 250 mg/kg bw/d) following administration of acetaldehyde by i.p. injection (Hynes et al. (2002).

These studies demonstrated that acetaldehyde has mutagenic potential in somatic cells. Although the i.p. dose route is non-physiological, it is widely considered acceptable to use this in the *in vivo* mammalian micronucleus test to ensure that the target tissue is adequately exposed. The bone marrow and/or peripheral blood cells are a surrogate for all somatic tissues, including those at sites of initial contact following exposure by physiological routes.

A further study (Kunugita *et al.* 2008) is available in which gene mutations and micronuclei were assayed in mice exposed to acetaldehyde via inhalation (125 or 500 ppm, continuously for two weeks) or orally (100 mg/kg bw/ day for two weeks). This study compared the effect of acetaldehyde in mice genetically engineered to lack the ALDH2 enzyme with that in wild-type mice. Mutations to the T-cell receptor gene (TCR) in lymphocytes and increased numbers of reticulocytes with micronuclei were evident in the "knock-out" mice but not on the wild type. It has been speculated that the knock-out mice in this study represent humans who have a mutated form of ALDH2. However, no data are available on the blood levels/half-life of acetaldehyde in such humans or the knock-out mice; therefore it cannot be judged whether the two are comparable. Although a negative result was found in the wild type mice, this does not detract from the positive results described above from the study that employed the i.p. route of administration.

Potentially genotoxic lesions have been found in the nasal mucosa of male F344 rats exposed to acetaldehyde by inhalation. Dose-dependent increases in the number of DNA-protein crosslinks occurred in the nasal respiratory mucosa following a single inhalation exposure to acetaldehyde (100, 300, 1,000 and 3,000 ppm) and in the nasal respiratory and olfactory mucosa following short-term repeated inhalation (1,000 ppm, 6 hours per day, 5 days per week).

Additional studies reported increased SCE in the bone marrow cells of Chinese hamsters and male mice following administration of acetaldehyde by i.p. injection. These results appear to support the micronucleus test data.

In summary, there is clear evidence that acetaldehyde has the potential to induce genetic damage, including micronuclei, to the somatic cells of laboratory animals. However, only limited data are available to indicate whether systemic exposure following inhalation of acetaldehyde (or oral or dermal uptake) can result in increased acetaldehyde levels and damage at locations distant from the initial site of contact. This is limited to a study in genetically engineered mice lacking the ALDH enzyme in which mutations and micronuclei were seen in circulating lymphocytes and reticulocytes, respectively, following inhalation exposure.

In vivo data – germ cells

Two studies were presented in the CLH report, both of which involved the administration of acetaldehyde to mice by i.p. injection.

The first study investigated the induction of meiotic micronuclei in mouse spermatids

(Lähdetie, 1988). Mice (4 animals per treatment group) were given a single dose of 0, 125, 250, 375 or 500 mg/kg bw acetaldehyde and killed 13 days later. Mice in the positive control groups received cyclophosphamide or adriamycin. In the 500 mg/kg bw group, all animals died due to acute toxicity, whereas all survived at lower doses. Spermatids at stage 1 of mouse spermatogenesis were harvested and investigated by fluorescence microscopy for the presence of meiotic micronuclei (1,000 spermatids scored per animal).

Effect of acetaldehyde on the frequency of micronuclei in early spermatids in mice (following i.p. injection)

Substance	Dose (mg/kg)	No. of mice	Frequency of micronuclei in 1,000 early spermatids (mean ± S.E., range)
Acetaldehyde	0	7	1.57 ± 0.61 (0-4)
	125	4	1.50 ± 0.50 (0-2)
	250	4	1.25 ± 0.48 (0-2)
	375	4	1.00 ± 0.71 (0-3)
	500	-	-
Cyclophosphamide	75	4	4.75 ± 0.75(2-9)
Adriamycin	6	4	4.75 ± 3.77 (0-16)

Compared to the vehicle control, the number of spermatids with micronuclei did not increase after acetaldehyde treatment, whereas there was a clear increase in the positive control samples. In a separate experiment, the author also investigated the sperm morphology in mice treated with acetaldehyde for a short period (62.5, 125 or 250 mg/kg bw/d for 5 days). Acetaldehyde did not decrease sperm count, testis weight or seminal vesicle weight, nor did it induce abnormal sperm. The highest administered dose was lethal to half of the animals treated. The results of this study are clearly negative, and suggest that acetaldehyde does not have mutagenic potential *in vivo* in germ cells. Use of the i.p. route (although not a physiological route of exposure) is assumed to have ensured the highest possible exposure of the germ cells.

The second study investigated the induction of SCE in mouse spermatogonial cells (Madrigal-Bujaidar *et al.,* 2002). This was conducted to determine the effect of disulfiram (a drug which inhibits ALDH enzyme activity and is used in the treatment of alcohol abuse) on the genotoxic potential of acetaldehyde. In the first part of the experiment, male mice (4-7 per group) were injected with acetaldehyde at concentrations of 0, 0.4, 4, 40 and 400 mg/kg bw or cyclophosphamide (positive control). Animals given the top dose of acetaldehyde exhibited piloerection, respiratory failure and lethargy (as part of the preliminary work for the study, the LD_{50} for acetaldehyde was determined to be 560 mg/kg bw). In the second study, dose groups were included in which mice were pre-treated with disulfiram.

Fifty-three hours after dosing, the animals were killed and the tunica albuginea removed from each testis to obtain spermatogonial cells from the seminiferous tubules. The

number of SCE per cell was determined; the results are presented in the table below.

Effect of acetaldehyde on the frequency of SCE in spermatogonial cells in mice (exposed by i.p. injection)

Substance	Dose (mg/kg)	Mice number	$\bar{X}$SCE/cell ± S.D.	SCE increase
Distilled water	-	7	1.9 ± 0.16	n/a
Acetaldehyde	0.4	5	2.9 ± 0.33*	1.1
	4	5	4.1 ± 0.34*	2.2
	40	5	4.6 ± 0.51*	2.7
	400	4	5.1 ± 0.8*	3.2
Cyclophosphamide	50	5	6.0 ± 0.1*	4.1
Additional phase with disulfiram				
Distilled water	-	5	2.2 ± 0.21	
Acetaldehyde	0.004	5	2.2 ± 0.12	0
	0.04	5	2.4 ± 0.12	0.2
Disulfiram	150	5	2.4 ± 0.16	0.1
Disulfiram and acetaldehyde	150 and 0.004	5	2.9 ± 0.19*	0.69
Disulfiram and acetaldehyde	150 and 0.04	5	3.7 ± 0.19*	1.41
Cyclophosphamide	50	5	6.5 ± 0.24*	4.3

- * Statistically significant difference compared to control, $p < 0.05$

At the lowest dose levels of acetaldehyde, no effect on SCE incidence was observed. At doses ≥0.4 mg/kg acetaldehyde, there was a statistically significant increase in the number of cells with SCE in treated mice compared to controls. However, only a marginal increase in SCE frequency was observed as the dose was increased from 4 to 400 mg/kg. In contrast, the variation in the data from animal to animal (standard deviation: S.D.) appeared to increase with dose of acetaldehyde. In animals pre-treated with the ALDH inhibitor, sensitivity to acetaldehyde appeared to increase slightly. The results in the negative and positive controls were similar across experiments.

Although this study does not conform to a regulatory standard, it does appear to have shown that acetaldehyde has the potential to reach the germ cells following i.p, administration and to interact with genetic material.

Interpretation of the biological relevance of the findings with acetaldehyde is not straightforward. In genotoxicity testing, SCEs have previously been used as a potential

indicator of DNA damage but increased incidences of SCE have been reported in rats, mice and humans *in vivo* that have not been exposed to genotoxic substances (e.g., Fischman and Kelly, 1987; Fischman *et al.,* 1996; Silva 1999; Silva *et al.,* 2002). The mechanisms by which they arise are not well understood and as a consequence their significance is unclear. As such, it should not be concluded from this study that acetaldehyde is mutagenic in germ cells, or that it induces abnormal zygotes.

The DS concluded that the SCE study provides limited evidence of acetaldehyde genotoxicity in the germ cells of mice. The DS suggested that the difference in results between the two *in vivo* studies (i.e., negative micronucleus, positive SCE) could be due to a difference in sensitivity between the two assays, or related to the breakdown of acetaldehyde in the body by ALDH. Due to these uncertainties, and to the non-physiological route of exposure, the DS stated that it cannot be concluded that acetaldehyde is genotoxic in germ cells based on these results alone. RAC agrees with this assessment.

RAC considers that the result of the *in vivo* germ cell micronucleus study is negative. The micronucleus test is a well-established assay for the assessment of mutagenicity, and RAC notes that the author was based within an established genetics laboratory (which had published many papers using this technique); this provides confidence in the result. In the SCE study, an increase in the incidence of SCE was observed following i.p. injection. The author of this study is also based within a genetics laboratory, and has published other papers using the SCE technique. However, the mechanism and biological significance of SCE formation is not fully understood, and this parameter does not provide a robust indicator of DNA damage or mutagenicity. Therefore, the results of this study are difficult to interpret in the context of classification for heritable germ cell mutagenicity. Given that it was only a single study and that the dose-response was unclear, RAC concludes that the SCE study does not provide conclusive evidence that acetaldehyde can reach the germ cells and interact with the DNA following exposure via the i.p. route.

Furthermore, there is no direct evidence from the available toxicokinetic data that acetaldehyde reaches the germs cells, testes or ovaries following exposure via oral, dermal or inhalation exposure. Taking into account all the available information, it is not possible to conclude whether the endogenous background levels in testes are increased after exposure to acetaldehyde by these relevant, physiological routes of exposure.

Conclusion and Comparison With Criteria

The DS has proposed classification of acetaldehyde in category 1B for germ cell mutagenicity. During the public consultation, comments were received in favour of classification in either category 1B or category 2, or for no classification for this endpoint.

As discussed above, acetaldehyde has mutagenic potential in mammalian cells *in vitro* and *in vivo*. The reproducible positive results seen in the *in vivo* mouse micronucleus test, supported by numerous *in vitro* mutagenic and clastogenic observations are sufficient to justify classification of this substance in at least category 2 for germ cell mutagenicity. Although it may be possible to identify a threshold for this mutagenic activity, as raised during the public consultation, a case cannot be made for no classification in accordance with the CLP criteria.

Substances which are known to induce heritable mutations or are to be regarded as if

they induce heritable mutations in the germ cells of humans may be classified in category 1. As no data are available from human epidemiological studies, or from *in vivo* heritable germ cell mutagenicity tests in mammals, classification in category 1A would be inappropriate.

Two *in vivo* studies have investigated the effects of acetaldehyde *in vivo* on germ cells. Unfortunately, both have limitations. The micronucleus test employed a robust, well established endpoint but its sensitivity may have been limited due to the small number of animals employed in each dose group and/or the short period between dosing and sampling. On the other hand, regarding the second study, SCE is not regarded as a reliable endpoint for the investigation of mutagenicity or genotoxicity in germ cells. In the SCE study, acetaldehyde an increased frequency of SCE was seen with acetaldehyde compared to the solvent control group, but the magnitude of the effect did not change with a 100-fold increase in dose. In the absence of supporting information, the positive test result is therefore to be interpreted with caution.

Toxicokinetic information shows that acetaldehyde distributes widely in the body, although no direct evidence of germ cell exposure is available. The enzyme ALDH contributes to the detoxification of acetaldehyde in animals. It is polymorphic in humans, but clear information on the consequences of this polymorphism for individuals exposed to acetaldehyde appears to be lacking. Overall, without evidence to the contrary, it appears that acetaldehyde has a rapid metabolism in humans and a short half-life in the body.

Given the lack of a definitive genetic toxicity study in germ cells and the absence of toxicokinetic information to demonstrate that acetaldehyde can reach the relevant target tissues, RAC is not in agreement with the DS about classification in category 1B for mutagenicity. This is illustrated against the relevant criteria in the following table:

Criteria supporting a Category 1B classification	RAC Opinion
Positive results from *in vivo* heritable germ cell mutagenicity tests in mammals, Or:	No data available
Positive results from *in vivo* somatic cell mutagenicity tests in mammals, In combination with:	Yes, mouse micronucleus tests (supported by *in vitro* data)
Some evidence that the substance has potential to cause mutations to germ cells	There are 2 germ cell studies, both maximised acetaldehyde exposure of the target tissues by using the i.p. route of administration, but they gave conflicting results. The more conventional method gave a negative result. The induction of SCE in the second study is not straightforward to interpret. Toxicokinetic and toxicodynamic information, other than the germ cell SCE test, provides only very limited indirect evidence that

	acetaldehyde or its metabolites can reach the germ cells and interact with the genetic material.

RAC conclusion: Criteria for Category 1B are not met; **classification for germ cell mutagenicity in Category 2 is warranted for acetaldehyde**.

4.10 Carcinogenicity

4.10.1 Non-human information

Data on animal carcinogenicity studies are summarized in Table 21.

Table 21 Summary of animal carcinogenicity studies on acetaldehyde exposure.

Species	Design	Exposure levels	Observations and remark	References
Oral administration				
Rats, Sprague Dawley	50 animals/sex/group; animals kept in observation until spontaneous death (last animal died in week 161); gross necroscopy and histopathological examinations.	0 – 50 – 250 – 500 - 1,500 - 2,500 mg acetaldehyde/L drinking water (*ad libitum*; dose in kg/kg bw not given).	*Klimisch-score*: 2 *General*: No difference between control and exposed animals on consumption, body weight and survival. *Lesions*: Number of malignant tumour-bearing animals did not differ significantly from controls; Number of tumours per 100 animals was statistically significantly increased at 50 (females only), and at 2,500 mg/L (males – female – both sexes, **p*<0.05): - 0 mg/L: 34% – 46% – 40% - 50 mg/L: 52% - 82%* - 67% - 2,500 mg/L: 66%*- 78%*- 72% *Remark*: The EFSA noted that the animals may have been infected with *mycoplasma pulmonis*. Therefore, DECOS considers the study of questionable relevance.	Soffritti et al., 2002(87)
Rats, Wistar	10 male animals/ group; study duration 8 months; immuno-histochemistry and histopathological examination of the tongue, epiglottis, and forestomach; no other tissue examined.	0 or 120 mM in drinking water (*ad libitum*; dose in kg/kg bw not given).	*Klimisch-score*: 3 (only one dose used, short exposure period, limited examination of tissues) *General*: No difference between control and exposed animals on consumption, body weight and survival. *Lesions*: No cancerous or dysplastic lesions observed. Microscopic examination revealed hyperplasia in basal layers of squamous epithelia in the examined tissues of exposed animals.	Homann et al., 1997(88)
Rat F344	19-20 male animals/ group: Intraperitoneal injection of DEN1) as	2.5 and 5% (equivalent to 1.66 and 2.75	*Klimisch-score: 3* No increase in the GST-P positive cell foci in the liver	Ikawa et al. 1986 (89)

	initiator, followed by acetaldehyde administration for 4 weeks from 2 weeks after the start of study	mg/kg/day) in drinking water		
Inhalation				
Rats, Wistar	105 animals/sex/ group; six hours/day, five days/week for 28 months; gross necroscopy and histopathological examination.	0 - 1,350 - 2,700 - 5,400 mg/m^3; due to toxicity, the highest exposure level was reduced to 1,800 mg/m^3 over a period of 11 months.	*Klimisch-score*: 2 *General*: lower survival and body weights were observed in exposed animals compared to controls. *Lesions*: exposure induced malignant tumour in the respiratory tract. See main text and Table 17. *Note*: only the respiratory tract was examined for the presence of abnormalities.	Woutersen et al., 1986(8)
Rats	Number of animals not given, exposure for 52 weeks followed by 26 weeks (n=20) and 52 weeks (n=10) recovery	750, 1500, 3000/1000 ppm	*Klimisch-score: 3* Increased incidence of nasal tumors	Woutersen and Feron, 1987 (90)
Hamster, Syrian golden	36 animals/sex/group; seven hours/day, five days/week for 52 weeks, week 53-81, post-exposure period; gross necroscopy and histopathological examination; 6 animals/sex were killed for interim examination.	4,500 mg/m^3 (week 1-9), 4,050 mg/m^3 (week 10-20), 3,600 mg/m^3 (week 21-29), 3,240 mg/m^3 (week 30-44) and 2,970 mg/m^3 (week 45-52); due to considerable growth retardation and to avoid early death, exposures were reduced gradually during experiment.	*Klimisch-score*: 2 (no standard procedure of doses applied) *General*: from week 4 onwards, exposed animals showed significant reduced body weight compared to controls; reduction diminished partly in the post-exposure period. *Lesions*: exposure induced rhinitis, hyperplasia and metaplasia in the nasal, laryngeal and tracheal epithelium. Also laryngeal and nasal carcinomas and polyps were observed; respiratory tract tumours: 0/30–8/29 (male, control-exposed) 0/28–5/29 (female, control-exposed)	Feron et al., 1982(7)
Hamster, Syrian golden	35 animals/group (males only); 7 hours/day, five days/week for 52 weeks, animals killed after 78 weeks; at week 52, 5 animals were killed for interim examination; gross necroscopy and histopathological	0 or 2,700 mg/m^3	*Klimisch-score*: 2 (only one sex used, only one dose applied) *General*: in exposed animals, body weights were slightly lower than in controls. In the last part of the exposure period mortality increased more rapidly in exposed animals than in controls. *Lesions*: no substance-related tumours found. Acetaldehyde induced hyperplastic, metaplastic and	Feron et al., 1979(91)

	examination.		inflammatory changes. *Note*: exposure level may have been too low to induce adverse health effects.	
Dermal exposure				
Rats	14 to 20 animals; subcutaneous injection	(Total) dose not known; repeated injections.	*Klimisch-score*: 4 (data from secondary source; original study in Japanese; no abstract available)) *General*: no data. *Lesions*: spindle-cell sarcomas at site of injections (in four animals that survived the period up to 554 days).	Watanabe and Sugimoto 1956(92)
Intratracheal installation				
Hamsters, Syrian golden	35 animals/sex/group; weekly installations for 52 weeks, experiment was terminated at week 104.	0 or 2% acetaldehyde (installation volume, 0.2 mL)	*Klimisch-score*: 3 (only one dose applied; experiment not performed according to today's standard methods). *General*: no clear effects on body weight or mortality. *Lesions*: No substance-related tumours found. Hyperplastic and inflammatory changes observed in the bronchioalveolar region of exposed animals.	Feron et al., 1979(91)

4.10.1.1 Carcinogenicity: oral

Male and female Sprague-Dawley rats (50 animals/sex/group) were exposed to 0, 50, 250, 500, 1500 and 2500 mg/L acetaldehyde in drinking water (dose in kg bw not given), beginning at six weeks of age (Soffritti et al., 2002).(87) Animals were kept under observation until spontaneous death. In various organs and tissues neoplastic lesions were observed. However, no clear increase in number of tumour-bearing animals was found in any of the exposed groups compared to the control group. The investigators reported a significantly increased total number of tumours (per 100 animals) in groups exposed to 50 mg/L (females only), and 2,500 mg/L (males; females). There was a lack of statistical analysis, and the limited examination of non-neoplastic end-points. Furthermore, the European Food Safety Authority (EFSA) has evaluated the studies performed by the European Ramazzi Foundation of Oncology and Environmental Sciences, who performed this study, and noted that the animals used by this foundation, may have been infected with Mycoplasma pulmonis. This may have resulted in chronic inflammatory changes.(93) For these reasons, the findings of the study are considered of questionable relevance.

Homann et al. (1997) have given male Wistar rats (N=10/group) either water containing acetaldehyde (120 mM) or tap water to drink for eight months.(88) Animals were then sacrificed, and of each animal tissue samples were taken from the tongue, epiglottis, and forestomach. No tumours were observed. However, in these organs, microscopic examination revealed statistically significant hyperplasia of the basal layers of squamous epithelia in rats receiving acetaldehyde (compared to controls). Furthermore, in the three organs of the treated animals, cell proliferation

was significantly increased, and the epithelia were significantly more hyperplastic, than in control animals.

4.10.1.2 Carcinogenicity: inhalation

In a carcinogenicity study by Woutersen et al. (1986), Wistar rats (105 animals/sex/group) inhaled acetaldehyde at a concentration of 0, 750, 1,500 or 3,000 ppm (0, 1,350, 2,700 or 5,400 mg/m^3) for six hours a day, five days per week for a maximum of 28 months.(8) The highest exposure level was reduced progressively over a period of eleven months to 1,000 ppm (1,800 mg/m^3) due to toxicity. The study focussed on lesions in the respiratory tract.

In general, animals exposed to acetaldehyde showed lower survival rates and body weights compared to controls. This was most pronounced in males exposed to the highest concentration of acetaldehyde. Gross examination at autopsy did not reveal acetaldehyde-related lesions, except for decolourisation of the fur and nasal swellings in all exposed groups. Microscopic examination revealed several non-neoplastic lesions in the respiratory tract of males and females, such as: hyperplasia in the respiratory nasal and olfactory epithelium; squamous metaplasia in the respiratory nasal epithelium; and, squamous metaplasia/hyperplasia in the larynx. These lesions were mainly noted in the mid and/or high exposure groups, and were statistically significantly increased compared to controls. No lesions were found in the lungs.

In a second publication on the same study (Woutersen & Feron, 1987), the progression and regression of nasal lesions were studied. Major compound-related nasal lesions found at the end of the exposure period comprised thinning of the olfactory epithelium with loss of sensory and sustentacular cells at all concentrations; this condition was accompanied by focal basal cell hyperplasia in low- and mid-concentration animals. The top concentration group showed hyper- and metaplasia of the respiratory epithelium frequently accompanied by keratinisation and occasionally by proliferations of atypical basal cells and rhinitis in several top-concentration rats (90).

In the nose, also exposure-related neoplastic lesions were observed (see Table 21). It concerned squamous cell carcinoma in the respiratory epithelium of the nose, and adenocarcinomas in the olfactory epithelium. The relative lower tumour incidences in the high exposure groups were explained by the investigators by early mortality due to other causes than cancer. According to the authors, the observations support the hypothesis that nasal tumours arise from degeneration of the nasal epithelium. The same research group reported earlier on degeneration of the olfactory epithelium in rats inhaling acetaldehyde for four weeks, under comparable experimental conditions (Appelman et al., 1986).85

In a separate publication, the same authors reported on the interim results obtained in the first 15 month of the study (Woutersen et al. 1984).(94) In short, nasal lesion were reported in exposed animals, indicating chronic and permanent inflammation.

In a study by Feron et al. (1982), Syrian golden hamsters (n=36/sex/group) inhaled decreasing concentrations of acetaldehyde (from 2,500 ppm to 1,650 ppm (equal to 4,500 to 2,970 mg/m^3)) or clean room air, for seven hours a day, five days per week for 52 weeks.(7) The concentrations were reduced during the study because of considerable growth retardation and to avoid early death. Acetaldehyde induced rhinitis, hyperplasia and metaplasia of the nasal, laryngeal and tracheal epithelium. The exposed animals also developed laryngeal carcinomas with a few laryngeal polyps, and nasal polyps and carcinomas. The incidences of respiratory tract tumours were 0/30 (males, control), 8/29 (males, exposed), 0/28 (females, control) and 5/29 (females, exposed) (see Table 23).

It is noted that the study by Feron et al. supports the findings of the carcinogenicity study by Woutersen et al. (1986) with rats.

Male Syrian golden hamsters (n=35/group) were exposed to 1,500 ppm (2,700 mg/m^3) acetaldehyde combined with weekly intratracheal instillations of benzo[a]pyrene (0.0625, 0.125, 0.25, 0,5 or 1 mg/kg bw) (Feron et al., 1979).(91) The exposure was for seven hours a day, five days per week for 52 weeks. No tumours were found in hamsters exposed to acetaldehyde alone, whereas in animals treated with benzo[a]pyrene alone, or with a combination of acetaldehyde and benzo[a]pyrene, a dose-related increase in respiratory-tract tumours were found.

Table 22 Respiratory tract tumour incidences in rats, which were exposed by inhalation to acetaldehyde for 28 months.(8)

Exposure level (ppm)	**0**	**750**	**1,500**	**3,000-1,000**
Male animals				
Nose:				
Papilloma	0/49	0/52	0/53	0/49
Squamous cell carcinoma	1/49	1/52	*10/53	**15/49
Carcinoma in situ	0/49	0/52	0/53	1/49
Adenocarcinoma	0/49	**16/52	**31/53	**21/49
Larynx: carcinoma in situ	0/50	0/50	0/51	0/47
Lungs: poorly differentiated adenocarcinoma	0/55	0/54	0/55	0/52
Female animals				
Nose:				
Papilloma	0/50	1/48	0/53	0/53
Squamous cell carcinoma	0/50	0/48	5/53	**17/53
Carcinoma in situ	0/50	0/48	3/53	5/53
Adenocarcinoma	0/50	*6/48	**26/53	**21/53
Larynx: carcinoma in situ	0/51	0/46	1/47	0/49
Lungs: poorly differentiated adenocarcinoma	0/53	1/52	0/54	0/54

Fischer exact test: * $p<0.05$, ** $p<0.001$.

Table 23 Respiratory tract tumour incidences in hamsters, which were exposed by inhalation to acetaldehyde for 52 weeks (Feron et al., 1982).(7)

	Incidence of tumours: males		**Incidence of tumours: females**	
	Control	**Acetaldehyde**	**Control**	**Acetaldehyde**
Nose				
Adenoma	0/24	1/27	0/23	0/26
Adenocarcinoma	0/24	0/27	0/23	1/26

Anaplastic carcinoma	0/24	1/27	-	-
Larynx				
Polyp/papilloma	0/20	1/23	0/22	1/20
Carcinoma in situ	0/20	3/23	0/22	0/20
Squamous cell carcinoma	0/20	2/23	0/22	1/20
Adeno-squamous cell carcinoma	-	-	0/22	2/20
Total	0/30	8/29*	0/28	5/29

* Statistical significance (Fisher's exacttest).

4.10.1.3 Carcinogenicity: dermal

Watanabe *et al*. (1956) reported on the induction of sarcomas in rats given acetaldehyde by subcutaneous injections.(92) However, the study design had limitations, such as the small number of animals and the lack of a control group.

4.10.1.4 Carcinogenicity: other routes of exposure

No tumours were found in Syrian golden hamsters (n=35/sex/dose), which were given acetaldehyde by intratracheal installations, weekly or biweekly, for 52 weeks, followed by a recovery period for another 52 weeks (Feron et al., 1979).(91) Doses applied were 0.2 mL of 2% or 4% solutions. In positive controls, which were given benzo[a]pyrene and N-nitrosodiethylamine, a variety of tumours in the respiratory tract were found.

4.10.2 Human information

No human studies addressing the carcinogenicity of acetaldehyde alone have been retrieved from public literature.

In East-Germany, nine cancer cases were found in a factory where the main process was dimerization of acetaldehyde, and where the main exposures were to acetaldol, acetaldehyde, butyraldehyde, crotonaldehyde and other higher, condensed aldehydes, as well as to traces of acrolein.(95, 96) Of these cancer cases, five were bronchial tumours and two were carcinomas of the oral cavity. All nine patients were smokers. The relative frequencies of these tumours were reported to be higher than those observed in the population of East-Germany. A matched control group was not included. The combined exposure with other potential carcinogenic substances, the small number of cases, and the poorly defined exposed population have been considered when evaluating this study.

4.10.3 Other relevant information

Alcohol consumption

Regarding the general population, some investigators suggest a role for acetaldehyde in cancer development (and other disorders) in humans after alcohol consumption, in particular in people with

a genetic predisposition of one of the enzymes that are involved in ethanol metabolism.(2, 3, 97-103) Acetaldehyde is the major metabolite of ethanol (ethyl alcohol).(3,92,96-98) First, ethanol is oxidized by alcohol dehydrogenase (ADH) to acetaldehyde, and subsequently acetaldehyde is converted by aldehyde dehydrogenase (ALDH2) to acetate. Both enzymes show genetic polymorphisms. This means that depending on the genotype, the enzymes may lead to a faster breakdown of ethanol to acetaldehyde, and/or to a slower breakdown of acetaldehyde to acetate. Thus, people having unfavourable genotypes of these enzymes are likely to be exposed internally to higher levels of acetaldehyde after alcohol consumption than would be the case when not having one of these isoenzymes. This would increase the susceptibility to cancer development after alcohol consumption, since it is suggested that acetaldehyde possesses carcinogenic properties (see also Chapter 4.9).

Several studies reported on the association between genetic polymorphism and ethanol-related cancer development, all suggesting a role for acetaldehyde. As a result, a few meta-analyses have been performed to get more clarity. For instance, Chang et al. (2012) performed a meta-analysis to study the association between ADH1B[2] and ADH1C genotypes in head and neck cancer risk.(104) The analysis included twenty-nine studies. According to the authors, having at least one of the fast alleles ADH1B*2 or ADH1C*1 reduced the risk for head and neck cancer (odds ratios: 0.50 (95% confidence interval (CI), 0.37-0.68) for ADH1B*2; 0.87 (95%CI, 0.76-0.99).

Wang et al. (2012) performed a meta-analysis to derive a more precise estimate of the relationship between ADH1C genotypes, and breast cancer risk.(105) Twelve case-control studies were included in the analysis, covering 6,159 cases and 5,732 controls (all Caucasians). The investigators did not find any significantly increased breast cancer risk that could be related to any ADH1C genotype.

Boccia et al. (2009) reported on a meta-analysis to study the relationship between ALDH2 homozygous and heterozygous genotypes, alcohol consumption, and head and neck cancer.(106) The analysis included six case-control studies, covering 945 Japanese cases and 2,917 controls. For the analysis, the investigators used a Mendelian randomization approach. The homozygous genotype ALDH2*2*2 (unable to metabolize acetaldehyde) reduced the risk of head and neck cancer, whereas the heterozygous genotype ALDH2*1*2 (partly able to metabolize acetaldehyde) did significantly increase the risk compared to the homozygous ALDH2*1*1 genotype (able to metabolize acetaldehyde). According to the authors, the reduction of cancer risk in ALDH2*2*2 was most likely explained by the fact that people having this genotype consumed markedly lower levels of alcohol compared to the other genotypes, probably due to discomfort. Therefore, the authors conclude that their study supports the hypothesis that alcohol increases head and neck cancer risk through the carcinogenic action of acetaldehyde.

The same results were obtained by Fang et al. (2011), who carried out a meta-analysis of ALDH2 genotypes and esophageal cancer development.(107) Data from sixteen studies (hospital- or population-based, one multicenter study) were analysed, covering 2,697 Asian cases and 6,344 controls. The analysis showed that the heterozygous ALDH2*1*2 genotype increased the risk of esophageal cancer, whereas the homozygous ALDH2*2*2 genotype reduced the risk.

[2] ADH has seven isoenzymes, which are divided into five classes. Most relevant for alcohol metabolism in the liver of adults are the class one isoenzymes ADH1B and ADH1C (formerly known as ADH2 and ADH3 isoenzymes).104. Chang JS, Straif K, Guha N. The role of alcohol dehydrogenase genes in head and neck cancers: a systematic review and meta-analysis of ADH1B and ADH1C. Mutagenesis. 2012;27(3):275-86. For each isoenzyme two or three different alleles are known, leading to different genotypes and thus to functional polymorphism. The genotypes of the isoenzyme ADH1B are expressed as ADH1B*1, ADH1B*2 and ADH1B*3; those for the isoenzyme ADH1C are expressed as ADH1C*1 and ADH1C*2. The metabolic speed is highest for homozygote genotypes ADH1B*2, ADH1B*3 and ADH1C*1. ADH1B*1 and ADH1C*2 are considered slow metabolisers.

Yokoyama and Omori (2005) reviewed a number of case-control studies (including those performed by themselves) on the relationship of genetic polymorphism of ADH1B, ADH1C and ALDH2 genotypes and esophageal, and head and neck cancer risk.(108) They found positive associations between the less-active ADH1B*1 genotype and inactive heterozygous ALDH2*1*2 genotype, and the risk for esophageal cancer in East Asian heavy drinkers. Light-to-moderate drinkers showed a higher vulnerability. According to the authors, some studies suggest similar associations for the risk for head and neck cancer in moderate-to-heavy-drinking Japanese. Data on ADH1C genotype were controversial.

It has to be emphasized that in none of the studies on genetic polymorphism and alcohol-related cancer risk, a direct association was found between acetaldehyde and cancer, although the indirect data are suggestive for this.

Cell transformation tests

Koivisto and Salaspuro (1998) reported on a transformation test in which human colon adenocarcinoma cell line Caco-2 were used to study changes in cell proliferation, cell differentiation, and adhesion due to exposure to acetaldehyde.(109) In the absence of cell cytotoxicity, on acute exposure (for 72 hours), acetaldehyde (0.5 or 1 mM) inhibited the cell proliferation rate, but on chronic exposure (for five weeks) it stimulated cell proliferation. Furthermore, acetaldehyde clearly disturbed the cell differentiation (concentration applied was 1 mM for 7, 14 or 21 days); and, a clear decrease of adhesion of Caco-2 cells to collagens was observed when acetaldehyde was applied to the cells at a concentration of 0.5 or 1 mM for four days. According to the authors, the increased proliferation rate, disturbed differentiation, and reduced adhesion, would *in vivo* predict more aggressive and invasive tumour behaviour.

Eker and Sanner (1986) used a rat kidney cell line in a two-stage cell transformation assay.(66) Acetaldehyde (up to 3 mM) did not affect cytotoxicity nor did it induce colony formation of the cells. When acetaldehyde treatment (3 mM) was followed by a tumour promoter 12-O-tetradecanoylphorbol-13-acetate (TPA), the ability of the cells to form colonies was increased.

In a poorly reported study by Abernathy et al. (1982), acetaldehyde (10 – 100 µl/ml (LC_{50}, 25 µg/ml)) induced cell transformation in C3H/10T½ cells, in the presence of TPA.(65) Treatment with acetaldehyde alone did induce transformed foci.

It should be emphasized that the value of transformation test in assessing carcinogenic potential is under debate. Therefore, little value is attached to the outcomes of these tests.

4.10.4 Summary and discussion of carcinogenicity

Epidemiological studies are not available. In the literature, it is suggested that acetaldehyde may play a role in cancer development in humans after alcohol consumption, in particular in combination with a genetic predisposition for enzymes that convert ethanol in acetaldehyde, and for enzymes that convert acetaldehyde in acetate. It should be emphasized that in none of the studies on genetic polymorphism and alcohol-related cancer risk, a direct association was found between acetaldehyde and cancer, although the indirect data are suggestive for this.

Regarding animal carcinogenicity studies, chronic inhalation of acetaldehyde induced squamous cell carcinomas and adenocarcinomas in the nose of male and female rats. In hamsters, inhaling the substance, one study showed the presence of laryngeal and nasal tumours, whereas in another study - using a lower exposure concentration - no tumours were observed at all.

4.10.5 Comparison with criteria

For epidemiological data, there is little or no data to support statements concerning an association between exposure to acetaldehyde and cancer. Therefore, it is considered that human data are insufficient to make a final conclusion on the carcinogenic potential of acetaldehyde in humans. For animal data, there is sufficient evidence of carcinogenicity, since a causal relationship was established between malignant tumours in animals and chronic inhalation to acetaldehyde in two studies (Woutersen et al. 1986, Feron et al. 1982), the main route of exposure in an occupational environment.(7, 8) According to the CLP classification criteria, acetaldehyde should, therefore, be classified as "presumed to have carcinogenic potential for humans", which corresponds to classification in category 1B. Supporting evidence for its carcinogenic potential is that the substance has mutagenic properties.

4.10.6 Conclusions on classification and labelling

It is concluded that acetaldehyde is *presumed to be carcinogenic to man*, and recommended to classify the substance in category 1B.

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

The DS presented evidence from several animal carcinogenicity studies, a brief summary of an epidemiological study involving workers at a factory in the former East Germany, and a discussion of the potential role of acetaldehyde formation in cancer in the general population related to alcohol consumption.

Animal carcinogenicity studies

In a carcinogenicity by Woutersen *et al.* (1986), **Wistar rats** inhaled acetaldehyde at different concentrations for six hours a day, five days per week for a maximum of 28 months. Exposed animals showed lower survival rates and body weights compared to controls. This was most pronounced in males exposed to the highest concentration (3,000 ppm). Gross examination at autopsy did not reveal acetaldehyde-related lesions, except for decolourisation of the fur and nasal swellings in all exposed groups. Microscopic examination revealed several non-neoplastic lesions in the respiratory tract, namely hyper- and metaplasia of the respiratory epithelium of males and females. These lesions were mainly noted in the mid and/or high exposure groups and were statistically significantly increased compared to controls. No lesions were found in the lungs. Major exposure-related nasal lesions were found at the end of the exposure period, which comprised thinning of the olfactory epithelium with loss of sensory and sustentacular cells at all concentrations. Exposure-related neoplastic lesions were observed in the nose. The relative lower tumour incidences in the high exposure groups were explained by early

mortality due to other causes than cancer. In a follow-up publication, the same authors reported on the interim results obtained in the first 15 months of the study. Nasal lesions were reported in exposed animals, indicating chronic and permanent inflammation.

In a carcinogenicity study published in 1982 (Feron *et al*., 1982), **Syrian golden hamsters** inhaled various concentrations of acetaldehyde or clean room air, for seven hours a day, five days per week for 52 weeks. Acetaldehyde induced rhinitis, hyperplasia and metaplasia of the nasal, laryngeal and tracheal epithelium. The exposed animals also developed laryngeal carcinomas with a few laryngeal polyps, nasal polyps and carcinomas.

In another study, published in 1979 (Feron, 1979), male **Syrian golden hamsters** were exposed by inhalation to 1,500 ppm (2,700 mg/m^3) acetaldehyde alone or combined with weekly intratracheal instillations of benzo[a]pyrene. No tumours were found in hamsters exposed to acetaldehyde alone.

Further studies have involved oral exposure via drinking water

In a carcinogenicity study from 2002 (Soffritti *et al.*, 2002), with male and female **Sprague-Dawley rats**, there were no clear increases in the number of tumour-bearing animals in any of the exposed groups compared to the control group. A significantly increased total number of tumours (per 100 animals) in groups exposed to 50 mg/L (females only), and 2,500 mg/L (males; females). There was a lack of statistical analysis, and the limited examination of non-neoplastic end-points. For these reasons, the findings of the study are considered of questionable relevance.

In another study (Homann *et al.*, 1997) with exposure via drinking water, male **Wistar rats** were exposed to acetaldehyde for eight months. No tumours were observed in tongue, epiglottis and forestomach. Cell proliferation was significantly increased in these three organs, and the epithelia were significantly more hyperplastic than in control animals.

Additionally, no tumours were found in **Syrian golden hamsters** given acetaldehyde by intratracheal installation weekly or biweekly for 52 weeks, followed by a recovery period for another 52 weeks (Feron, 1979).

Human information

In a study conducted in the former East Germany, nine cancer cases were found in a factory where the main process was dimerization of acetaldehyde. However, given the combined exposure with other potential carcinogens, the small number of cases, and the poorly defined exposed population, no firm conclusions were taken from this study. No other human studies addressing the carcinogenicity of acetaldehyde alone were retrieved from public literature.

The DS summarised several studies investigating genetic polymorphism and alcohol-related cancer risk, and emphasised that a direct association between acetaldehyde exposure and cancer was not reported in any of these studies. However, the DS commented that indirect data are suggestive for the carcinogenicity of acetaldehyde.

Conclusion

According to the DS there were no reliable epidemiological data available to inform on the carcinogenicity of acetaldehyde to humans. Acetaldehyde may play a role in cancer

development in humans after alcohol consumption, in particular in combination with genetic predisposition for enzymes that convert ethanol to acetaldehyde, and acetaldehyde to acetate. It should be emphasised that in none of the studies on genetic polymorphism and alcohol-related cancer risk, a direct association was found between acetaldehyde formation and cancer, although the indirect data are suggestive of this.

Regarding animal carcinogenicity studies, chronic inhalation of acetaldehyde induced squamous cell carcinoma and adenocarcinoma in the nose of male and female rats. In a study in hamsters, inhalation of acetaldehyde led to the presence of laryngeal and nasal tumours, whereas in another study (at lower exposure concentration) no such tumours were seen. In conclusion, there is little or no epidemiological data to support statements concerning an association between exposure to acetaldehyde and cancer. Therefore, it is considered that human data are insufficient to make a final conclusion on the carcinogenic potential of acetaldehyde in humans. In laboratory studies, there is sufficient evidence of acetaldehyde carcinogenicity, indicated by a causal relationship between malignant tumours in animals and chronic inhalation to acetaldehyde, the main route of exposure in an occupational environment. According to the DS, acetaldehyde should be classified as "presumed to have carcinogenic potential for humans", which corresponds to classification in category 1B. Supporting evidence for its carcinogenic potential is that the substance has mutagenic properties.

Comments received during public consultation

Three member states supported the proposal to classify acetaldehyde as a category 1B carcinogen.

One MS made some additional comments for consideration. They noted that in the 1986 rat study (Woutersen *et al.*, 1986) doses in the top dose group where reduced over time but the differences in body weights, between control group, top dose group and partly the mid dose group exceeded the value of approximately 10% reduction in body weight gain clearly. They recommended discussing the relevance of the lower survival rate of the top dose group. Furthermore, they considered that acetaldehyde likely induced tumours via a local genotoxic activity as indicated from mutagenic properties in somatic cells and the production of DNA protein cross links in cells at the sites of exposure. Also, there are differences in enzyme activities of ALDH in different regions of the respiratory tract. Its activity is more than 2-fold higher in the respiratory epithelium than in the olfactory epithelium. At high concentrations of intracellular acetaldehyde, ALDH activity will not be sufficient to oxidise all acetaldehyde to acetic acid and acetaldehyde may accumulate. Saturation of metabolism of acetaldehyde by ALDH indicating limited enzyme capacity is suggested to occur at acetaldehyde concentrations of 300 ppm (Stanek and Morris, 1999). They mentioned the ubiquitous occurrence of ALDH in organs/tissues (including the upper gastrointestinal tract) with regards to the human relevance of animal data.

Three industry associations proposed no change in the current classification for carcinogenicity, arguing that there is limited relevant new data available. In one set of comments, it was stated that particularly for an endogenous, ubiquitous compound like acetaldehyde, it is critically important to consider latest version of the CLP guidance (June 2015). The CLP guidance provides for a Category 2 classification of substances that induce cancer through excessive toxicity leading to cell death with associated regenerative hyperplasia. Acetaldehyde fits the description of such a substance as it is a skin, eye and respiratory tract irritant. The nature of acetaldehyde's nasal injury following

chronic inhalation exposure at high concentrations suggests degenerative changes initially followed by hyperplastic and metaplastic transformation, along with cell proliferation at higher exposure concentrations; these changes precede tumour development. Indeed, all concentrations of acetaldehyde in the rat inhalation studies induced chronic tissue damage in the respiratory tract. They cited Woutersen *et al.* (1986) who concluded that "These observations strongly support the hypothesis that the nasal tumours arise from epithelium which is damaged by acetaldehyde, via the olfactory epithelium in the low concentration group and both the olfactory and the respiratory epithelium in the mid- and top-concentration groups." Carcinogenicity of acetaldehyde in laboratory animals was a multistep process involving local cytotoxicity with regenerative cell proliferation as a key step.

One individual noted that a category 2 classification seems more appropriate taking into account that there are no reliable studies by the oral or dermal routes of exposure for this end point and only two reliable studies by the inhalation route, one each in hamsters and rats. All the studies were from the 1970s/1980s and therefore none are likely to meet current protocols. The rat study shows significant effects in the nose and not the larynx and the hamster study shows the reverse. The test doses in both studies exceeded the MTD, and therefore the high dose findings should not be taken into consideration. It was pointed out that in the Feron *et al.* (1982) study no individual tumour reached statistical significance. Statistical significance was only reached (males only) when all tumours were combined. In the study published by Woutersen and co-authors (1986), the only statistically significant findings in both males and females were nasal adenocarcinomas (seen at all dose levels). Squamous cell carcinoma of the nose reached statistical significance in males only at the mid dose level. The final conclusion in this comment was that there is not sufficient evidence to warrant a classification as Category 1B.

Additional key elements

In a study on the effects of acetaldehyde inhalation on rat pulmonary mechanics male **Wistar rats** were exposed to 243 ppm acetaldehyde for 8 hours a day, 5 days a week during 5 weeks. The authors report on intense subacute inflammatory reactions with olfactory epithelium hyperplasia and polymorphonuclear and mononuclear infiltration of the submucosa. Histopathological preparations failed to demonstrate any meaningful change other than in the nasal cavities. Study of the lower respiratory tract and the pulmonary parenchyma showed no differences between the control and exposed animals. In mice erosion and degeneration of the nasal and laryngeal epithelium was found after exposure to 125 ppm acetaldehyde for 24 hours over 14 consecutive days.

Several studies addressed genotoxic endpoints (DNA protein crosslinks [DPX] and adduct formation) following inhalation exposure of mice and rats to acetaldehyde. In one study, DPX were found in rat respiratory mucosa after exposure to 50 and 1,500 ppm for 6 hours a day, 5 days a week over 65 days. However, no dose dependency was established in this study and no increase in DPX was found after exposures to 150 and 500 ppm. Another study showed significant increase of DPX in the respiratory mucosa of rats after single exposure for 6 hours to 1,000 ppm acetaldehyde and in olfactory mucosa after repeated exposure for 6 hours daily on five consecutive days. However, yet another study found no significant increase of DPX in rat respiratory epithelium after a single

exposure for 6 hours to 1,500 ppm. After continuous exposure over 14 days three times more DNA adducts were found in the nasal epithelium of ALDH2 deficient mice compared to mice with a functional ALDH2 gene.

Assessment and comparison with the classification criteria

RAC agrees with the DS that the classification of acetaldehyde should be based essentially on evidence presented in animal carcinogenicity studies, also taking into account it's mutagenic and genotoxic potential.

There are no epidemiological studies available to assess whether humans exposed to acetaldehyde are at increased risk of cancer. An epidemiological study from former East-Germany reported nine cancer cases in workers at a factory where the main process was dimerization of acetaldehyde. However, as described by the DS, the study was highly confounded by smoking, multi-substance exposure and lack of control data. Therefore, the study is regarded as unreliable and irrelevant for classification.

At present, acetaldehyde is classified in Category 2 for carcinogenicity. This classification dates back to 1991 and was based on the data from the studies presented by the DS in their proposal. RAC's assessment of these studies follows below.

Inhalation exposure

Study	Tumour findings		Other findings and study limitations
Rat, Wistar Wouterson, 1986; additional information provided in Wouterson and Feron, 1987 28 month exposure 6 h/day, 5 days/week. Interim groups exposed for 52 weeks, with up to 52 weeks recovery. Initial exposure groups: 0 - 750 – 1,500 – 3,000 ppm; due to toxicity, the highest exposure level was reduced to 1,000 ppm over a period of 11 months.	Nasal tumours in animals of all exposed groups originating from olfactory epithelium males: papilloma 0/49-0/52-0/53-0/49 squamous cell carcinoma 1/49-1/52-10/53-15/49 carcinoma *in situ* 0/49-0/52-0/53-1/49 adenocarcinoma 0/49-16/52-31/53-21/49 females: papilloma 0/50-1/48-0/53-0/53 squamous cell carcinoma 0/50-0/48-5/53-17/53 carcinoma *in situ* 0/50-0/48-3/53-5/53 adenocarcinoma 0/50-6/48-26/53-21/53 Laryngeal tumour (carcinoma *in situ*) observed: Males 0/50-0/50-0/55-0/52 Females 0/51-0/46-1/47-0/49 Lungs (poorly differentiated adenocarcinoma): Males 0/55-0/54-0/55-0/52 Females 0/53-1/52-0/54-0/54		Hyper-/metaplasia in respiratory/ olfactory epithelium and larynx of animals in exposed groups No lesions in lungs, no substance related neoplasms outside respiratory tract Lower body weights and survival in exposed animals Follow up information: -increased incidence of nasal tumours confirmed; -observed regeneration of the olfactory epithelium in low and mid dose groups High and mid dose clearly exceed MTD (bw gain~10% lower than in controls) Nasal swellings in all exposed groups Non-standard protocol

Hamster, Syrian Golden Feron *et al*, 1982 52 week 7 h/day, 5 days/week, plus 29 weeks recovery Exposure groups of 0 and 2,500 ppm, exposures reduced gradually during experiment to 1650 ppm	Nasal tumours males, adenoma 0/24-1/27 males, adenocarcinoma 0/24-0/27 males, anaplastic carcinoma 0/24-1/27 females, adenoma 0/23-0/26 females, adenocarcinoma 0/23-1/26 Laryngeal tumours males, polyp/papilloma 0/20-1/23 males, carcinoma *in situ* 0/20-3/23 males, squamous cell carcinoma 0/20-2/23 females, polyp/papilloma 0/20-1/20 females, carcinoma *in situ* 0/22-0/20 No substance related tumours in other tissues		Only 1 exposure group. Increased early mortality in exposed group (*data not provided by DS*). Severe irritation/ inflammation of exposed tissues - rhinitis, hyper- and metaplasia. Significantly reduced body weights in exposed animals Statistical significance only for all male laryngeal tumours (including polyp/papilloma) combined. Non-standard protocol
Hamster, Syrian Golden Feron, 1979 52 weeks + 26 recovery Exposure groups of 0 and 1,500 ppm	No substance related tumours		Only one dose/ sex Hyper- and metaplasia in examined tissues Inflammatory/ hyperplastic changes in bronchoalveolar region of exposed animals (intratracheal exposure) Slightly reduced body weights in exposed animals, in the last part of exposure period mortality increased more rapidly in exposed animals Non-standard protocol

In the Wistar rat study (Wouterson *et al.*, 1986), the authors reported nasal swellings and hyper- and metaplasia in the respiratory and olfactory as well as laryngeal epithelium in exposed animals. Animals of all exposed groups showed increased mortality and growth retardation compared to control-group. The high and mid doses exceeded the MTD and the exposure concentrations in the high dose group had to be reduced. After 102 weeks, all top-concentration rats had died. When the study was terminated after 121 weeks, in the mid-concentration group only about 20% of the animals were still alive compared to 40% males and 50% females in the control group. Squamous cell carcinoma was seen in males in all dose-groups as well as in the control-group. Due to accompanying pathological changes, the findings in the low dose group appear to have been unrelated to treatment. Adenocarcinomas derived from the olfactory epithelium were found in all exposed groups in males and females. The incidence of this tumour was

highest in the mid dose group. In the low concentration group the incidence was higher in males than in females. There were no treatment-related neoplasms found in organs outside the respiratory tract in this study. The earliest nasal tumours were not only found in rats receiving the highest concentration of acetaldehyde but also in the low- and mid-concentration groups, suggesting that the latency period of nasal tumours was independent of the acetaldehyde exposure concentration.

Although acetaldehyde produced a dose dependent carcinogenic response in this study, the relevance of the data from the high and mid dose groups to humans is uncertain given the high level of toxicity observed. However, as noted by the study authors, rodents are obligatory nose breathers whilst humans are likely to inhale through the mouth and nose. As such, the observed effects may be over-predictive for human nasal tissue but could be more relevant for a cancer hazard in distal parts of the human respiratory tract.

The studies in Syrian golden hamsters show that very high exposure concentrations of acetaldehyde may present a cancer hazard in this species. Whilst no increased tumours were seen in the first study (exposure level 1,500 ppm), a slight increase in laryngeal tumours was evident in males in the second study (exposure level 2,500 ppm, reduced to 1,650 ppm during the study due to increased early mortality). Given the very slight increase in tumour frequency in this study, it seems likely that repeated exposure to highly irritant, toxic concentrations of acetaldehyde was a factor in the carcinogenic response seen.

In conclusion, high inhalation exposures in rats and hamsters have been found to produce increased tumour rates at sites of initial contact with the body.

Oral exposure

The only available investigation of carcinogenicity following long-term oral exposure to acetaldehyde involved its application in drinking water to Sprague-Dawley rats. The key findings from this study are summarised in the following table; additional details to those provided by the DS were taken from Scientific Committee on Consumer Safety (SCCS) Opinion on Acetaldehyde published in 2012.

Study	Tumour findings	Other findings and study limitations
Rat, Spraque-Dawley Soffritti *et al*, 2002 Study design: treatment for 104 weeks; terminated at when last animal died at 161 weeks. Acetaldehyde in drinking water ad libitum, dose groups: 0 - 50 - 250 - 500 - 1500 - 2500 mg/L (equivalent to approx. 5,25,49,147 and 246 mg/kg in males)	Number of total malignant tumour-bearing animals did not increase in exposed groups when compared to the concurrent control. One tissue-specific, but not dose-related increase in tumour incidence significant in low and high dose groups were cranial osteosarcoma: 0/50-5/50-1/50-2/50-0/50-7/50	Study design not guideline compliant. Dosing may have been compromised by volatility of test substance. No difference in survival or body weight gain observed between groups. Findings in other tissues not dose dependent and not statistically significant DS commented that EFSA in their evaluation noted the rats may have been infected with *Mycoplasma pulmonis*.

There were no significant differences in food consumption, behaviour, body weight or survival in the exposed groups of rats compared to the control group. There was no significant difference in the number of total malignant tumour-bearing animals between exposed animals and control group. However, there was a statistically significant increased incidence of cranial osteosarcomas at the lowest and highest doses in males only. Findings in other tissues were neither dose dependent nor statistically significant. Although this study was conducted under GLP-conditions, it does not meet the standard protocol defined in OECD guideline 451. The extension of the study duration to allow for the natural lifetime of all the subject animals makes the tumour findings in the cranium difficult to interpret. Given the absence of a dose-response relationship, it seems unlikely that they were treatment related. Moreover, findings are poorly reported and several international bodies (including EFSA) have noted that the rats in this study may have been infected with *Mycoplasma pulmonis*. In RAC's opinion, this study is of questionable relevance and does not provide any reliable evidence of acetaldehyde carcinogenicity.

The DS also presented a drinking water study (Homann *et al.*, 1997) in male Wister rats of 8 months duration. This included only a single dose group, receiving water containing 120 mmol/L acetaldehyde *ad libitum*. The only tissues investigated at the end of the study were the tongue, epiglottis and forestomach. Microscopic examination revealed increased cell proliferation and statistically significant hyperplasia in the basal layers of squamous epithelia of these tissues in the treated rats, but no cancerous or dysplastic lesions were seen. No meaningful conclusions about the carcinogenicity of acetaldehyde can be made from this study.

A mechanistic study in which Fischer F344 rats received acetaldehyde in the drinking water for 4 weeks after an intra-peritoneal injection of the tumour initiator diethynitrosamine was also included in the CLH report. No acetaldehyde -related increase

was seen in the GST-P positive rat liver cell foci in this study; it is uninformative about the potential carcinogenicity of acetaldehyde.

Other routes of exposure

The study of acetaldehyde inhalation in Syrian golden Hamsters (Feron, 1979) also included groups of 35 animals given 52 weekly intratracheal installations of a solution containing 0.2% acetaldehyde or a sample without acetaldehyde. After a further 52 weeks, the study was terminated. There were no clear effects on survival or body weight. Although hyperplastic and inflammatory changes were observed on the bronchio-alveolar region of the lungs in exposed animals, no acetaldehyde-related tumours were reported. Increased tumour incidences were seen in positive control groups that received the carcinogens benzo(a)pyrene or diethylnitrosamine.

A study from 1956 (Watanabe *et al.*, 1956) involved repeated subcutaneous injections of acetaldehyde being administered to rats (doses not known). Apparently spindle cell sarcoma was evident at the injection sites, but insufficient details about the study protocol and the results are available to enable a robust assessment of this study.

Comparison with criteria

Given that there are no epidemiological data showing a carcinogenic response in humans, classification in Category 1A would be inappropriate.

It then has to be considered whether the tumour findings in animals exposed to acetaldehyde justify classification in category 1B or category 2. RAC notes that acetaldehyde is already classified in Category 2, but provides here an independent assessment of the findings against the criteria provided in the CLP Regulation.

To be considered a Category 1B carcinogen, acetaldehyde should show a carcinogenic hazard in animals that can be presumed of clear relevance to humans. Classification of a substance in this category depends on strength of evidence and is warranted when a causal relationship has been established between the substance and an increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms. However, a single positive carcinogenicity study in one species and sex in combination with positive *in-vivo* somatic call mutagenicity data would be considered to provide sufficient evidence of carcinogenicity. In contrast, a substance shall be classified as a Category 2 carcinogen when the available human and/or animal evidence is not sufficient for Category 1A or 1B. Such evidence may be derived either from limited evidence of carcinogenicity in human studies or from limited evidence of carcinogenicity in animal studies.

The clearest evidence of acetaldehyde carcinogenicity is found in the rat inhalation study conducted in 1986 (Woutersen et al., 1986). This showed a statistically significantly increased incidence of nasal adenocarcinomas in all exposed groups in males and females, but animals of mid and high dose groups showed significantly increased mortality and growth retardation compared to the controls. Therefore, RAC considers only the findings in the low dose group to be reliable.

As discussed above, there are no other studies in which a clear carcinogenic response to acetaldehyde was found. There was an indication in hamsters of increased laryngeal cancer, but the low numbers of animals affected and a lack of a dose-response relationship prevent a firm conclusion from being reached.

The mechanistic basis for the increased incidence of tumours only at the initial site of

contact with acetaldehyde in exposed animals has not been established. It is possible that both the irritant nature of acetaldehyde and its genotoxicity were key factors.

In both carcinogenicity studies by the inhalation route, tumours were found at acetaldehyde concentrations which were clearly irritating to the nasal and laryngeal tissue (≥ 750 ppm). Lower concentrations were not tested. Erosion and degeneration of the nasal and laryngeal epithelium was seen in mice after exposure to markedly lower concentrations (125 ppm) of acetaldehyde. In rats same is true for inflammation and histological changes in the nasal epithelium (243 ppm). However, some studies also indicate genotoxic effects at these low concentrations. In combination with the findings on the mutagenic properties of acetaldehyde, a genotoxic mechanism of tumour formation cannot be ruled out.

Therefore, considering tumours in two species and sexes, genotoxic responses at low doses and mutagenic properties in somatic cells, RAC concludes, concurring with the DS, that there is sufficient data to categorise acetaldehyde as **Carc. 1B (H350)**.

4.11 Toxicity for reproduction

Not evaluated in this dossier.

4.12 Other effects

Not evaluated in this dossier.

5 ENVIRONMENTAL HAZARD ASSESSMENT

5.1 Degradation

Not evaluated in this dossier.

5.2 Environmental distribution

Not evaluated in this dossier.

5.3 Aquatic Bioaccumulation

Not evaluated in this dossier.

5.4 Aquatic toxicity

Not evaluated in this dossier.

5.5 Comparison with criteria for environmental hazards (sections 5.1 – 5.4)

5.6 Conclusions on classification and labelling for environmental hazards (sections 5.1 – 5.4)

6 OTHER INFORMATION

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