Committee for Risk Assessment

RAC

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

Adopted

16 September 2016

16 September 2016

CLH-O-0000001412-86-120/F

OPINION OF THE COMMITTEE FOR RISK ASSESSMENT ON A DOSSIER PROPOSING HARMONISED CLASSIFICATION AND LABELLING AT EU LEVEL

In accordance with Article 37 (4) of Regulation (EC) No 1272/2008, the Classification, Labelling and Packaging (CLP) Regulation, the Committee for Risk Assessment (RAC) has adopted an opinion on the proposal for harmonised classification and labelling (CLH) of:

Chemical name: **acetaldehyde; ethanal**

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

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

The proposal was submitted by **The Netherlands** and received by RAC on **22 June 2015.**

In this opinion, all classification and labelling elements are given in accordance with the CLP Regulation.

PROCESS FOR ADOPTION OF THE OPINION

The Netherlands has submitted a CLH dossier containing a proposal together with the justification and background information documented in a CLH report. The CLH report was made publicly available in accordance with the requirements of the CLP Regulation at ***http://echa.europa.eu/harmonised-classification-and-labelling-consultation/*** on **28 July 2015**. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **11 September 2015**.

ADOPTION OF THE OPINION OF RAC

Rapporteur, appointed by RAC: **Andrew Smith**

Co-Rapporteur, appointed by RAC: **Ralf Stahlmann**

The opinion takes into account the comments provided by MSCAs and concerned parties in accordance with Article 37(4) of the CLP Regulation and the comments received are compiled in Annex 2.

The RAC opinion on the proposed harmonised classification and labelling was adopted on **16 September 2016** by **consensus.**

Classification and labelling in accordance with the CLP Regulation (Regulation (EC) 1272/2008)

	Index No	International Chemical Identification	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors	Notes
					Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard state-ment Code(s)	Suppl. Hazard statement Code(s)		
Current Annex VI entry	605-003-00-6	acetaldehyde; ethanal	200-836-8	75-07-0	Flam. Liq. 1 Eye Irrit. 2 STOT SE 3 Carc. 2	H224 H319 H335 H351	GHS02 GHS07 GHS08 Dgr	H224 H319 H335 H351			
Dossier submitters proposal	605-003-00-6	acetaldehyde; ethanal	200-836-8	75-07-0	**Retain** Flam. Liq. 1 Eye Irrit. 2 STOT SE 3 **Add** Muta. 1B **Modify** Carc. 1B	**Retain** H224 H319 H335 **Add** H340 **Modify** H350	**Retain** GHS02 GHS07 GHS08 Dgr	**Retain** H224 H319 H335 **Add** H340 **Modify** H350			
RAC opinion	605-003-00-6	acetaldehyde; ethanal	200-836-8	75-07-0	**Retain** Flam. Liq. 1 Eye Irrit. 2 STOT SE 3 **Add** Muta. 2 **Modify** Carc. 1B	**Retain** H224 H319 H335 **Add** H341 **Modify** H350	**Retain** GHS02 GHS07 GHS08 Dgr	**Retain** H224 H319 H335 **Add** H341 **Modify** H350			
Resulting Annex VI entry if agreed by COM	605-003-00-6	acetaldehyde; ethanal	200-836-8	75-07-0	Flam. Liq. 1 Carc. 1B Muta. 2 STOT SE 3 Eye Irrit. 2	H224 H350 H341 H335 H319	GHS02 GHS07 GHS08 Dgr	H224 H350 H341 H335 H319			

GROUNDS FOR ADOPTION OF THE OPINION

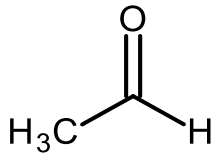
RAC general comment

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.

HUMAN HEALTH HAZARD EVALUATION

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**	**$\overline{X}$ 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**.

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.

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)**.

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ANNEXES:

Annex 1 The Background Document (BD) gives the detailed scientific grounds for the opinion. The BD is based on the CLH report prepared by the Dossier Submitter; the evaluation performed by RAC is contained in 'RAC boxes'

Annex 2 Response to comments document (RCOM) to the Opinion proposing harmonised classification and labelling at EU level of acetaldehyde; ethanal

Annex 3 Records of the targeted public consultation on the influence of acetaldehyde dehydrogenase (ALDH2) polymorphism on the physiological levels of acetaldehyde