CLH report

Proposal for Harmonised Classification and Labelling

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

Substance Name: Cyanamide

EC Number: **206-992-3**

CAS Number: **420-04-2**

Index Number: **615-013-00-2**

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Part A.

Part A.

1 PROPOSAL FOR HARMONISED CLASSIFICATION AND LABELLING

1.1 Substance

Table 1: Substance identity

Substance name:	***Cyanamide***
EC number:	206-992-3
CAS number:	420-04-2
Annex VI Index number:	615-013-00-2
Degree of purity:	***> 95% (w/w)***
Impurities:	No impurity is considered relevant for the classification of the substance Cyanamide

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	Acute Tox. 3* / H301 Acute Tox. 4* / H312 Eye Irrit. 2 / H319 Skin Irrit. 2 / H 315 Skin. Sens. 1 / H317
Current proposal for consideration by RAC	*Addition of* STOT-RE 1 / H372 Repr. 2 / H361f Repr. 2 / H361d Aquatic Chronic 1 / H 410 Chronic M-factor 1 *Re-evaluation of* Acute tox 3* / H301 ⇨ Acute tox 3 / H301 Acute Tox. 4* / H312 ⇨ Acute Tox. 3 / H311 Eye Irrit. 2 / H319, Skin Irrit 2 / H315 ⇨ Skin Corr 1 B / H314 Skin sens. 1 / H317 / ⇨ Skin sens. 1B / H317
Resulting harmonised classification (future entry in Annex VI, CLP Regulation)	Acute Tox. 3 / H301 Acute Tox. 3 / H311 Skin Corr. 1 B / H314 Skin. Sens. 1 B / H317 STOT-RE 1 / H372 (thyroid) Repr. 2 / H361f Repr. 2 / H361d Aquatic Chronic 1; H 410 Chronic M-factor 1

[+] Minimum classification

1.3 Proposed harmonised classification and labelling based on CLP Regulation and/or DSD criteria

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				conclusive but not sufficient for classification
2.2.	Flammable gases				Data lacking
2.3.	Flammable aerosols				Data lacking
2.4.	Oxidising gases				Data lacking
2.5.	Gases under pressure				Data lacking
2.6.	Flammable liquids				Data lacking
2.7.	Flammable solids				conclusive but not sufficient for classification
2.8.	Self-reactive substances and mixtures				conclusive but not sufficient for classification
2.9.	Pyrophoric liquids				Data lacking
2.10.	Pyrophoric solids				conclusive but not sufficient for classification
2.11.	Self-heating substances and mixtures				conclusive but not sufficient for classification
2.12.	Substances and mixtures which in contact with water emit flammable gases				conclusive but not sufficient for classification
2.13.	Oxidising liquids				Data lacking
2.14.	Oxidising solids				conclusive but not sufficient for classification
2.15.	Organic peroxides				Data lacking
2.16.	Substance and mixtures corrosive to metals				conclusive but not sufficient for classification
3.1.	Acute toxicity - oral	H301		H301*	
	Acute toxicity - dermal	H311		H312*	
	Acute toxicity - inhalation				conclusive but not sufficient for classification
3.2.	Skin corrosion / irritation	H314		H315	
3.3.	Serious eye damage / eye irritation	H314		H319	
3.4.	Respiratory sensitisation				Data lacking
3.4.	Skin sensitisation	H317		H317	
3.5.	Germ cell mutagenicity				conclusive but not sufficient for classification
3.6.	Carcinogenicity				conclusive but not sufficient for

					classification
3.7.	Reproductive toxicity	H361fd			
3.8.	Specific target organ toxicity –single exposure				conclusive but not sufficient for classification
3.9.	Specific target organ toxicity – repeated exposure	H372			
3.10.	Aspiration hazard				Data lacking
4.1.	Hazardous to the aquatic environment	H410	M=1		
5.1.	Hazardous to the ozone layer				

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

2 BACKGROUND TO THE CLH PROPOSAL

2.1 History of the previous classification and labelling

2.2 Short summary of the scientific justification for the CLH proposal

Based on the findings in the acute oral and dermal toxicity studies, a classification for acute toxicity category 3 (H301 and H311) is proposed (corresponding to T/R25 and Xn/R21). The test material was corrosive in an in vitro skin study (category 1B, H314, corresponding to C/R34) and irritating to eyes (category 2, H319, corresponding to Xi/R36) in an in vivo study in rabbits. However as the substance is skin corrosive, specific classification as an eye irritant is not necessary, because it is already included implicitly in the classification as skin corrosive. Therefore, no classification and labeling for eye irritation is necessary. Considering the findings in thyroid which were observed in the repeat-dose toxicity studies, a classification into STOT-RE category 1 (H372, corresponding to R48/25) is proposed. There were indications for adverse effects on fertility and development of the unborn offspring leading to the proposal for classification into category 2 (H361fd, corresponding to Repr. category 3/R62-R63 according to DSD). Based on the available toxicological study results, the dossier submitter considers no further classification for toxicological hazards as necessary.

2.3 Current harmonised classification and labelling

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

Table 4: Current classification in Annex VI, Table 3.1

Index number:	Classification	Wording
Hazard classes, Hazard categories	Acute Tox. 3* Acute Tox. 4* Skin Irrit. 2 Eye Irrit. 2 Skin Sens. 1	
Hazard statements	H301 H312 H315 H319 H317	Toxic if swallowed Harmful in contact with skin Causes skin irritation Causes serious eye irritation May cause an allergic skin reaction

* Minimum classification

Table 5: Current labelling in Annex VI, Table 3.1

Index number:	Labelling	Wording
Pictograms	GHS06	
Signal Word	Danger	
Hazard statements	H301 H312 H319 H315 H317	Toxic if swallowed Harmful in contact with skin Causes serious eye irritation Causes skin irritation May cause an allergic skin reaction
Suppl. Hazard statements	-	-
Precautionary statements	None listed in Annex VI	

2.4 Current self-classification and labelling

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

Self-classification according CLP with regard to toxicological hazards (according to the updated registration dossier): H301-H311-H314-H317-H318

3 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

The substance is a biocide, regulated under Directive 98/8/EC, and must therefore be classified at Community Level (cf. Art. 36(3) of CLP Regulation).

Part B.

SCIENTIFIC EVALUATION OF THE DATA

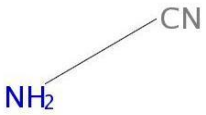
1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substance

Table 6: Substance identity

EC number:	206-992-3
EC name:	Cyanamide
CAS number (EC inventory):	420-04-2
CAS number:	420-04-2
CAS name:	Cyanamide
IUPAC name:	Cyanamide
CLP Annex VI Index number:	615-013-00-2
Molecular formula:	CH_2N_2
Molecular weight range:	42.04 g/mol

Structural formula:



1.2 Composition of the substance

The confidential information can be found in the "Confidential Annex" or the technical dossier.

Table 7: Constituents (non-confidential information)

Constituent	Typical concentration	Concentration range	Remarks
Cyanamide	**Min. > 95 % w/w**		

Current Annex VI entry:

Table 8: Impurities (non-confidential information)

Impurity	Typical concentration	Concentration range	Remarks
confidential			

Table 9: Additives (non-confidential information)

Additive	Function	Typical concentration	Concentration range	Remarks
confidential				

1.3 Physico-chemical properties

Table 10: 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	Colourless, odourless solid	Anonymous, 2006	
Melting/freezing point	46.1 °C	Wenighofer, T., 2007	
Boiling point	Decomposes before boiling	Wenighofer, T., 2007	
Relative density	$d_4^{20} = 1.23\,(21.4°C)$	Tognucci, 2000	
Vapour pressure	20°C: 0.5 Pa 25°C: 1.0 Pa	Eskötter, 1991	
Surface tension	72.86 mN/m (20 °C)	Wenighofer, 2007	
Water solubility	pH 7: > 560 g/L (preliminary test)	Eskötter, 1990	
Partition coefficient n-octanol/water	log $P_{o/w}$ = -0.72 (20 °C, dist. pH 6.8)	Turner, 2005	
Flash point	Testing can be waived, Cyanamide F 1000 is a solid substance.	BAM-2.2 (2011)	*Data Waiver*
Flammability + Explosiveness: Properties of a self-reactive substance (UN Test Series A to F)	The investigated test samples are not able to propagate a detonation or a deflagration and the explosive power is insignificant according to the criteria as defined in the UN MTC (4th rev. ed.) relating to UN Tests A.1, C.1, C.2, E.1, E.2, E.3 and F.3. Cyanamide should not be assigned to the group of self-reactive substances according to GHS.	BAM-2.2 (2006)	*Expert opinion*
Flammability on contact with water	Testing can be waived in accordance with REACH Column 2 of Annex VII, 7.10: The classification procedure needs not to be applied because the organic substance does not contain metals or metalloids.	BAM-2.2 (2011)	*Data Waiver*
Flammability: pyrophoric properties	Testing can be waived in accordance with REACH Column 2 of Annex VII, 7.10: The	BAM-2.2 (2011)	*Data Waiver*

	classification procedure needs not to be applied because the organic substance is known to be stable into contact with air at room temperature for prolonged periods of time (days).		
Explosive properties	Cyanamide was neither shock sensitive nor friction sensitive nor thermally sensitive according to the criteria of EEC test method A.14.	Budde, K., 2009	Study report
Self-ignition temperature	The study does not need to be conducted if the substance is completely molten at 160 °C. The thermal stability of the substance is quantitatively given by the SADT test.	BAM-2.2 (2011)	*Data Waiver*
Oxidising properties	Testing can be waived in accordance with REACH Column 2 of Annex VII, 7.13: The classification procedure needs not to be applied because there are no chemical groups present in the molecule which are associated with oxidising properties.	BAM-2.2 (2011)	*Data Waiver*
Granulometry	d10: 235 µm d50: 548 µm d90: 1006 µm	Mekelburger, H.-, 2009	
Stability in organic solvents and identity of relevant degradation products	-	-	
Dissociation constant	Cyanamide does not dissociate in water.	Tognucci, 2000	
Viscosity	Cyanamide is a solid substance at room temperature	-	
Thermal storage stability	UN Tests H.2 and H.4 were used to determine the SADT of Cyanamide F 1000 with the results that in both cases a SADT of 50 °C for a container containing 50 kg of the substance an admissible maximum temperature of 40°C would result for transports according to the dangerous goods	BAM-2.2 (2006)	*Expert opinion*

	regulations.		
Corrosive to metals	Cyanamide L 500 was found to be not corrosive to aluminium and steel.	Staber, H., 1998	Study report

2 MANUFACTURE AND USES

2.1 Manufacture

2.2 Identified uses

3 CLASSIFICATION FOR PHYSICO-CHEMICAL PROPERTIES

Not covered in this dossier.

4 HUMAN HEALTH HAZARD ASSESSMENT

Cyanamide was evaluated as a PPP active ingredient under directive 91/414/EEC (non-inclusion of cyanamide in Annex I to Council Directive 91/414/EEC, Commission Decision of 18 September 2008) and is currrently subject to the evaluation under BPD (directive 98/8/EC). In both procedures Germany acts as RMS. Studies submitted under these procedures were used to prepare the present CLP report.

In the evaluation process of cyanamide (CAS No 420-04-2) as a PPP active ingredient it was considered that cyanamide is manufactured as a technical concentrate. It was reported that the active substance content should be in the range of 488 g/kg to 530 g/kg or 520 g/L to 564 g/L (min. 854 g/kg calculated on dry weight basis). There is currently no FAO specification for cyanamide. It was established that cyanamide contains no known relevant impurities.

Furthermore it was considered that the technical active substance cyanamide is an aqueous solution containing at least 488 g of pure cyanamide per kg solution. The technical active substance was tested under the following product/brand names: Cyanamide L 500, aqueous cyanamide solution, SKW Cyanamide L 500, aqueous hydrogen cyanamide and aqueous cyanamide.

The technical active substance was regarded as identical to the plant protection products ALZODEF (formerly registered in Germany as a herbicide and as a plant growth regulator for sucker control in hops) and DORMEX (registered in various EU countries as a plant growth regulator for breaking bud dormancy). The only differences between the technical active substance and the products ALZODEF and DORMEX consist in the addition of a minor amount (20 ppm) of the triphenylmethane dye Acid Blue 9, which gives the products ALZODEF and DORMEX a slightly blue tint, and the addition of the emetic (taste aversive agent) Denatonium benzoate (50 ppm). It was concluded that for this reason it is not necessary to distinguish between the technical active substance and the plant protection products ALZODEF and DORMEX: Studies performed with the technical active substance were used without any restrictions for the evaluation of the products and vice versa.

Both procedures, the evaluation of cyanamide under PPP and BPD, were based on the same experimental toxicological studies. These studies were conducted from the 70s to 2009. The purity of cyanamide used in these studies ranged from about 20 % to nearly 100 %. In most studies an aqueous solution of cyanamide of about 50 % was applied. Doses, NOAELs, LOAELs, and reference values were calculated and reported as pure cyanamide. In the evaluation process of cyanamide the results of these studies were considered as adequate to evaluate the hazard of cyanamide.

In the registration dossier, it is stated that “Calcium cyanamide is rapidly and quantitatively converted to hydrogen cyanamide upon contact with water (as was already shown in a report "Conversion rate of calcium cyanamide (technical grade) to hydrogen Cyanamide", Doc. No. 593-001). Consequently, data obtained from toxicological tests performed with calcium cyanamide are also applicable to hydrogen Cyanamide.” (cited from Endpoint study record “420-04-2_cyanamide

(calcium cyanamide)_Health surveillance data_1976 (Gfaller).[1] A similar bridging statement was included in the dossier under the PPP procedure, which was accepted during the PPP peer review process. The dossier submitter agrees with this bridging concept (at least for non-acute endpoints), hence studies conducted with calcium cyanamide are used to judge on the hazard of cyanamide.

Information from a registration dossier and a CLP notification as of March 2014 (last update of the registration dossier October 25, 2010) were taken into account.

The assessment prepared by Germany under the PPP prodecure is available under http://dar.efsa.europa.eu/dar-web/provision and is attached to the technical dossier. The assessment under BPD is not yet publically available. On request from the European Commission the EFSA conducted a focused peer review and delivered its conclusions on cyanamide.[2] The outcome of the EFSA peer-review regarding classification and labelling is indicated, when discussing the need for a classification for the individual endpoints/hazards.

Changed criteria according to Commission Regulation (EU) No 286/2011 were taken into account, when assessing the study results.

4.1 Toxicokinetics (absorption, metabolism, distribution and elimination)

4.1.1 Non-human information

Studies on the absorption, distribution, metabolism and excretion were conducted with [^{14}C]-radiolabelled test substance in rats (see Table 11). The results from the metabolism study in rats with [^{14}C]-cyanamide demonstrate that the compound is completely absorbed after oral administration and was rapidly excreted. The percent excreted in the urine ranged from approximately 79.0 to 97.7 % of the applied dose at 168 hours (faeces 2.76 - 4.15 % and expired CO_2 1.45 - 10 %). Regardless of the route of administration a total of approximately 67 % to 92 % was excreted by all routes in the first 24 h postdose. The major metabolic reaction is acetylation of the nitrogen. After oral administration of hydrogen cyanamide, the major urinary metabolite formed in a variety of species, including man, is N-acteylcyanamide. If at all, other metabolites seem to play a minor role. A minor biotransformation pathway of cyanamide is the formation of hydroxycyanamide as a product of microsomal oxidation, as shown by *in vitro* studies. Hydroxycyanamide is an intermediate instable metabolite, which decomposes to cyanide and nitroxyl. However, no metabolic degradation of cyanamide to cyanide was found in men. All tissues (blood, bone, brain, fat, heart, kidneys, liver, lungs, muscle, ovaries, spleen, thyroid and uterus) collected 168 h after postdose contained 0.03 % or less of the radioactivity, except liver and kidney containing 0.14 to 1.18 % and 0.02 to 0.09 %, respectively. These results indicated no tendency for accumulation of cyanamide. The half-life ($t_{1/2}$) of cyanamide is very short with approximately 1 h (after i.v. administration of 35 mg/kg bw).

[1] All studies conducted with calcium cyanamide were excluded from the *updated* registration dossier.

[2] European Food Safety Authority; Conclusion on the peer review of the pesticide risk assessment of the active substance cyanamide. EFSA Journal 2010;8(11):1873. [61 pp.] doi:10.2903/j.efsa.2010.1873. Available online: www.efsa.europa.eu/efsajournal.htm

Table 11: Overview of studies on adsorption, distribution, excretion and metabolism in mammals

Study type	Species / strain	Vehicle	Comments	Results	Reference
Metabolism of [^{14}C]-hydrogen cyanamide in rats	Sprague Dawley rat	Ammonium phosphate buffer and sodium chloride	Complete absorption after oral administration, no tendency for accumulation	Major metabolite: N-acetylcyanamide in urine	Sturble, 1993 Doc. No. 512-001
Bioavailability of cyanamide in fasted and unfasted rats	Sprague Dawley rat	Distilled water	Toxicokinetic data	Absorption of cyanamide almost complete after oral administration. Very short half life (approximately 1 hour)	Obach, 1986 Doc. No. 592-019
Investigations on the absorption, metabolism and excretion of hydrogen cyanamide in rat and human	Wistar rat and male human volunteers	Distilled water Drinking water		N-acetyl-cyanamide: main urinary metabolite in man and rat	Gloxhuber, 1989 Doc. No. 512-004

4.1.2 Human information

No other relevant information is available.

4.1.3 Summary and discussion on toxicokinetics

Following oral intake, cyanamid was completely absorbed after oral administration and was rapidly excreted. Total elimination rate of radioactivity reached an amount of approx. 79.0 to 97.7 % within 7 d following treatment. The results indicated no tendency for accumulation of cyanamide. After oral administration of hydrogen cyanamide, the major urinary metabolite formed in a variety of species, including man, is N-acteylcyanamide. No metabolic degradation of cyanamide to cyanide was found in men.

4.2 Acute toxicity

4.2.1 Non-human information

The acute oral LD_{50} was determined in a former study in rats to be 142 mg/kg bw cyanamide (Engels, 1973). Mortality mainly occurred during the first day after administration. Cyanamide is considered to be toxic via the oral route in this study. Additionally, a modern study was carried out in accordance to a recent guideline under GLP. An oral LD_{50} of 223 mg/kg bw cyanamide was obtained. Signs of intoxication included lethargy, hunched posture, uncoordinated movements, tremors, piloerection and breathing difficulties (Daamen, 1994). It is concluded that the study by Daamen (1994) is a reliable study as it was performed according to GLP and OECD 401 and thus relevant details and results are provided with the report. The study by Engel (1973) is considered to be of supplementary value for the purpose of classification and labelling. The results of this study

cannot be excluded to derive the LD_{50}. Therefore, the oral LD_{50} (sex combined) is considered to be 142 - 223 mg/kg bw leading to classification and labelling with T, R25.

The dermal LD_{50} was found to be between approximately 2120 - 3180 mg/kg bw of cyanamide in an elder study. In a newer study according to recent guidelines the acute dermal toxicity of an aqueous cyanamide solution was re-examined. Based on the observed mortality pattern in this study, it was concluded that the LD_{50} of pure active substance cyanamide is 848 mg/kg bw combined for the sexes. Therefore, cyanamide is considered to be harmful in contact with skin.

Although a four hour exposure to 1.0 mg pure active substance cyanamide /L air (the highest attainable concentration) was considered to be supplementary, the study was suitable to determine the LC_{50} in rats. No mortality or severe injury was observed. Therefore, the LC_{50} of cyanamide was greater than 1.0 mg/L air.

Table 12: Summary table of relevant acute toxicity studies

Study type	Species / strain	Comments	Results m /f	Reference
LD_{50} oral	Wistar rat	Mortalities mainly at day 2; many clinical signs; necropsy: changes in digestive tract, haemorrhages in body cavities, lungs, thymus urinary bladder	222 / 226 mg/kg bw 223 mg/kg bw combined	Daamen, 1994 Doc-No. 521-002 TOX2001-421
LD_{50} oral	Wistar rat	Mortalities mainly at day 1; clinical signs: only convulsions in rats that died	142 mg/kg bw combined	Engels, 1973 Doc-No, 521-001 TOX2004-349
LD_{50} dermal	New Zealand White rabbit	Dark red lungs or dark red lung areas	901 / 741 mg/kg bw 848 mg/kg bw combined	Osheroff, 1988 Doc-No. 522-002 TOX2004-358
LD_{50} dermal	New Zealand White rabbit	Only 1 animal/sex/dose group with intact skin	between 2120 and 3180 mg/kg bw	van Beek, 1973 Doc-No. 522-001 TOX2004-358
LC_{50} inhalation	Wistar rat	No mortalities in the highest attainable concentration of 1 mg/L cyanamide	LC_{50} >1 mg/L	Kruysse, 1973 Doc-No. 523-001 TOX2004-360

4.2.1.1 Acute toxicity: oral

Title: Daamen (1994): Assessment of the acute oral toxicity with cyanamide in the rat; Notox, Doc. No. 521-002; Netherlands (NOTOX project 101688), unpublished

Guidelines: OECD 401 (1987), EEC 92/69- B1

Deviations: None

GLP: Yes

Acceptability: The study is considered to be acceptable.

Material and Methods:

The test substance was identified as follows:

Cyanamide (supplied as 50 % aqueous solution)

Batch number: 08/11/93

Purity: 53 % (w/v)

Cyanamide was orally administered in distilled water via gavage to five male and five female Albino Wistar rats per dose group at dosage levels of 150; 200; 250; 320 and 400 mg/kg bw. The applied volume was 5 mL/kg bw. The rats were provided by BRL Ltd., Basel, Switzerland. The animals were observed for treatment-related effects on the day of dosing and for a subsequent 14-day observation period. Mortality, clinical observations, body weights and gross necropsy findings were recorded.

Findings:

Mortalities occurred between 4 hours and day 7 after treatment. Most of the mortalities occurred on day 2. Mortality and time of deaths in the acute oral study are shown in Table 13.

Table 13: Mortality in the acute oral toxicity study in Wistar rats with cyanamide

Males			Females		
Dose	**Mortality**	**Time of death**	**Dose**	**Mortality**	**Time of death**
150 mg/kg	0/5	-	150 mg/kg	2/5	Day 2 and 3
200 mg/kg	3/5	Day 2	200 mg/kg	0/5	-
250 mg/kg	2/5	Day 2	250 mg/kg	2/5	Day 2
320 mg/kg	5/5	Day 1, 2, 5 and 7	320 mg/kg	5/5	Day 2
400 mg/kg	5/5	Day 2	400 mg/kg	5/5	Day 1 and 2

Clinical signs of toxicity were seen at all doses showing a clear dose response. Among the majority of animals lethargy, tremors, piloerection, hunched posture and uncoordinated movements were noted. Some animals had also abnormal gait, breathing difficulties and ventro-lateral recumbency. Male rats showed erythema (day 7 - 12), scales (day 9 - 13) and scabs (day 14 - 15) on the tail. All surviving animals had recovered between day 5 and 13 except those which showed scabs on the tail. Macroscopic post mortem examination of the animals that died during the study included enlarged stomach, haemorrhages in glandular stomach, thickened limiting ridge in stomach, watery and haemorraghic fluid contents in body cavities, dark red appearance of lungs, red foci and haemorrhages in lungs, haemorrhages in thymus, haemorrhages in wall of urinary bladder. There were no changes due to treatment upon post-mortem examination of the surviving animals.

Conclusions:

The acute oral LD_{50} of cyanamide in rats was 223 mg/kg bw for the sexes combined and 222 mg/kg bw for males alone. An LD_{50} value of 226 mg/kg bw cyanamide could be estimated for the females due to the mortality distribution.

Title:	Engel, C. (1973): Determination of the acute oral toxicity of cyanamide in Albino rats; Doc. No. 521-001; Centraal Instituut voor Voedingsonderzoek, Netherlands; unpublished Additional, more detailed report to the study: Spanjers, M.T. (1986)
Guidelines:	No. Study is conducted following OECD 401 and is performed to reveal acute oral toxicity of hydrogen cyanamide in rats.
Deviations:	Applied volume was not constant. Body weight determination only at day 1 and at termination.
GLP:	No (study was conducted prior to the implementation of GLP)
Acceptability:	The study is considered to be supplementary. Although there are some deviations from OECD 401, the study is suitable to determine the LD_{50}.

Material and Methods:

The test substance was identified as follows:

SKW Cyanamide L 500 (supplied as 50 % aqueous solution)

Batch: Not mentioned

Purity: Unclear

Cyanamide was orally administered in a 5 % (v/v) aqueous dilution to four groups of Albino Wistar rats (source not mentioned), each group with five males and five females, at dose levels of 100 to 175 mg/kg bw by gavage. For details of dosing see Table 14. The dose selection was based upon a range-finding study using groups of one to five male and female rat/test group: undiluted technical cyanamide was dosed at 1.0, 2.0 or 5.0 mL /kg bw followed by dosages of 4.0, 6.0 and 8.0 mL of a 10 % (v/v) aqueous dilution (corresponding to 0.4, 0.6 and 0.8 mL test substance/kg bw). Mortality was seen in all rats dosed with 0.6 mL test substance/kg bw or more shortly after dosing with signs of convulsion. All females rats dosed with 0.4 mL/kg bw survived while all males of this dose group died. Clinical signs were lachrymation, apnoea and coma.

Table 14: Dosing scheme - acute oral toxicity study in Wistar rats with cyanamide

Number of rats/sex	Applied volume 5% dilution (mL/kg bw)	Applied dose test substance (mL/kg bw)	Applied dose pure active substance cyanamide (mg/kg bw)
5	4	0.2	100
5	5	0.25	125
5	6	0.3	150
5	7	0.35	175

The animals were observed for treatment-related effects during the first 4 h and for a subsequent 14-day observation period. Mortality, clinical observations, body weights and gross necropsy findings were recorded.

Findings:

Mortality was observed in all dose levels with a higher incidence in males and occurred mainly during the first day between 6 and 24 h after dosing. One male died on the third post treatment day (see Table 15).

Table 15: Mortality in the acute oral toxicity of cyanamide

Males			Females		
Dose	**Mortality**	**Time of death**	**Dose**	**Mortality**	**Time of death**
100 mg/kg	1/5	Day 1	100 mg/kg	0/5	-
125 mg/kg	4/5	Day 1	125 mg/kg	0/5	-
150 mg/kg	5/5	Day 1 and 3	150 mg/kg	2/5	Day 1
175 mg/kg	5/5	Day 1	175 mg/kg	1/5	Day 1

The only clinical symptoms were convulsions in rats that died. Surviving rats had no significant changes at necropsy.

Conclusion:

The acute oral LD_{50} of pure active substance cyanamide in rats was estimated to be 142 mg/kg bw for the sexes combined.

A position paper on the appropriate LD_{50} for acute oral toxicity and the classification and labelling of cyanamide regarding acute oral toxicity was submitted by the notifier (Moeller & Hofer, 2009). The results of the position paper are presented in the following.

Classification and Labelling of cyanamide with R25, T was proposed in the DAR. The notifier disagrees. He proposed that classification and labelling should be based on the most valid acute oral toxicity study. A summary of acute oral toxicity is provided in the following.

Two studies, i.e. Engel (1973) and Daamen (1994), on acute oral toxicity of Cyanamide L500 (aqueous solution 50 % a.s.) were performed with the rat. In addition, one study on the influence of N-acetylcysteine (NAC, one dose group +/- post-administration) on acute oral toxicity of cyanamide was performed by Daamen (1994).

The study by Daamen (1994) was performed according to GLP and OECD 401. The study was performed with albino Wistar rats soon after the test substance (batch reported) arrived at the test facility and stored refrigerated. The test substance was diluted in distilled water and stability was reported to be at least 96 h in this vehicle. Formulations of L500 (50 % a.s.) were prepared immediately prior to dosing by weighing the test substance into glass flasks on an analytical balance and by adding the vehicle (w/w). Homogeneity in vehicle was obtained by mechanical stirring. Animals were acclimatised for 5 d and dosed with a constant dosing volume (as required by OECD 401) and thus with increasing cyanamide concentrations in the formulation with increasing dose levels (150, 200, 250, 320, 400 mg/kg bw). Food and water quality control analysis is reported. LD_{50} (combined) was 223 mg/kg bw.

Daamen (1994) performed a few weeks later a further study with one dose level (230 mg/kg bw) cyanamide and an additional group with a post-administration of N-acetylcysteine (NAC). The study was performed in the same way as the former study and according to GLP. A mortality rate of 40 % is reported for the concentration of 230 mg/kg bw, in line with the previously obtained LD_{50} of 223 mg/kg bw.

The results of the acute oral studies are summarised in the following table.

Table 16: Acute oral toxicity of Cyanamide

Dose volume (conc. a.s.)	Dose level (mg/kg bw a.s.)	Mortality (5 animals /sex/group) ♂	♀	Clinical signs Dead	survivors	Necropsy Dead	survivors	Study (GLP/GL) (yes/no)
4 mL (2.5 %)	100	1	0	convulsions	n.a.	n.p.	n.a.	Engel 1973 521-001 (n/n)
5 mL (2.5 %)	125	4	0	convulsions	n.a.	n.p.	n.a.	Engel 1973 521-001 (n/n)
6 mL (2.5 %)	150	5	2	convulsions	n.a.	n.p.	n.a.	Engel 1973 521-001 (n/n)
5 mL (3 %)	150	0	2	lethargy, tremors, piloerection, hunched posture, uncoordinated movements	see dead animals, but recovery	Local findings stomach and systemic findings	n.a.	Daamen 1994 521-002 (y/y)
7 mL (2.5 %)	175	5	1	convulsions	n.a.	n.p.	n.a.	Engel 1973 521-001 (n/n)
5 mL (4 %)	200	3	0	lethargy, quick breathing, piloerection, hunched posture, uncoordinated movements, chromodachryorrhoea	see dead animals, but recovery	Local findings stomach and systemic findings	n.a.	Daamen 1994 521-002 (y/y)
5 mL (4.6 %)	230	2	2	lethargy, tremors, ventro-lateral recumbency, hunched posture, uncoordinated movements, piloerection, chromodacryorrhoea, ptosis and emaciation	see dead animals, but recovery	Local findings stomach and systemic findings	n.a.	Daamen 1994 521-003 (y/n)
5 mL (5 %)	250	2	2	lethargy, tremors, piloerection, hunched posture, uncoordinated movements, abnormal gait, erythema, scales and scabs on the tail	see dead animals, but recovery	Local findings stomach and systemic findings	n.a.	Daamen 1994 521-002 (y/y)
5 mL (6.4 %)	320	5	5	lethargy, tremors, piloerection, hunched posture, uncoordinated movements, ventro-lateral recumbency, labored respiration, chromodachryorrhoea	see dead animals, but recovery	Local findings stomach and systemic findings	n.a.	Daamen 1994 521-002 (y/y)
5 mL (8 %)	400	5	5	lethargy, tremors, piloerection, hunched posture, uncoordinated movements, ventro-lateral recumbency	see dead animals, but recovery	Local findings stomach and systemic findings	n.a.	Daamen 1994 521-002 (y/y)

n.a. no abnormalities, n.p. not performed, GL = guideline

Mortality rates for female animals in the three studies are consistent. Toxicity markedly increased between 320 and 400 mg/kg bw. For males, mortalities observed by Engel (1973) were unexpectedly high which was not observed by Daamen (1994). Notably, animals dying in the older study (Engel, 1973) had strong convulsions before dying mainly on the first day. The survivors had

no convulsions. For both dead animals and survivors, no further clinical signs were observed. As necropsy was not performed for the dead animals, an identification of local effects in the stomach or systemic effects is lacking. Thus, an explanation for this unusual study outcome cannot be provided.

Daamen (1994) described various clinical signs in the animals for both, dead animals and survivors receiving the same amount of test substance. Survivors recovered over the period of the study. Body weight (gain) was reduced for animals dosed with 200 mg/kg bw and more, recovering in the second week of the study. With increasing dose, animals died earlier or more animals died. None of these animals had convulsions before dying. Necropsy established local stomach effects and systemic findings for the animals found dead during the study.

In summary, the study by Daamen (1994) described dose dependent systemic toxic effects which were confirmed for one dose level by the subsequent study with NAC which was performed independently by Daamen (1994). Engel (1973) described extraordinary high mortality rates for male animals. A reason for this outcome cannot be provided as reporting is very limited and necropsy was not reported for dead animals. Notably, convulsions before dying of the animals were not confirmed by Daamen (1994 and 1994), although higher concentrated dose formulations of cyanamide were administered. In this study, increasing concentrations lead to increasing systemic effects, but even at the highest concentration (8 % L500, 400 mg a.s./kg bw) inducing 100 % mortality, no convulsions were observed.

It is concluded by Moeller & Hofer (2009), that the study by Daamen (1994) is the reliable study as it was performed according to GLP and OECD 401, and thus relevant details and results are provided with the report. The authors concluded that the study by Engel (1973) is not valid for the purpose of classification and labelling. Therefore, the authors proposed that the oral LD_{50} (sex combined) is 223 mg/kg bw, leading to classification and labelling with Xn, R22.

Conclusion of the Rapporteur Member State (RMS):

It is concluded that the study by Daamen (1994) is a reliable study as it was performed according to GLP and OECD 401, and thus relevant details and results are provided with the report. The study by Engel (1973) is considered to be of supplementary value for the purpose of classification and labelling. The results of this study cannot be excluded to derive the LD_{50}. Therefore, the oral LD_{50} (sex combined) is considered to be 142 - 223 mg/kg bw leading to classification and labelling with T, R25.

4.2.1.2 Acute toxicity: inhalation

Title:	Kruysse (1973): Acute inhalation toxicity study with SKW Cyanamide L 500 in rats; Doc. No. 523-001; Centraal Instituut voor Voedingsonderzoek, Netherlands; unpublished
Guidelines:	The study complies to some extend with OECD 403.
Deviations:	Report deficiencies: E.g. temperature of air, humidity of air, equipment for measuring temperature, humidity and particulate aerosol. No data of body weight, individual clinical signs and gross pathological findings
GLP:	No (study was conducted prior to implementation of GLP)

Acceptability: The study is considered to be supplementary.

Material and methods:

The test substance was identified as follows:

Cyanamide (supplied as 50 % aqueous solution)

Batch: Not mentioned

Purity: 53 % (w/v)

The toxicity of cyanamide by the inhalation route (whole-body) was investigated in five male and five female Wistar rats (Central Institute for the Breeding of Laboratory Animals TNO, Zeist, Netherlands). Body weight at the study initiation was about 200 g for males and about 180 g for females. The rats were exposed to the mist of technical active cyanamide in a concentration of 2.0 mg/L of air (corresponding to the highest technical attainable concentration) for 4 h (see Table 17). The animals were observed for 14 d. They were checked for mortality, clinical symptoms and gross necropsy findings.

Table 17: Experimental procedure LC_{50} determination in Wistar rats with cyanamide

Parameters	Value
Flow rate	50 L/min
Analytical concentration	2.0 mg/L
Particle size (mass median aerodynamic diameter, MMAD) < 3 µm	99 %

Findings:

No mortalities were recorded during the study. During the exposure, a lumback behaviour and a rapid shallow respiration with frequent coughing and swallowing were observed. Within a few hours after exposure, all animals were completely recovered and showed normal behaviour. No visible lesions were observed at gross necropsy.

Conclusions:

The inhalative LC_{50} of cyanamide was > 1mg/L in male and female rats after 4 h inhalation (corresponding to technical active Cyanamide L 500 > 2.0 mg/L).

4.2.1.3 Acute toxicity: dermal

Title: Osheroff (1988): Acute dermal toxicity study in rabbits with aqueous hydrogen cyanamide; Doc. No. 522-002; Hazelton Laboratories (HLA Study no 2319-122), USA; unpublished.

Guidelines: U.S. EPA 40 CFR Part 158.81.2

Deviations: Not applicable

GLP: Yes

Acceptability: The study is considered to be acceptable.

Material and methods:

The test substance was identified as follows:

Cyanamide (supplied as 50 % aqueous solution)

Batch: No 07/07/87

Purity: 53 % (w/v)

The test substance was applied dermally to the shaved intact skin (approximately 10 % of the total body surface) of five male and five female rabbits per dose group. The New Zealand White Rabbits were provided by Hazelton Research Products Inc., Denver Pennsylvania. Based on the results of a preliminary test with an acute median lethal dermal dose of cyanamide between 1 and 3 mL/kg bw of 50 % w/w hydrogen cyanamide technical solution 1.0, 2.0 and 4.0 mL/kg bw (corresponding to 530, 1060 and 2120 mg/kg bw pure active substance cyanamide) were applied. The liquid test material was held in contact with the skin for a 24 h period with impervious rubber damming. The animals were evaluated for treatment related effects on the day of dosing and for a subsequent 14-day observation period. Mortality, clinical observations, dermal findings, body weight and gross necropsy findings were recorded.

Findings:

Mortality and time of death in the acute dermal study are shown in Table 18.

Table 18: Mortality in the acute dermal toxicity study in New Zealand White rabbits with cyanamide

Males			Females		
Dose	**Mortality**	**Time of death**	**Dose**	**Mortality**	**Time of death**
530 mg/kg bw	0/5	-	530 mg /kg bw	1/5	Day 1
1060 mg/kg bw	5/5	Day 1 and 2	1060 mg/kg bw	5/5	Day 2
2120 mg/kg bw	4/5	Day 2	2120 mg/kg bw	5/5	Day 2

Mortality was observed in all dose groups, especially at mid (1060 mg/kg bw) and high dose (2120 mg/kg bw) levels and occurred between day 1 and day 2 after treatment. No clinical observations were noted at the mid dose as all animals died on day 1.

Clinical signs of toxicology included tremors, ataxia, anorexia, depression and were observed at the low and high dose group. No signs of dermal irritations were noted in the low dose group, whereas all surviving animals of the high dose group showed a well defined erythema and a very slight oedema on day 1. All other animals died before primary dermal irritation scoring could be performed. In rabbits that survived, no pathologic changes at necropsy were observed. In rabbits found dead during the observation period, changes mainly concerning the lung were observed (dark red lungs or dark red lung areas). In one animal of the high dose group a pale liver was noted.

Conclusions:

The acute dermal LD_{50} was 901 mg/kg bw in males, 742 mg/kg bw in females and 848 mg/kg bw combined for the sexes pure active substance cyanamide in rabbits (corresponding to about 1.6

mL/kg bw for the sexes combined, 1.7 mL/kg bw for males and 1.4 mL/kg bw for females of the 50 % aqueous hydrogen cyanamide solution).

Title:	van Beek, L. and Dreef-van der Meulen, H.C. (1973): Acute dermal toxicity study with SKW Cyanamide L 500 in rabbits; Doc. No. 522-001; Centraal Instituut voor Voedingsonderzoek, Netherlands; unpublished
Guidelines:	No, however study complies to a great extent with OECD Guideline 402.
Deviations:	2 animals per dose group (1 of each sex) with intact skin and 2 animals per dose group (1 of each sex) with abraded skin (however, a dermal toxicity study should not be performed with abraded skin); no data about time of deaths, clinical signs, gross pathological findings, dermal irritations
GLP:	No; study was conducted prior to the implementation of GLP
Acceptability:	The study is considered to be supplementary.

Materials and methods:

The test substance was identified as follows:

SKW Cyanamide L 500 (50 % aqueous solution)

Batch: Not mentioned

Purity: Unclear.

Cyanamide L 500 was applied dermally to the shaved intact skin (approximately 10 % of the total body surface) of two male and two female New Zealand White Albino rabbits at a dosage level of 2.0, 4.0 and 6.0 mL/kg bw (corresponding to 1060, 2120 and 3180 mg/kg bw pure active cyanamide). Half the number of the animals received the test substance on the intact skin, the other half on the abraded skin. The test sites were covered with a thin layer of cellulose sheet. Approximately 24 h after application, the test substance was removed and the sites were wiped dry with towels. The animals were evaluated for treatment effects on the day of dosing and for subsequent 14-day observation period. Mortality, clinical observations, dermal findings, body weights and gross necropsy findings were recorded. Samples of liver, spleen, treated and untreated skin were examined microscopically.

Findings:

Mortality and time of death in the acute dermal study are shown in Table 19.

Table 19: Acute dermal toxicity of cyanamide

Males			Females		
Dose	**Mortality**	**Time of death**	**Dose**	**Mortality**	**Time of death**
1060 mg/kg bw	0/2[+]	Not mentioned	1060 mg/kg bw	0/2	Not mentioned
2120 mg/kg bw	1/2	Not mentioned	2120 mg/kg bw	0/2	Not mentioned
3180 mg/kg bw	2/2	Not mentioned	3180 mg/kg bw	2/2	Not mentioned

[+] intact/abraded skin

Mortality was seen in three animals treated with 3180 mg/kg bw within 24 h after treatment. Two animals (2120/ 3180 mg/kg bw) were killed when moribund.

Clinical signs of toxicology were observed in all test animals during or at the end of the 24 h exposure period and included apathy, dilation of the pupils, erythema, oedema and haemorrhages in the treated skin area. Pareses was noted in the mid and high dose group. Haemorrhages and pareses observed in the animals treated at the abraded skin were more pronounced than in those treated at the intact skin. After one and two weeks, scaliness and necrosis were observed in all surviving animals. There was no difference in reaction between male and female rabbits. Gross examination at autopsy revealed skin lesions in all test animals and swollen livers and haemorrhagic erosions in the stomach in the rabbits that died. Hyper- and parakeratosis and oedema in the skin, enlarged hepatocytes in the liver, atrophy of the white pulp in the spleen and erosions in the stomach were observed upon microscopic examination.

Growth, food and water intake and haematology showed no distinct differences between treated and control animals.

Conclusion:

The dermal LD_{50} of SKW Cyanamide L 500 in male and female New Zealand White rabbits was found to be between 2120 and 3180 mg/kg bw cyanamide.

4.2.1.4 Acute toxicity: other routes

No other relevant information is available.

4.2.2 Human information

No relevant information is available.

4.2.3 Summary and discussion of acute toxicity

It is concluded that a sufficient data package on acute toxicity of cyanamide is available.

Two studies, i.e. Engel (1973) and Daamen (1994), on acute oral toxicity of cyanamide L500 (aqueous solution 50 % a.s.) were performed with the rat. In addition, one study on the influence of N-acetylcysteine (NAC, one dose group +/- post-administration) on acute oral toxicity of cyanamide was performed by Daamen (1994).

The acute oral LD_{50} was determined in the former study in rats to be 142 mg/kg bw cyanamide (Engels, 1973). Mortality mainly occurred during the first day after administration. Cyanamide is considered to be toxic via the oral route in this study.

The study by Daamen (1994) was performed according to GLP and OECD 401. Animals were dosed with a constant dosing volume (as required by OECD 401) and thus with increasing cyanamide concentrations in the formulation with increasing dose levels (150, 200, 250, 320, 400 mg/kg bw). An oral LD_{50} of 223 mg/kg bw cyanamide was obtained. Signs of intoxication included lethargy, hunched posture, uncoordinated movements, tremors, piloerection and breathing difficulties (Daamen, 1994).

A few weeks later, Daamen (1994) performed a further study with one dose level (230 mg/kg bw) cyanamide and an additional group with a post-administration of N-acetylcysteine (NAC). The study was performed in the same way as the former study and according to GLP. A mortality rate of 40 % is reported for the concentration of 230 mg/kg bw, in line with the previously obtained LD_{50} of 223 mg/kg bw.

The study by Daamen (1994) described dose dependent systemic toxic effects which were confirmed for one dose level by the subsequent study with NAC which was performed independently by Daamen (1994). Engel (1973) described extraordinary high mortality rates for male animals. A reason for this outcome cannot be provided as reporting is very limited and necropsy was not reported for dead animals. Notably, convulsions before dying of the animals were not confirmed by Daamen (1994 and 1994), although higher concentrated dose formulations of cyanamide were administered. In this study, increasing concentrations led to increasing systemic effects, but even at the highest concentration (8 % L500, 400 mg a.s./kg bw) inducing 100 % mortality, no convulsions were observed.

It is concluded, that the study by Daamen (1994) is a reliable study as it was performed according to GLP and OECD 401, and thus, relevant details and results are provided with the report. The study by Engel (1973) is considered to be of supplementary value for the purpose of classification and labelling. The results of this study cannot be excluded to derive the LD_{50}. Therefore, the oral LD_{50} (sex combined) is considered to be 142 - 223 mg/kg bw leading to classification and labelling with T, R25.

Although a four hour exposure to 1.0 mg pure active substance cyanamide /L air (the highest attainable concentration) was considered to be supplementary, the study was suitable to determine the LC_{50} in rats. No mortality or severe injury was observed. Therefore, the LC_{50} of cyanamide was greater than 1.0 mg/L air.

The dermal LD_{50} was found to be between approximately 2120 - 3180 mg/kg bw of cyanamide in an elder study. In a newer study according to recent guidelines the acute dermal toxicity of an aqueous cyanamide solution was re-examined. Based on the observed mortality pattern in this study, it was concluded that the LD_{50} of pure active substance cyanamide is 848 mg/kg bw combined for the sexes (901 mg/kg bw in males, 742 mg/kg bw in females).

4.2.4 Comparison with criteria

Table 20 presents the toxicological results in comparison with DSD and CLP criteria.

Table 20: Results of acute toxicity studies in comparison with DSD and CLP criteria

Toxicological result	DSD criteria	CLP criteria
Oral LD_{50}, rat: 142 - 223 mg/kg	Harmful: LD_{50} per oral, rat: $25 < LD_{50} \leq 200$ mg/kg	Cat. 3: $> 50 < LD_{50} \leq 300$ mg/kg (oral)
Inhalation LC_{50}, rat: > 1 mg/l (highest attainable conc., aerosol, 4-h)	Harmful: LC_{50} inhalation, rat, for aerosols or particulates: $1 < LC_{50} \leq 5$ mg/litre/4h	Cat.3: $2,0 < LC_{50} \leq 10,0$ mg/l (vapours) $0.5 < LC_{50} \leq 1.0$ (dusts and mists) Cat. 4: $10,0 < LC_{50} \leq 20,0$ mg/l (vapours) $1.0 < LC_{50} \leq 5.0$ (dusts and mists)
Dermal LD_{50}: 901 mg/kg bw in males, 742 mg/kg bw in females	Harmful: LD_{50} dermal, rat or rabbit: $400 < LD_{50} \leq 2\,000$ mg/kg	Cat. 3: $200 < LD_{50} \leq 1\,000$ mg/kg (dermal)

4.2.5 Conclusions on classification and labelling

The acute oral toxicity of cyanamide meets the DSD and CLP criteria. According to the criteria in Dir. 67/548, based on the results of the acute oral toxicity studies, cyanamide has to be classified as toxic and assigned the symbol “T” and the indication of danger “Toxic if swallowed” accordingly. The following risk phrase should be assigned: “R25 Toxic if swallowed”.

According to the criteria in Reg. 1272/2008, based on the results of the acute oral toxicity studies cyanamide has to be classified as “Acute toxicity, cat. 3”. The following risk phrase should be assigned: “H301 Toxic if swallowed”.

Rats were exposed to the mist of technical active cyanamide in a concentration corresponding to the highest technical attainable concentration. No mortality or severe injury was observed. Therefore, the LC_{50} of cyanamide was greater than 1.0 mg/L air. In summary, the results of the acute inhalation toxicity studies do not meet the DSD and CLP criteria to be classified as upon inhalative exposure.

The acute dermal toxicity of cyanamide meets the DSD and CLP criteria. According to the criteria in Dir. 67/548, based on the results of the acute dermal toxicity studies cyanamide has to be classified as toxic and assigned the symbol “Xn” and the indication of danger “Harmful in contact with skin” accordingly. The following risk phrase should be assigned: “R21 Harmful in contact with skin”.

According to the criteria in Reg. 1272/2008, based on the results of the acute dermal toxicity studies cyanamide has to be classified as “Toxic in contact with skin, cat. 3”. The following risk phrase should be assigned: “H311 Toxic in contact with skin”.

This proposal is in agreement with the outcome of the EFSA peer-review.

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

4.3.1 Summary and discussion of Specific target organ toxicity – single exposure

No toxicity to a specific organ in the absence of lethality was observed in acute oral, inhalation or dermal toxicity studies in animals.

Cyanamide exposure (ingestion or inhaltion) alone when handled improperly or more pronounced in combination with alcohol consumption induces vasomotoric reactions in humans, known as "Cyanamide Flush"; including several clinical symptoms, e.g. facial flushing, tachycardia, dyspnea, hypotensia, headache, nausea, vomiting, tightness in the chest and sensation of coldness in the extremities. In general these symptoms disappear with no residual effects on general health without specific treatment. In the cases of exposure to larger quantities (gram range/day) severe irrritating properties of hydrogen cyanamide to the mucous membranes were also observed. Additional effects such as trembling, convulsion, salivation, danger of aspiration, pains behind the sternum and in the epigastrum, unconsciousness and final exits can occur. (see section 4.12.1.6 “Human information, Cyanamide in alcohol therapy”).

Calcium cyanamide has been intensively used worldwide as a drug to deter drinking in alcoholics. The consumption of alcoholic beverages after intake of cyanamide leads to intolerances with the aforementioned symptoms. It is presumed that the vasomotoric reactions are due to an inhibition of acetaldehyde dehydrogenase thus leading to a retardation in ethanol breakdown which stops on the stage of acetaldehyde accumulating in the blood. Intolerance reactions towards alcohol occur in man after daily cyanamide doses higher than 20 mg. In general, daily doses of more than 0.4 – 1 mg/kg bw cyanamide have been used in the alcohol aversion therapy (see section 4.12.1.6 “Human information, Cyanamide in alcohol therapy”).

4.3.2 Comparison with criteria

Well-substantiated human data are available showing adverse health consequences that might be attributed to a single exposure of cyanamide.

Table 21: Classification criteria for Categories 1 and 2 of specific target organ toxicity-single exposure

Toxicological data on cyanamide	CLP criteria	
Cyanamide exposure (ingestion or inhalation) alone or more pronounced in combination with alcohol consumption induces vasomotoric reactions, known as "Cyanamide Flush". It is presumded that the vasomotoric reactions are due to an inhibition of acetaldehyde dehydrogenase thus leading to a retardation in ethanol breakdown which stops on the stage of acetaldehyde accumulating in the blood. Symptoms disappear with no residual effects on general health without specific treatment. Intolerance reactions towards alcohol occur in man after daily cyanamide doses higher than 20 mg. In general, daily doses of more than 0.4 – 1 mg/kg bw cyanamide have been used in the alcohol aversion therapy.	Category 1	Substances that have produced significant toxicity in humans or that, on the basis of evidence from studies in experimental animals, can be presumed to have the potential to produce significant toxicity in humans following single exposure - reliable and good quality evidence from human cases or epidemiological studies; or - observations from appropriate studies in experimental animals in which significant and/or severe toxic effects of relevance to human health were produced at generally low exposure concentrations.
	Category 2	Substances that, on the basis of evidence from studies in experimental animals can be presumed to have the potential to be harmful to human health following single exposure - observations from appropriate studies in experimental animals in which significant toxic effects, of relevance to human health, were produced at generally moderate exposure concentrations.

There is evidence from human experience/incidents that the described toxicity by single exposure of cyanamide is not only restricted to reports of adverse health consequence, but provides the scientific detail that can be obtained from human data in alcohol therapy (clinical trials and case reports). There is no uncertainty about exposure conditions. Intolerance reactions towards alcohol occur in man after daily cyanamide doses higher than 20 mg. In general, daily doses of more than 0.4-1 mg/kg bw cyanamide have been used in the alcohol aversion therapy (see 4.12.1.6 Human information, Cyanamide in alcohol therapy). The mechanism of the vasomotoric reactions, known as "Cyanamide Flush" can be defined as an inhibition of acetaldehyde dehydrogenase.

However, these effects are most pronounced in combination with alcohol consumption. The combination effect of Cyanamide and alcohol does not justify STOT-SE.

From animal studies with Cyanamide no specific organ can be identified to trigger STOT-SE. Neither animal data nor human findings require a classification for specific target organ toxicity after single exposure (STOT-SE). Additionally, according to the guidance document to CLP regulation, “STOT-SE should be considered where there is clear evidence of toxicity to a specific organ especially when it is observed in the absence of lethality” (chapter 3.8.1). Effects observed in animals after acute exposure are considered to be covered by classification into acute toxicity category 3.

4.3.3 Conclusions on classification and labelling

According to the criteria in Reg. 1272/2008 and considering the results of animal studies and human experience/incidents, it is not proposed to classify cyanamide with STOT-SE category 1 or 2.

This proposal is in agreement with the outcome of the EFSA peer-review.

4.4 Irritation

4.4.1 Skin irritation

Three studies were performed to investigate the skin irritating potential of cyanamide. The results were contradictory: a newer study with an aqueous hydrogen cyanamide solution (49 % w/w) did not show any irritating potential, whereas two elder studies (SKW Cyanamide L 500 and preparation ALZODEF (> 25 %)) revealed a skin irritating potential of cyanamide. Regarding the dermal effects of the 21-day dermal toxicity study in rats (Murugan, S.S. et al. (1996), see section 4.7.1.3) and the result of skin sensitisation by the method of Buehler (Mercier (1988), see section 4.6.1.1), an overall weight of evidence suggests at least a skin irritating potential.

Table 22: Summary table of relevant skin irritation studies

Study type	Species / strain	Comments	Results m /f	Reference
Skin irritation	New Zealand White rabbit		Not irritating	Ligget, 1989 Doc-No. 565-002 TOX2004-361
Skin irritation	New Zealand White rabbit	Dermal reading 4 and 52 h after treatment	Irritating	van Beek, 1982 Doc-No 565-001 TOX2004-517
Skin irritation	New Zealand White rabbit	ALZODEF in 3 concentrations; highest concentration (25 % as)	Irritating (25 % active substance)	van Beek, 1984 Doc-No. 565-004 TOX2001-422

4.4.1.1 Non-human information

Title: Liggett (1989): Irritant effects on rabbit skin of aqueous hydrogen Cyanamide 49 % w/w, Doc. No. 565-002, (HRC report no 891330D/STB 4/Se); Huntingdon Research Centre Ltd., UK, unpublished

Guidelines: OECD 404 (1981); EEC 92/69- B4

Deviations: None

GLP: Yes

Acceptability: The study is considered to be acceptable.

Material and methods:

The test substance was identified as follows:

Cyanamide (supplied as aqueous solution)

Batch: 110889

Purity: 49 % (w/w), equivalent to 520 g/L of active substance cyanamide

Six albino rabbits (New Zealand white rabbits, supplied by Froxfield Farms Ltd.; Petersfield, Hampshire, England, body weight in the range of 2.2 to 2.9 kg at the age of 10 to 13 weeks) were exposed to an aliquot of 0.5 mL of aqueous hydrogen cyanamide which was applied onto the clipped skin (area approximately 10 cm^2) under a 2.5 cm^2 gauze pad to one intact skin site on each animal. The treatment site was occluded with a semi-occlusive adhesive dressing for a 4 h period. At the end of the exposure period the treatment site was washed with water to remove any residual test substance. Examinations on the treated skin were made 0.5, 24, 48 and 72 h after removal of the test substance. All animals were observed daily for clinical signs and mortality.

Findings:

The individual and mean scores of erythema and oedema are given in Table 23.

Table 23: Results of the primary skin irritation test with cyanamide in Albino rabbits

	Erythema						**Oedema**					
Animal no	**449**	**450**	**451**	**452**	**453**	**454**	**449**	**450**	**451**	**452**	**453**	**454**
0.5 h*	1	2	1	1	1	1	0	1	1	1	1	1
24 h*	0	1	0	0	1	0	0	0	0	0	0	0
48 h*	0	0	0	0	0	0	0	0	0	0	0	0
72 h*	0	0	0	0	0	0	0	0	0	0	0	0
Mean score 24 - 72 h	0.11						0					

* after end of exposure period

Half an hour after application, very slight oedema occurred in 5/6 animals and very slight to well-defined erythema were observed in 6/6 animals. After the first reading interval, very slight erythema was still observed in 2/6 animals, however, 24 h after application no oedema were observed. These reactions had resolved by the following day. The study was terminated on day 4, where no findings at all were observed.

Conclusion:

Cyanamide is not irritant to the skin under the test conditions according to the criteria specified in council directive 67/548/EEC.

Title: Beek, L. (1982): Primary dermal irritation/corrosion test with SKW Cyanamide L 500; Doc. No. 565-001; CIVO Institute TNO; Netherlands; No. B82-0061-4, unpublished.

Guidelines: The study follows the recommendations of OECD guideline 404 broadly.

Deviations: Skin reactions were scored at 4 and 52 h after end of exposure instead of 24, 48 and 72 h. Skin irritation study should not performed with abraded skin.

GLP: No (study was conducted prior to the implementation of GLP)

Acceptability: The study is considered to be supplementary.

Materials and methods:

The test substance was identified as follows:

SKW Cyanamide L 500

Batch: Not mentioned

Purity: Not mentioned

0.5 mL of the undiluted test substance was applied to the intact and abraded skin of six New Zealand White rabbits. The application sites were covered with gauze bandaging secured with several wrappings of adhesive tape and provided semi-occlusive dressing. Approximately 4 h after dosing the bandages and the test substance were removed. Effects of dermal irritation were recorded. Dermal readings were taken at approximately 4 h after bandage removal and 52 h after dosing.

Findings:

The individual scores of erythema and oedema are given in Table 24.

Table 24: Results of the primary skin irritation test with SKW Cyanamide in New Zealand White rabbits with intact skin

	Erythema						Oedema					
Animal no	**1016**	**1017**	**1018**	**1019**	**1020**	**1021**	**1016**	**1017**	**1018**	**1019**	**1020**	**1021**
after 4 h	1	3	1	3	2	4	3	2	2	3	2	2
after 52 h	2	4	2	4	4	4	1	1	1	1	2	2

Very slight to moderate erythema, slight ischemia and slight to severe oedema were observed 4 h after application. After 52 h, well-defined erythema, slight ischemia, distinct incrustation and very slight or slight oedema were noted. The treated skin areas had a slightly purple colour suggesting the presence of haemorrhages. One week after the treatment period, the greater part of the skin areas showed slight to distinct necrosis.

Conclusion:

It was concluded that SKW Cyanamide was primary skin irritant in Albino rabbits in this study.

Title: van Beek, L. (1984): Primary skin irritation tests with three aqueous dilutions of ALZODEF in Albino rabbits; Doc. No. 565-004; CIVO institutes TNO, Netherlands; No. B 83-0061/48; unpublished.

Guidelines: The study follows to a great extent OECD guideline 404.

Deviations: Reporting deficiencies (housing and feeding conditions); only 50 % of technical active substance cyanamide were tested.

GLP: No, however the study seems to follow the GLP principles throughout experimental phase and reporting.

Acceptability: The study is considered to be supplementary.

Materials and methods:

The test substance was identified as follows:

ALZODEF (50 % aqueous dilution of cyanamide)

Batch: 9.11.84

Purity: Unclear

The test substance was diluted with water to ALZODEF dilutions with a cyanamide content of 1, 5 and 25 %. The ALZODEF dilutions were applied on the intact skin of six New Zealand White rabbits. The application sites were covered with gauze bandaging secured with several wrappings of adhesive tape and provided semi-occlusive dressing. Approximately 4 h after dosing the bandages and the test substance were removed. Effects of dermal irritation were recorded. Dermal readings were taken approximately at the end of exposure period and at 24, 48 and 72 h after dosing.

Findings:

The individual and mean scores of erythema and oedema for ALZODEF 1 % are given in Table 25, for ALZODEF 5 % in Table 26 and for ALZODEF 25 % in Table 27.

Table 25: Results of the primary skin irritation test with cyanamide (ALZODEF 1 %) in New Zealand White rabbits

	Erythema						Oedema					
Animal no	**1871**	**1872**	**1873**	**1874**	**1875**	**1876**	**1871**	**1872**	**1873**	**1874**	**1875**	**1876**
after 4 h*	2	1	1	1	1	1	1	0	0	1	1	1
after 24 h*	1	0	1	1	1	0	0	0	0	1	0	0
after 48 h*	1	0	0	1	1	0	0	0	0	0	0	0
after 72 h*	0	0	0	1	1	0	0	0	0	0	0	0
mean score 24 - 72 h*	0.5						0.06					

* after end of exposure

Table 26: Results of the primary skin irritation test with cyanamide (ALZODEF 5 %) in New Zealand White rabbits

	Erythema						Oedema					
Animal no	**1871**	**1872**	**1873**	**1874**	**1875**	**1876**	**1871**	**1872**	**1873**	**1874**	**1875**	**1876**
after 4 h	1	1	1	1	1	1	0	1	1	1	0	0
after 24 h*	0	1	1	1	1	0	0	1	0	0	0	0
after 48 h*	0	1	1	1	0	0	0	0	0	0	0	0
after 72 h*	0	1	1	1	0	0	0	0	0	0	0	0
Mean score 24 - 72 h*	0.6						0.06					

* after end of exposure

Table 27: Results of the primary skin irritation test with cyanamide (ALZODEF 25 %) in New Zealand White rabbits

	Erythema						Oedema					
Animal no	**1871**	**1872**	**1873**	**1874**	**1875**	**1876**	**1871**	**1872**	**1873**	**1874**	**1875**	**1876**
after 4 h	2	1	1	4	1	4	0	1	1	1	1	1
after 24 h*	1	1	1	2	1	2	0	1	0	1	1	1
after 48 h*	1	1	1	2	1	1	0	0	0	0	0	0
after 72 h*	0	1	1	2	1	1	0	0	0	0	0	0
mean score 24 - 72 h*	1.2						0.2					

* after end of exposure

The ALZODEF dilutions containing 1 or 5 % cyanamide caused very slight erythema with or without very slight oedema and slight scaliness of the treated skin. The ALZODEF dilution containing 25 % cyanamide caused erythema, oedema and some slight scaliness. The erythema was well defined 4 h after treatment. 24 h after treatment, the erythema was slight in two animals and very slight in four animals. Two rabbits showed very slight ischaemia at the end of the exposure period. All reactions had cleared up completely during the 7-day period following the exposure period.

Conclusion:

The test substance does not induce skin irritancy at 1 % and 5 % concentrations under the test conditions. At 25 % concentration severe primary skin irritancy was observed 4 h after treatment. Therefore it was concluded, that ALZODEF was skin irritant in this study at the concentration of 25 %.

A position paper on the validity of acute toxicity studies was submitted by the notifier (Moeller & Hofer, 2009). The results of the position paper concerning skin irritation are presented in the following.

The available studies for skin irritation, i.e. Ligget (1989; GLP and guideline compliant) and van Beek (1982) and van Beek (1984), are considered as sufficient for classification and labelling although the studies of van Beek are only considered as supplementary by the RMS due to guideline deviations and non-GLP status. Van Beek (1982) investigated skin irritation/corrosion of undiluted Cyanamide L500 on intact and abraded skin, the latter one not recommended according to guideline. The study is considered supplementary as there were deviations from OECD 404. Skin reactions were not scored after 24, 48 and 72 h, instead after 4 and 54 h. This deviation did not impact the result of the study as reactions were already observed after 4 h. Van Beek (1984) investigated also the irritancy of Alzodef (50 % cyanamide)-dilutions containing 1, 5 and 25 % cyanamide, nevertheless, the study followed OECD 404 to a great extent and is thus considered as supplementary. Although results are not consistent, the studies are considered sufficient for the purpose of classification and labelling.

Conclusion of the Rapporteur Member State (RMS):

In agreement with the notifier it is concluded, that sufficient data on skin irritation of cyanamide are available.

4.4.1.2 Human information

Severe irritation can occur in humans after dermal exposure (see section 4.12.1.6 "Human information, Cyanamide in alcohol therapy").

4.4.1.3 Summary and discussion of skin irritation

Three studies were performed to investigate the skin irritating potential of cyanamide. The results were contradictory: a newer study with an aqueous hydrogen cyanamide solution (49 % w/w) did not show any irritating potential, whereas two elder studies (SKW Cyanamide L 500 and preparation ALZODEF (> 25 %)) revealed a skin irritating potential of cyanamide.

Regarding the dermal effects of the 21-day dermal toxicity study in rats (Murugan, S.S. et al. (1996), see section 4.7.1.3) and the result of skin sensitisation by the method of Buehler (Mercier (1988), see section 4.6.1.1), an overall weight of evidence suggests at least a skin irritating potential.

In the study of Beek, L. (1982) after dermal application of New Zealand White rabbits very slight to moderate erythema, slight ischemia and slight to severe oedema were observed 4 h after application. After 52 h well-defined erythema, slight ischemia, distinct incrustation and very slight or slight oedema were noted. The treated skin areas had a slightly purple colour suggesting the presence of haemorrhages. One week after the treatment period the greater part of the skin areas showed slight to distinct necrosis.

In the study of van Beek, L. (1984) after dermal application of New Zealand White rabbits The ALZODEF dilution containing 25 % cyanamide caused erythema, oedema and some slight scaliness. The erythema was well defined 4 h after treatment. 24 h after treatment the erythema was slight in two animals and very slight in four animals. Two rabbits showed very slight ischaemia at the end of the exposure period. All reactions had cleared up completely during the 7-day period following the exposure period.

After dermal exposure severe irritation can occur in humans (see section 4.12.1.6 "Human information, Cyanamide in alcohol therapy").

Further relevant information on skin effects upon single exposure is reported in section 4.5 (corrosion).

4.4.1.4 Comparison with criteria

Please refer to section 4.5 (corrosion).

4.4.1.5 Conclusions on classification and labelling

The results of the studies of Ligget, 1991 and van Beek, 1974 meet the DSD (Xi, R38) and CLP criteria (skin irritation cat. 2, H315). Based on an in vitro skin corrosion test with Cyanamid L 500 using EpiDerm reconstructed skin membranes (Reus, 2011; see chapter 4.5.1), the test substance has to be classified as corrosive (Skin Corr. 1B, H314).

Please refer also to section 4.5 (corrosion).

4.4.2 Eye irritation

Aqueous hydrogen cyanamide 49 % w/w was an eye irritant to one New Zealand white rabbit under the test conditions. Due to the severity of the reaction no further animals were exposed to the test substance. In a second study a 50 % aqueous dilution of cyanamide (SKW Cyanamide L 500) was irritating to the eyes of six New Zealand white rabbits. One week after the end of exposure some recovery was observed. After 7 d, slight conjunctivitis was still noted in all animals.

Table 28: Summary table of relevant eye irritation studies

Study type	Species / strain	Comments	Results m /f	Reference
Eye irritation	New Zealand White rabbit	Due to moderate/ severe ocular lesions only one animal was exposed	Irritating	Ligget, 1991 Doc-No. 566-002 TOX2001-423
Eye irritation	New Zealand Whit rabbit	Light opacity, mild iritis, moderate redness and moderate to severe swelling of conjunctiva	Irritating	van Beek, 1974 Doc. No. 566-001 TOX2001-423

4.4.2.1 Non-human information

Report: Liggett (1991): Eye irritation to the rabbit of aqueous hydrogen cyanamide 49 % w/w; Doc. No. 566-002; Huntingdon Research Centre Ltd., UK; (HRC report no 91660/STB 11/SE); unpublished

Guidelines: EPA 81-4 (claimed by the author)

Deviations: No major deviations from OECD 405

GLP: Yes

Acceptability: The study is considered to be acceptable.

Material and methods:

The test substance was identified as follows:

Cyanamide (supplied as aqueous solution)

Batch: 06/20/91

Purity: 49 % (w/w), equivalent to 520 g/L of active substance hydrogen cyanamide

The test substance was applied in a single dose of a 0.1 mL aliquot into the lower everted lid of one eye of an adult New Zealand white rabbit (Foxfields Rabbits; Petersfield, Hampshire, England, body weight 3 kg, approximately 14 weeks old). The eye lids were then gently hold together for one second before releasing. The contralateral eye remained untreated and served as a control. Examination of the eye was made 1 h as well as 1, 2, 3, 4 and 7 d after the end of exposure. The animal was observed daily for clinical signs and behaviour.

Findings:

The results of the primary eye irritation test of technical cyanamide are summarised in Table 29.

Table 29: Results of the primary eye irritation test in a rabbit with cyanamide

Time	Cornea	Iris	Conjunctiva		
			redness	chemosis	discharge
1 h	D*	0	2	3	3
24 ho	2	0	2	3	3
48 ho	2	1	2^+	2	2
72 h	2	1	2^+	2	2
96 h	1	0	1^+	1	1
7 d	0	0	0	0	0
mean scores 24 - 72 h	2	0.67	2	2.3	

D = Dulling of the cornea
$^+$ haemorrhage of nictating membrane

1 h after the end of exposure, the cornea was dull and by the following day an opacity had developed which persisted for the following 3 d and resolved 7 d after instillation. Iritis was observed 2-3 d after the end of exposure. A diffuse conjunctiva redness was accompanied by swelling with the lids half closed and a copious discharge. There was also evidence of haemorrhage on the nictating membrane. All lesions had resolved 7 d after the end of exposure.

Due to the severity of the reaction no further animals were exposed to the test substance.

Conclusion:

According to the criteria specified in council directive 67/548/EEC cyanamide was an eye irritant to one New Zealand white rabbit under the test conditions.

Title: van Beek, A.P. (1974): Eye irritation test with SKW Cyanamide L 500; Doc. No. 566-001; Centraal Instituut voor Voedingsonderzoek; Netherlands, No. 4398; unpublished.

Guidelines: Not mentioned; however, the study complies to a great extent with OECD Guideline 405.

Deviations: No eye examination 1 h after instillation.

GLP: No (study was conducted prior to the implementation of GLP)

Acceptability: Study is considered to be acceptable.

Materials and methods:

The test substance was identified as follows:

SKW Cyanamide L 500 (50 % aqueous dilution of cyanamide)

Batch: Not mentioned

Purity: Unclear

The test substance cyanamide was applied in a single dose of a 0.1 mL aliquot into the lower everted lid of one eye of six adult New Zealand white rabbits. The eye lids were gently held together for one second before releasing. The other eye remained untreated and served as a control. The eyes were not washed following instillation. The eyes are examined 24, 48, 72 h and 7 d after the end of exposure. The animals were observed daily for clinical signs and behaviour.

Findings:

The results of the primary eye irritation test of SKW Cyanamide L 500 are summarised in Table 30.

Table 30: Results of primary eye test of Cyanamide L 500

	Cornea						**Iris**						**Conjunctiva-redness**						**Conjunctiva-chemosis**					
Time / Rabbit	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
24 hours	1	1	1	1	2	1	1	1	1	1	1	1	>2	2	2	2	2	2	3	3	2	3	2	2
48 hours	1	1	1	1	2	1	1	1	1	1	1	1	>2	2	2	2	2	2	3	3	2	2	2	2
72 hours	1	1	0	2	1	1	0	1	0	1	1	1	2	2	2	2	1	1	2	3	3	2	2	1
7 days	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0	1	1	1	1	1
mean scores 24-72 h	1.1						0.9						1.9						2.3					

The test substance caused eye irritation consisting of light opacity, mild iritis, moderate redness and moderate to severe swelling of the conjunctiva in all test rabbits after 24 h. One week after the end of exposure some recovery was observed. After 7 d, slight conjunctivitis was still noted in all animals.

Conclusion:

Under the experimental conditions chosen cyanamide (SKW Cyanamide L 500) was irritating to the eyes of New Zealand white rabbits according to the criteria specified in council directive 67/548/EEC.

A position paper on the validity of acute toxicity studies was submitted by the notifier (Moeller & Hofer, 2009). The results of the position paper concerning eye irritation are presented in the following.

Two studies for eye irritation are available, i.e. Ligget (1991) and van Beek (1974). Both studies are considered acceptable since there were no major deviations from OECD 405. Although the study of van Beek was performed prior to implementation of GLP and the eye was not examined after 1 h, the result is considered acceptable. Both studies show eye irritating properties and are considered sufficient for the purpose of classification and labelling.

Conclusion of the Rapporteur Member State (RMS):

In agreement with the notifier it is concluded that sufficient data on eye irritation of cyanamide are available.

4.4.2.2 Human information

No relevant data are available.

4.4.2.3 Summary and discussion of eye irritation

Cyanamide was an eye irritant to New Zealand white rabbits in two independent studies.

4.4.2.4 Comparison with criteria

Table 31 presents the toxicological results in comparison with DSD and CLP criteria.

Table 31: Toxicological results of relevant eye irritation studies in comparison with DSD and CLP criteria

Toxicological result	DSD criteria	CLP criteria
Mean Score*: corneal opacity: 2 iris lesion: 0.67 conjunctival redness: 2 oedema of the conjunctivae (chemosis): 2.3	Irritating to eyes: cornea opacity: ≥ 2 - < 3 iris lesion: ≥ 1 - < 1,5 redness of the conjunctivae: ≥ 2,5 oedema of the conjunctivae (chemosis): ≥ 2	Irritating to eyes (Category 2): corneal opacity: ≥ 1 iritis: ≥ 1 conjunctival redness: ≥ 2 conjunctival oedema (chemosis): ≥ 2
Mean Score**: corneal opacity: 1.1 iris lesion: 0.9 conjunctival redness: 1.9 oedema of the conjunctivae (chemosis): 2.3		

* Ligget, 1991, Doc-No. 566-002, TOX2001-423 ** van Beek, 1974, Doc. No. 566-001, TOX2001-423

4.4.2.5 Conclusions on classification and labelling

The results of the studies of Ligget, 1991 and van Beek, 1974 meet the DSD (Xi, R36) and CLP criteria (eye irritation cat. 2, H319).

However as the substance is skin corrosive (cat. 1B, H314 according to CLP criteria and C, R 34 according to DSD criteria), specific classification as an eye irritant is not necessary, because it is already included implicitly in the classification as skin corrosive. Therefore, no classification and labeling for eye irritation is necessary.

Please refer also to section 4.5 (corrosion).

This proposal is not in agreement with the outcome of the EFSA peer-review, because the respective studies driving the assessment were not available in that procedure.

4.4.3 Respiratory tract irritation

4.4.3.1 Non-human information

In the acute (4-hour) inhalation toxicity study in rats with cyanamide no mortalities were recorded during the study. During the exposure a lumback behaviour and a rapid shallow respiration with frequent coughing and swallowing were observed. Within a few hours after exposure all animals were completely recovered and showed normal behaviour. No visible lesions were observed at gross necropsy (Kruysse, 1973).

In the 14-day inhalation study in Wistar rats, no mortality and no clinical signs of toxicity were observed. There were liver and kidney weight changes in males and females. Histopathological changes revealed consistent recurring lesions in the brain (oedema), liver (centrilobular cloudy swelling, hyperaemia), heart and lungs in the high dose group in both sexes. Bronchiectasis was observed in 2 males and 1 female (5 of each sex) (Kumar et al., 1996).

There is no evidence of respiratory tract irritation in the available studies.

4.4.3.2 Human information

No relevant data.

4.4.3.3 Summary and discussion of respiratory tract irritation

There are no relevant human data. The only relevant findings with respect to respiratory tract irritation were clinical signs (lumback behaviour and a rapid shallow respiration with frequent coughing and swallowing) in an acute inhalation study. No gross findings were reported in the study report.

4.4.3.4 Comparison with criteria

Considering that the findings were restricted to clinical signs which were not corroborated by histological findings, it is concluded that the findings were not sufficient to classify with STOT-SE cat. 3.

4.4.3.5 Conclusions on classification and labelling

Classification and labelling is not required.

4.5 Corrosivity

4.5.1 Non-human information

Title: Reus, A.A. (2011): In vitro skin corrosion test with Cyanamid L 500 using EpiDerm reconstructed skin membranes; TNO Triskelion, AV Zeist, The Netherlands; Internal Study Code: V20018/01; Doc. No. 565-005 (unpublished).

Guidelines: The study follows to:

- Council regulation (EC) No. 440/2008. Official Journal of the European Union L142/394 – 399. 31 May 2008. Annex 40 Bis: In vitro skin corrosion: Human skin model test.

- Organisation for Economic Co-operation and Development (OECD). Guideline no. 431. OECD Guideline for the testing of chemicals. In vitro skin corrosion: Human skin model test. Adopted 13 April 2004.

Deviations: No

GLP: Yes

Acceptability: The study is considered to be acceptable.

Materials and methods:

Lot/Batch number:	103401
Specification:	As given in section 2
Description:	Clear colourless liquid
Purity:	50.2 % a.s.c. (w/w)
Stability	No stability data indicated; not required as test substance was immediately used in this test.

The test substance was applied as provided.

Test System	Species Human (neonates)
Type:	Normal human epidermal keratinocytes (NHEK) from one single donor, derived from neonatal-foreskin tissue.

The study was designed to examine the potential of Cyanamid L 500 to cause skin corrosion in vitro using EpiDerm™ reconstructed skin membranes.

The EpiDerm™ skin models were exposed to one dose level of the test substance for 3 min and for 60 min. Negative and positive controls were run in parallel. The MTT test was performed to determine the viability of the skin models immediately after exposure. The general principle for the

determination of viability via the MTT test is the conversion of the yellow tetrazolium salt (MTT) to the purple coloured product formazan by mitochondrial enzymes. The formation of formazan was measured using a spectrophotometer.

The EpiDermTM (EPI-200) skin model consists of normal human epidermal keratinocytes (NHEK) from one single donor, derived from neonatal-foreskin tissue. The keratinocytes are plated on chemically modified, collagen-coated, 9 mm ID cell culture inserts (surface area 0.64 cm^2). The skin models are commercially available and were obtained form MatTek Corporation, USA. Upon arrival at TNO Triskelion on 23 February 2011, the EPI-200 skin models were pre-incubated in 0.9 mL assay medium, (lot. 021711TTA) provided with the kit, for 60 min in a humidified incubator (ca. 37 ºC and 5 % CO_2). At the end of the pre-incubation period, the skin models were transferred to a new 6-well plate containing 0.9 mL assay medium and kept in a humidified incubator until the start of the treatment. The certificate of analysis (CoA) and quality control data (QC) of the EpiDerm™ reconstructed skin model are provided in Appendices 2 and 3, respectively. The MTT-100 assay kit was obtained form MatTek Corporation, USA. Upon arrival at TNO Triskelion, the MTT concentrate was stored at < −18 ºC and the MTT diluent was stored at 2-10 ºC until use.

The in vitro corrosive potential of the test substance was determined from the relative mean tissue viabilities compared to the negative control tissues, using the following prediction model:

Table 32: Prediction model

Mean tissue viability (% of negative control) 3 min	60 min	Prediction
< 50 %	-	Corrosive
≥ 50 %	< 15 %	Corrosive
≥ 50 %	≥ 15 %	Non-Corrosive

Findings:

The test substance Cyanamid L 500 was examined for its in vitro skin corrosion potential using EpiDerm™ reconstructed skin membranes. Optical density of the negative control (Milli-Q) and positive control (8 M KOH) were within the acceptable ranges and correctly indicated non corrosivity and corrosivity, respectively. After 60 min exposure to the positive control (8 M KOH), the viability of was 15 ± 14 % resulting in a relatively high coefficient of variation (CV) of 91 %. In the range between 20 % and 100 % mean tissue viability the CV was ≤ 30 %. Therefore the study was considered valid.

Table 33: Formazan production in EpiDerm™ skin membranes exposed to the negative control, test substance and positive control

Test group	Study substance	A_{570} (% of control) 3 min	60 min
NC	Negative control (Milli-Q)	100 ± 2	100 ± 7
A	Cyanamid L 500	85 ± 0	7 ± 0
PC	Positive control (8 M KOH)	10 ± 1	15 ± 14

Conclusion:

Based on the results presented in the table above, the test substance Cyanamid L 500 was classified as corrosive.

Further information from the registration dossier:

Van Beek 1982[3]: A skin irritation study similar to OECD TG 404 was conducted with Cyanamide F1000. The registrant considered this study as "reliable with restrictions" (because skin reactions were scored at 4 and 52 h after end of exposure instead of 24, 48 and 72 h which was the case for all skin irritation studies by Van Beek, *c.f.*, section 4.4.1). Following executive summary is provided in the dossier:

> "0.5 g of the test substance was applied to the intact or abraded skin of six New Zealand White rabbits. The application sites were covered with gauze bandaging secured with several wrappings of adhesive tape and provided semi-occlusive dressing. Approximately 4 h after dosing the bandages and the test substance were removed. Dermal readings were taken at approximately 4 h after bandage removal and 52 h after dosing.
>
> The observations showed after 4 h very slight to moderate erythema, focal haemorrhages and slight or moderate edema; after 52 h, well-defined to moderate erythema, slight ischemia, very slight incrustation and very slight edema were observed. The treated skin areas had a purple colour suggesting the presence of haemorrhages. After one week the greater part of the treated skin areas showed slight to distinct necrosis, therefore it can be concluded according to the CLP/GHS regulations that KW-Cyanamide F 1000 is a severe primary skin irritant and that it is corrosive to the skin of albino rabbits when brought into contact with the skin for 4 h.

4.5.2 Human information

After dermal exposure severe irritation can occur in humans (see section 4.12.1.6 "Human information, Cyanamide in alcohol therapy").

[3] van Beek, L. (1982): Primary dermal irritation/corrosion test with SKW-Cyanamide F 1000 in Albino rabbits; Testing laboratory: CIVO Institutes TNO; Report no: B82-0061-4

This study was not submitted for the evaluations under PPP directive or BPD.

4.5.3 Summary and discussion of corrosivity

The potential of cyanamide was examined to cause skin corrosion in vitro using EpiDerm™ reconstructed skin membranes (Reus, 2011). The method is sufficiently validated for classification according to CLP. The test refers to the production of irreversible tissue damage in the skin following the application of a test material [as defined by the Globally Harmonised System for the Classification and Labelling of Chemical Substances and Mixtures (GHS)]. The test method does not normally provide adequate information on skin irritation, nor does it allow the subcategorisation of corrosive substances as permitted in the Globally Harmonised Classification System (GHS). However, the positive control was potassium hydroxide. According OECD Guideline no. 431 potassuim hydroxide is a corrosive reference chemical. Positive in vitro results do not require further testing and can be used for classification. In comparison to potassuim hydroxide the results of the assay demonstrated that cyanamid is corrosive.

In a skin irritation study similar to OECD TG 404 conducted with Cyanamide F1000, well-defined to moderate erythema, slight ischemia, very slight incrustation and very slight edema were observed after 52 h. The treated skin areas had a purple colour suggesting the presence of haemorrhages. After one week the greater part of the treated skin areas showed slight to distinct necrosis. Hence the seen effects were not completely reversible with in the observation period.

After dermal exposure severe irritation can occur in humans (see section 4.12.1.6 “Human information, Cyanamide in alcohol therapy”).

Table 34: Summary table of relevant skin irritation studies

Study type	Species / strain	Comments	Results m /f	Reference
In vitro skin corrosion test	EpiDerm™ reconstructed skin membranes	In vitro EpiDerm assay, human epidermal keratinocytes	Corrosive	Reus, 2011
In vivo skin irritation/corrosion	Rabbit	Skin effects not reversibel up to 52 h post-administration	Corrosive	Van Beek, 1982

4.5.4 Comparison with criteria

Comparison with criteria in DSD:

The criteria for classification with R35 (Causes severe burns) are:
“if, when applied to healthy intact animal skin, full thickness destruction of skin tissue occurs as a result of up to three minutes exposure, or if this result can be predicted.”

The criteria for classification with R34 (Causes burns) are:
“if, when applied to healthy intact animal skin, full thickness destruction of skin tissue occurs as a result of up to four hours exposure, or if this result can be predicted […]“

Skin effects indicative for full thickness destruction (incrustations after 52 h and necrosis after 1 week) were described the in vivo study with Cyanamide F1000. There was an exposure period of 4 h. These effects lead to a classification with R34. Even though, in vitro methods are not mentioned in the criteria, the study results by Reus (2011) support the classification with R34.

Comparison with criteria in CLP regulation:

Based on the results of the in vitro skin corrosion test with Cyanamid L 500 using EpiDerm reconstructed skin membranes (Reus, 2011) cynamide is corrosive. According the Guidance to

Regulation (EC) No 12772/2008 human skin models like this test (OECD 431) are accepted at present to allow a classification into Skin Corrosion Category 1 A to 1 C on its own. Accordingly, the same criteria as used to classify based on animal studies shall be applied. Considering that a corrosive effct was noted after exposure durations of longer than 3 min and shorter than 1 h, a classification into category 1 B (H314) can be selected.

The skin effects (necrosis) in the in vivo study with Cyanamide F1000 persisted until the end of the observation period. There was an exposure period of 4 h leading to a classification with skin corrosion category 1 C (H314).

Overall, the classification into category 1 is required. The results of the in vitro study are preferred compared to the results of the in vivo study, based on the better study conduct (i.e., limited times of scoring of the animal's skin reactions), hence a classification into category 1 B is proposed.

4.5.5 Conclusions on classification and labelling

According to the criteria in Dir. 67/548, cyanamide has to be classified as "corrosive" and assigned the symbol "C". The indication of danger "R34 Causes burns" should be assigned.

According to the criteria in Reg. 1272/2008, cyanamide has to be classified as "Skin Corrosion Category 1 B". The following risk phrase should be assigned: "H314: Causes severe skin burns and eye damage".

This proposal is not in agreement with the outcome of the EFSA peer-review, because the respective study driving the assessment was not available in that procedure.

4.6 Sensitisation

4.6.1 Skin sensititsation

4.6.1.1 Non-human information

Title:	Til, H.P. (1982): Sensitisation Test with SKW Cyanamide F 1000 in Guinea Pig; Doc. No. 567-003; CIVO Institute TNO, Netherlands; B 82-0063/1; unpublished.
Guidelines:	The study follows to a great extent OECD guideline 406 (Guinea Pig Maximisation Test).
Deviation:	None
GLP:	Yes
Acceptability:	The study is considered to be acceptable.

Materials and methods:

The test substance was identified as follows:

SKW Cyanamide F 1000

Batch: Not mentioned

Purity: Not mentioned

The dermal sensitisation potential of SKW Cyanamide F 1000 (pure active substance) was evaluated in the Guinea Pig Maximisation Test (GPMT) according to the method of Magnusson and Kligman in 15 male albino guinea pigs (Central Institute for the breeding of Laboratory animals TNO; Netherlands). The test conditions are shown in Table 35.

Table 35: Test conditions of SKW Cyanamide F 1000 – Guinea Pig Maximisation Test (GPMT)

Intradermal induction	10 % (v/v) in water
Dermal induction (one week after intradermal induction)	5 % (w/w) in vaseline
Dermal challenge (two weeks after dermal induction)	1 % (w/w) in vaseline (left flank) 2.5 % (w/w) in vaseline (right flank)

Approximately 24 and 48 h after the challenge phase, the test sites were evaluated for signs of elicited sensitisation. The same procedures were carried out on a contemporaneous control group, where the test articles was replaced by water (vehicle control).

Findings:

Induction: The intradermal injection caused slight erythema and abscesses in all test animals. The dermal applications induced slight erythema in 4/10 test animals.

The results obtained during the challenge treatment of the 1 % dilution are summarised in Table 36 and for the 2.5 % dilution of SKW Cyanamide F 1000 are listed in Table 37.

Table 36: Results – Guinea Pig Maximisation Test (GPMT) of 1 % SKW Cyanamide F 1000

Control group animals	Directly after patch removal	24 h after patch removal	48 h after patch removal	Test group animal	Directly after patch removal	24 h after patch removal	48 h after patch removal
1	1/0	0/0	0/0	1	3/1	3/1	3/1
2	0/0	0/0	0/0	2	2/1	2/1	2/1
3	0/0	0/0	0/0	3	1/1	0/0	1/0
4	0/0	0/0	0/0	4	2/1	1/1	1/0
5	0/0	0/0	0/0	5	1/0	1/1	1/1
-	-	-	-	6	1/0	2/0	0/0
-	-	-	-	7	2/1	2/1	2/1
-	-	-	-	8	1/0	0/0	0/0
-	-	-	-	9	2/1	1/1	0/0
-	-	-	-	10	1/0	2/1	1/0

Score: Grade of erythema/oedema

The challenge treatment of the left flank (1 % dilution) provoked slight to moderate erythema in all test animals. The erythema was visible immediately after removal of the dressing. At the same time one of the control animals reacted slight positive. After 24 and 48 h most of the test animals still

showed slight to moderate erythema (see Table 37). None of the control animals showed a positive reaction at this time. No test substance-related clinical signs of toxicity were observed.

Table 37: Results – Guinea Pig Maximisation Test (GPMT) of 2.5 % SKW Cyanamide F 1000

Control group animals	Directly after patch removal	24 h after patch removal	48 h after patch removal	Test group animal	Directly after patch removal	24 h after patch removal	48 h after patch removal
1	1/0	0/0	0/0	1	4/1	4/1	4/1
2	0/0	0/0	0/0	2	3/1	3/1	3/1
3	0/0	0/0	0/0	3	2/1	3/0	2/0
4	0/0	0/0	0/0	4	2/1	1/1	1/0
5	0/0	0/0	0/0	5	2/1	3/1	3/1
-	-	-	-	6	2/1	2/1	2/1
-	-	-	-	7	2/1	2/1	2/1
-	-	-	-	8	3/1	2/1	2/0
-	-	-	-	9	3/1	2/1	2/1
-	-	-	-	10	3/1	4/1	4/1

Score: grade of erythema/oedema

All ten test animals challenged with 2.5 % dilution of the test substance showed well-defined to severe erythema immediately after removing of the dressing. At the same time one of the control animals reacted slight positive. After 24 and 48 h the test animals still showed clear positive reactions. None of the control animals showed a positive reaction at this time. No test substance-related clinical signs of toxicity were observed.

Conclusion:

Based on the results of the Guinea Pig Maximisation Test (GPMT) according to the method of Magnusson and Klingman, the active substance hydrogen cyanamide is a sensitiser.

Report: Mercier (1988): Test to evaluate the sensitising potential by topical applications in the guinea-pig (Buehler test); Doc. No. 567-001; Hazelton Institute Francais de Toxicologie, ST- Germain-sur-l`Arbresle; France, (report no 807313); unpublished

Guidelines: OECD 406 (1981); EEC 92/69- B6

Deviations: None. However, based on EU evaluation practice 9 induction applications instead of only 3 are recommended for Buehler test.

GLP: Yes

Acceptability: The study is considered to be supplementary.

Material and methods:

The test substance was identified as follows:

Cyanamide (aqueous solution)

Batch: 07/07/87

Purity: 49 % (w/w), equivalent to 520 g/L of active substance hydrogen cyanamide

Test animals were Dunkin Hartley guinea-pigs, supplied by Iffa Credo; l´Arbresle, France.

A preliminary test was performed to define the irritative threshold. In the main study 20 animals (10 per sex) were tested with 0.5 mL of a 40 % solution of the test substance in distilled water which was found to be maximum non-irritant in the preliminary test. This concentration was used for the induction on day 1, 8 and 15 and for the challenge on day 29. The suitability of the test system and study design was confirmed by analogous investigations with the known sensitising compound 2,4-dinitrochlorbenzene (DNCB) in 20 animals (10/10) as a positive control. During the induction and challenge period DNCB was applied at the concentration of 0.5 %.

Histopathological examinations of the skin were carried out with all positive control animals and in treated animals with macroscopic reactions.

Findings:

The results of the Buehler test in males are depicted in Table 38 and in females in Table 39

Table 38: Results in the Buehler test of cyanamide in males

Control group animals	24 h after patch removal	48 h after patch removal	Test group animal	24 h after patch removal	48 h after patch removal
1	0/0	0/0	1	0/0	0/0
2	0/0	0/0	2	0/0	0/0
3	0/0	0/0	3	0/0	0/0
4	0/0	0/0	4	0/0	0/0
5	0/0	0/0	5	0/0	0/0
6	0/0	0/0	6	0/0	0/0
7	0/0	0/0	7	0/0	0/0
8	0/0	0/0	8	0/0	0/0
9	0/0	0/0	9	1/0	not applicable
10	0/0	0/0	10	1/0	not applicable

Score: grade of erythema/oedema

Table 39: Results in the Buehler test of cyanamide in females

Control group animals	24h after patch removal	48h after patch removal	Test group animal	24h after patch removal	48h after patch removal
1	0/0	0/0	1	1/0	not applicable
2	0/0	0/0	2	0/0	0/0
3	0/0	0/0	3	0/0	0/0
4	0/0	0/0	4	0/0	0/0
5	0/0	0/0	5	0/0	0/0
6	0/0	0/0	6	1/0	not applicable
7	0/0	0/0	7	0/0	0/0
8	0/0	0/0	8	0/0	0/0
9	0/0	0/0	9	0/0	0/0
10	0/0	0/0	10	0/0	0/0

Score: grade of erythema/oedema

In both sexes there were no test substance related signs of clinical intoxication or impairment of body weight development. During the induction period, no oedema or erythema were observed. Skin reactions did not occur after the challenge application in control and in most of the treated animals. Two males (see Table 38) and two females (see Table 39) of the treated group showed slight erythema. The histopathological examination showed evidence of a reaction of orthoergic

type (corresponding to a reaction of an irritant type) in one male. No abnormalities was found in the other 3 animals.

The animals of the positive control group showed effects of "delayed hypersensitivity type" in 13/19 animals. Histopathological examinations revealed evidence of images of the allergic type (spongiosis) in 3/19 animals; 16 other animals showed orthoergic reactions. DNCB thus provoked "delayed hypersensitivity" in 13 animals at minimum. The caustic potential of DNCB did not allow the histopathological examinations to specify its allergenicity.

Conclusion:

Under the conditions described in the study, cyanamide induced some skin reactions after challenge application although the result does not clearly indicate a sensitising potential of cyanamide.

A position paper on the validity of acute toxicity studies was submitted by the notifier (Moeller & Hofer, 2009). The results of the position paper concerning skin sensitisation are presented in the following.

Skin sensitisation was investigated by Til (1982) and Mercier (1988). Til (1982) performed a GLP-compliant study according to Magnusson and Kligman 1970, and thus complies to a great extent to OECD 406. The result shows sensitising properties of cyanamide and is considered acceptable. The study by Mercier (1988) follows the Buehler method and OECD 406 (1981). Although following guidelines, GLP and reporting of purity and batch, the study is considered as supplementary since only 3 inductions were performed. Nevertheless, the Magnusson & Kligman study by Til (1982) is considered sufficient for the purpose of classification and labelling.

Conclusion of the Rapporteur Member State (RMS):

In agreement with the notifier it is concluded that sufficient data on skin sensitisation of cyanamide are available.

4.6.1.2 Human information

A few cases of dermal sensitisation in humans have been reported. However, based on the information from the cyanamide production Degussa AG (formerly SKW Trostberg AG) that no cases of confirmed or suspected allergy towards cyanamide occurred. Even though these persons were heavily exposed, there is very limited evidence that cyanamide causes skin sensitisation in humans (see section 4.12.1.6 "Human information").

4.6.1.3 Summary and discussion of skin sensitisation

According to the classification by Magnusson and Kligman, cyanamide had skin-sensitising properties: after dermal application of SKW Cyanamide F 1000 (pure active substance) all Albino guinea pigs showed a positive response in the challenge test. According to the method of Buehler an aqueous solution of cyanamide (53 % w/v) induced some positive skin reactions after the challenge application (4/20). Indications for a severe irritating effect of cyanamide was noticed in one of these rabbits due to histological examination, whereas no histological findings showed any sign of delayed hypersensitivity. Thus, the results of the Buehler test were inconclusive. In contrast, the more sensitive M&K test clearly demonstrated a potential for skin sensitisation.

Table 40: Summary table of relevant skin sensitisation studies

Study type	Species / strain	Comments	Results m /f	Reference
Skin Sensitisation Magnusson and Kligman	Albino guinea pig	Pure active substance (1; 2.5 %) used as test item; positive response with the 1 % and 2.5 % concentration	Sensitising	Til, 1982 Doc-No. 567-003 TOX2004-364
Skin Sensitisation Buehler test	Albino guinea pig	Study did not reveal clear indications for a sensitising potential. Irritation observed.	Equivocal	Mercier, 1988 Doc-No. 567-001 TOX2004-365

4.6.1.4 Comparison with criteria

Table 41 presents the toxicological results in comparison with DSD and CLP criteria.

Table 41: Results of Magnusson and Kligman test in comparison with DSD and CLP criteria

Toxicological result	DSD criteria	CLP criteria
Til, 1982 (M&K): 10/10 animals positive (1 % challenge) 10/10 animals positive (2.5 % challenge) 5 % intra dermal induction concentration	Adjuvant type test method: ≥ 30 % of the animals positive	**Guinea pig maximisation test** Category 1A: ≥ 30 % responding at ≤ 0,1 % intradermal induction dose or ≥ 60 % responding at > 0,1 % to ≤ 1 % intradermal induction dose Categrory 1B: ≥ 30 % to < 60 % responding at > 0,1 % to ≤ 1 % intradermal induction dose or ≥ 30 % responding at > 1 % intradermal induction dose
Mercier, 1988 (Buehler): 4/20 (20 %) animals positive 40 % dermal induction concentration	Other test method: ≥ 15 % of the animals positive	**Buehler assay** Category 1A: ≥ 15 % responding at ≤ 0,2 % topical induction dose or ≥ 60 % responding at > 0,2 % to ≤ 20 % topical induction dose Categrory 1B: ≥ 15 % to < 60 % responding at > 0,2 % to ≤ 20 % topical induction dose or ≥ 15 % responding at > 20 % topical induction dose

4.6.1.5 Conclusions on classification and labelling

The results of the Buehler test were inconclusive. In contrast, the more sensitive M&K test clearly demonstrated a potential for skin sensitisation. Based on the results in the study according to M&K design, Cyanamide fulfills the criteria in DSD to be classified as a skin sensitiser (R43).

Based on the results in the study according to M&K design, Cyanamide fulfills the criteria in CLP regulation to be classified as a skin sensitiser (H317, sub-category 1B).

This proposal is in agreement with the outcome of the EFSA peer-review.

4.6.2 Respiratory sensitisation

No data were submitted by the notifier.

4.7 Repeated dose toxicity

4.7.1 Non-human information

4.7.1.1 Repeated dose toxicity: oral

Oral administration (28–day study) in the rat

Title:	Osheroff, M.R. (1988): 28-day repeated dose oral toxicity study with aqueous hydrogen cyanamide in rats; Doc. No. 532-001; Hazelton Laboratories America Inc., Rockwell, Maryland USA; unpublished
Guidelines:	OECD 407 (1981); EEC B7
Deviations:	None
GLP:	Yes
Acceptability:	The study is considered to be acceptable.

Materials and methods:

The test substance was identified as follows:

Hydrogen cyanamide (aqueous solution)

Batch: 04/22/87

Purity: 50 % w/w solution (53 % w/v of active substance hydrogen cyanamide)

In a 28-day study, hydrogen cyanamide was administered by gavage to male and female Sprague-Dawley rats (5 rats/sex/concentration) at dose levels of 10, 20, 40 and 80 mg/kg bw/day, corresponding to 5, 10, 20 and 40 mg/kg bw/day active substance hydrogen cyanamide. An additional group of 5 males and 5 females received the vehicle (distilled water) and served as the concurrent control group. The test substance was prepared weekly and administered daily by gavage at a dose volume of 10 mL/kg.

Animals were observed twice daily for mortality and/or moribundity and once daily for overt signs of toxicity. Body weight and food consumption measurements were performed once per week. At termination, clinical parameters (haematology, clinical chemistry and thyroid function test) were evaluated. Following 28 d of treatment, all animals were sacrificed and subjected to a complete gross necropsy. Organ weight evaluations (absolute and relative) and histopathological examinations were performed on all animals.

Findings:

General observations:

The stability of the dosing solution over a 8 h period was demonstrated. The correctness of the concentrations in the vehicle was analytically verified.

The death of one female animal at 40 mg/kg bw/day on day 29 is considered to be due to the general decrease in its health status. Rough hair coat was observed in three males and one female in the high dose group at the week 4 physical examination. All other clinical observations noted appeared to be incidental in nature and were not contributed to the administration of cyanamide. The body weight data at day 28 are presented in Table 42

Table 42: Body Weights

Pure active substance cyanamide (mg/kg bw/d) (n = 5 each sex)	**0**	**5**	**10**	**20**	**40**
Males					
Start	179.0	190.6	183.5	181.1	185.9
Week 4	369.3	388.5	356.1	313.8*	275.3*
Females					
Start	156.2	161.1	163.2	157.6	158.8
Week 4	244.3	241.9	233.2	214.2	196.5*

* Significantly different from control by the Dunnets test, criteria, $p < 0.05$

As depicted in Table 42, the mean body weights at termination were statistically significantly decreased at 20 mg/kg bw/day in males (-15 %) and at 40 mg/kg bw/day in both sexes (-25.4 % for males and -19.6 % for females), when compared to the control groups. Mean body weight gain values for weeks 0 - 4 (see Table 43) were significantly decreased for males and females at 20 (-30.3 % in males, -35.9 % in females) and at 40 mg/kg bw/day (-53.0 %/-57.3 %) when compared to the corresponding control.

Table 43: Body weight gain/food consumption

Pure active substance cyanamide (mg/kg bw/d) (n = 5 each sex)	**5**	**10**	**20**	**40**
Males				
Body weight gain, week 0 - 4 (% control)	100	90.7	69.7*	47.0*
Food consumption, week 0 - 4 (% control)	100	100	86.8*	75.2*
Females				
Body weight gain, week 0 - 4 (% control)	98.2	79.5	64.1*	42.7*
Food consumption, week 0 - 4 (% control)	100	97.1	87.3	84.4

* Significantly different from control by the Dunnets test, criteria, $p < 0.05$

Total food consumption for weeks 1 - 4 was decreased at 20 and 40 mg/kg bw/day in females and males when compared to the corresponding control (Table 43).

Haematology and clinical chemistry:

Evaluation of the haematology data revealed a decrease in red cell parameters (RBC, Hct and Hb) in the high-dose animals at week 4, that was statistically significant in the males and in the females

(except RBC in females, see Table 44). This reduction was associated with statistical significant decreases in MCH and MCHC in males of the highest dose. Monocytes were statistical significant increased in the high dose group of males and females.

Table 44: Haematology and clinical chemistry

Pure active substance cyanamide (mg/kg bw/day) (n = 5 each sex)	**0**	**5**	**10**	**20**	**40**
Males					
RBC (10^6/µL)	8.22	7.78	8.09	8.37	7.48*
Hb (g/dL)	17.2	16.6	16.5	17.3	14.5*
HCT (%)	47.4	46.0	46.2	47.6	41.0*
MCH (pg)	20.9	21.4	20.5	20.7	19.3*
MCHC (g/dL)	36.2	36.2	35.8	36.3	35.3*
MCV (fl)	57.7	59.1	57.2	56.9	54.8
WBC (10^3/µL)	9.5	10.5	11.3	12.3	12.7
Lymphocytes (%)	8.3	9.0	9.7	11.0	10.5
Monocytes (%)	0.1	0.0	0.0	0.2	0.4*
Thrombocytes (10^3/µL)	1299	1143	1068	1019	960
Total Bilirubin (mg/dL)	0.0	0.0	0.0	0.0	0.2*
Total Prot (g/dL)	5.3	5.3	5.0	4.9*	5.0
Globulin (g/dL)	1.9	1.9	1.8	1.6*	1.6*
Blood Urea Nitrogen (mg/dL)	11	11	11	13	19*
Calcium (mg/dL)	9.5	9.6	9.5	9.4	9.2
Females					
RBC (10^6/µL)	7.94	8.05	8.39	8.32	7.47
Hb (g/dL)	16.4	16.9	17.3	16.9	15.1*
HCT (%)	45.7	46.6	47.2	46.5	42.5*
MCH (pg)	20.6	21.0	20.7	20.3	20.3
MCHC (g/dL)	35.9	36.3	36.7	36.4	35.6
MCV (fl)	57.5	57.9	56.3	55.9	56.9
WBC (10^3/µL)	7.3	8.0	9.6	9.4	11.6
Lymphocytes (%)	6.4	6.8	8.4	8.2	9.9
Monocytes (%)	0.1	0.1	0.2	0.2	0.3*
Thrombocytes (10^3/µL)	1165	1266	1108	1142	923
Total Bilirubin (mg/dL)	0.1	0.1	0.1	0.1	0.2
Total Prot (g/dL)	5.4	5.1	5.4	5.1	5.2
Globulin (g/dL)	1.8	1.7	1.7	1.7	1.6
Blood Urea Nitrogen (mg/dL)	11	16	15	15	18
Calcium (mg/dL)	9.6	9.5	9.7	9.0*	9.5

* Significantly different from control by the Dunnets test criteria, $p < 0.05$

As shown in Table 44, several statistical significant changes were noted in clinical chemistry data: the male mean globulin levels were decreased at 20 and 40 mg/kg bw/day, while the mean total bilirubin and blood urea nitrogen values were elevated at 40 mg/kg bw/day. Those effects were seen in females without statistical significance. Other incidental changes included a decrease in thrombocytes and an increase in WBC and lymphocytes almost most pronounced in the highest dose groups of both sexes. Decreased total protein in males and decreased calcium in females were statistically significant decreased only at 20 mg/kg bw/day.

Table 45 gives information about the thyroid function of the treated rats at termination.

Table 45: Thyroid function test at week 4

Pure active substance cyanamide (mg/kg bw/day) (n = 5 each sex)	0	5	10	20	40
Males					
T3 (ng/dL)	46.4	49.5	47.2	55.5	55.6
T4 (µg/dL)	4.7	5.5	5.3	5.1	3.5
TSH (ng/mL)	1.3	1.2	1.3	1.1	2.6
Females					
T3 (ng/dL)	56.0	52.2	56.2	46.2	45.7
T4 (µg/dL)	3.2	3.8	2.7	2.7	2.3
TSH (ng/mL)	0.8	1.1	1.0	0.9	1.9

At 40 mg/kg bw/day the mean TSH values were increased by 100 %, whereas T4 concentrations were decreased by 28 % in both sexes compared to control animals.

Gross pathology, organ weights and histopathology:

No gross pathology findings were attributed to the administration of cyanamide. Organ weights data are depicted in Table 46:

Table 46: Organ weights (absolute/relative[a])

pure active substance cyanamide (mg/kg bw/day) (n = 5 each sex)	0	5	10	20	40
Males					
Brain with stem: absolute weight (g)	2.01	2.02	1.95	1.90	1.87
Brain with stem: relative weight (%)	0.6	0.577	0.606	0.678	0.751*
Kidney: absolute weight (g)	2.75	2.85	2.70	2.55	2.62
Kidney: relative weight (%)	0.818	0.81	0.833	0.906*	1.043*
Liver: absolute weight (g)	9.81	11.09	10.44	10.07	11.86
Liver: relative weight (%)	2.926	3.139	3.205	3.560*	4.742*
Thyroid/parathyroid: absolute weight (g)	0.022	0.020	0.025	0.021	0.024
Thyroid/parathyroid: relative weight (%)	0.0065	0.0057	0.0079	0.0076	0.0097*
Testes: absolute weight (g)	4.09	3.98	4.04	3.50*	3.48*
Testes: relative weight (%)	1.227	1.138	1.256	1.251	1.395
Females					
Brain with stem: absolute weight (g)	1.85	1.87	1.87	1.78	1.70
Brain with stem: relative weight (%)	0.842	0.864	0.890	0.910	0.984
Kidney: absolute weight (g)	1.76	1.78	1.74	1.72	1.70
Kidney: relative weight (%)	0.797	0.821	0.830	0.881*	0.980*
Liver: absolute weight (g)	6.68	6.26	6.21	6.02	6.18
Liver: relative weight (%)	3.008	2.880	2.955	3.083	3.570*
Thyroid/parathyroid: absolute weight (g)	0.020	0.015	0.015	0.016	0.019
Thyroid/parathyroid: relative weight (%)	0.0088	0.0070	0.0073	0.0081	0.0111

a Relative organ weight: Absolute organ weight to body weight in %

* Significantly different from control by the Dunnets test criteria, $p < 0.05$

Mean relative kidney weights (females and males) and mean relative liver weights (males) were statistical significantly increased already at 20 mg/kg bw/day, whereas the absolute testes weight was statistical significantly decreased. Most of those effects were pronounced in the high dose group. Additionally, the mean relative weight of the brain with stem in male rats and the mean relative liver weight in females were statistically significantly increased in the highest dose.

Compound-related histopathological lesions were observed in sections of thyroid, spleen and liver (see Table 47). In the thyroid, the incidence of follicular cell hyperplasia was increased at 10 mg/kg bw/day and higher in male rats. A decreased colloid content and small and closely packed follicles were seen already, even though to a marginal extent, at 5 mg/kg bw/day in the males. The females were less sensitive: dose-dependent thyroid effects were seen at 20 and 40 mg/kg bw/day. In the spleen, the incidence of pigmented macrophages was increased in the high dose group in male rats and in the female rats at 10, 20 and 40 mg/kg bw/day. In the liver, the incidence of biliary hyperplasia was increased dose-dependently at 10 mg/kg bw/day and higher in male rats. No other compound-related histomorphological lesions were observed.

Table 47: Incidences of histopathological findings

pure active substance cyanamide (mg/kg bw/day) (n = 5 each sex)	**0**	**5**	**10**	**20**	**40**
	Male/female	**male/female**	**male/female**	**male/female**	**male/female**
Liver, bile duct hyperplasia	0/0	0/0	1/0	2/0	5/0
Spleen, pigment	0/0	0/0	0/2	0/4	5/5
Thyroid: decreased colloid	0/0	1/0	5/0	5/1	5/4
Thyroid: follicular cell hyperplasia	0/0	0/0	5/0	5/1	5/4
Small and closely packed follicles	0/0	2/0	5/0	5/2	5/4

Conclusion:

The NOAEL was 5 mg/kg bw/day in this 28-day oral repeated dose toxicity study in rats for the pure active substance cyanamide based on the findings in the thyroid seen in all males at 10 mg/kg bw/day and higher. In contrast, marginal histopathological effects seen at 5 mg/kg bw/day were not accompanied by follicular cell hyperplasia and were therefore not assessed as adverse. In females, thyroid changes were obvious in the mid and high dose group with a dose-dependent increase of incidences. At 10 mg/kg bw/day and above, additional histopathological findings were found in the liver of males (bile duct hyperplasia) and in splenic pigmentation in females, which was seen as well in males, but only at the highest dose of cyanamide. Anaemia occured with statistical significance in both sexes at 40 mg/kg bw/day.

Oral 90-day study in rats

Title: Til, H.P. et al. (1975): Sub-chronic (90-day) toxicity study with Cyanamide L 500 in albino rats; Doc. No. 533-001; CIVO Institutes, TNO Central Institute for Nutrition and Food Research, Zeist, Netherlands; unpublished

Guidelines: None

Deviations: Stability of the test substance, correctness of the dietary preparations as well as homogeneity of the test substance in the dietary preparation not proven; no individual data; no ophthalmological examination;

	determination of food consumption during the first 4 weeks and in week 10 to 12, instead of a weekly determination throughout the study; no determination of water consumption; no determination of haematocrit, platelet count and blood clotting time/potential; no determination of sodium, potassium, glucose, total cholesterol, urea, blood urea nitrogen, creatinine; no determination. of volume, osmolality, specific gravity and blood/blood cells during urinalysis; no absolute organ weights; no histopathological investigation of brain, pituitary parathyroid, thymus, spleen, heart, female mammary gland, skin and bone marrow.
GLP:	No; study was conducted prior to the implementation of GLP.
Acceptability:	The study is considered to be supplementary.

Materials and methods:

The test substance was identified as follows:

Cyanamide L 500 (Alzogur)

Batch: Not mentioned

Purity: Not mentioned

In a 90-day feeding study, Cyanamide L 500 was administered to weanling albino rats from the CIVO colony (Wistar derived). 10 males and 10 females were used per concentration at dietary dose levels of 0, 20, 60 and 180 ppm (equivalent to 0, 10, 30 and 90 ppm pure active substance cyanamide) corresponding to mean daily intakes of 0, 1, 3 and 9 mg/kg bw/day (equivalent to 0, 0.5, 1.5 and 4.5 mg/kg bw/day pure active substance cyanamide).

The animals were observed for clinical symptoms and mortalities. Body weights were recorded weekly and food intake of each group was measured during the first four weeks and in week 10 to 12. Haematological investigations were performed during week 12 in all rats (erythrocyte count, haemoglobin, packed cell volume, leucocytes, lymphocytes, differential blood count), urinalysis (appearance, pH, glucose, protein, occult blood, ketones and microscopy of the sediment) was done in week 13. At autopsy in week 14, clinical chemical investigations were performed in all rats: glutamic-oxalacetic transaminase, glutamic-pyruvic transaminase, alkaline phosphatase, total serum protein and serum albumin. All animals were subjected to gross macroscopic investigation. The weights of the heart, kidneys, liver, spleen, brain, gonads, thymus, thyroid and adrenals were determined. Histopathology was confined to all rats of the highest dosage group and of the control groups.

Findings:

General observations:

A test substance-related mortality did not occur. No increase in the incidence of clinical signs was observed in any of the groups fed with cyanamide in the diet.

The body weights of all test groups (Table 48) were generally slightly higher compared to the control groups in correspondence with the food consumption (Table 49) but without a tendency of a dose-related response.

Table 48: Body weights

Pure active substance cyanamide (mg/kg bw/day) (n = 10 each sex)	Males Body weight (g) day 0	Males Body weight (g) day 91	Females Body weight (g) day 0	Females Body weight (g) day 91
0	54.8	311	51.7	187
0.5	54.9	326	51.6	193
1.5	54.8	327	51.5	196
4.5	54.8	320	51.3	193

Table 49: Food consumption (in g)

Pure active substance cyanamide (mg/kg bw/day) (n = 10 each sex)	Males week 0 - 2	Males week 3 - 4	Males week 11 - 12	Females week 0 - 2	Females week 3 - 4	Females week 11 - 12
0	20.2	31.2	31.5	15.7	21.7	21.7
0.5	20.9	33.2	33.5	17.0	22.6	23.1
1.5	21.5	31.3	32.0	17.5	23.1	23.4
4.5	21.7	33.2	33.5	17.1	23.7	24.9

The results of the haematological investigations showed a significant increase in erythrocyte counts in males of the highest dose group (see Table 50). A slightly decreased haematocrit value in males was only obvious in the mid dose group. No statistical significant changes in the leucocyte and differential blood count were seen.

Table 50: Erythrocytes indices

Pure active substance cyanamide (mg/kg bw/day) (n = 10 each sex)	0	0.5	1.5	4.5
Males				
RBC (10^6/µL)	7.7	7.8	8.0	8.2**
Hb (g/dL)	16.1	15.5	16.2	16.5
Hct (%)	49.2	47.6	46.2*	48.7
Females				
RBC (10^6/µL)	7.7	7.9	7.7	7.8
Hb (g/dL)	16.4	16.8	16.4	16.3
Hct (%)	47.8	48.0	48.1	47.2

* Significantly different from control, $p < 0.05$
** Significantly different from control, $p < 0.01$

Clinical chemical investigations demonstrated no significant changes in serum enzyme activities or in serum protein values. There were no effects on urinalysis.

Gross pathology, organ weights and histopathology:

No test substance-related gross lesions were observed at necropsy. Organ weight determinations revealed a significant increase in the relative liver weight in males and a significant decrease in the relative weight of the thymus in female rats receiving 4.5 mg/kg bw/day. The results are summarised in Table 51.

Table 51: Organ weights

Pure active substance cyanamide (mg/kg bw/day) (n = 10 each sex)	0	0.5	1.5	4.5
Males				
Relative[a] liver weight (%)	3.26	3.35	3.28	3.44*
Relative[a] thymus weight (%)	0.93	0.92	0.103	0.86
Females				
Relative[a] liver weight (%)	3.05	3.00	3.06	3.18
Relative[a] thymus weight (%)	0.148	0.152	0.142	0.123*

a Relative weight is defined as the absolute organ weight to body weight (%)
* Significantly different from control by the (test type) criteria, $p < 0.05$

Histopathological investigations (see Table 52) revealed treatment-related changes in the thyroids.

Table 52: Incidences of histopathological findings in the thyroid

Pure active substance cyanamide (mg/kg bw/day) (n = 10 each sex)	0	0.5	1.5	4.5
	male/female	male/female	male/female	male/female
Thyroid: predominantly small follicular lumens without colloid, separated by proliferating epithelial cells and interfollicular cells.	0/0	0/0	1/0	3/2

Thyroid effects were clearly seen in the high dose groups. The effects in one male at 1.5 mg/kg bw were not seen as an adverse effect, since the incidence was 1/20 rats. The histopathological changes observed in other organs were equally distributed between control and test groups or occurred only in a single animal.

Conclusion

The NOAEL in the 90-day feeding study in rats was 1.5 mg/kg bw/day pure active substance cyanamide (equivalent to 30 ppm in the diet) based on the histopathological findings in the thyroid at 4.5 mg/kg bw/day cyanamide (equivalent to 90 ppm in the diet) in males and females.

Oral 90-day study in dogs

Title: Til, H.P. et al., 1982, Sub-chronic (90-day) oral toxicity study with Alzodef in dogs; Doc. No. 533-002; CIVO Institutes, TNO Central Institute for Nutrition and Food Research, Zeist, Netherlands; unpublished

Guidelines: None

Deviations: Stability, correctness of the concentrations and homogeneity of the test substance not determined; no ophthalmological examination pre-treatment; calcium, phosphorus, chloride, sodium and potassium not investigated; osmolarity or specific gravity not performed in urinalysis; gall bladder, epididymides and uterus not weighed at autopsy.

GLP: Yes

Acceptability: The study is considered to be acceptable with reservations.

Materials and methods:

The test substance was identified as follows:

Alzodef (50 % aqueous solution of cyanamide)

Batch: Not mentioned

Purity: 50 ± 1 %

In a 90-day oral study, Alzodef was administered to 4 male and 4 female beagle dogs, about 4 months old, per test group over a period of 3 months. During the first week, Alzodef was administered with the diet at levels of 0, 30, 100 or 300 ppm. Starting on day 8, the test substance was given via gavage since the dogs of the top-dose group were very reluctant to eat their food. The dose levels were 1.2, 4 and 12 mg/kg bw/day, equivalent to 0.6, 2.0 and 6.0 mg/kg bw/day of active substance cyanamide given as a solution in distilled water. Each dog received a single volume of 0.25 mL/kg bw. Food consumption and body weight of the animals was determined weekly. The dogs were checked daily for signs of toxicity. Ophthalmalogical examinations were carried out in week 13 of the study. Clinical chemistry and haematological examinations were carried out pre-treatment, at day 44 and day 85. Urinalysis was performed on day 0, 43 and 78. Following tests were carried out: bromsulfophthalein test for liver function on day 87, phenolsulfonephthalein test for kidney function on day 80 and thyroid function tests on days 0, 8, 15, 28, 57 and 85 in all dogs of the highest dose group and of the control.

After sacrification all dogs were examined gross-pathologically and subsequently histopathologically.

Findings:

General observations:

No deaths occurred during the course of the study. In the high dose group, all dogs and in the mid-dose group a few dogs showed resistance to dosing. In some cases a part of a daily amount of test substance was probably lost due to spitting, coughing or kecking. Some dogs of the mid and high dose groups showed redness of the buccal mucosa. The authors assumed that it is probably a result of the dosing and/or irritancy of the test substance.

No ophthalmological differences between dogs of the control group and the treatment groups were obtained.

As shown in Table 53, mean body weights were lower in the highest dose group when compared to the controls with statistical significance in females. Mean food intake was slightly decreased in females in the highest dose group. In males, food intake in all test groups was slightly lower than in the control, but without evidence of a dose-related response.

Table 53: Body weights[+]/Body weights gain

Pure active substance cyanamide (mg/kg bw/day) (n = 4 each sex)	Males Body weight (kg)	Males Bw gain (kg)	Females Body weight (kg)	Females Bw gain (kg)
0	13.9	5.5	10.9	3.9
0.6	12.9	4.8	11.0	3.9
2.0	13.3	5.0	10.6	3.7
6.0	12.7	4.5	9.5*	2.7

[+] Body weight at day 91
*Significantly different from control by Dunnetts test, $p < 0.05$

As shown in Table 54, a statistical significant decrease in haemoglobin content, haematocrit and red blood cell count was seen in the high dose groups. A lower reticulocyte count could not be seen as an effect. The monocyte count was dose-dependently increased at day 85 in the mid and high dose groups with statistical significance (except males of the mid dose group). Thrombocytes were decreased in both upper dose groups, but not with a clear dose-dependency. PTT was decreased at the high dose groups in males. The results are summarised in Table 54.

Table 54: Haematology and clinical chemistry

Pure active substance cyanamide (mg/kg bw/day) (n = 4 each sex)	0		0.6		2.0		6.0	
	day 44	day 85	day 44	day 85	day 44	day 85	day 44	day 85
Males								
RBC (10^6/µL)	5.7	5.9	5.9	5.6	5.6	5.2	5.6	5.3*
Hb (mmol/L)	8.4	9.1	8.3	8.6	8.2	8.3	7.8	7.8*
Hct (%)	43.6	45.1	43.0	43.1	41.9	41.1	40.6	40.3*
Reticulocytes (/10^3RBC)	8.7	14.8	-	-	-	-	7.5	7.0
WBC (10^9/L)	13.2	12.1	15.3	12.9	13.3	12.8	12.9	12.1
Monocytes (WBC x %)	4.0	2.8	2.8	2.0	6.8	5.8	3.3	9.3*
Thrombocytes (10^9/L)	372	322	340	288	258*	189*	308	197
PTT	27.9	28.2	25.5	24.9	24.3	24.8	23.2*	23.8*
Plasma albumin (g/L)	39.3	38.3	35.8	35.0*	37.8	37.0	36.3	37.5
GOT (U/L)	32.8	32.3	34.0	33.5	31.5	30.0	33.5	30.3
Plasma cholesterol (mmol/L)	3.2	3.3	3.3	3.4	3.8	3.7	3.7	3.9
Females								
RBC (10^6/µL)	5.7	5.8	5.8	5.6	5.6	5.9	5.3	5.3
Hb (mmol/L)	8.1	8.9	8.4	8.5	8.3	9.2	7.9	7.9
Hct (%)	42.5	44.8	43.4	43.5	42.5	46.1	41.1	40.6
Reticulocytes (/10^3RBC)	10.0	18.0	-	-	-	-	5.0	9.0
WBC (10^9/L)	13.9	14.2	12.2	12.0	12.3	10.0	13.0	14.8
Monocytes (WBC x %)	3.3	3.5	3.7	4.3	6.8	6.8*	6.2	10.8*
Thrombocytes (10^9/L)	367	338	341	271	246	204	258*	203
PTT	24.9	24.6	24.4	27.2	23.6	25.8	24.2	24.7
Plasma albumin (g/L)	37.3	38.5	39.0	38.0	36.5	37.5	35.0	35.8*
GOT (Units/L)	33.0	33.5	34.3	33.8	34.0	32.8	34.5	29.0*
Plasma cholesterol (mmol/L)	2.4	2.5	3.2	2.8	3.2*	3.0	3.1*	3.2*

* Significantly different from control by the Mann/Whitney U-Test, criteria, $p < 0.05$.
\- not performed

Regarding the serum chemistry data, the plasma albumin level was relatively low in females at 6 mg/kg bw on day 44 and 85. Plasma glutamic-oxalacetic transaminase activity (GOT) was slightly, but statistically significant decreased in females at the high dose group at the end of the study. Regarding the concentration of GOT (26.3 U/L) at day 0 the decrease may be not attributed to the substance. The plasma cholesterol levels were higher in all test groups in female dogs

compared to the control with statistical significance in the highest dose group at day 44 and 85 and additionally, on day 44 in the mid-dose group. Almost the same effects in cholesterol concentration were seen in males, but without statistical significance.

The thyroid function test (see Table 55) showed no treatment-related differences in plasma T3-uptake between the test groups of both sexes and the controls.

Table 55: Thyroid function tests

Pure active substance cyanamide (mg/kg bw/day) (n = 4 each sex)	**0**	**0.6**	**2.0**	**6.0**
	male/female	**male/female**	**male/female**	**male/female**
T3 uptake in plasma (%)				
day 0	45/46.8	47.3/45.6	46.3/46.4	46.5/46.7
day 28	46.2/47.8	50.0/48.8	46.8/47.7	47.5/50.5
day 85	44.4/46.5	46.0/47.0	44.8/46.4	45.1/47.0
T3 content in the plasma (pmol/L)				
day 0	1241/1094	1142/1186	1128/1120	1071/1178
day 28	1442/1346	889*/1190	1191/1211	1117/1184
day 85	1358/1322	1141/1233	1026/1108	850/840
T4 content in the plasma (nmol/ L)				
day 0	35.4/33.0	27.2/34.9	35.7/37.2	29.6/37.3
day 28	42.6/42.6	25.8/34.2	30.7/37.7	31.8/35.1
day 85	38.1/37.8	26.0/31.9	23.5/25.5	20.1/32.4

Plasma mean T3-content was generally higher in the control group of the males compared with all treated male groups at day 0. No statistical significant differences were revealed but the lowest mean T3 values were clearly seen in both sexes at 6.0 mg/kg bw/d at day 85 (-35 %).

Plasma T4-content was generally dose-dependent decreased at day 85 in the males. At 2.0 mg/kg bw the decrease revealed –38 % and in the high dose group –47 % compared with the control group (see also Table 56).

Table 56: Thyroid hormones

Pure active substance cyanamide (mg/kg bw/day) (n = 4 each sex)	**0**		**0.6**		**2.0**		**6.0**	
Males	**day 0**	**day 85**	**day 0**	**day 85**	**day 0**	**day 85**	**day 0**	**day 85**
T3 (pmol/ L)	1241	1358	1142	1141	1128	1026	1071	850
T4 (nmol/ L)	35.4	38.1	27.2	26.0	35.7	23.5	29.6	20.1
Females								
T3 (pmol/ L)	1094	1322	1186	1233	1120	1108	1178	840
T4 (nmol/ L)	33.0	37.8	34.9	31.9	37.2	25.5	37.3	32.4

Gross pathology, organ weights and histopathology

There were no macroscopic test substance related changes. Organ weight data are depicted in Table 57.

Table 57: Organ weights

Pure active substance cyanamide (mg/kg bw/day) (n = 4 each sex)	0	0.6	2.0	6.0
Males				
Absolute heart weight (g)	110.78	98.88	97.25	89.72
Relative[a] heart weight (g/kg)	7.21	7.67	7.33	7.04
Absolute testes weight (g)	14.998	11.900	14.205	9.158
Relative[a] testes weight (g/kg)	1.07	0.91	1.07	0.70
Females				
Absolute heart weight (g)	82.17	88.01	77.04	71.03
Relative[a] heart weight (g/kg)	7.51	7.97	7.30	7.47
Absolute thymus weight (g)	12.912	15.210	12.887	7.415
Relative[a] thymus weight (g/kg)	1.17	1.40	1.21	0.76
Absolute liver weight (g)	351.75	330.00	290.00	274.00
Relative[a] liver weight (g/kg)	31.9	29.8	27.5	29.2
Absolute lungs weight (g)	90.25	91.50	86.25	79.75
Relative[a] lungs weight (g/kg)	8.31	8.40	8.15	8.42

a Relative weight is defined as the organ to body weight ratio

Both the absolute and relative testes weights were clearly reduced in the high dose group when compared to the control. At this dose, one dog showed small testes and the other one unilateral cryptorchidism.

Incidental decreases of mean absolute organ weights were seen in the heart of both sexes and of thymus, liver and lungs in females. Additionally, the mean relative weight of the thymus was reduced in females of the high dose group.

Only the microscopic examination of the testes/epididymidis revealed effects, which are listed in Table 58. Other histopathological effects were not contributed to the substance since they were incidental and not dose-dependent.

Table 58: Incidences of histopathological effects in the testes/epididymidis

Pure active substance cyanamide (mg/kg bw/day) (n = 4 each sex)	0	0.6	2	6
Testes				
Multinucleated giant cells one or few	3[a]	4	3	1
Multinucleated giant cells several	0	0	0	2
Spermatogenesis, slight to moderate reduced	0	2	1	0
Spermatogenesis, absent	0	0	1	3
Atrophic seminiferous tubules	0	1	2	1
Atrophy testis unilateral	0	0	0	1
Atrophy testes bilateral	0	0	0	1
Epididymidis				
Reduced number of sperms	0	2	2	0
Absent number of sperms	0	2	2	3

a Number of organs with microscopic change

In general, dose-related effects were seen in reduced or absent spermatogenesis, accompanied by reduced or absent sperms in the epididymidis and in atrophy of seminiferous tubules and the testes. As shown in Table 58, one or a few multinucleated giant cells were found in the testes of all dose groups, whereas several cells were only seen in the high dose group.

The severity of the impact on spermatogenesis showed a clear dose-relationship. Already in the low-dose group all dogs revealed an effect on spermatogenesis (see Table 59): Two dogs showed

reduced spermatogenesis in the testes accompanied by absent amounts of sperms in the epididymidis (B6, B8) and two other dogs showed a reduced number of sperms in the epididymidis (B2, B4). The impact of cyanamide on spermatogenesis was more severe in the mid-dose group showing one dog without any spermatogenesis (C6). At 6 mg/kg bw, three dogs showed no spermatogenesis and therefore no sperms in the epididymidis. Testes atrophy was a further effect in the high dose group.

In contrast, historical data showed a slight to moderate reduced spermatogenesis in 2 of 8 dogs (25 %) and absent spermatogenesis in 1 of 8 dogs of the same strain and age. The distribution of these effects between the dogs is not mentioned. Since atrophic seminiferous tubules were seen in 2 of 8 dogs of the historical control, the atrophy of seminiferous tubules at 0.6 mg/kg bw cyanamide in this study was not attributed to the substance.

No effects on spermatogenesis were seen in the control group.

Table 59: Individual histopathological effects in the testes/ epididymidis

Pure active substance cyanamide (mg/kg bw/day) (n = 4 each sex)	historical controls	0	0.6	2	6
Spermatogenesis, slight to moderate reduced	2/8		dog B6, B8	dog C6	
Spermatogenesis, absent	1/8			dog C8	dog D4,6,8
Atrophic seminiferous tubules	2/8		dog B4	dog C4, C8	dog D4
Atrophy testis unilateral	n.m.				dog D4
Atrophy testes bilateral	n.m.				dog D 6
Reduced number of sperms in epididymidis	n.m.		dog B2, B4	dog C2, C4	
Absent number of sperms	n.m.		dog B6, B8	dog C6, C8	dog D4, D6, D8

n.m. = not mentioned

A peer review on male reproductive organs on the 90-day study of Til (1982) was performed by Weber and Creasy (2009) and submitted by the notifier. The result of the peer review is presented in the following.

No histopathological sections were available anymore. Therefore, the original microscopic findings in the final study report were reviewed. The study was conducted using immature dogs at the age of 4-5 months at study start. Cyanamide was administered to the animals by daily single oral gavage at doses 0, 0.6, 2.0 and 6.0 mg/kg bw/day. At the end of the study, the dogs were 7-8 months of age, which is borderline for attainment of sexual maturation.

Four 4-5 month old male beagles were used per group at study start. At this age, spermatogenesis has not yet begun and the animals would be completely immature at study start. The oldest animals at the end of the study reached an age of 8 months. Dogs can become sexually mature anytime between 7-12 months of age, so it is probable that many of the testes of these dogs were still immature, some were peripubertal and others were mature. The characteristics of immature and peripubertal testes (reduced organ weight, degenerating (multinucleate) germ cells, incomplete or reduced spermatogenesis and sloughed testicular germ cells and debris in the epididymal lumens) are indistinguishable from the degenerative changes that occur with testicular toxicants and so it is usually not possible to evaluate testicular toxicity reliably in animals of this age. Despite the difficulties outlined above, this age of animal is recommended by the OECD guidelines.

On the basis of the diagnoses presented in the study report, there appears to be reduced spermatogenesis and sometimes atrophic tubules in the testes of all cyanamide treated groups. The testicular findings were generally associated with decreased numbers of spermatozoa (incorrectly designated spermatocytes in the report) and increased cell debris in the epididymis. These findings would be consistent with normal peripubertal testes. Some of the cyanamide treated dogs had testes

ith marked reduction of spermatogenesis and absence of spermatozoa in the epididymis; this is also within normal range for this age of dog and would reflect dogs just beginning spermatogenesis. Based on the dogs' age of 7-8 months, combined with the wide age variability over which dogs mature, plus the small number of dogs in each group, it is quite possible that there was an uneven distribution of dogs (with respect to sexual maturity status) between the control and the cyanamide treated groups in this study. However, since there appeared to be a dose related severity with respect to the testes weight, the reduction in spermatogenesis and the presence of spermatozoa in the epididymis, an alternative explanation might be that there was a delay in sexual maturation in the cyanamide treated animals. Regarding testicular weight it has to be noted that dog No. 4 was a cryptorchid dog that changed the summarised testicular weights. This could be secondary to the significant reduction in body weight (21 % reduction compared with controls, at 6 mg/kg/day). Body weight loss and general stress are known to cause decreases in testosterone secretion through reduced levels of GnRH (Bergendahl et al 1989; Bergendahl et al. 1991; Dong et al. 1994; Dong et al. 1994). Such effects have been shown to retard sexual maturation, but are reversible (Grewal et al 1971; Glass et al. 1986).

It was concluded that it is so difficult to interpret testicular changes in this age of dog and, therefore, it is more relevant to base any interpretation on the supplementary study where cyanamide was administered for 90 days to mature dogs.

Table 60: Summarised results in the report on male reproductive organs from TNO Study No. V 82.084 (90-day) with cyanamide in dogs

Lesion	Group 1 (Control)	Group 2 (Low dose)	Group 3 (Mid dose)	Group 4 (High dose)
No. dogs examined	4	4	4	4
Testes				
Multinucleated giant cells one or few	3	4	3	1
Multinucleated giant cells several	0	0	0	2
Red. spermatogenesis slight/moderate	0	2	1	0
Spermatogenesis absence	0	0	1	3
Atrophic seminiferous tubules one/few	0	1	2	1
Atrophy unilateral	0	0	0	2#
Atrophy bilateral	0	0	0	1
Prostate				
Prostatitis	1	1	1	1
Multinuclear giant cells	1	1	0	0
Epididymides				
Debris in lumen	4	1	0	0
Increased debris in lumen	0	3	4	4
Spermatocytes absent	0	2	2	3
Epithelial cysts	0	0	1	0
Multinuclear giant cells	0	0	0	1
#at cryptorchism site in animal no. D4				

The expert statement of Schilling (2009) was performed to address questions raised in respect to the relevant NOAEL for testicular findings and related to the distribution of testicular findings and epidydimides considering historical control data and its consequence for the NOAEL. The conclusion for the 90-day toxicity study in dogs of Til (1982) is presented in the following.

Finally, the peer review pathologists concluded that as it is so difficult to interpret testicular changes in this age of dog it is more relevant to base any interpretation on the supplementary study where cyanamide was administered for 90 days to mature dogs.

The absolute and relative weight of testes was decreased for the high dose animals with one animal of this dose group showing a congenital cryptorchidism. No dose-response relationship was observed for testes weights of the low and mid dose.

The histopathological investigations as described in the study report showed no dose-response relationship in the incidence of multinucleated giant cells in the testes.

The incidence of animals with slightly/moderately reduced spermatogenesis did also not follow any dose-response relationship. In addition the incidences in the low (1) and mid dose group (2) were within the historical control range of 2 animals out of 8. The incidence of animals with absent spermatogenesis in testes is within the historical control range for the mid dose animals and slightly above the historical control range for the high dose animals.

The number of animals with atrophic seminiferous tubules did also not follow any dose-response relationship. One high dose animal with congenital cryptorchidism showed unilateral atrophic tubules and one other animal showed bilateral atrophic tubules. The incidences were within the historical control range. The findings in the animal with the congenital cryptorchidism are difficult to evaluate and considered as of limited value for the overall assessment.

With regard to a comment received during PPP peer-review, it is agreed that the number of historical control animals is low. However, the incidences confirmed that either 1 or 2 dogs out of 8 showed reduced or absent spermatogenesis and 2/8 atrophic seminiferous tubules. Thus, the incidences in the study of 0/4, 1/4, 2/4 and 1/4 at 0, 0.6, 2.0, and 6.0 mg cyanamide/kg bw/d demonstrated clearly no substance-related effect. In addition, there was no dose-dependency and 1 high dose dog showed unilateral cryptorchidism, a congenital abnormality. The finding in this cryptorchide dog accounts also for the unilateral testis atrophy and findings in spermatology, and thus, these findings are clearly not substance-related.

In conclusion the NOAEL in this study is considered to be 0.6 mg/kg bw/d based on slight anaemia and hypothyroidism in the mid and high dose animals. Also, a slight monocytosis in the mid and top dose group was found in both sexes.

Conclusion of the Rapporteur Member State (RMS):

In a 90-day oral study of Til et al. (1982), Alzodef was administered with the diet at levels of 0.6, 2.0 and 6.0 mg/kg bw/day of active substance cyanamide to 4 male and 4 female beagle dogs, about 4 months old, per test group over a period of 3 months. To derive a NOAEL the following effects are considered:

In the top-dose group, body weight gain and food intake were decreased, specially in females.

Table 61: Body weights[+]/Body weights gain

Pure active substance cyanamide (mg/kg bw/day) (n = 4 each sex)	**Males Body weight (kg)**	**Males Bw gain (kg)**	**Females Body weight (kg)**	**Females Bw gain (kg)**
0	13.9	5.5	10.9	3.9
0.6	12.9	4.8	11.0	3.9
2.0	13.3	5.0	10.6	3.7
6.0	12.7	4.5	9.5*	2.7

[+] Body weight at day 91

*Significantly different from control by Dunnetts test, $p < 0.05$

In the top-dose group, slight anemia was observed. The percentage of monocytes was increased in females of the mid-dose group and in males and females of the top-dose group and cannot be excluded as substance-related effect.

Table 62: Haematology and clinical chemistry

Pure active substance cyanamide (mg/kg bw/day) (n = 4 each sex)	**0**		**0.6**		**2.0**		**6.0**	
	day 44	**day 85**	**day 44**	**day 85**	**day 44**	**day 85**	**day 44**	**day 85**
Males								
RBC (10^6/µL)	5.7	5.9	5.9	5.6	5.6	5.2	5.6	5.3*
Hb (mmol/L)	8.4	9.1	8.3	8.6	8.2	8.3	7.8	7.8*
Hct (%)	43.6	45.1	43.0	43.1	41.9	41.1	40.6	40.3*
Reticulocytes (/10^3RBC)	8.7	14.8	-	-	-	-	7.5	7.0
WBC (10^9/L)	13.2	12.1	15.3	12.9	13.3	12.8	12.9	12.1
Monocytes (WBC x %)	4.0	2.8	2.8	2.0	6.8	5.8	3.3	9.3*
Thrombocytes (10^9/L)	372	322	340	288	258*	189*	308	197
PTT	27.9	28.2	25.5	24.9	24.3	24.8	23.2*	23.8*
Plasma albumin (g/L)	39.3	38.3	35.8	35.0*	37.8	37.0	36.3	37.5
GOT (U/L)	32.8	32.3	34.0	33.5	31.5	30.0	33.5	30.3
Plasma cholesterol (mmol/L)	3.2	3.3	3.3	3.4	3.8	3.7	3.7	3.9
Females								
RBC (10^6/µL)	5.7	5.8	5.8	5.6	5.6	5.9	5.3	5.3
Hb (mmol/L)	8.1	8.9	8.4	8.5	8.3	9.2	7.9	7.9
Hct (%)	42.5	44.8	43.4	43.5	42.5	46.1	41.1	40.6
Reticulocytes (/10^3RBC)	10.0	18.0	-	-	-	-	5.0	9.0
WBC (10^9/L)	13.9	14.2	12.2	12.0	12.3	10.0	13.0	14.8
Monocytes (WBC x %)	3.3	3.5	3.7	4.3	6.8	6.8*	6.2	10.8*
Thrombocytes (10^9/L)	367	338	341	271	246	204	258*	203
PTT	24.9	24.6	24.4	27.2	23.6	25.8	24.2	24.7
Plasma albumin (g/L)	37.3	38.5	39.0	38.0	36.5	37.5	35.0	35.8*
GOT (Units/L)	33.0	33.5	34.3	33.8	34.0	32.8	34.5	29.0*
Plasma cholesterol (mmol/L)	2.4	2.5	3.2	2.8	3.2*	3.0	3.1*	3.2*

* Significantly different from control by the Mann/Whitney U-Test, criteria, $p < 0.05$.
\- not performed

Albumin level and glutamic-oxalacetic transaminase (GOT) activity in the plasma were decreased in the top-dose group only. Cholesterol level was increased in females of the 2 and 6 mg/kg bw groups. Plasma K level was decreased in the top-dose group in males only. The decreases in plasma albumin and potassium levels and in plasma GOT activities were observed only in one of the sexes in the top-dose group und could not be correlated with any pathomorphological or functional change. Therefore, the significance of these findings is not clear.

Hypothyroidism appeared from the values of the thyroid function tests. These tests revealed that plasma T3 content was relatively low in the 2 and 6 mg/kg bw groups in both sexes, and plasma T4 content was decreased in males of the 6 mg/kg bw group as well as in males of the 2 mg/kg bw group at the end of the administration period.

Table 63: Thyroid function tests

Pure active substance cyanamide (mg/kg bw/day) (n = 4 each sex)	**0**	**0.6**	**2.0**	**6.0**
	male/female	**male/female**	**male/female**	**male/female**
T3 uptake in plasma (%)				
day 0	45/46.8	47.3/45.6	46.3/46.4	46.5/46.7
day 28	46.2/47.8	50.0/48.8	46.8/47.7	47.5/50.5
day 85	44.4/46.5	46.0/47.0	44.8/46.4	45.1/47.0
T3 content in the plasma (pmol/L)				
day 0	1241/1094	1142/1186	1128/1120	1071/1178
day 28	1442/1346	889*/1190	1191/1211	1117/1184
day 85	1358/1322	1141/1233	1026/1108	850/840
T4 content in the plasma (nmol/ L)				
day 0	35.4/33.0	27.2/34.9	35.7/37.2	29.6/37.3
day 28	42.6/42.6	25.8/34.2	30.7/37.7	31.8/35.1
day 85	38.1/37.8	26.0/31.9	23.5/25.5	20.1/32.4

Table 64: Thyroid hormones

Pure active substance cyanamide (mg/kg bw/day) (n = 4 each sex)	**0**		**0.6**		**2.0**		**6.0**	
Males	**day 0**	**day 85**	**day 0**	**day 85**	**day 0**	**day 85**	**day 0**	**day 85**
T3 (pmol/ L)	1241	1358	1142	1141	1128	1026	1071	850
T4 (nmol/ L)	35.4	38.1	27.2	26.0	35.7	23.5	29.6	20.1
Females								
T3 (pmol/ L)	1094	1322	1186	1233	1120	1108	1178	840
T4 (nmol/ L)	33.0	37.8	34.9	31.9	37.2	25.5	37.3	32.4

Table 65: Thyroid hormones: Individual values for the T3 content in plasma (pmol/L)

Pure active substance cyanamide (mg/kg bw/day)	**0**		**0.6**		**2.0**		**6.0**	
Males	**day 0**	**day 85**	**day 0**	**day 85**	**day 0**	**day 85**	**day 0**	**day 85**
	1340 *A2*	1439 *A2*	1288 *B2*	1153 *B2*	1225 *C2*	1071 *C2*	1258 *D2*	778 *D2*
	1113 *A4*	1273 *A4*	1135 *B4*	1213 *B4*	1467 *C4*	1036 *C4*	993 *D4*	722 *D4*
	1218 *A6*	1476 *A6*	1219 *B6*	942 *B6*	918 *C6*	1131 *C6*	1141 *D6*	1043 *D6*
	1185 *A8*	1242 *A8*	927 *B8*	1256 *B8*	903 *C8*	865 *C8*	890 *D8*	858 *D8*
Females								
	1081 *A1*	1230 *A1*	1456 *B1*	1484 *B1*	1029 *C1*	857 *C1*	1330 *D1*	825 *D1*
	1231 *A3*	1407 *A3*	1225 *B3*	1125 *B3*	1253 *C3*	1167 *C3*	1038 *D3*	867 *D3*
	1246 *A5*	1617 *A5*	930 *B5*	1144 *B5*	1287 *C5*	1255 *C5*	1289 *D5*	600 *D5*
	817 *A7*	1033 *A7*	1131 *B7*	1179 *B7*	909 *C7*	1151 *C7*	1053 *D7*	1069 *D7*

Table 66: Thyroid hormones: Individual values for the T4 content in plasma (nmol/L)

Pure active substance cyanamide (mg/kg bw/day)	**0**		**0.6**		**2.0**		**6.0**	
Males	**day 0**	**day 85**	**day 0**	**day 85**	**day 0**	**day 85**	**day 0**	**day 85**
	33.5 *A2*	44.3 *A2*	32.4 *B2*	28.5 *B2*	30.1 *C2*	25.5 *C2*	30.4 *D2*	12.3 *D2*
	39.8 *A4*	32.4 *A4*	29.0 *B4*	28.1 *B4*	52.0 *C4*	25.2 *C4*	32.1*D4*	24.1*D4*
	34.8 *A6*	33.4 *A6*	29.3 *B6*	20.8 *B6*	24.8 *C6*	24.8 *C6*	31.3 *D6*	23.3 *D6*
	33.5 *A8*	42.3 *A8*	18.1 *B8*	26.7 *B8*	35.9 *C8*	18.3 *C8*	24.6 *D8*	20.6 *D8*
Females								
	26.1 *A1*	24.0 *A1*	47.0 *B1*	47.7 *B1*	43.7 *C1*	23.0 *C1*	46.1 *D1*	27.2 *D1*
	33.5 *A3*	38.1 *A3*	23.5 *B3*	12.2 *B3*	44.7 *C3*	36.2 *C3*	23.1 *D3*	25.4 *D3*
	47.1 *A5*	45.7 *A5*	29.6 *B5*	30.7 *B5*	30.2 *C5*	19.8 *C5*	44.8 *D5*	27.4 *D5*
	25.3 *A7*	43.4 *A7*	39.5 *B7*	36.8 *B7*	30.1 *C7*	22.9 *C7*	35.1 *D7*	49.5 *D7*

Since the test substance was administered daily by gavage, the decrease in food intake cannot be ascribed to decreased palatibility, but might be ascribed to the hypothyroidism in this group. The slight anemia may also be correlated with the observed slight hypothyroidism in the mid- and top-dose groups. The increased plasma cholesterol levels in the top-dose group in females might be ascribed to the hypothyroidism observed in this group, too.

Microscopic examination revealed changes in the testes. Signs of atrophy and/or reduced spermatogenesis were observed in male dogs. No such effects were observed in the 4 control animals. The severity of the impact on spermatogenesis showed a clear dose-relationship (Table 67 and

Table 68. These changes were accompanied by reduced testes weights in the high-dose group.

Table 67: Incidences of histopathological effects in the testes/epididymidis

Pure active substance cyanamide (mg/kg bw/day) (n = 4 each sex)	**0**	**0.6**	**2**	**6**
Testes				
Multinucleated giant cells one or few	3[a]	4	3	1
Multinucleated giant cells several	0	0	0	2
Spermatogenesis, slight to moderate reduced	0	2	1	0
Spermatogenesis, absent	0	0	1	3
Atrophic seminiferous tubules	0	1	2	1
Atrophy testis unilateral	0	0	0	1
Atrophy testes bilateral	0	0	0	1
Epididymidis				
Reduced number of sperms	0	2	2	0
Absent number of sperms	0	2	2	3

a Number of organs with microscopic change

Already in the low-dose group all dogs revealed an effect on spermatogenesis: Two dogs showed reduced spermatogenesis in the testes accompanied by absence of spermatozoa in the epididymis (B6, B8) and two other dogs showed a reduced number of sperm in the epididymis (B2, B4).

Table 68: Individual histopathological effects in the testes/ epididymidis

Pure active substance cyanamide (mg/kg bw/day) (n = 4 each sex)	historical controls	0	0.6	2	6
Spermatogenesis, slight to moderate reduced	2/8		dog B6, B8	dog C6	
Spermatogenesis, absent	1/8			dog C8	dog D4,6,8
Atrophic seminiferous tubules	2/8		dog B4	dog C4, C8	dog D4
Atrophy testis unilateral	n.m.				dog D4
Atrophy testes bilateral	n.m.				dog D6
Reduced number of sperms in epididymidis	n.m.		dog B2, B4	dog C2, C4	
Absent number of sperms	n.m.		dog B6, B8	dog C6, C8	dog D4, D6, D8

n.m. = not mentioned; Dog D4 was a cryptorchid dog

In evaluating the toxicological significance of these findings the following aspects presented by the study authors and the peer reviewer are taken into consideration:

- The authors of the study explained that both the degree of spermatogenesis and the extent of atrophic changes may vary considerably in dogs of the strain used, without indications of a treatment-effect relationship (Til, H.P. et al., 1982).

- Weber and Creasy (2009) pointed out that 4-5 month old male beagles were used per group at study start. At this age spermatogenesis has not yet begun and the animals would be completely immature at study start. The authors reported that the characteristics of immature and peripubertal testes are indistinguishable from the degenerative changes that occur with testicular toxicants. The authors explained that it is usually not possible to evaluate testicular toxicity reliably in animals of this age.

The registrant submitted historical data in dogs about atrophy and/or reduced spermatogenesis in dogs (Woutersen, R.A.; Bruijntjes, J.P., 2005;). Woutersen and Bruijntjes reported that in the period 1977 up to 1985 at TNO CIVO eight other subchronic oral toxicity studies have been performed with the same strain of dogs (Beagles) from the same supplier (CPB), used in the subchronic oral toxicity study in dogs of Til et al. (1982). The historical data presented by Woutersen and Bruijntjes show atrophy and/or reduced spermatogenesis in the testes of dogs up to a maximum of 1 of 4 dogs (25 %). No abnormalities in the epididymides were observed. The age of the animals varied considerable per study. In most of the studies the animals were older than in the study of Til et al. (1982). Therefore, the data are not very well comparable due to the different age of the animals and cannot support the statement of the study authors about the variability of the degree of spermatogenesis and the extent of atrophic changes in dogs of the strain used. For further details see Addendum 2 to the DAR of 31 August 2007.

However, the statement of Weber and Creasy (2009) is supported by study results of Goedeken et al. (Spontaneous and Age-Related Testicular Findings in Beagle Dogs, Toxicol. Pathol. 2008; 36; 465, 2008). Goedken et al. (2008) found that in male beagle dogs hypospermatogenesis was observed in 75 % of dogs six to seven months of age and declined to fewer than 10 % in dogs over eleven months of age. Atrophy/hypoplasia of seminiferous tubules was observed in 25 to 40 % of dogs under twelve months of age, decreasing with age to 14 to 17 % in dogs twelve to thirty-six months of age. Six- and seven-month-old dogs had lower testicular weights, less filling of the epididymal tails with sperm, and a two-fold higher incidence of abnormal epididymal content compared to dogs more than eight months of age. Most male beagles were histologically sexually mature by eight to nine months of age. The study confirms published reports (Rehm, S., Spontaneous Testicular Lesions in Purpose-Bred Beagle Dogs, Toxicol. Pathol. 2000; 28; 782) that dogs at least ten months of age at necropsy usually are adequate for routine microscopic evaluation of the testes.

- Weber and Creasy (2009) reported that there appeared to be a dose-related severity with respect to the testis weight, the reduction in spermatogenesis and the presence of spermatozoa in the epididymis. The authors concluded that there might be a delay in sexual maturation in the cyanamide treated animals. In line with this explanation, a substance related effect in the cyanamide treated animals concerning a delay in sexual maturation cannot be excluded.

In summary it is concluded that in the study of Til et al. (1982) the severity of the changes in testes and epididymidis observed in the high dose group exceeds that of the changes observed in the low- and intermediate-dose groups and also the severity of the changes observed in control dogs in other experiments. In evaluating the toxicological significance of these findings it has to be taken into account that dogs less than eight months of age have high incidences of hypospermatogenesis, lower testicular weights, and incomplete filling of epididymal tails with sperm, all compatible with immaturity. Therefore, it is difficult to evaluate the observed testicular toxicity reliably. In agreement with the study authors Til et al. (1982) it seems justified to relate the more severe changes in testes and epididymidis observed in the high-dose group to the administration of cyanamide, whereas the slight changes found in the lower dose groups are regarded as findings unrelated to treatment of which degree and incidence disappear within the background. In conclusion, the NOAEL in this study is considered to be 0.6 mg/kg bw/d based on decreased T3 and T4 in the mid and high dose animals.

Oral 90-day study in dogs

Title:	Til, H. P. and Beems, R., 1986, Supplementary (90 day) oral toxicity study with a 49 % aqueous Cyanamide solution in dogs; Doc. No. 533-003; CIVO Institutes, TNO Toxicology and Nutrion Institute, Zeist, Netherlands; V 86.137/250626; unpublished
Guidelines:	Not applicable
Deviations:	3 groups (1 control, 2 dose groups); males only; examinations limited to clinical findings, testes weights, gross pathology, histopathology limited to testes, epididymides, prostate.
GLP:	Yes
Acceptability:	The study is considered to be supplementary.

Materials and methods:

The test substance was identified as follows:

Aqueous cyanamide solution (purity: 49 %)

Batch: Not indicated

Purity: About 49 %

Stability of test compound:

The test compound was analytically determined after storage in a refrigerator at 5 °C for 7 weeks. Some reduction in the active substance content in the low dose solution was observed but as the

solutions for dosing were stored only for a maximum of two weeks, the reductions were considered as negligible.

No vehicle was used.

Test animals:

Species:	Dogs, immunised against distemper, hepatitis, leptospirosis, parvo infection, rabies
Strain:	Beagle, 12 purebred
Age:	52 – 58 weeks
Source:	TNO Central Institute for breeding of Laboratory Animals, Zeist, Netherlands
Acclimation period:	not indicated
Diet:	Each animal was fed with a basal diet, pelleted by adding a molasses/water mixture (1:1) as a binding agent (50 g/kg diet).

The dogs were fed ad libitum from weighed feeders, which were filled up with diet twice a week

Water:	ad libitum
Housing:	Animals were individually caged
Environmental conditions:	
Temperature:	17 ± 3 °C
Humidity:	At least 40 %
Air changes:	10 per hour
Photoperiod:	12 hours light, 12 hours dark

The study was started on 26 November 1985 and lasted until 27 February 1986.

The test substance was administered to 4 male Beagle dogs per test group over a period of 3 months via gavage. The dose levels were 0, 1.2 and 12 mg/kg bw/day, corresponding to 0, 0.6 and 6.0 mg/kg bw/day of active substance cyanamide.

Solutions of cyanamide in distilled water, containing 0.48 % or 48 % (w/v) of the test substance, were freshly prepared once weekly. Each dog was given 0.25 mL/kg bw/day of one of the two solutions by gavage. The control dogs received 0.25 mL water /kg bw/day. The amounts of the solutions given to each dogs were adapted to body weight each week.

The test solutions were analysed after a storage period of 7 weeks in a refrigerator to determine the stability of the test item in the solutions. The analyses were carried out at TNO, Zeist, Netherlands according to a method provided by the sponsor.

Body weights and testes weights were evaluated by analysis of (co-)variance followed by multiple comparison tests (Dunnett). Food intake values were evaluated by analysis of variance followed by LSD test.

The animals were observed daily for health, condition and behaviour. All signs of ill-health or reaction to treatment were recorded. Body weights were recorded at the start and then once weekly. Food consumption was recorded weekly. Ophthalmoscopic examination, haematology and clinical chemistry, and urinalysis were not performed.

On day 93 the animals were sacrificed after anaesthesia by intravenous injection of Nembutal® (40 – 50 mg/kg bw) and then exsanguinated. Thereafter, the dogs were subjected to autopsy. The testes of all dogs were weighed. The following tissues and organs were preserved in a neutral aqueous phosphate-buffered 4 % solution of formaldehyde: Abdominal wall, adrenals, anal sac, aorta, axillary lymph nodes, brain, caecum, cervical lymph nodes, circumanal glands, colon, diaphragma, duodenum, epididymides, eyes, gall bladder, heart, ileum, jejunum, kidneys, liver, lungs, mesenteric lymph nodes, esophagus, pancreas, popliteal lymph nodes, pituitary, prostate, rectum, salivary glands, sciatic nerve, skeletal muscle, skin, spinal cord, spleen, sternum, stomach, testes, thymus, thyroid with parathyroid, tonsils, tongue, trachea, ureter, urinary bladder, all gross lesions. Sections of the following tissues from all animals were examined by microscopy following staining with hematoxylin and eosin: testes, epididymides, prostate.

Findings:

General observations:

In the high dose group 2/4 dogs showed redness of the buccal mucosa. No further treatment-related effect occurred. No mortalities were observed during the study. Mean body weights of the cyanamide treated dogs were generally slightly lower but differences were not statistically significant when compared to the corresponding controls.

Table 69: Body weights/body weight gain in males

Pure active substance cyanamide in mg/kg bw/day	0	0.6	6
Day 0 (kg)	11.7	11.6	12.1
Day 28 (kg)	13.6	12.9	12.2
Day 56 (kg)	14.0	12.9	12.8
Day 84 (kg)	13.8	13.2	13.2
Day 91 (kg)	14.1	12.9	12.8
Grand means	13.5	12.8	12.4

Mean food intake was slightly decreased in males of the highest dose group.

Table 70: Food consumption in males

Pure active substance cyanamide in mg/kg bw/day	0	0.6	6
Day 7 (kg/dog/week)	4.54	4.22	2.73
Day 28 (kg/dog/week)	2.11	2.20	2.65
Day 56 (kg/dog/week)	3.24	3.61	2.49
Day 84 (kg/dog/week)	3.41	3.59	3.3
Day 91 (kg/dog/week)	4.05	4.09	3.18
Grand means (kg/dog/week)	3.53	3.58	2.92

As substance application was by gavage, the complete dose was taken up as intended.

Both the mean absolute and mean relative weights of the testes in the high dose group were reduced when compared to those of the other groups and to the controls. However, the difference to the

control dogs was not statistically significant and was predominantly caused by severe reductions of only 2/4 dogs at this dose level.

Table 71: 90 day feeding study in dogs: organ weights

Pure active substance cyanamide in mg/kg bw/day	0	0.6	6
Body weight at day 91 (kg)	14.1	12.9	12.8
Absolute testes weight (g)	19.34	19.71	14.81
Relative[a] testes weight (g/kg)	1.47	1.60	1.24
[a] Relative weight is defined as the organ to body weight ratio			

Macroscopic test substance related changes consisted of small prostate and testes in 2/4 dogs of the high dose level. Microscopic examination of the reproductive organs revealed treatment-changes in 2/4 dogs of the high-dose group only. The changes consisted of degeneration of the germinal epithelium of the seminiferous tubules in the testes, reduced spermatogenesis and occurrence of multinucleate giant cells and were accompanied in the epididymides by a decreased number of spermatocytes and intraluminar debris.

Table 72: Incidences of microscopic effects in males

Pure active substance cyanamide in mg/kg bw/day	0	0.6	6
Number of dogs/group	4	4	4
Testes			
No abnormalities detected	3	3	2
Single atrophic tubule	0	1	0
Few atrophic tubule	1	0	0
Reduced spermatogenesis Moderate Severe	 0 0	 0 0	 1 1
Multinucleate giant cell(s)	0	0	1
Degeneration of germinal epithelium Slight Moderate	 0 0	 0 0	 1 1
Prostate			
No abnormalities detected	4	4	4
Epididymides			
No abnormalities detected	4	4	2
Intraluminar cellular debris	0	0	2
Reduced number of spermatocytes	0	0	2
Epididymitis	0	0	1
Epithelial hyperplasia	0	0	1

A histopathological peer review on male reproductive organs of the supplementary 90-day study of Til and Beems (1986) was performed by Weber and Creasy (2009) and submitted by the notifier. The result of the peer review is presented in the following.

Along with a copy of the final report, sections of the testes were delivered and microscopically reviewed.

Animals were > 1 year (52-58 weeks of age) at study start. All dogs would be expected to be sexually mature at this age. All originally recorded lesions as well as lesions recorded during the re-evaluation are presented in the peer review report and figures demonstrate slide quality and are

representative for recorded lesions. The diagnostic criteria for the terms used by the peer reviewer are defined as:

- Decreased spermatids: partial depletion of spermatids (mostly elongating but also some round spermatids)
- Tubular degeneration: partial depletion of germ cells associated with degenerating germ cells
- Hypospermatogenesis: Depletion of one or more layers of germ cells, with features resembling maturation depletion

One control dog (A008) had occasional tubules close to the rete testis that had tubular degeneration characterised by tubular vacuolation, loss or degeneration of germ cells and sperm stasis. This is quite a common background finding in dogs. Occasional tubules in testes from control and cyanamide treated dogs also showed hypospermatogenesis, which is a common background finding in Beagle dogs (Rehm, 2000; Goedken et al., 2008). The finding was minimal and focal or multifocal in most of the dogs.

The only findings of note were in two high dose (6 mg/kg/day) dogs (C2 and C4). Dog C2 had moderate diffuse acute (neutrophilic) inflammatory infiltrate of the epididymis, atrophy of the prostatic acini, reduced numbers of spermatids in the testes and reduced sperm in the epididymis. Acute inflammation of the epididymis is an unusual finding which is not commonly seen as a background lesion. The inflammation was diffuse, mostly interstitial but occasionally within the epididymal ducts. Since it was not associated with any evidence of tissue damage or necrosis, the most common explanation for an acute inflammation of this kind would be as a response to a bacterial infection. Based on the presence of similar lesions in the testes and epididymides of a number of the animals in the 52 week study and on the specific characteristics of the lesions in all these dogs the inflammation is considered to be due to infection with canine brucellosis and not related to administration of cyanamide. The other testicular findings in this dog (atrophy of the prostatic acini, reduced numbers of spermatids in the testes and reduced sperm in the epididymis) are consistent with the effects of a reduction in testosterone level. Secretory activity in the prostate is an androgen dependant function, which is very sensitive to reductions in testosterone levels. Atrophy of the acini strongly suggests reduced testosterone levels. Depletion of elongating spermatids and reduction in epididymal sperm would also be consistent with the effect of decreased testosterone on spermatogenesis. Testosterone depletion can be a primary test article related effect, but it can also be secondary to stress (Rivier and Rivest 1991). Since there were no other cyanamide treated dogs that showed these specific changes and since this dog had inflammatory changes consistent with a bacterial infection, the depletion of spermatids and sperm and the atrophic changes in the prostate are considered secondary to stress induced decrease in testosterone.

Dog C4 had moderate diffuse tubular degeneration/depletion of the testes, decreased sperm in the epididymis and an increase in the numbers of sloughed germ cells/cell debris in the epididymis. The prostatic acini of this animal showed normal secretory activity, indicating normal testosterone levels. This finding is considered potentially test article related.

All testes, epididymides and prostates were normal (similar to controls) from the 0.6 mg/kg/day dosed animals.

Table 73: Summarised results of the peer review on the supplementary study (90-Day) with cyanamide in dogs

Lesion	Group 1 (Control)	Group 2 (Low dose)	Group 4 (High dose)
No. dogs examined	4	4	4
Testes			
Focal/multifocal apoptosis	1	1	0
Hypospermatogenesis	1	3	3
Tubular degeneration	1	0	1
Decreased spermatids	0	0	1
Prostate			
Atrophy	0	0	1
Epididymides			
Epididymitis	0	0	1
Reduced sperm	0	0	2
Cellular debris	0	0	2

In conclusion, one of four dogs from the high dose group (6 mg/kg/day) had tubular degeneration/depletion in the testes, sloughed germ cells/debris in the epididymis and reduced sperm in the epididymis. Although these changes could be incidental, their possible relationship to cyanamide administration cannot be excluded. There were no findings in the testes or epididymides of dogs dosed with 0.6 mg/kg/day that were considered to be potentially treatment related.

The expert statement of Schilling (2009) was prepared to adress questions raised (1) in respect to the relevant NOAEL for testicular findings and (2) related to the distribution of testicular findings and epidydimides considering historical control data and its consequence for the NOAEL. The conclusion for the supplementary 90-day study of Til and Beems (1986) is presented in the following.

The recently performed histopathological peer review (Weber & Creasy, 2009) emphasised that the results from this supplementary 90-day study in mature dogs revealed one high dose (6 mg/kg/day) dog with testicular and prostatic changes suggestive of reduced testosterone levels. This dog also had epididymal inflammatory changes suggestive of a bacterial infection and the findings in this animal are likely stress induced. One other high dose dog had degeneration of the seminiferous tubules and reduced epididymal sperm that is considered potentially related to cyanamide administration. No lesions that were outside the range of background alterations were recorded.

All testes, epididymides and prostates were normal (similar to controls) from the 0.6 mg/kg/day dosed animals.

The peer review pathologists concluded that one of four dogs from the high dose group (6 mg/kg/day) had tubular degeneration/depletion in the testes, sloughed germ cells/debris in the epididymis and reduced sperm in the epididymis. Although these changes could be incidental, their possible relationship to cyanamide administration cannot be excluded. There were no findings in the testes or epididymides of dogs dosed with 0.6 mg/kg/day that were considered to be potentially treatment related.

All dogs treated with the high dose of cyanamide showed a retarded body weight gain and reduced food consumption compared to control.

Treatment related effects on the male reproductive organs were limited to the high dose level of 6 mg/kg bw/day pure active substance. It is noteworthy to mention that, as in the previous 90-day

study, again not all animals were affected but only 2/4 male dogs. The mean absolute and relative weight of testes was decreased for the high dose animals but this was related to the weight decrease of only 2/4 dogs.

In conclusion, the NOAEL in this supplementary 90-day dog study was 0.6 mg/kg bw/day pure active substance based on retarded body weight gain, reduced food consumption as well as on limited evidence of testicular findings at the high dose level of 6 mg/kg bw/day.

Conclusion of the Rapporteur Member State (RMS):

In a supplementary (90 day) oral toxicity study with dogs (Til, H. P. et Beems, R., 1986) 0, 0.6 and 6.0 mg/kg bw/day of cyanamide were administered to mature male beagle dogs. One of four dogs from the high dose group (6 mg/kg/day) had tubular degeneration/depletion in the testes, sloughed germ cells/debris in the epididymis and reduced sperm in the epididymis. Although these changes could be incidental, their possible relationship to cyanamide administration cannot be excluded. The NOAEL was 0.6 mg/kg bw/day and was based on retarded body weight gain, reduced food consumption as well as evidence on testicular damage at the high dose level of 6 mg/kg bw/day.

Oral 1-year toxicity study in dogs

Title:	Osheroff; M.R. (1989): Chronic toxicity Study in dogs with aqueous hydrogen cyanamide; Doc. No. 537-002; Hazelton Laboratories America, Inc.; unpublished
Guidelines:	OECD 452 (1981) U.S. EPA 83-1
Deviations:	None
GLP:	Yes
Acceptability:	The study is considered to be acceptable.

Materials and methods:

The test substance was identified as follows: aqueous hydrogen cyanamide, batch: 07-07-87, purity: 50 % w/w, 53 % w/v active substance.

Aqueous hydrogen cyanamide was administered via gavage over a period of one year to 4 male and 4 female dogs of each sex and group, which were 6 - 8 months old at study begin. The pure-bred beagle dogs were received from Hazelton Research Products, Inc., Cumberland, Virginia. For the first two weeks the dose levels were 0, 0.2, 1.0 and 5.0 mg/kg bw/day corresponding to 0, 0.1, 0.5 and 2.5 mg/kg bw/day pure active substance hydrogen cyanamide. The remaining 50 weeks doses of 0, 0.4, 2.0 and 10.0 mg/kg bw/day (corresponding to 0, 0.2, 1.0 and 5.0 mg/kg bw/day pure active substance hydrogen cyanamide) were administered. An additional group of 4 dogs/sex was administered the vehicle (distilled water) and thus served as the concurrent control group.

All animals were observed twice daily for mortality and moribundity and once daily for clinical signs. Individual body weights and food consumption were recorded weekly for weeks 0 - 16, and every fourth week thereafter. Ophthalmoscopic examinations were performed pre-treatment to initiation and at termination. Evaluation of clinical pathology parameters (haematology, serum

chemistry, thyroid function tests and urinalysis) were evaluated prior to initiation of treatment and at weeks 13, 26 and 52.

After 52 weeks of compound administration, all animals were sacrificed and subjected to complete gross examination. Organ weight evaluations (absolute and relative) and histomorphological examinations were performed on selected organs.

Findings:

General observations

Results of analytical chemistry analysis indicated that the test solutions were generally prepared within the acceptable range of target concentrations. Stability evaluation of the low and high-dose solutions were established and showed stability over the entire range.

There were no deaths during the course of this study. As compound related clinical sign, salivations were noted in all high dose animals and in one medium-dosed female as well as tremors which were were seen in three high-dosed males and two high dose females. At termination of the study, no ophthalmological abnormalities occurred related to the test compound.

Table 74: Body weight/ Body weight gain at week 13 and 52

Pure active substance cyanamide (mg/kg bw/day) (n = 4 each sex)	**Males Body weight (kg)**		**Males Bw gain (kg)**		**Females Body weight (kg)**		**Females Bw gain (kg)**	
Week	week 13	week 52	week 13	week 52	week 13	week 52	week 13	week 52
0	10.9	12.0	1.0	2.1	9.2	9.9	1.3	2.0
0.2	10.9	12.2	0.9	2.2	10.4	11.7	1.9*	3.2
1.0	11.3	12.4	1.1	2.2	8.6	9.1	1.2	1.6
5.0	10.3	10.3	0.6	0.7*	8.5	8.2	0.2*	0.0*

* Significantly different from control by the Dunnets test criteria, $p < 0.05$

Mean body weights were reduced at week 13 and more pronounced in week 52 in both sexes at 5.0 mg/kg bw, when compared to the control groups (Table 74). The males showed no gain of body weights between those weeks, whereas the females revealed a decrease of mean body weight.

Compared with the control animals, mean cumulative body weight gain, an indication of growth, was statistical significant decreased in the high-dose groups. Mean food consumption values were generally comparable between each dose group and control.

Haematology and clinical chemistry:

Haematological data at week 13 and at week 52 are shown in Table 75.

Table 75: Haematology in the dog (week 13 and 52)

Pure active substance cyanamide (mg/kg bw/day) (n = 4 each sex)	**0**		**0.2**		**1.0**		**5.0**	
week	week 13	week 52	week 13	week 52	week 13	week 52	week 13	week 52
Males								
Erythocytes (10^6/µL)	7.07	6.90	6.84	6.93	7.03	7.72	6.68	7.03
Haemoglobin (g/dL)	16.1	16.3	15.5	15.8	15.8	17.0	13.8	14.1
Haematocrit (%)	46.2	46.3	44.1	45.3	45.1	49.0	40.5	41.5
MCV (fl)	65.5	67.0	64.6	65.2	64.2	63.5*	60.7	58.9*
MCH (pg)	22.9	23.6	22.6	22.7*	22.4	22.1*	20.7*	20.1*
MCHC (g/dL)	34.9	35.1	35.1	34.8	35.0	34.8	34.1	34.1*
Leucocytes$^+$ (10^3/µL)	9.8	8.7	nd$^\#$	nd$^\#$	nd$^\#$	nd$^\#$	9.2	7.4*
Segmented neutrophils	6.2	6.3	nd$^\#$	nd$^\#$	nd$^\#$	nd$^\#$	3.3*	3.0*
Lymphocytes	2.8	1.8	nd$^\#$	nd$^\#$	nd$^\#$	nd$^\#$	5.0*	3.6
Monocytes	0.3	0.3	nd$^\#$	nd$^\#$	nd$^\#$	nd$^\#$	0.5	0.6*
Females								
Erythocytes (10^6/µL)	6.63	6.16	6.53	6.72	6.92	7.06	6.38	6.36
Haemoglobin (g/dL)	15.6	14.5	15.2	15.6	15.3	15.1	13.5*	13.0
Haematocrit (%)	44.3	41.3	43.5	44.5	43.9	43.9	39.3*	38.4
MCV (fl)	66.8	67.1	66.6	66.3	63.4	62.3*	61.6*	60.4*
MCH (pg)	23.6	23.5	23.3	23.2	22.2	21.5*	21.1*	20.5*
MCHC (g/dL)	35.3	35.0	34.9	35.0	34.9	34.5	34.3*	33.9*
Leucocytes$^+$ (10^3/µL)	9.9	9.0	nd$^\#$	nd$^\#$	nd$^\#$	nd$^\#$	11.3	11.8
Segmented neutrophils	6.5	6.6	nd$^\#$	nd$^\#$	nd$^\#$	nd$^\#$	5.0	7.4
Lymphocytes	2.8	1.6	nd$^\#$	nd$^\#$	nd$^\#$	nd$^\#$	5.2*	3.6*
Monocytes	0.3	0.3	nd$^\#$	nd$^\#$	nd$^\#$	nd$^\#$	0.8	0.9*

* Significantly different from control, by Dunnetts test criteria, $p < 0.05$
$^+$ corrected count, $^\#$ no data

Anaemia was seen in the female dogs of the high dose-group at week 13 with statistical significance based on reduced haemoglobin and haematocrit. The data of the males and of both sexes at week 52 were even suspected for anaemia. All erythrocyte parameters (MCV, MCH, MCHC) were reduced. The statistical significant decreases of MCV and MCH at 1.0 mg/kg bw at week 52 were not contributed to be substance-related, since no anaemia occurred at this dose.

Additionally, lymphocytes were decreased at week 13 and more pronounced at week 52 in the high-dose groups of both sexes. Leucocytes and segmented neutrophiles were reduced in the high dose males. Monocytosis was seen at 5.0 mg/kg bw in both sexes.

Differences in clinical chemistry-parameter compared to the control group are shown in Table 76.

Table 76: Clinical chemistry (week 13 and 52)

Pure active substance cyanamide (mg/kg bw/day) (n = 4 each sex)	**0**		**0.2**		**1.0**		**5.0**	
week	week 13	week 52	week 13	week 52	week 13	week 52	week 13	week 52
Males								
Total cholesterol (mg/dL)	165	136	176	132	191	153	249*	189
Serum albumine (g/dL)	3.8	3.8	3.7	3.7	3.7	3.6	3.3*	3.2*
Globulin (g/dL)	2.5	2.7	2.7	2.8	2.6	2.8	3.0	3.3
Phosphor (mg/dL)	5.2	3.5	5.4	3.8	5.1	3.8	5.3	4.4*
AST (U/L)	28	30	25	30	25	27	16*	25
ALT (U/L)	54	72	42	41	56	64	26*	55
Females								
Total cholesterol (mg/dL)	158	153	195	165	218*	230	222*	215
Serum albumine (g/dL)	3.7	3.6	3.7	3.6	3.6	3.5	3.1*	2.8*
Globulin (g/dL)	2.4	2.8	2.5	2.9	2.8	2.9	3.3*	3.5
Phosphor (mg/dL)	5.2	8.6	5.9	5.6	5.6	5.0	5.6	4.1
Serum glucose (mg/dL)	98	87	102	90	96	88	87	71*
Blood urea nitrogen (mg/dL)	14	15	11	12	13	13	9	8*
Creatinine (mg/dL)	0.9	0.9	0.9	0.8	0.9	0.8	0.8*	0.6*
Calcium (mg/dL)	10.6	9.8	10.6	9.6	10.7	9.8	9.8*	8.9*
AST (U/L)	28	30	27	28	26	37	17*	23
ALT (U/L)	58	45	31	33	33	31	23*	25

* Significantly different from control, by Dunnetts test criteria, $p < 0.05$

Following parameters were decreased in the high dose groups in week 13 and more pronounced in week 52: Serum albumine in both sexes and calcium in the female. Globulin was increased in week 13 and 52. Glucose and urea blood nitrogen was statistically significantly decreased in females – and phosphorus in males – when compared to control animals. Statistically significant decreases were noted for aspartate aminotransferase (AST) in the high dose animals and for alanine aminotransferase (ALT) in the high dose males and females only at week 13. A statistically significant increase of cholesterol was seen at week 13 at 1 mg/kg bw in females and at 5 mg/kg bw in both sexes.

Table 77: Thyroid hormones

Pure active substance cyanamide (mg/kg bw/day) (n = 4 each sex)	**0**			**0.2**			**1.0**			**5.0**		
week	**-2**	**13**	**52**	**-2**	**13**	**52**	**-2**	**13**	**52**	**-2**	**13**	**52**
Males												
T3 (ng/dL)	75.6	61.9	54.0	58.9	66.3	56.7	66.0	64.6	66.2	75.5	48.3	62.6
T4 (µg/dL)	3.1	2.5	1.8	2.6	2.5	1.9	2.9	2.2	1.6	2.6	1.8	1.0*
Females												
T3 (ng/dL)	72	56.1	56.0	75.3	69.7	61.5	77.4	62.6	65.8	77.8	39.3	41.4
T4 (µg/dL)	3	2.6	2.2	3.3	3.2	2.3	2.8	2.4	2.4	3.8	1.4	1.3

Decreased thyroxine values (T4) were observed in the high dose groups with statistically significance in the male at week 52 (-45 % vs –41 % in females). At 13 weeks the decrease was 28 % in the males and 46 % in the females compared to the control dogs. T3 values were lower in the high-dosed groups, but without statistical significance. The data of TSH were not shown, since not all animals were tested in the groups (Table 77).

Results of urinalysis were generally unremarkable.

Gross pathology, organ weights and histopathology

A pale area on the spleen was seen in one medium dose male and in two high dose females. Other findings were not related to treatment with the test compound.

The only significant finding in organ weight data was an increased thyroid/parathyroid weight-to-terminal body weight ratio value in the high dose females (see Table 78).

Table 78: Organ weights

Pure active substance cyanamide (mg/kg bw/day) (n = 4 each sex)	0	0.2	1	5
Males				
Absolute liver weight (g)	287	266	278	291
Relative liver weight (%)	2.5	2.2	2.3	2.9
Absolute thyroid/parathyroid weight (g)	1.16	0.88	0.93	1.13
Relative[a] thyroid/parathyroid weight (%)	0.010	0.007	0.009	0.011
Females				
Absolute liver weight (g)	214	250	207	221
Relative liver weight (%)	2.3	2.2	2.3	2.9
Absolute thyroid/parathyroid weight (g)	0.87	0.92	0.84	1.08
Relative[a] thyroid/parathyroid weight (%)	0.009	0.008	0.009	0.014*

a Relative weight is defined as the organ to body weight ratio.
* Significantly different from control by the Dunnets test criteria, $p < 0.05$.

Compound-related histomorphologic alterations were observed in the high-dose groups (see Table 79). Thymic atrophy noted in one high dose female was accompanied by demodicosis. An increased incidence and severity of brown pigment was present in Kupfer cells of the livers in the high dose of both sexes, increased extramedullary hematopoiesis was present in the spleen of two high dose males and microcholeliths were present in the gall bladder of one high dose male and two high dose females. Other findings in high dose dogs were inflammation of the testes and decreased spermatogenic activity. Thymic atrophy was noted in all four high dose males and in one high dose female that also showed demodicosis.

Other histopathological effects were not attributed to the treatment since they were incidental and not dose-dependent.

Table 79: Incidences of histopathological effects

Pure active substance cyanamide (mg/kg bw/day) (n = 4 each sex)	0	0.2	1	5
	male/female	male/female	male/female	male/female
Liver: brown pigment in kupffer cells	1/1	0/0	1/1	3/4
Spleen: increased extramed. hematopoiesis	0/0	0/0	0/0	2/0
Gallbladder: microcholeliths	0/0	0/0	0/0	1/2
Thymusatrophy	1/0	0/0	0/0	4/1
Testes: bilateral aspermatogenesis	0	0	0	1
Testes: bilateral hypospermatogenesis	0	0	0	2
Testes:chronic active inflammation	0	0	0	2
Epididymidis:hypospermia	0	0	0	2
Epididymidis: immature sperms	0	0	1	1

A Histopathological Peer Review on Male Reproductive Organs of the 1-year study performed with dogs by Osheroff (1989) was performed by Weber and Creasy (2009) and submitted by the notifier. The result of the peer review is presented in the following.

All originally recorded lesions as well as lesions recorded during the re-evaluation are presented in the peer review report. Representative illustrations of the recorded lesions are provided.

The diagnostic criteria for the terms used by the peer reviewer are defined as:

- Segmental tubular hypoplasia: Focal area of contracted tubules lined only by Sertoli cells.
- Tubular degeneration: partial depletion of germ cells associated with degenerating germ cells
- Sperm stasis: presence of sperm plugs within the tubular lumen, generally located close to the rete testis
- Hypospermatogenesis: Depletion of one or more layers of germ cells, in a pattern resembling maturation depletion
- Inflammation, chronic active: Presence of mixed inflammatory infiltrate, mostly interstitial but sometimes infiltrating seminiferous tubules. Focally associated with vascular necrosis

A number of the testes from control and cyanamide treated dogs showed minimal or slight hypospermatogenesis and segmental hypoplasia which are both common background findings in Beagle dog testes (Rehm, 2000; Goedken et al., 2008). One control dog also had a number of tubules close to the rete that showed tubular degeneration and sperm stasis. This is also a common background finding in Beagle dog testes. The incidence and severity of these findings showed no relationship to treatment.

The only unusual findings were present in the high dose (5 mg/kg/day) group dogs. Two high dose males (25293 and 25294) had marked to severe (grade 4 or 5) tubular degeneration which was accompanied by a chronic (active) inflammatory infiltrate, consisting of lymphocytes, plasma cells and neutrophils. There were also areas of vascular necrosis with fibrinous exudate in the interstitium. It is very unusual to observe inflammation in the testis, since it is an immunologically protected tissue (to keep immune cells away from the antigenically foreign sperm). In most cases, tubular degeneration (whether background or chemically induced), is not associated with an inflammatory response. The most common cause of vascular necrosis and inflammatory infiltrate in the dog testis is due to Beagle arteritis (Beagle Pain Syndrome). This is an arteritis of unknown etiology that affects multiple tissues including the testis and epididymis. However, this etiology was excluded following evaluation of all organs from the respective animals, since they showed no evidence of arteritis in any other tissues. The most likely alternative explanation for the findings is as a response to a bacterial infection such as canine Brucellosis. Although this is rarely seen in dogs these days, it was a relatively common infection of dogs 20-30 years ago (Hollett, 2006; Sýkora et al., 1977; von Kruedener, 1976; Yamauchi et al, 1974) when these studies were conducted. There are many reports in the literature that provide histopathological descriptions of the testicular and epididymal lesions associated with this infection (Shin and Carmichael, 1999) and they are consistent with the changes seen in these two dogs from the high dose group and also with the acute epididymal inflammation seen in one dogs from the 90-day supplementary study described in chapter MIIA 5.3.3. The presence of the infection in high dose animals and not in controls is likely explained by increased susceptibility to infection due to stress induced decrease in immunological status. This is supported by the fact that these dogs had significantly reduced body weight gain, as well as evidence of mite infestation and increased severity of thymic atrophy (noted during microscopic review of the other tissues). There were also decreased neutrophils and increased lymphocytes in the systemic circulation.

An additional dog (25295) from the high dose group had moderate tubular degeneration/depletion in the testis, increased sloughed germ cells/cell debris in the epididymis and decreased sperm in the epididymis. These changes were not associated with any inflammation and are potentially related to cyanamide.

Table 80: Summarised results of peer review on chronic toxicity study in dogs with aqueous hydrogen cyanamide

Lesion	Group 1 (Control)	Group 2 (Low dose)	Group 3 (Mid dose)	Group 4 (High dose)
Pure active substance cyanamide (mg/kg bw/day)	0	0.2	1	5
No. dogs examined	4	4	4	4
Testes				
Tubular dilation	1	0	0	0
Hypoplastic tubules	1	2	1	2
Hypospermatogenesis	2	2	1	2
Inflammation	0	0	0	2
Tubular degeneration	1	0	0	3
Sperm stasis	1	0	0	0
Prostate				
Focal mononuclear infiltration	1	0	2	0
Inflammation	1	1	0	1
Epididymides				
Focal mononuclear infiltration	1	0	3	0
Neutrophils in epidydimal duct	0	0	1	0
Hypospermia	0	0	0	2
Cellular debris	0	0	0	1
Acute inflammation	0	0	0	2

In conclusion, two of the four high dose dogs had lesions in the testes and/or epididymis that were considered to be secondary to a bacterial infection, probably canine brucellosis. A third dog from the high dose group had moderate degeneration of seminiferous tubules in the testes, which may be associated with cyanamide administration but could also be within the background range of testicular lesions in Beagle dogs.

The expert statement of Schilling, 2009 was prepared to adress questions raised in respect to the testicular findings and effects on spermatogenesis and its consequence for the NOAEL. The conclusion for the the 1-year study performed with dogs by Osheroff (1989) is presented in the following.

The histopathology of the male reproductive organs was recently peer reviewed by Weber & Creasy (2009). These peer review pathologists emphasised that in this study 2 of 4 high dose (5 mg/kg/day) males revealed a multifocal chronic active inflammation (orchitis) in the testes and acute inflammation of the epididymides. It is deemed that this inflammation is most likely due to bacterial infection secondary to stress induced decreased immunological status. These 2 dogs also had skin infestation with mites, increased severity of thymic atrophy, significantly less body weight gain, and decreased neutrophils and increased lymphocytes. One of the two remaining high dose dogs had moderate degeneration of the seminiferous tubules in the testes which could potentially be test article related but could also be within the background range of testicular lesions in beagle dogs. No other lesions showed significant differences between controls and high dose animals.

The peer review pathologists concluded that two of the four high dose dogs had lesions in the testes and/or epididymis that were considered to be secondary to a bacterial infection, probably canine brucellosis. A third dog from the high dose group had moderate degeneration of seminiferous tubules in the testes, which may be associated with cyanamide administration but could also be within the background range of testicular lesions in Beagle dogs.

Test substance related effects were observed in dogs administered 5.0 mg/kg bw/day of cyanamide (highest dose group). The body weight of male dogs was reduced in high dose animals (5 mg/kg bw/d) by 14 % compared to control at the study termination. The body weight gain was statistically significantly reduced by 67 % compared to control values. This indicates that a distinct systemic toxicity occurred at this dose level.

Haematological changes revealed anaemia, leucopenia, lymphocytosis and monocytosis. Anaemia was seen in the female dogs of the high dose-group at week 13 with statistical significance based on reduced haemoglobin and haematocrit. Erythrocyte parameters (MCV, MCH, MCHC) were reduced. The histopathological findings in liver and spleen seen in the high dose-group supported the anaemic signs. However, the statistically significant decreases of MCV and MCH at 1.0 mg/kg bw/day at week 52 were not contributed to be substance-related, since no anaemia occurred at this dose and no histopathological findings were seen to support anaemia.

The author concluded that the NOAEL of the 1-year study in dog is 1.0 mg/kg bw/day based on the reduced body weight/gain, the haematological findings and the testicular findings in one dog which could not be excluded with certainty as treatment-related.

Conclusion of the Rapporteur Member State (RMS):

The peer review pathologists Weber and Creasy (2009) concluded that two of the four high dose dogs had lesions in the testes and/or epididymis that were considered to be secondary to a bacterial infection, probably canine brucellosis. A third dog from the high dose group had moderate degeneration of seminiferous tubules in the testes, which may be associated with cyanamide administration but could also be within the background range of testicular lesions in Beagle dogs.

It is concluded that the NOAEL of the 1-year study in dog is 1.0 mg/kg bw/day based on the reduced body weight/gain in both sexes, the haematological findings in the female dogs and the testicular findings in one dog which could not be excluded with certainty as treatment-related.

Conclusion of the Rapporteur Member State (RMS) about the appropriate overall NOAEL for the dog regarding especially the findings in the testes:

For the derivation of the reference values for cyanamide the evaluation of testes effects observed in dog studies is crucial. Therefore, the RMS DE commented on the following question:

> What is the appropriate overall NOAEL for the dog regarding especially the findings in the testes?

Further information about the observed testes effects in dogs and a detailed justification to derive an overall NOAEL of 1.0 mg/kg bw/day from dog studies is provided below.

1. Differences in the evaluation of the dog studies by RMS and PRAPeR Meeting 79

In the recent evaluation of cyanamide the experts of the PRAPeR Meeting 79[2] agreed in line with the Additional Report, 2010[1] on the NOAEL of 0.6 mg/kg bw/day and the LOAEL of 6.0 mg/kg bw based on the 90-day oral toxicity study with mature male Beagle dogs which were 12-15 months at study begin (Til, H. P. et Beems, R., 1986[1]) and on the NOAEL of 1.0 mg/kg bw/day and the LOAEL of 5.0 mg/kg bw/day of the 1-year study in Beagle dogs (Osheroff, M.R., 1989[1]) which were 6-8 months old at study begin[2].

However, the experts of the PRAPeR Meeting 79 concluded the dose of 0.6 mg/kg bw/day in the 90-day oral study of Til et al. (1982[1]) in dogs, 4-5 month old at study begin, is a LOAEL[2]. The experts argued that the findings at 0.6 mg/kg bw/day exceed the concurrent control and the range of historical control data (HCD)[2]. This is contradictory to the results reported by the RMS.[1] In particular, the historical control data of male dogs concerning reproductive organs and its consequence for the derivation of a NOAEL/LOAEL of the 90-day oral study in dogs of Til et al. (1982; TOX2001-425[1]) have not been considered appropriately by the experts.

Further details about the testes effects observed in the 90-day oral study in dogs of Til et al. (1982[1]) and other dog studies are given below in Table 81.

Table 81: Overview of oral dog studies with cyanamide[1]

Study type / species/ dose levels	Comments	NOAEL (RMS)	NOAEL (PRAPeR 79)	Reference
90-day gavage study in Beagle dogs with 0.6, 2 and 6 mg/kg bw/day pure active substance cyanamide*	T3 and T4 decrease at 2 and 6 mg/kg bw; histopathological findings in testes and epididymidis regarding spermatogenesis most pronounced at 6 mg/kg bw/day accompanied by reduced testes weights; anaemia at 6 mg/kg bw in both sexes	0.6 mg/kg bw/d	<0.6 mg/kg bw/d	Til et al., 1982 Doc-No 533-002 TOX2001-425
90-day oral toxicity study in dogs (mature males only) with 0, 0.6 and 6.0 mg/kg bw/day pure active substance cyanamide**	Test substance related effects in male dogs at 6.0 mg/kg bw/day (retarded body weight gain, reduced food consumption as well as evidence on testicular damage)	0.6 mg/kg bw/d	0.6 mg/kg bw/d	Til, H. P. et Beems, R., 1986 Doc. No. 533-003 Z32048 / Z32032
52-weeks oral gavage study in Beagle dogs with 0, 0.2, 1.0 and 5.0 mg/kg bw/day pure active substance cyanamide***	Test substance related effects in female and male dogs at 5.0 mg/kg bw/day (e.g. anaemia, several parameter of serum-chemistry, T4 decrease, histopathological changes in liver, spleen, gallbladder, thymus and testes/ epididymidis)	1 mg/kg bw/d	1 mg/kg bw/d	Osheroff, 1989 Doc-No 537-002 TOX2004-368

* 4-5 month old at study beginn ** 12-15 months at study begin *** 6-8 months at study begin

2. Details on testes findings in the dog studies with cyanamide

In the 90-day oral study of Til et Beems (1986[1]), conducted with dogs about 12-15 months old at the start of the study, one of four dogs from the high dose group (6 mg/kg/day) had tubular degeneration/depletion in the testes, sloughed germ cells/debris in the epididymis and reduced sperm in the epididymis. Although these changes could be incidental, their possible relationship to cyanamide administration cannot be excluded. The NOAEL of 0.6 mg/kg bw/day is based on retarded body weight gain, reduced food consumption as well as evidence on testicular damage at the high dose level of 6 mg/kg bw/day.[1]

In a 1-year study by Osheroff, M.R. (1989[1]), conducted with dogs about 6-8 months old at study begin, the NOAEL of 1.0 mg/kg bw/day is based on the reduced body weight/gain in both sexes, the haematological findings in the female dogs and testicular findings in one dog, which could not be excluded with certainty as treatment-related[1].

In the 90-day oral study of Til et al. (1982[1]), cyanamide was administered with the diet at levels of 0.6, 2.0 and 6.0 mg/kg bw/day to 4 male and 4 female beagle dogs per test group, about 4-5 months old at study begin, over a period of 3 months. The dogs were obtained from the Central Institute for the Breeding of Laboratory Animals TNO, Zeist, the Netherlands. At termination, when the dogs were about 7-8 months of age, the microscopic examination revealed testes atrophy in 2 animals of the high dose group and/or reduced spermatogenesis in some dogs from all dose groups, whereas no such findings were observed in the 4 control animals. The severity of the impact on spermatogenesis showed a clear dose-relationship (Table 82). These changes were accompanied by reduced testes weights in the high-dose group (Table 83).

Table 82: Incidences of histopathological findings in the testes/epididymidis (Til et al., 1982;)[1]

Pure active substance cyanamide (mg/kg bw/day) (n = 4)	**0**	**0.6**	**2**	**6**
Testes				
Multinucleated giant cells one or few	3[a]	4	3	1
Multinucleated giant cells several	0	0	0	2
Spermatogenesis, slight to moderate reduced	0	2	1	0
Spermatogenesis, absent	0	0	1	3
Atrophic seminiferous tubules	0	1	2	1
Atrophy testis unilateral	0	0	0	1
Atrophy testes bilateral	0	0	0	1
Epididymidis				
Reduced number of sperms	0	2	2	0
Absent number of sperms	0	2	2	3

a Number of organs with microscopic change

Table 83: Individual histopathological findings in the testes/ epididymidis (Til et al., 1982)[1]

Pure active substance cyanamide (mg/kg bw/day) (n = 4)	**0**	**0.6**	**2**	**6**
Spermatogenesis, slight to moderate reduced		dog B6, B8	dog C6	
Spermatogenesis, absent			dog C8	dog D4,6,8
Atrophic seminiferous tubules		dog B4	dog C4, C8	dog D4
Atrophy testis unilateral				dog D4
Atrophy testes bilateral				dog D6
Reduced number of sperms in epididymidis		dog B2, B4	dog C2, C4	
Absent number of sperms		dog B6, B8	dog C6, C8	dog D4, D6, D8

n.m. = not mentioned; Dog D4 was a cryptorchid dog

Already in the low-dose group all dogs revealed reduced spermatogenesis when compared to controls: Two dogs showed reduced spermatogenesis in the testes accompanied by absence of spermatozoa in the epididymis (B6, B8), and two other dogs showed a reduced number of sperm in the epididymis (B2, B4) (Table 83).

Table 84: Testes weights (Til et al., 1982)[1]

Pure active substance cyanamide (mg/kg bw/day) (n = 4)	0	0.6	2.0	6.0
Males				
Absolute testes weight (g)	14.998	11.900	14.205	9.158
Relative[a] testes weight (g/kg)	1.07	0.91	1.07	0.70

a Relative weight is defined as the organ to body weight ratio

Both the absolute and relative testes weights were clearly reduced in the high dose group when compared to the control (

Table 84). At this dose, one dog showed small testes and another one unilateral cryptorchism.

3. Historical control data

In evaluating the toxicological significance of the above-mentioned findings, additional control data and aspects presented by the study authors Til et al. (1982[1]), the registrant (Woutersen, R.A.; Bruijntjes, J.P., 2005[4]) and the peer reviewer Weber and Creasy (2009[1]) are taken into consideration.

3.1 Control data from Til et al. (1982)

The authors of the study explained that both the degree of spermatogenesis and the extent of atrophic changes may vary considerably in dogs of the strain used, without indications of a treatment-effect relationship at the low- and intermediate dose groups (Til et al., 1982[1]). In the study report, historical control data from eight control dogs of the same strain and age from studies performed in the same institute (TNO CIVO) were provided.

3.2. Control data from Woutersen and Bruijntjes (2005)

The registrant submitted additional historical control data about atrophy and/or reduced spermatogenesis in dogs from a further 8 studies (Woutersen, R.A.; Bruijntjes, J.P., 2005, Letter report historical data 3 studies with Cyanamide performed at in 1982, 1986 and 1989; Toxicology and Applied Pharmacology Zeist, Netherlands; unpublished[4]). Woutersen and Bruijntjes reported that in the period 1977 up to 1985 at TNO CIVO eight other subchronic oral toxicity studies have been performed with the same strain of dogs (Beagles) from the same supplier (CPB), used in the subchronic oral toxicity study in dogs of Til et al. (1982). The historical data of controls from these studies are summarised in Table 90 and Table 93.

The historical data presented by Woutersen and Bruijntjes show atrophy and/or reduced spermatogenesis in the testes of dogs up to a maximum of 1 of 4 dogs (25 %). No abnormalities in the epididymides were observed. The age of the animals varied considerable per study. In some of the studies the age of the animals was older than in the study of Til et al. (1982) (Table 90). Therefore, the data are not very well comparable due to the different age of the animals. In the Addendum to the Draft Assessment Report (2005), it was concluded that the historical data presented by Woutersen and Bruijntjes do not allow to discount any effects in the dose groups of the study of Til et al. (1982) [4] (Table 93).

Table 85: Historical data at TNO CIVO from subchronic oral toxicity studies with dogs (Beagles) from the supplier CPB (Woutersen and Bruijntjes, 2005)[4]

Study no.	Start date	End exp. date	Report date	Animals/ group	Age in months	Reduced spermatogenesis/ atrophy observed in controls
1.	05-1977	08-1977	04-1978	4	7	not observed (0/4)
2.	09-1977	12-1977	05-1979	4	4-5	1/4 bilateral in the testes; not in the epididymides
3.	09-1977	12-1977	09-1980	4	4-5	not observed (0/4)
4.	03-1977	06-1977	09-1978	4	2	not observed (0/4)
5.	02-1978	05-1978	05-1980	4	4-5	not observed (0/4)
6.	03-1980	06-1980	02-1981	4	4-5	1/4 showed a few adult sperm cells in the testes; no abnormalities in the epididymides
7.	12-1980	03-1981	11-1982	4	7-8	1/4 showed reduced spermatogenesis in the testes; no abnormalities in the epididymides
8.	11-1985	02-1986	12-1986	4	5-6	not observed (0/4)

3.3. Control data of Rehm (2000)

Rehm (2000)[6] assessed spontaneous testicular lesions in 50 control purpose-bred male Beagle dogs used in 13 toxicologic studies at SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania. The dogs were purchased from Marshall Animal Farms, Inc, North Rose, NY. They were selected from 13 toxicology studies conducted over the period 1988-1999. Age of the dogs at study termination varied from 8-20 months with an average age of 13 months.

Regardless of age, the most significant finding was bilateral segmental hypospermatogenesis in 15/50 (30%) of the dogs (Table 86). Cross sections of tubules with hypospermatogenesis were distributed randomly throughout the testes and were characterized by reduced proportions of germ cells, tubular shrinkage, and Sertoli cell prominence. These changes were occasionally associated with giant cells, with cellular debris, and in 6/15 (40%) with atrophic tubules devoid of germ cells, indicating a degenerative process. Focal subcapsular tubular atrophy or hypoplasia (tubules lined by Sertoli cells only) was also found in 9/35 (26%) of dogs without hypospermatogenesis. Inhibited spermiation with retention of mature sperm in tubules was seen in 6/50 dogs, 3 of which also showed hypospermatogenesis. Other findings of high incidence but low prevalence included tubules with multinucleated giant cells, swollen spermatocytes, or apoptotic germ cells. The author assumed that these latter changes are probably a constituent of normal spermatogenesis.

The conclusion of Rehm (2000)[6] is that about 30% of control beagle dogs show segmental hypospermatogenesis, which may be associated with degenerative changes, and an additional 18% of the dogs exhibit focal tubular atrophy/hypoplasia in otherwise normal testes. The author explained that these changes have to be distinguished from compound-related toxic effects.

Table 86: Testicular findings observed in control beagle dogs from toxicology studies performed in 1988-1999 (Rehm, 2000)[6]

Testicular lesions	8–11 months[a] n = 11	12–13 months n = 13	14–17 months n = 16	18–20 months n = 10	Total n = 50
Hypospermatogenesis[b] (n dogs affected)					
Mild	2	2	4	1	9
Moderate	0	3	0	2	5
Severe	1	0	0	0	1
Total incidence n (%)	3 (27%)	5 (38%)	4 (25%)	3 (30%)	15/50 (30%)
Tubular atrophy/hypoplasia[c]					
Incidence n dogs (%)	5 (45%)	4 (31%)	4 (25%)	3 (30%)	15/50 (30%)
Bilateral occurrence n (%)	1/5	3/4	2/4	1/3	6/15 (40%)
No. affected areas/testis in affected dogs	5/8[d] (0.6/testis)	11/8 (1.4/testis)	16/8 (2/testis)	7/6 (1.2/testis)	39/30 (1.6/testis)
Tubules with multinucleated giant cells					
Incidence n dogs (%)	11 (100%)	13 (100%)	16 (100%)	9 (90%)	49/50 (98%)
Bilateral occurrence n dogs (%)	11/11	11/13	15/16	8/9	45/49 (92%)
No. affected tubules/testis in affected dogs	123/20[d] (6/testis)	152/26 (6/testis)	127/32 (4/testis)	98/18[d] (5.5/testis)	500/96 (5/testis)
Tubules with swollen spermatocytes					
Incidence n (%)	11 (100%)	13 (100%)	14 (88%)	10 (100%)	48/50 (96%)
Bilateral occurrence n (%)	11/11	9/13	6/14	6/10	32/46 (70%)
No. affected tubules/testis in affected dogs	64/20[d] (3/testis)	48/26 (2/testis)	36/32 (1/testis)	61/18[d] (3/testis)	209/96 (2/testis)
Retained sperm					
Incidence n (%)	5/11 (45%)	0	1/14 (6%)	0	6/50 (12%)

a = Age at necropsy.
b = Bilateral occurrence in all cases.
c = Tubules lined predominantly by Sertoli cells.
d = Single dog was excluded from enumeration because of widespread involvement of both testes.

3.4. Control data from Goedeken et al. (2009)

The notifier submitted a peer review from Weber and Creasy (2009[1]) in March 2009 that was not considered in the Draft Assessment Report, 2005[3]. The peer reviewer pointed out that in the 90-day dog study of Til et al. (1982) 4-5 month old male beagles were used at study begin. They concluded that at this age spermatogenesis has not yet begun and the animals would be completely immature at study start. The authors reported that the characteristics of immature and peripubertal testes are indistinguishable from the degenerative changes that occur with testicular toxicants. The authors explained that it is usually not possible to evaluate testicular toxicity reliably in animals of this age. The conclusion of the peer review is supported by the results of Goedeken et al. (Spontaneous and Age-Related Testicular Findings in Beagle Dogs, Toxicol. Pathol. 2008; 36; 465, 2008) that were not considered in the Draft Assessment Report, 2005[3].

Goedken et al. (2008)[5] conducted a study to characterize spontaneous testicular and epididymal microscopic findings in beagle dogs. Testes from eighty control beagle dogs (aged six to thirty-six months) were obtained from toxicology studies conducted between 2000 and 2005 at Pfizer Global Research and Development, Groton, Connecticut. All dogs were obtained from Marshall Farms (North Rose, NY).

Table 87: Incidence of hypospermatogenesis and atrophy/hypoplasia by age group (Goedken et al., 2008, 1857527)[5]

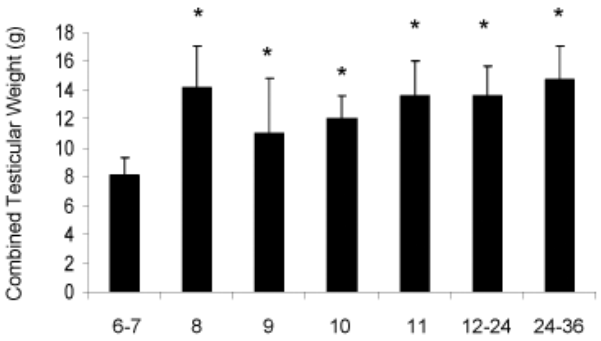
Age (mo.)	n	Finding	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4	No. with both	Total incidence	Percentage incidence
6–7	8	Hypospermatogenesis	2	0	2	1	3	2	6/8	75
		Atrophy/hypoplasia	5	0	1	1	1		3/8	37.5
8	5	Hypospermatogenesis	3	0	1	1	0	2	2/5	40
		Atrophy/hypoplasia	3	0	1	1	0		2/5	40
9	15	Hypospermatogenesis	12	0	2	1	0	3	3/15	20[a]
		Atrophy/hypoplasia	10	1	2	2	0		5/15	33.3
10	8	Hypospermatogenesis	5	1	1	1	0	2	3/8	37.5
		Atrophy/hypoplasia	6	0	1	1	0		2/8	25
11	14	Hypospermatogenesis	14	0	0	0	0	0	0/14	0[a]
		Atrophy/hypoplasia	10	1	1	2	0		4/14	29
12–23	23	Hypospermatogenesis	21	0	0	2	0	2	2/23	8.7[a]
		Atrophy/hypoplasia	19	0	1	3	0		4/23	17.3
24–36	7	Hypospermatogenesis	7	0	0	0	0	0	0/7	0[a]
		Atrophy/hypoplasia	6	1	0	0	0		1/7	14.3

Note: There were no statistical differences among dogs with atrophy/hypoplasia. There were no statistical differences comparing dogs with atrophy/hypoplasia and hypospermatogenesis at any age.
a = Statistically different from six- to seven-month-old dogs with hypospermatogenesis.

The authors found that in male Beagle dogs hypospermatogenesis was observed in 75 % of dogs six to seven months of age and declined to fewer than 10 % in dogs over eleven months of age. Atrophy/hypoplasia of seminiferous tubules was observed in 25-40 % of dogs under twelve months old, decreasing with age to 14-17 % in dogs 12 to 36 months old (Table 87). Most dogs six to seven months of age had little or no sperm in their epididymal tails, and ducts of the epididymal head and body were incompletely expanded with sperm.

Significantly lower paired testicular weights were seen in 6-7 months old dogs (8.1 ± 1.2 g) compared to testicular weights from dogs aged 8-36 months (range: 11.0 ± 3.9 g to 14.8 ± 2.3 g) (Figure 1). Testicular weights appeared to plateau at 8 months of age, with ranges as follows: 6-7 months (6.5–10.4 g), 8 months (10.6–18.3 g), 9 months (5.5–16.2 g), 10 months (9.3–12.7 g), 11 months (9.3–17 g), 12-24 months (8.3–19.1 g), and 24-36 months (11.5–17.7 g). Dogs 6-7 months of age had less filling of the epididymal tails with sperm, and a two-fold higher incidence of abnormal epididymal content compared to dogs more than 8 months of age. Most male beagles were histologically sexually mature by 8-9 months of age.

Figure 1: Relationship of control beagle dog age and testes weight (Goedken et al., 2008)5



Testicular weight was measured in control beagle dogs of varying age (n = 5–23 dogs). Asterisks (*) represent a statistical difference ($p < .05$) compared to six- and seven-month-old dogs.

4. Conclusion

In the 90-day dog study of Til et al. (1982[1]), the findings in testes and epididymidis at the high dose group are of a greater severity than the findings in the concurrent control group and in historical controls from the same laboratory (Til et al., 1982[1], Woutersen and Bruijntjes, 2005[4]) and are therefore considered to be treatment-related. In contrast, the findings at the low and intermediate dose groups are of a comparable quality to those reported for control dogs of the same age from the same laboratory and from different laboratories and are therefore not considered to be treatment-related.

The peer reviewer (Weber and Creasy, 2009[1]) reported that there might be a delay in sexual maturation in the cyanamide treated animals. In line with this explanation a substance related effect in the cyanamide treated animals concerning a delay in sexual maturation can not be excluded.

In evaluating the toxicological significance of these findings, the study results of Goedeken et al. (2008)[5] and Rehm (2000)[6] have to be taken into account. They clearly demonstrate that Beagle dogs less than eight months of age have high incidences of hypospermatogenesis, lower testicular weights, and incomplete filling of epididymal tails with sperm, all compatible with immaturity. A direct numeral comparison of the historical control data found by Goedken et al. (2008)[5] and Rehm, S. (2000)[6] with the results found in the testes and epididymidis of the 90-day oral study of Til et al. (1982; TOX2001-425[1]) in dogs may not be appropriate because the sources of the used Beagle dogs are different and the studies have been conducted at a different period. However, it has to be taken into account that 4-5 months old male beagles used in the study of Til et al. (1982[1]) would be completely immature at study start. Therefore, it is difficult to evaluate the observed testicular toxicity reliably.

Table 88: Comparison of incidences of histopathological findings in testes and epididymidis (Til et al., 1982[1])

Pure active substance cyanamide (mg/kg bw/day)	**Til et al, 1982**					**Rehm, S. (2000)+**	**Goedken et al. (2008)***	**Woutersen & Bruijntjes, (2005)•**
	historical controls #	**0**	**0.6**	**2**	**6**			
Testes								
Spermatogenesis, slight to moderate reduced	25% (2/8)	0	50% (2/4)	25% (1/4)	0	30%[d]	75% [a] 40%	25% [f]
Spermatogenesis, absent	12.5% (1/8)	0	0	25% (1/4)	75% (3/4)			n.o.[f]
Atrophic seminiferous tubules	25% (2/8)	0	25% (1/4)	50% (2/4)	25% (1/4)	18%[e]	25–40% [b]	n.m.
Atrophy testis unilateral	n.m.	0	0	0	25% (1/4)			25% [f]
Atrophy testes bilateral	n.m.	0	0	0	25% (1/4)			
Epididymidis								
Reduced number of sperms	n.m.	0	50% (2/4)	50% (2/4)	0		[c]	n.o.[f]
Absent number of sperms	n.m.	0	50% (2/4)	50% (2/4)	75% (3/4)		[c]	n.o.[f]

n.m. = not mentioned; Dog D4 was a cryptorchid dog n.o. = not observed
+ = Spontaneous testicular lesions in purpose-bred Beagle dogs (Rehm, S., 2000)[5]
***** Spontaneous and age-related testicular findings in Beagle dogs (Goedeken et al., 2008)[4]
• Historical data at TNO CIVO from subchronic oral toxicity studies performed 1977 up to 1985 with dogs (Beagles) from the supplier CPB presented by Woutersen, R.A.; Bruijntjes, J.P., 2005[4]
# 8 controls from two similar studies of the same institute with dogs of the same strain and age (Til et al. (1982)[1]
a = Hypospermatogenesis was observed in 75% of dogs 6-7 months old and in 40% of dogs 8 months old
b = Atrophy/hypoplasia of seminiferous tubules was observed in 25-40 % of Beagle dogs under 12 months old.

c = Most Beagle dogs 6-7 months of age had little or no sperm in their epididymal tails, and ducts of the epididymal head and body were incompletely expanded with sperm.
d = 30% of control Beagle dogs 8-20 months of age show segmental hypospermatogenesis, which may be associated with degenerative changes.
e = An additional 18% of the Beagle dogs 8-20 months of age exhibit focal tubular atrophy/hypoplasia.
F = Atrophy and/or reduced spermatogenesis in the testes of Beagle dogs 4 to 8 month old up to a maximum of 1 of 4 dogs (25%)

Table 89: Comparison of testes weights (Til et al., 1982[1])

Pure active substance cyanamide (mg/kg bw/day) (n = 4)	**0**	**0.6**	**2.0**	**6.0**	**Goedken et al. (2008)***
Absolute testes weight (g) mean +/- SEM	14.998 +/- 2.668	11.900 +/- 2.081	14.205 +/- 2.005	9.158 +/- 3.227	about 14[a]
Absolute testes weight (g) indiviual range (lowest and highest organ weight)	11.1 - 22.7	8.7 - 17.5	10.1 - 19.6	3.9 - 17.9	10.6 - 18.3 [a]
Relative[a] testes weight (g/kg) mean	1.07	0.91	1.07	0.70	-

***** Spontaneous and age-related testicular findings in Beagle dogs (Goedeken et al., 2008[5])[4]
a = Testicular weights were seen in eight-month-old dogs

In agreement with the study authors Til et al. (1982[1]) it seems justified to relate the more severe changes in testes and epididymidis observed in the high-dose group to the administration of cyanamide, whereas the slight changes found in the lower dose groups are regarded as findings unrelated to treatment of which degree and incidence disappear within the background. In conclusion, the NOAEL in this study is considered to be 0.6 mg/kg bw/d based on decreased T3 and T4 in the mid and high dose animals.

References:

1 Additional Report, 04 January 2010, Cyanamide, Rapporteur Member State: Germany
2 Report of PRAPeR Expert Meeting 79 (12 - 16 July 2010), 16 July 2010, Cyanamide, 2. Mammalian Toxicology
3 Draft Assessment Report, 20 December 2005, Cyanamide
4 Addendum 2 to the Draft Assessment Report of 20 December 2005
5 Goedeken et al., Spontaneous and Age-Related Testicular Findings in Beagle Dogs, Toxicol Pathol 2008; 36; 465, 2008
6 Rehm, S., Spontaneous Testicular Lesions in Purpose-Bred Beagle Dogs, Toxicol Pathol 2000; 28; 782

4.7.1.2 Repeated dose toxicity: inhalation

Title: Kumar, T. et al. (1996): Subacute inhalation toxicity study of hydrogen cyanamide 50 % w/w formulation (Dormex) in Wistar rats; Doc. No. 532-003; Frederick Institute of Plant Protection and Toxicology, Padappai, India; unpublished

Guidelines: Gaitonde committee guidelines

Deviations: 14 days of exposure instead of 28 days; satellite group: Only 3 animals of each sex of the intermediate dose groups; actual

concentration spaces are to narrow between the low and mid dose groups.

GLP: No

Acceptability: The study is considered to be supplementary.

Materials and methods:

The test substance was identified as follows: Dormex (hydrogen cyanamide, aqueous solution), batch: 507401; purity: 50 % w/w formulation.

In a subacute inhalation study, Dormex (hydrogen cyanamide, 50 % w/w formulation) was administered by inhalation (head only exposure) to male and female Wistar rats for 14 days. 10 rats (5 males and 5 females) were exposed to 2.5 mg/L (low dose group) and 9.375 mg/L (high dose group) whereas 16 rats (8 males and 8 females) were exposed to 5.0 mg/L (intermediate dose group) nominal concentration of the active substance hydrogen cyanamide. An additional group of 8 males and 8 females was used as control. The aerosol of the test substance was generated by infusing it through the nebuliser which generates particles less than 5µm in diameter. During the exposure, samplings of the atmosphere from the inhalation chamber were done for the HPLC determination of actual concentrations of hydrogen cyanamide. Animals were exposed for 6 hours per day, 5 days a week for 2 weeks. On day 15 after start of the exposure 5 males and 5 females of each group were necropsied. The remaining animals (3 males and 3 females) of the control and intermediate dose group were maintained without any exposure for a further period of 2 weeks (satellite group).

Animals were observed daily for mortality and/or moribundity and during exposure every hour for toxic symptoms. Body weight and food consumption measurements were performed daily. Clinical pathology parameters (haematology and clinical chemistry) were determined in rats after 15 days of exposure in the main groups and after 29 days in the animals of the satellite group (control and intermediate dose). After the scheduled exposure regimen all animals except the satellite animals were necropsied and the lesions were noted grossly. Organ weight (absolute and relative) and histopathological examination were performed on all animals. The same procedure was performed in the remaining animals at day 29.

Findings:

General observations:

The actual concentrations of hydrogen cyanamide as active substance in the inhalation atmosphere sampled from the chamber during exposure were 0.1484 (range 0.0848-0.1916), 0.2629 (range 0.1489-0.3270) and 0.7991 (range 0.4080-1.1184) mg/L air.

No mortality was observed in rats belonging to any of the groups throughout the study period.

No clinical signs of toxicity were found in rats exposed to hydrogen cyanamide.

The results of body weight data at day 0, 1 and 14 are summarised in Table 90.

Table 90: Body weights

Pure active substance cyanamide (mg/L/day)	Males Body weight (kg)	Males Bw gain (g)	Females Body weight (kg)	Females Bw gain (g)
0 (n = 8)	160.3	64.7	161.6	65.1
0.15 (n = 5)	147.0*	55.0	147.6*	53.0
0.26 (n = 8)	141.8*	41.0	133.4*	35.8
0.8 (n = 5)	146.0*	51.8	128.6*	34.8

* Significantly different from control by the Student's t-test criteria, $p < 0.05$

There were statistical significant lower mean body weights in all treated animals with a dose-dependency in females that showed a decreased body weight gain of 18 % compared to the control already at the low dose. At higher concentrations, body weight gain were 45 % (0.26 mg/L/day) and 47 % (0.8 mg/L/day) less than the gain in control rats. During the post-treatment observation period, recovery was seen in both sexes.

Total food consumption was not mentioned. Clear substance-related effects were not seen in food consumption. No compound-related effects were found in haematological and blood chemistry parameters.

As depicted in Table 91, several absolute and relative organ weights in all treated groups in males and females were statistical significant different from the control group.

Table 91: Organ weights

Pure active substance cyanamide (mg/L/day) (n = 5 each sex)	0	0.15	0.26	0.8
Males				
Absolute heart weight (g)	0.443	0.501*	0.509	0.594**
Relative[a] heart weight (%)	0.278	0.337*	0.353*	0.405**
Absolute liver weight (g)	5.008	4.718	6.134*	7.532**
Relative[a] liver weight (%)	3.149	3.204	4.249**	5.153**
Absolute gonad weight – left (g)	0.557	0.835	0.916**	0.992**
Relative[a] gonad weight – left (%)	0.350	0.560*	0.634**	0.676**
Absolute gonad weight – right (g)	0.546	0.837*	0.915**	0.987**
Relative[a] gonad weight – right (%)	0.342	0.561*	0.633**	0.673**
Females				
Absolute heart weight (g)	0.463	0.483	0.490	0.492
Relative[a] heart weight (%)	0.288	0.326**	0.341**	0.389**
Absolute liver weight (g)	4.719	6.137**	6.987**	6.950**
Relative[a] liver weight (%)	2.934	4.152**	4.865**	5.486**

a Relative weight is defined as the organ to body weight ratio.
* Significantly different from control by the Student's test criteria, $p < 0.05$.
** Significantly different from control by the Student's test criteria, $p < 0.01$.

In the satellite group the absolute and relative liver and kidney was still increased in male animals. In female animals an increase in absolute and relative lung weight and an increase in relative kidney weight was observed.

Gross pathological lesions were observed in animals of all dose groups including the controls (see Table 92).

Table 92: Incidences of pathological findings

Pure active substance cyanamide (mg/L/day) (n = 5 each sex)	0	0.15	0.26	0.8
	male/female	male/female	male/female	male/female
Lung : congestion	0/0 [a]	0/0	0/0	1/0
Lung: sparse petechiae	1/1	0/0	0/0	1/1
Lung: marbling	0/0	1/1	0/0	1/1
Lung: emphysematous	2/2	1/1	2/2	0/3
Liver: mottling	0/0	1/0	0/0	0/1
Liver: foci of congestion	0/0	0/0	0/0	0/1
Kidneys: gray specks in cortices	0/0	0/0	0/0	0/1

As shown in Table 93 histopathological changes revealed consistent recurring lesions in the brain, liver, heart and lungs in the high dose group in both sexes.

Table 93: Incidences of histopathological findings

Pure active substance cyanamide (mg/L/day) (n = 5 each sex)	0	0.15	0.26	0.8
	male/female	male/female	male/female	male/female
Brain (cerebrum): microcavitations (oedema)	0/0	0/0	1/0	1/2
Brain (cerebellum): microcavitations (oedema)	0/0	0/0	0/1	2/2
Liver: centrilobular cloudy swelling	0/0	1/0	1/0	2/1
Liver: hyperaemia	0/0	0/0	0/0	1/1
Heart: edematous foci myocardium	0/0	0/0	0/0	1/1
Heart: focal hemorrhage	0/0	0/0	1/0	1/0
Lungs: bronchiectasis	0/0	0/0	0/0	2/1
Kidney: calcified foci	0/0	0/0	0/0	0/1
Ileum: enteritis	0/0	0/0	0/1	0/1

Conclusion:

Based on the reduced body weight/body weight gain, the NOAEL was below 0.15 mg/L air.

4.7.1.3 Repeated dose toxicity: dermal

Title: Murugan, S.S. et al. (1996): Subacute dermal toxicity study of hydrogen cyanamide 50 % w/w formulation (Dormex) in rabbits; Doc. No. 532-002; Fredrick Institute of Plant Protection and Toxicology, Padappai, India; unpublished

Guidelines: Gaitonde Committee guideline

Deviations: 21 days of exposure instead of 28 days; stability, homogeneity and correctness of the concentrations not verified analytically; 3 males and 3 females used per concentration instead of 5 males and 5 females; no sacrifice immediately after end of application, but after 14 days; body weight 1200 g ± 200 g instead of 2000 – 3000 g at the beginning of treatment; housing temperature 24 ± 2 °C instead of 20 ± 3 °C; no

	summary or individual data of clinical findings; less haematological and blood chemistry parameter compared to OECD 410; no individual organ weight data were submitted.
GLP:	No
Acceptability:	The study is considered to be supplementary with regard to dermal effects. It cannot be used for evaluation of systemic effects.

Materials and methods:

The test substance was identified as follows:

Dormex (hydrogen cyanamide, aqueous solution)

Batch: 507401

Purity: 50 % w/w formulation

Dormex was applied undiluted to the skin of three male and three female New Zealand White rabbits per dose level for a period of six hours per day on 5 days per week for three consecutive weeks followed by a 14 day post-application observation period. The concentrations were 0, 25, 50 and 75 mg/kg bw/day equivalent to 12.5, 25 and 37.5 mg/kg bw/day active substance cyanamide, which were selected according to the results of a pilot study. The control group did not receive any treatment with the test substance.

The animals were examined once daily for mortalities, clinical symptoms and local skin irritations. Data of body weight and food consumption of individual rabbits were recorded weekly. Clinical-chemical and haematological investigation and urinalysis were performed on day 0, 22 and 35.

All animals were sacrificed not immediately after the end of treatment, but after a 14-day recovery period. Therefore, the data received in organ weights, pathology and histopathology were not accepted for the evaluation.

Findings:

General observations:

There were no mortalities in any groups of the test material or post-application observation period. At the highest concentration, severe erythema of the skin and reduction in the fur density around the areas of application were observed. Slight erythema during the application period were observed at the mid dose of 25 mg/kg bw/day pure active substance cyanamide. Diarrhoea, dullness and lethargy were observed in few rabbits (dose groups not mentioned). These symptoms disappeared during the post-exposure observation period.

There were no significant differences in the body weight and feed consumption of animals treated with Dormex when compared to the control animals.

None of the haematological, clinical chemical and urine parameters of the treated groups showed any statistical significant variation as compared to the controls at each time of measurement. The data received after sacrifice of the rabbits 14 days after the end of treatment were not listed.

Conclusion:

Regarding the local skin effects at 25 mg/kg bw, the NOAEL for dermal effects is 12.5 mg/kg bw of Dormex in rabbits. A NOAEL for systemic effects is not applicable since the animals were not sacrificed and examined gross pathologically and histopathologically immediately after the end of treatment. These data were not acceptable.

4.7.1.4 Repeated dose toxicity: other routes

No data submitted by the notifier.

4.7.1.5 Human information

Medical surveillance on manufactoring plant personnel which also included special investigations of functional disorders regarding the testes and the thyroid gland and potential sensitising properties did not reveal any signs of diseases or health impairments caused by cyanamide.

In a human study it was investigated if there are effects of cyanamide exposure on the testes and the thyroid gland. According to this investigation, disturbances of the gonadal function and the thyroid function can be excluded (Mertschenk et al, 1993).

For further details see section 4.12.1.6 “Human information”.

Cyanamide in alcohol therapy

Calcium cyanamide has been intensively used worldwide as a drug to deter drinking in alcoholics. Intolerance reactions towards alcohol occur in man after daily cyanamide doses higher than 20 mg. In general, daily doses of more than 0.4 – 1 mg/kg bw cyanamide have been used in the alcohol aversion therapy. The duration of the treatment ranges from a few months to a few years. In some cases, patients have taken cyanamide for more than 10 years. No signs of reproductive disorders have been observed.

For further details see section 4.12.1.6 “Human information”

4.7.1.6 Other relevant information

4.7.1.7 Summary and discussion of repeated dose toxicity

A short-term oral toxicity study of cyanamide in the rat at doses of 0, 5, 10, 20, 40 mg/kg bw/day over a period of 28 days was characterised by a depression in body weight, body weight gain and food consumption at 20 and 40 mg/kg bw/day (Osheroff, 1988). A decrease in red cell parameters was obtained in males and females at the highest dose associated by decreases in MCH and MCHC and an incidental increase of total bilirubine. It was assumed that anaemia was caused by haemolysis. Splenic pigmentation in females was found at 10 mg/kg bw/day and higher, which was seen as well in males, but only at the highest dose of cyanamide. Histopathological thyroid findings obtained in the low dose group of male rats were not seen as an adverse effect, whereas the more severe changes at 10 mg/kg bw/day and up were attributed to cyanamide. In females, thyroid effects were obtained at 20 and 40 mg/kg bw/day. Other histopathological findings were seen at 10, 20 and 40 mg/kg bw/day in the liver of males (bile duct hyperplasia). The NOAEL for cyanamide was 5 mg/kg bw/day, based on the thyroid effects in males at 10 mg/kg bw.

A 90-day oral toxicity study was performed in rats with 0.5, 1.5 and 4.5 mg/kg bw/day of cyanamide (corresponding to 10, 30, 90 ppm; feeding study) (Til et al., 1975). At 4.5 mg/kg bw/day thyroid effects were seen in males as well in females. The changes in the thyroid were comparable to the effects found in the 4 week study. Histopathological changes in the thyroids in only one male (1/20) were not contributed due to an adverse effect of cyanamide at 1.5 mg/kg bw/day. Additionally, male rats showed – in contrast to the 28 day study – a slight increase in erythrocyte counts. The NOAEL was 1.5 mg/kg bw/day pure active substance cyanamide (equivalent to 30 ppm in the diet) in males and females.

In a 90-day oral study of Til et al. (1982), Alzodef was administered via gavage at levels of 0.6, 2.0 and 6.0 mg/kg bw/day of active substance cyanamide to 4 male and 4 female beagle dogs, about 4 months old, per test group over a period of 3 months. Histopathological findings in testes and epididymidis regarding spermatogenesis accompanied by reduced testes weights were most pronounced at 6 mg/kg bw/day. Slight changes in testes and epididymides found in the lower dose groups are regarded as findings unrelated to treatment of which degree and incidence disappear within the background. The experts of the PRAPeR Meeting 79 concluded the dose of 0.6 mg/kg bw/day in the 90-day oral study of Til et al. (1982[1]) is a LOAEL. However, in agreement with the study authors Til et al. (1982; TOX2001-425) it seems justified to relate the more severe changes in testes and epididymidis observed in the high-dose group to the administration of cyanamide, whereas the slight changes found in the lower dose groups are regarded as findings unrelated to treatment of which degree and incidence disappear within the background (discussed in *Conclusion of the Rapporteur Member State (RMS) about the appropriate overall NOAEL for the dog regarding especially the findings in the testes*). In conclusion, the NOAEL in this study is considered to be 0.6 mg/kg bw/d based on decreased T3 and T4 in the mid and high dose animals.

In a supplementary (90 day) oral toxicity study with dogs (Til, H. P. et Beems, R., 1986) 0, 0.6 and 6.0 mg/kg bw/day of cyanamide were administered to mature male beagle dogs (12 – 15 months at study begin). One of four dogs from the high dose group (6 mg/kg/day) had tubular degeneration/depletion in the testes, sloughed germ cells/debris in the epididymis and reduced sperm in the epididymis. Although these changes could be incidental, their possible relationship to cyanamide administration cannot be excluded. The NOAEL was 0.6 mg/kg bw/day and was based on retarded body weight gain, reduced food consumption as well as evidence on testicular damage at the high dose level of 6 mg/kg bw/day.

In a 1-year study by Osheroff, M.R. (1989) 0, 0.2, 1 and 5 mg/kg bw/day of cyanamide were administered via oral gavage to 4 beagle dogs of each sex and group (6 - 8 months old at study begin). It is concluded that the NOAEL of the 1-year study in dog is 1.0 mg/kg bw/day based on the reduced body weight/gain in both sexes, the haematological findings in the female dogs and the testicular findings in one dog which could not be excluded with certainty as treatment-related.

A 21-day dermal study in rabbits revealed local skin effects at 25 mg/kg bw and higher after an application of Dormex. Therefore, the NOAEL for dermal effects was 12.5 mg/kg bw. A NOAEL for systemic effects was not applicable, since the animals were not sacrificed and examined gross pathologically and histopathologically immediately after the end of treatment, but with a period of 14 days after. Data received in pathology and histopathology are not acceptable for evaluation of systemic effects.

A 14-day inhalation study revealed reduced body weights and body weight gain at 0.15 mg/L air, which was clear dose-dependent in females at higher doses. Thus, the NOAEL was below 0.15 mg cyanamide/L air in this study in rats.

For further details about human data see 4.12.1.4.

Table 94: Summary table of relevant repeated dose toxicity studies

Method	Remarks	Results	Reference
28-day gavage study in Sprague-Dawley rats with 0, 5, 10, 20 and 40 mg/kg bw/day pure active substance cyanamide	Depression in the body weights, body weight gains and food consumption at 20 and 40 mg/kg bw/day; relevant histopathological findings in thyroid at 10 mg/kg bw/day and higher in males	NOAEL: 5 mg/kg bw/day of cyanamide	Osheroff, 1988 Doc-No 532-001 TOX2004-366
90-day dietary study in Wistar rats with 0.5, 1.5 and 4.5 mg/kg bw/day pure active substance cyanamide (0, 10, 30 and 90 ppm)	Marked effects in the thyroid in males and in females at 4.5 mg/kg bw/day; additionally increases in erythrocyte counts	NOAEL: 1.5 mg/kg/day of cyanamide	Til et al., 1975 Doc-No 533-001 TOX2004-367
90-day gavage study in beagle dogs* with 0.6, 2 and 6 mg/kg bw/day pure active substance cyanamide * 4 - 5 month old at study begin	T3 and T4 decrease at 2 and 6 mg/kg bw; histopathological findings in testes and epididymidis regarding spermatogenesis most pronounced at 6 mg/kg bw/day accompanied by reduced testes weights; anaemia at 6 mg/kg bw in both sexes	NOAEL (RMS): 0.6 mg/kg bw/day of cyanamide NOAEL (PRAPeR 79): < 0.6 mg/kg bw/day of cyanamide	Til et al., 1982 Doc-No 533-002 TOX2001-425
Supplementary (90 day) oral toxicity study in dogs** (mature males only) with 0, 0.6 and 6.0 mg/kg bw/day pure active substance cyanamide ** 12 – 15 months at study begin	Test substance related effects in male dogs at 6.0 mg/kg bw/day (retarded body weight gain, reduced food consumption as well as evidence on testicular damage)	NOAEL: 0.6 mg/kg bw/day of cyanamide	Til, H. P. et Beems, R., 1986 Doc. No. 533-003 Z32048 / Z32032
52-weeks oral gavage study in Beagle dogs*** with 0, 0.2, 1.0 and 5.0 mg/kg bw/day pure active substance cyanamide *** 6 - 8 months at study begin	Test substance related effects in female and male dogs at 5.0 mg/kg bw/day (e.g. anaemia, several parameter of serum-chemistry, T4 decrease, histopathological changes in liver, spleen, gallbladder, thymus and testes/ epididymidis)	NOAEL: 1 mg/kg bw/day of cyanamide	Osheroff, 1989 Doc-No 537-002 TOX2004-368
21-day dermal study in New Zealand rabbits with 12.5, 25 and 37.5 mg/kg bw/day pure active substance cyanamide	No conclusion about systemic effects after 21 days, since animals not sacrificed immediately after end of treatment, but with a period of 14 days after; local skin effects at 25 mg/kg bw and higher	NOAEL: 12.5 mg/kg bw of Dormex for dermal effects. NOAEL for systemic effects not applicable	Murugan et al., 1996 Doc-No 532-002 TOX2001-426
14-day inhalation study in Wistar rats with 0.1485, 0.2629 and 0.7991 mg/L air	Decreased body weights and body weight gain; clear dose-dependent in females	NOAEL: < 0.15 mg/L	Kumar et al., 1996 Doc-No 532-003 TOX2001-427

4.7.1.8 Summary and discussion of repeated dose toxicity findings relevant for classification according to DSD

Repeated dose toxicity findings are discussed with respect to reproductive toxicity in section 4.11. For the discussion of other effects see section 4.8.

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

4.8.1 Summary and discussion of repeated dose toxicity findings relevant for classification as STOT RE according to CLP Regulation

Concerning specific target organ toxicity, thyroid effects were seen in rats after repeated dosing.

A short-term oral toxicity study of cyanamide in the Sprague-Dawley rat at doses of 0, 5, 10, 20, 40 mg/kg bw/day over a period of 28 days (Osheroff, 1988) was characterised by an increased incidence of follicular cell hyperplasia at 10 mg/kg bw/day and higher in male rats. Decreased colloid content and small and closely packed follicles were seen already at 5 mg/kg bw/day in the males, albeit to a marginal extent. The females were less sensitive: thyroid effects were seen dose-dependently at 20 and 40 mg/kg bw/day. At 40 mg/kg bw/day, the mean TSH values were increased by 100 %, whereas T4 concentrations were decreased by 28 % in both sexes compared to control animals.

A supplementary 90-day oral toxicity study was performed in Wistar rats with 0.5, 1.5 and 4.5 mg/kg bw/day of cyanamide (corresponding to 10, 30, 90 ppm; feeding study) (Til et al., 1975). At 4.5 mg/kg bw/day in the thyroid predominantly small follicular lumens without colloid, separated by proliferating epithelial cells and interfollicular cells were seen in males as well in females, comparable to the effects found in the 4-week study. The effects in one male at 1.5 mg/kg bw were not seen as an adverse effect since the incidence was 1/20 rats.

4.8.2 Comparison with criteria of repeated dose toxicity findings relevant for classification as STOT RE

In short-term oral toxicity studies, significant toxic effects in the thyroid were observed in males and females of two strains of rats. The morphological changes in the thyroid in both studies were comparable. They have toxicological significance and are considered to be of relevance to human health. The lowest effect dose was seen at 4.5 mg/kg bw/day in a 90-day dietary study. In a 28-day gavage study the lowest effect dose was at 10 mg/kg bw/day in male rats and at 20 mg/kg bw/day in female rats. Thus, it can be concluded that classification for this endpoint is required because the effect levels were below the threshold dose levels set in DSD and CLP regulation. According to CLP criteria, category 1 is proposed.

Table 95: Toxicological results in comparison with criteria of specific target organ toxicity – repeated exposure

Toxicological result	DSD criteria	CLP criteria
28-day gavage study in Sprague-Dawley rats with 0, 5, 10, 20 and 40 mg/kg bw/day pure active substance cyanamide (Osheroff, 1988; Doc-No 532-001)	Danger of serious damage to health by prolonged exposure Serious damage (clear functional disturbance or morphological change which has toxicological significance)	Category 1: Substances that have produced significant toxicity in humans or that, on the basis of evidence from studies in experimental animals, can

Results: Thyroid: increased incidence of follicular cell hyperplasia at ≥ 10 mg/kg bw/day in male rats increased incidence of follicular cell hyperplasia at ≥ 20 mg/kg bw/day in female rats decreased colloid content and small and closely packed follicles ≥ 5 mg/kg bw/day in the males decreased colloid content and small and closely packed follicles ≥ 20 mg/kg bw/day in the females 90-day dietary study in Wistar rats with 0.5, 1.5 and 4.5 mg/kg bw/day pure active substance cyanamide (0, 10, 30 and 90 ppm) (Til et al., 1975; Doc-No 533-001) Results: Thyroid: increased incidence of predominantly small follicular lumens without colloid, separated by proliferating epithelial cells and interfollicular cells in males and females at 4.5 mg/kg bw/day	is likely to be caused by repeated or prolonged exposure by an appropriate route. Guide values can apply when severe lesions have been observed: Oral, rat: <u>Threshold for "harmful"</u> 28-day: ≤ 150 mg/kg bw/d 90-day: ≤ 50 mg/kg bw/d <u>Threshold for "toxic"</u> Substances and preparations are classified at least as toxic when these effects are observed at levels of one order of magnitude lower (i.e. 10-fold) than those set out above (for harmful)	be presumed to have the potential to produce significant toxicity in humans following repeated exposure. Substances are classified in Category 1 for target organ toxicity (repeat exposure) on the basis of: reliable and good quality evidence from human cases or epidemiological studies; or observations from appropriate studies in experimental animals in which significant and/or severe toxic effects, of relevance to human health, were produced at generally low exposure concentrations. Equivalent guidance values for 28-day and 90-day studies: Oral, rat: 28-day: ≤ 30 mg/kg bw/d 90-day: ≤ 10 mg/kg bw/d
		<u>Category 2</u>: Substances that, on the basis of evidence from studies in experimental animals can be presumed to have the potential to be harmful to human health following repeated exposure. Substances are classified in category 2 for target organ toxicity (repeat exposure) on the basis of observations from appropriate studies in experimental animals in which significant toxic effects, of relevance to human health, were produced at generally moderate exposure concentrations. Guidance dose/concentration values are provided below (see 3.9.2.9) in order to help in classification. In exceptional cases human evidence can also be used to place a substance in Category 2. Equivalent guidance values for 28-day and 90-day studies: Oral, rat: 28-day: ≤ 300 mg/kg bw/d 90-day: ≤ 100 mg/kg bw/d

4.8.3 Conclusions on classification and labelling of repeated dose toxicity findings relevant for classification as STOT RE

The short-term oral toxicity of cyanamide meets the DSD and CLP criteria.

According to the criteria in Dir. 67/548, based on the results of the short-term oral toxicity studies cyanamide has to be classified as "Toxic" and assigned the symbol "T" and the indication of danger "Danger of serious damage to health by prolonged exposure" accordingly. The following risk phrase should be assigned: "R48/25 Toxic: danger of serious damage to health by prolonged exposure if swallowed".

According to the criteria in Reg. 1272/2008, based on the results of the short-term oral toxicity studies cyanamide has to be classified as "specific target organ toxicity after repeated exposure, cat. 1". The following risk phrase should be assigned: "H372: Causes damage to the thyroid through prolonged or repeated exposure".

This proposal is not in agreement with the outcome of the EFSA peer-review, however, based on a detailed reconsideration and reanalysis of the available data and study results, the dossier submitter came to the proposal which was presented above.

4.9 Germ cell mutagenicity (Mutagenicity)

4.9.1 Non-human information

4.9.1.1 In vitro data

Gene mutation in bacterial cells

Title:	Jagannath, D.R. and Myhr, C. (1987): Mutagenicity test on hydrogen cyanamide in the Ames Salmonella/Microsome reverse mutation assay; Doc. No. 557-001; Hazelton Laboratories America, Inc., Kensington, Maryland, U.S.A; published: unpublished
Guidelines:	U.S. EPA FIFRA Guideline 84-2 (1982); complies to a great extent to EEC B 14
Deviations:	None
GLP:	Yes, U.S. EPA FIFRA Good Laboratory Practice
Acceptability:	The study is considered to be acceptable**.**

Materials and methods:

The unlabelled test substance was identified as

Hydrogen cyanamide

Batch: 02/10/86

Purity: 50 % w/w (equivalent to 53 % w/v)

Hydrogen cyanamide was tested for mutagenicity in the reverse mutation assay in bacteria (standard plate incorporation method) both, with and without metabolic activation (S9 mix from the liver of Aroclor 1254 induced male Sprague-Dawley rats). The *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538 were exposed to the test substance (dissolved in aqua bidest., 169 mg/mL cyanamide) at concentrations ranging from 0.1 to 15.0 µL/plate, corresponding to 20 to 2540 µg/plate with and without S9 mix. Two independent experiments with and without S9 mix (experiment I and II) were conducted with all strains used. The concentrations selected in the main experiments were obtained from the results of a pre-experiment performed with identical conditions with tester strain TA 100.

For control purposes solvent (aqua bidest.) and positive controls (without S9 mix: 10 µg/plate 2-nitrofluorene for strains TA98 and TA1538; 10 µg/plate sodium azide for strains TA100 and TA1535; 5 µg/plate quinacrine mustard for strain TA1537; with S9 mix: 2.5 µg/plate 2-amino-anthracene for strains TA98, TA100, TA1535, TA1537 and TA1538) were used concurrently.

Findings:

In the pre-experiment with TA100 without metabolic activation, bacteriotoxic effects evidenced by reduced background bacterial lawn and reduced revertant rate were observed at concentrations of 18.8 µL/plate and higher. In the main experiments slightly reduced revertant rates were observed in all indicator organisms at the highest investigated concentration (15 µL/plate, corresponding to 2540 µg/plate) without metabolic activation in both experiments.

No relevant increase in the number of histidine (his^+) revertants was observed in the bacterial strains TA98, TA100, TA 1535, TA1537 and TA1538 tested either with or without S9 mix. The sensitivity of the test system used was demonstrated by the induction of an increased number of revertants by the positive controls.

Conclusion:

Hydrogen cyanamide did not exhibit mutagenic effects in this bacterial reverse mutation assay in the presence and absence of S9 mix in all strains used.

Title:	Cadena, A. et al. (1984): Evaluation de posible mutagenicidad de la cianamida mediante las pruebas de ames y devoret; Doc. No. 592-007; Departemento de microbiologia – Laboratorios LASA, Sant Feliu de Llobregat (Barcelona), Espana; published: Boll. Chim. Farm. 123, 74-82
Guidelines:	Not indicated.

Deviations:	Limited compliance to EEC B 13 and B 14 • only one experiment was performed, instead of two • only two replicates were used per concentration, instead of three • the *E. coli* strains contain different genetic modifications compared to those described in the guideline • no positive control was used in *Salmonella* strain TA 1538 without S9 mix
GLP:	No, study is a publication in a scientific journal
Acceptability:	The study is considered to be supplementary**.**

Materials and methods:

The test substance was identified as

Cyanamide

Batch: not indicated

Purity: not indicated

Cyanamide was tested for mutagenicity in the reverse mutation assay in bacteria (pre-incubation method, 37 °C, 30 min) both, with and without metabolic activation (S9 mix from the liver of Aroclor 1254 induced male Sprague-Dawley rats). The *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538 and the *E. coli* strains GY 4015 and GY 5027 were exposed to the test substance (dissolved in aqua bidest.) at concentrations ranging from 10 to 5000 µg/mL with and without S9 mix.

For control purposes solvent (aqua bidest.) and positive controls (with and without S9 mix: N-methyl-N´-nitro-N-nitrosoguanidin for strain TA 1535; 9-aminoacridine for strain TA 1537; 2-aminofluorene for strain TA 1538 only with S9 mix; 4-nitroquinoline-N-oxide for strains TA 98 and TA 100 and mitomycin C in *E. coli* strains GY 4015 and GY 5027) were used concurrently.

Findings:

No bacteriotoxic effects were observed up to the highest investigated concentrations in all strains used with and without S9 mix.

No relevant increase in the number of revertants was observed in the *Salmonella* strains TA 98, TA 100, TA 1535, TA1537 and TA1538 and the *E. coli* strains GY 4015 and GY 5027 tested either with or without S9 mix. The sensitivity of the test system used was demonstrated by the induction of an increased number of revertants by the positive controls.

Conclusion:

Cyanamide did not exhibit mutagenic effects in this bacterial reverse mutation assay in the presence and absence of S9 mix in all strains used. However, this publication should be regarded as a supplementary study, due to the deviations mentioned above.

Gene mutation in mammalian cells

Title:	Wollny, H.E.; Arenz, M. (2000): Gene mutation assay in Chinese hamster V79 cells *in vitro* (V79/HPRT) with Cyanamide L 500, Doc. No. 557-004, RCC Cytotest Cell Research GmbH, Roßdorf, Germany, unpublished
Guidelines:	U.S. EPA FIFRA 40 CFR, OECD 476 (1997); EEC 87/301 (1987)
Deviations:	None
GLP:	Yes; OECD and Chemicals Act Germany
Acceptability:	The study is considered to be acceptable**.**

Materials and methods:

The test substance was identified as follows:

Cyanamide L 500

Batch: Tank 77/2 – Lab Registry No. 99/14492

Purity: 51.1 % (w/w), aqueous solution

The potential mutagenic effect of Cyanamide L 500 in mammalian cells was examined by assaying the induction of 6-thioguanine resistant mutants in Chinese hamster V79 cells. In a preliminary cytotoxicity assay (by means of a growth inhibition test), the test substance was applied at a maximum concentration of 1000.0 µg/mL (≈ 10 mM) and 8 lower concentrations. Thereafter, four independent main assays were conducted. The treatment period was 4 h in the first experiment with and without S9 mix and 24 h in the second experiment without S9 mix. A third experiment using 4 h treatment in the presence of S9 mix was required since the toxic concentration range was not reached. This experiment had to be repeated due to technical reasons (experiment four). Without S9 mix the concentration range was 31.3 to 1000.0 µg/mL in experiment 1 and 15.6 to 500.0 µg/mL in experiment 2. Concentrations from 1.25 to 30.0 µg/mL (experiment 1) and from 2.0 to 250.0 µg/mL (experiment 2) were investigated with metabolic activation. S9 mix was obtained from the liver of rats induced with phenobarbital and β-naphthoflavone. The test substance was dissolved in deionised water which was also used as solvent control, whereas the positive control substances ethylmethane sulfonate (EMS, not requiring activation) and 7,12-dimethylbenz(a)anthracene (DMBA, acting only after metabolic activation) were diluted in water and DMSO respectively. The effects of treatment were examined at expression time of 7 days.

Findings:

According to the results of the pre-experiment the top concentration in experiment I (4 h treatment interval) were selected to be 1000.0 µg/mL (without S9 mix) and 30 µg/mL (with S9 mix), respectively. Without metabolic activation moderate toxic effects, evidenced by a reduction of the cell density at the first subcultivation were observed at the maximum concentration. However, in the experiment with metabolic activation, in contrast to the pre-experiment no relevant toxic effects occurred up to the highest concentration. Therefore, this experiment had to be repeated.

The second experiment (24 h treatment interval) without S9 mix was carried out with 500 µg/mL as a top concentration. Strong toxic effects were obtained at 250.0 and 500.0 µg/mL. These effects were more distinct at the highest concentration where the cultures could not be continued.

The repeat experiment with metabolic activation was performed with 250 µg/mL as the maximum concentration. In this experiment strong toxic effects occurred at 62.5 µg/mL and above, with more distinct effects at 125 and 250 µg/mL where the cultures could not be continued.

A comparison of the mutation rates found in the groups treated with Cyanamide L 500 with the negative and solvent controls did not show any relevant increase of gene mutations. Cyanamide L 500 did not induce a reproducible concentration-related increase in mutant colony numbers. EMS (0.3 mg/mL) and DMBA (2.5 µg/mL) were used as positive controls and showed distinct increases in induced mutant colony number.

Conclusion:

In conclusion, it can be stated that in this mutagenicity assay and under the experimental conditions reported, Cyanamide L 500 did not induce gene mutations at the HPRT-locus in V79 cells.

Title:	Enninga, I.C. (1988): Evaluation of the mutagenic activity of aqueous hydrogen Cyanamide in an *in vitro* mammalian cell gene mutation test with L5178Y mouse lymphoma cells; Doc. No. 557-008; RCC Notox V., s-Hertogenbosch, The Netherlands; published: no
Guidelines:	OECD 476 (1984); EEC B 17
Deviations:	No statistical analysis
GLP:	Yes; OECD and U.S. EPA
Acceptability:	The study is considered to be acceptable**.**

Materials and methods:

The test substance was identified as follows:

Hydrogen cyanamide

Batch: 28/03/88

Purity: 49 % w/w ≈ 52 % w/v

The potential mutagenic effect of hydrogen cyanamide in mammalian cells was examined by assaying the induction of forward mutations at the thymidine kinase locus in L5178Y mouse lymphoma cells. In a preliminary cytotoxicity assay (by means of a growth inhibition test), the test substance was applied at a maximum concentration of 2600.0 µg/mL and 8 lower concentrations. Thereafter, two independent main assays were conducted both with 2 h treatment period. Without S9 mix the concentration range was 50 to 1600.0 µg/mL in experiment 1 and 50.0 to 1000.0 µg/mL in experiment 2. Concentrations from 5 to 35.0 µg/mL (experiment 1) and from 5.0 to 22.5 µg/mL

(experiment 2) were investigated with metabolic activation. S9 mix was obtained from the liver of Sprague Dawley rats induced with Aroclor 1254. The test substance was dissolved in culture medium which was also used as solvent control, whereas the positive control substances ethylmethane sulfonate (not requiring activation) and dimethylnitrosamine (acting only with activation) were diluted in culture medium. The cultures were subcultured at least every other day. The expression period for BrdU-resistant mutants was 3 days. After 13 days the formed microcolonies were counted. No distinction between small and large colonies as an indication for chromosomal and point mutations, respectively, was conducted.

Findings:

According to the results of the pre-experiment the top concentration in experiment 1 (2 h treatment interval) were selected to be 1600.0 µg/mL (without S9 mix) and 35 µg/mL (with S9 mix), respectively in experiment 1. Without metabolic activation distinct toxic effects, evidenced by a reduction of the cloning efficiency at day 0 (after treatment of the test substance) was observed at the maximum concentration. In the experiment with metabolic activation, in contrast to the pre-experiment, no relevant toxic effects occurred up to the highest concentration.

The second experiment (2 h treatment interval) without S9 mix was carried out with 1000 µg/mL as a top concentration. Strong toxic effects were obtained at the highest investigated concentration.

The second with metabolic activation was performed with three concentrations of the test substance with 22.5 µg/mL as the maximum concentration. In this experiment strong toxic effects occurred at 22.5 µg/mL where the cultures could not be continued. The two lower concentrations were in the range of the corresponding solvent control. The results of cytotoxicity are summarised in Table 96.

Table 96: Cytotoxic and mutagenic effects of aqueous hydrogen cyanamide in the mouse lymphoma L5178Y test system

Dose µg/mL	C.E. at day 0 (% of control)	C.E. at day 3 (absolute %)	Mean no. of mutants per plate	Mutation Frequency x 10^{-5}
Experiment 1 without S9 mix				
Solvent control	100	79	1.6	2.0
50	95	73	1.2	1.7
160	94	70	2.4	3.4
500	68	72	2.3	3.2
1600	25	67	3.2	4.8
2 mM EMS	78	72	8.1	11.3
Experiment 1 with S9 mix				
Solvent control	100	73	1.9	2.6
5	91	76	1.8	2.4
16	85	71	2.0	2.8
25	79	70	3.1	4.5
35	82	70	2.1	3.0
0.5 mM DMN	81	46	6.1	13.3
Experiment 2 without S9 mix				
Solvent control	100	62	1.5	2.4
50	114	58	2.1	3.7
160	93	74	1.5	2.0
500	58	63	2.9	4.6
1000	11	35	1.8	5.1
2 mM EMS	89	56	10.2	18.4
Experiment 2 with S9 mix				
Solvent control	100	64	2.3	3.6
5	94	61	2.3	3.8
15	90	63	1.7	2.7
22.5	1	-	-	-
0.5 mM DMN	81	53	10.3	19.4

- = not investigated
C.E. = Cloning Efficiency

A comparison of the mutation rates found in the groups treated with hydrogen cyanamide with the solvent controls showed a weak increase in the induction of gene mutations without metabolic activation at the highest investigated concentration. A mutation factor of 2.4 (experiment 1) and 2.1 (experiment 2), respectively was obtained. No increase in the mutation frequency was obtained with metabolic activation in both independent experiments. EMS (2 mM) and DMN (0.5 mM) were used as positive controls and showed distinct increases in induced mutant colony number. No historical control data were mentioned in this study. The results of mutagenicity are summarised in Table 96.

The effects at the maximum concentrations in experiment 1 and 2 without metabolic activation are slightly above the mutation factors of 2 (2.4 and 2.1, respectively). The maximum concentration of

1.600 µg/mL in the first experiment is above the level of 10 mM for hydrogen cyanamide (10 mM ≈ 1000.0 µg/mL). This level is a limit for mutagenicity tests carried out in cell cultures in order to avoid effects of osmolarity which can lead to false positive results. The maximum concentration in experiment 2 (1000 µg/mL) represents exactly the level of 10 mM. Due to this fact the obtained weak mutagenic effect at the upper concentrations in experiment 1 and 2 is questionable.

Conclusion:

In conclusion, aqueous hydrogen cyanamide was found to be weakly mutagenic without metabolic activation in this mutagenicity assay at concentrations equal or above the limit of 10 mM, where false positive results due to unfavourable culture conditions (e.g. high osmolarity) could not be excluded. Therefore, the obtained results are questionable.

Clastogenicity in mammalian cells

Title:	Ivett, J.L. (1987): Mutagenicity test on hydrogen cyanamide in an *in vitro* cytogenetic assay measuring chromosomal aberration frequencies in Chinese hamster ovary (CHO) cells; Doc. No. 557-010; Hazelton Laboratories America, Inc., Kensington, Maryland, USA; published: no
Guidelines:	U.S. EPA FIFRA Guideline 84-2 (1984); complies to a great extent to EEC B 14
Deviations:	None
GLP:	Yes, U.S. EPA
Acceptability:	The study is considered to be acceptable**.**

Materials and methods:

The test substance was identified as follows:

Hydrogen cyanamide

Batch: 02/10/86

Purity: 50 % w/w (equivalent to 53 % w/v)

Hydrogen cyanamide was assessed for its potential to induce structural chromosome aberrations in Chinese hamster ovary (CHO) cells *in vitro*. Hydrogen cyanamide was tested in the presence and absence of metabolic activation (S9 mix prepared from Aroclor 1254 induced male Sprague-Dawley rat liver). The test article was dissolved in culture medium. Duplicate cultures of CHO cells were exposed to the test substance for 20 hours at concentrations of 42.4, 56.5, 141, 283 and 424 µg/mL in the non-activation assay and for 2 hours at 438, 875, 1310 and 1750 µg/mL (20 hours preparation interval) and 321 and 428 µg/mL(10 hours preparation interval) in the presence of metabolic activation. Cells were harvested at 10 (with S9 mix) and 20 hours (with and without S9 mix), respectively after treatment had commenced. For the final 2.5 hours prior to harvest, cultures were exposed to colcemide. These test conditions had been selected on the basis of a preliminary range-finding test which was also described in the original report. Following harvest, cells were fixed on slides, stained and examined for chromosomal aberrations. 100 cells from each duplicate

culture were analysed, except at 283 µg/mL without and 1.310 µg/mL with metabolic activation at the 20 hour preparation interval where only 50 cells were investigated. Mitomycin C (80 ng/mL for the non-activation set) and cyclophosphamide (17.5 and 50 µg/mL, respectively requiring activation) served as positive control substances. An untreated negative control (culture medium) was also included in the testing.

Findings:

In the preliminary concentration finding test, CHO cells were exposed to culture medium and to various concentrations of hydrogen cyanamide ranging from 17.7 µg/mL to 5.300 µg/mL in the absence and in the presence of S9 mix. Without metabolic activation a significant cell cycle delay was obtained beginning 53.0 and 177 µg/mL. Complete toxicity was obtained above concentrations of 530.0 µg/mL without S9 mix. Therefore, a 20 hour preparation interval was selected for testing a dose range of 28.2 µg/mL to 565 µg/mL without metabolic activation.

In the presence of metabolic activation complete toxicity was observed at 1750 µg/mL. A cell cycle delay was obtained at 530 µg/mL. Therefore, two harvest times were selected for the aberration assay with a dose range of 437 to 1750 µg/mL at the 20 hour preparation interval and a dose range of 107 µg/mL to 428 µg/mL at 10 hour preparation interval.

In the main experiment without metabolic activation there was complete toxicity at 424 and 565 µg/mL and a reduction in observable mitotic cells was obtained at 283 µg/mL. 4 concentrations were analysed beginning at 42.4 up to 283 µg/mL. A significant increase was obtained at 141 and 283 µg/mL and a dose-response was established. With metabolic activation complete toxicity was obtained at 1.750 µg/mL and few mitotic cells were discernible at 1.310 µg/mL. Three concentrations (438, 875 and 1.310 µg/mL) were evaluated at the 20 hour preparation interval. In this experiment a significant dose-dependent increase in aberrant cells was obtained beginning at the lowest concentration. In the 10 hour preparation interval 2 concentrations 321 and 428 µg/mL were evaluated with no observable toxicity at the highest concentration. In this experiment a slight but not significant increase in cells with chromosomal aberrations was obtained at the highest investigated concentration. The results are summarised in Table 97.

Table 97: Chromosome aberrations in Chinese hamster ovary (CHO) cells

Test groups/Concentrations	Cells scored	% Cells with aberrations without gaps
Fixation interval 20 h after treatment without S9 mix		
Negative and Solvent control	200	2.0
Mitomycin C 80 ng/mL	25	28.0*
42.4	200	1.0
56.5	200	1.5
141.0	200	33.5*
283.0	50**	96.0*
424	-	-
Fixation interval 20 h after treatment with S9 mix		
Negative and Solvent control	200	1.0
Cyclophosphamide 17.5 µg/mL	25	36.0*
438.0	200	6.0*
875.0	200	42.0*
1310.0	50**	94.0*
1750.0	-	-
Fixation interval 10 h after treatment with S9 mix		
Negative and Solvent control	200	1.5
Cyclophosphamide 17.5 µg/mL	25	16.0*
321.0	200	1.5
428.0	200	5.0

* statistically significant by Fisher's Exact Test ($p < 0.01$)
** highly toxic effects
- no scorable metaphases

Positive control substances revealed statistically significant increases in chromosome aberration rate and demonstrated the sensitivity of the assay.

Conclusion:

Under the conditions of the assay described in this report, Hydrogen cyanamide induced an increase in structural chromosome aberrations in CHO cells with nonactivation and metabolic activation and should be considered as clastogenic in this test system.

Title: Enninga, I.C. and van de Waart, E.J. (1988): Evaluation of the ability of aqueous hydrogen cyanamide to induce chromosome aberrations in cultured peripheral human lymphocytes; Doc. No. 557-007; RCC Notox, s-Hertogenbosch, The Netherlands; published: No.

Guidelines: OECD 473 (1983), EEC 84/449 (1984)

Deviations: None

GLP: Yes; a GLP statement is included in the study

Acceptability: The study is considered to be acceptable.

Materials and methods:

The test substance was identified as follows:

Hydrogen cyanamide

Batch: 28/03/88

Purity: 49 % w/w (52 % w/v) aqueous solution

Hydrogen cyanamide was assessed for its potential to induce structural chromosome aberrations in cultured human lymphocytes *in vitro*. Hydrogen cyanamide was tested in the presence and absence of metabolic activation (S9 mix prepared from Aroclor 1254 induced male Sprague-Dawley rat liver). The test article was diluted in culture medium. Duplicate cultures of human lymphocytes cells were exposed to the test substance for 24 hours at concentrations of 1.0, 3.3, 10.0, 33.3, 100.0, 333.0, 1000.0, 3330 and 5000 µg/mL in the non-activation assay and for 2 hours at concentrations of 0.1, 0.3, 1.0, 3.3, 10.0, 33.3, 100.0, 333.0, 1000.0, 3330.0 and 5000 µg/mL culture medium in the presence of metabolic activation. For both assays, cells were harvested at 24 hours after treatment had commenced. For the final 3 hours prior to harvest, cultures were exposed to colcemide. An appropriate range and number of dose levels was chosen, instead of a preliminary range-finding test. Following harvest, cells were fixed on slides, stained and examined for chromosomal aberrations. 100 cells from each duplicate culture were analysed. Mitomycin C (0.1 µg/mL for the non-activation assay) and cyclophosphamide (15 µg/mL requiring activation) served as positive control substances. An untreated solvent control (culture medium) was also included in the testing.

Findings:

Since no preliminary range-finding test was performed a variety of concentrations in an appropriate range were chosen. In the absence of S9 mix the mitotic index was reduced by 46 % at 33.3 µg/mL and in the presence of S9 mix at 333.3 µg/mL by 61 %. Based on these observations the following doses were selected for the scoring of chromosome aberrations: 3.3, 10.0 and 33.3 µg/mL without S9 mix and 10.0, 33.3, 100.0 and 333.0 µg/mL in the presence of S9 mix.

A statistically significant increase in numerical chromosomal aberrations (exclusive gaps) was observed in the absence and in the presence of S9 mix at the highest concentration (33.3 µg/mL) without S9 mix and at 33.3 and 333.3 µg/mL with metabolic activation. The results of both experiments are summarised in Table 98and in Table 99.

Table 98: Cells with aberration with metabolic activation

Conc. µg/mL	**0**	**10.0**	**33.3**	**100.0**	**333.0**
Number of cells with aberrations (inclusive gaps) in 200 cells	2	8*	7*	12**	25***
Number of cells with aberrations (exclusive gaps) in 200 cells	2	6	7*	6	22***

* Significantly different from control by the Chi-square test criteria, $p < 0.05$
** Significantly different from control by the Chi-square test criteria, $p < 0.01$
*** Significantly different from control by the Chi-square test criteria, $p < 0.001$

Table 99: Cells with aberration without metabolic activation

Conc. µg/mL	0	3.3	10.0	33.3
Number of cells with aberrations (inclusive gaps) in 200 cells	9	6	13	20*
Number of cells with aberrations (exclusive gaps) in 200 cells	6	6	8	19**

* Significantly different from control by the Chi-square test criteria, $p < 0.05$
** Significantly different from control by the Chi-square test criteria, $p < 0.01$
*** Significantly different from control by the Chi-square test criteria, $p < 0.001$

The positive control substances revealed statistically significant increases in chromosome aberration rate and demonstrated the sensitivity of the assay.

Conclusion:

Under the conditions of the assay described in this report, hydrogen cyanamide induced a significant increase in structural chromosome aberrations with nonactivation and metabolic activation in human lymphocytes *in vitro* and should be considered as clastogenic in this test system.

DNA damage and repair

Title: de Raat, W.K. (1979): An investigation into the sister chromatid exchange induction in Chinese hamster ovary cells by a sample of "Calcium Cyanamide"; Doc. No. 557-005; TNO, Delft, The Netherlands; published: no

Guidelines: Not indicated, to a limited extend to EEC B 19.

Deviations: One culture per concentration instead of at least duplicate cultures only 20 metaphases were evaluated per culture, instead of 50.

GLP: No, study was performed prior to the implementation of GLP

Acceptability: The study is considered to be supplementary**.**

Materials and methods:

The test substance was identified as follows:

Calcium cyanamide

Batch: Not indicated

Purity: 23 %

Kalkstickstoff is a nitrogen fertiliser based on calcium cyanamide. Upon contact with water, the calcium cyanamide of Kalkstickstoff is quantitatively hydrolysed to cyanamide. Therefore, studies carried out with Kalkstickstoff can be used for assessing the risks of cyanamide. Calcium cyanamide was assessed for its potential to induce sister chromatid exchange in Chinese hamster

ovary (CHO) cells *in vitro*. Calcium cyanamide was tested in the presence and absence of metabolic activation (S9 mix prepared from Aroclor 1254 induced male Wistar rat liver). The test article was dissolved in DMSO. One culture of CHO cells was exposed to the test substance for 1 hour at concentrations of 10, 50, 250 and 500 µg/mL in the non-activation assay and in the presence of metabolic activation. Soon after fresh medium containing 5-bromodeoxyuridine (BrdU) was added. For both assays, cells were harvested 24 h after treatment had commenced. For the final 3 hours prior to harvest, cultures were exposed to colcemide. These test conditions had been selected on the basis of a preliminary range-finding test which was also described in the original report. Following harvest, cells were fixed on slides, stained with Hoechst 33258 flurorochrome and Giemsa and examined for sister chromatid exchange. 20 cells from one culture were analysed. Trenimon® (1.25 ng/mL for the non-activation set) and cyclophosphamide (5 µg/mL requiring activation) served as positive control substances. A solvent control (DMSO) was also included in the testing.

Findings:

In the preliminary concentration finding test (cloning efficiency) at concentrations up to 100 µg/mL the test substance did not influence the survival of the cells. Based upon these findings, concentrations of 10, 50, 250 and 500 µg/mL in the absence and presence of S9 mix were selected for further study in the main experiment.

No statistically significant increase in sister chromatid exchanges were observed neither in the absence nor the presence of S9 mix at any of the concentrations. Positive control substances revealed statistically significant increases in chromosome aberration rate and demonstrated the sensitivity of the assay.

Conclusion:

Under the conditions of the assay described in this report, calcium cyanamide did not induce an increase in sister chromatid exchanges in CHO cells and should be considered negative in this test system. However, the study should be regarded as supplementary, due to the deviation concerning guideline requirements.

Title:	Cifone, M.A. (1987): Mutagenicity test on hydrogen cyanamide in the rat primary hepatocyte unscheduled DNA synthesis assay; Doc. No. 557-002; Hazelton Laboratories America Inc., Kensington, Maryland, U.S.A; published: unpublished
Guidelines:	U.S. EPA FIFRA 84-2 (1982); complies to a great extent to EEC B 18.
Deviations:	None
GLP:	Yes; a GLP statement is included.
Acceptability:	The study is considered to be acceptable.

Materials and methods:

The test substance was identified as follows:

Hydrogen cyanamide

Batch: 02/10/86

Purity: 50 % w/w (equivalent to 53 % w/v)

The potential of hydrogen cyanamide to cause DNA damage was assessed by measuring unscheduled DNA synthesis (UDS) in rat primary hepatocytes *in vitro*. Five cultures, each containing the rat hepatocytes and ^{3}H-thymidine were exposed for 18 to 19 hours to hydrogen cyanamide dissolved in deionised water. Three of the cultures were used for the UDS assay and the other two cultures per concentration were used to assess cytotoxicity. Concentrations used for UDS analysis ranged from 5.95 µg/mL to 190 µg/mL of hydrogen cyanamide. Deionised water was used as the solvent control. 2-Acetyl aminfluorene (2-AAF) at 0.05 and 0.10 µg/mL, was used as the positive control. Following the exposure period, the cells were washed and mounted on cover slips, coated and stored for 10 days at 4 °C in light tight boxes with desiccant. Slides were then developed, fixed and stained in haemotoxylin-sodium acetate-eosin stain. Nuclear grains were counted in 50 cells in random areas on each of three cover slips per concentration. The net nuclear counts were determined by counting 1 nucleus-sized areas adjacent to each nucleus and subtracting the average cytoplasmic count from the nuclear count.

Findings:

The concentration range in the UDS assay was selected according to the results of a preliminary cytotoxicity assays in which cytotoxicity was observed at concentrations higher than 53.0 µg/mL. According to this result 5.95, 11.9, 23.8, 47.6, 71.4, 95.2, 143 and 190 µg/mL were selected for the main assay. In this assay the highest concentrations of hydrogen cyanamide (190 µg/mL) was highly toxic (two slides could not be analysed) while the remaining seven doses were slightly toxic or not toxic (relative survival 100.8 to 53.3 %). Hydrogen cyanamide did not significantly increase net nuclear grain counts at any of the treatment concentrations and no concentration response was observed. Both concentrations of the positive control compound, 2-AAF, induced significant increases in the average net nuclear count of silver grains indicating the sensitivity of the test system.

Conclusion:

The results of the UDS assay (rat primary hepatocytes) indicate that under the test conditions hydrogen cyanamide did not induce an UDS response at any dose level. Therefore, hydrogen cyanamide is considered negative in this study.

4.9.1.2 In vivo data

In vivo cytogenetic assay

Title: Willems, M.I. (1979): Evaluation of "Kalkstickstoff" and "Thioharnstoff" in the micronucleus test; Doc. No. 557-006; Central Institute for nutrition and food research, Zeist, The Netherlands; published: no

Guidelines: Not mentioned, however study was performed to a great extent to OECD 476 (1983) and EEC B12.

Deviations: None

GLP: No; study was conducted before the implementation of GLP.

Acceptability: The study is considered to be acceptable.

Materials and methods:

The test substance was identified as follows:

Calcium cyanamide

Batch: Not indicated

Purity: 23 %

Kalkstickstoff is a nitrogen fertiliser based on calcium cyanamide. Upon contact with water, the calcium cyanamide of Kalkstickstoff is quantitatively hydrolysed to cyanamide. Therefore, studies carried out with Kalkstickstoff can be used for assessing the risks of cyanamide. The ability of calcium cyanamide to cause chromosomal damage *in vivo* was investigated in the mouse micronucleus assay. Male and female Wistar rats were dosed orally by gavage with calcium cyanamide. The dose level used in the study was selected in the light of the acute oral LD_{50} of the test materials for rats (765 mg/kg bw). Only one concentration (153 mg/kg bw) was tested in the micronucleus test. The vehicle control (water) as well as the positive control Trenimon were also tested.

Five mice/sex were exposed twice with the test substance in an interval of 24. Six hours after the last treatment all animals were sacrificed. Only one sampling time was performed.

Bone marrow smear slides were prepared and stained. Approximately 2000 erythrocytes were examined microscopically for the presence of micronuclei. The ratio of poly- (PCE´s) to normochromatic erythrocytes (NCE´s) was determined to assess inhibition of erythropoesis.

Findings:

No moralities or abnormalities of condition or behaviour, attributable to treatment were observed in any of the animals during the exposure period.

No significant changes in the ratio of NCE´s to PCE´s were observed. The test substance, calcium cyanamide, induced no significant increases in micronucleated polychromatic erythrocytes over the levels observed in the negative controls in either sex. The positive control, Trenimon, induced significant increases in micronucleated PCEs in both sexes.

Conclusion:

Calcium cyanamide did not induce micronuclei in polychromatic erythrocytes of mice when orally (by gavage) treated up to 153 mg/kg body weight.

Title: Ivett, J.L. (1987): Mutagenicity test on hydrogen cyanamide in the *in vivo* mouse micronucleus assay; Doc. No. 557-003; Hazelton Laboratories America Inc., Kensington, Maryland, U.S.A; unpublished

Guidelines:	U.S. EPA FIFRA Guidelines 84-2; performed to a great extent to EEC B12.
Deviations:	None
GLP:	Yes; a GLP statement is included.
Acceptability:	The study is considered to be acceptable.

Materials and methods:

The test substance was identified as follows:

Hydrogen cyanamide in aqueous solution

Batch: 7/07/87

Purity: 53 % w/v

The ability of hydrogen cyanamide to cause chromosomal damage *in vivo* was investigated in the mouse micronucleus assay. Male and female ICR mice were dosed orally by gavage with a aqueous solution of hydrogen cyanamide. A dose rangefinding study was carried out to assess toxicity with dose levels of 75, 150, 300, 450 and 600 mg/kg bw. Based upon the results of this trial the dose levels selected for the micronucleus assay were 35, 175 and 350 mg/kg bw. The vehicle control as well as the positive control triethylenemelamine was also tested.

Five mice/sex were exposed for 24, 48 and 72 h at all dose levels. Only one sampling time, 24 hours after treatment, was performed with the negative and the positive control. A second group of animals was also assigned to the study and was dosed with the high dose of the test article. These animals were only used in the assay as replacements for any which died in the primary dose group.

Bone marrow smear slides were prepared and stained. Approximately 1000 polychromatic erythrocytes (PCE's) were examined for the presence of micronuclei. The ratio of poly- to normochromatic erythrocytes was determined to assess inhibition of erythropoesis and all animals were examined after dosing and periodically throughout the duration of the study for toxic effects and/or mortalities.

Findings:

The stability of the test substance throughout the study period was shown by reanalysis. The homogeneity was guaranteed by mixing before preparations of the test solutions. The stability of the test substance in water was verified analytically and the following concentrations were determined: 31.44, 157.4 and 330.5 mg/kg.

At the high dose group 3 male animals were found dead after 5 and 20 h, respectively. All males at this dose level had ruffled coats throughout the duration of the study. All other animals were apparently healthy until the appropriate sacrifice time. No significant changes in the ration of NCEs to PCEs were observed. The test substance, hydrogen cyanamide, induced no significant increases in micronucleated polychromatic erythrocytes over the levels observed in the negative controls in either sex or at any sampling interval. The positive control, triethylenmelamine, induced significant increases in micronucleated PCEs in both sexes.

Conclusion:

Hydrogen cyanamide did not induce micronuclei in polychromatic erythrocytes of mice when orally (by gavage) treated up to 350 mg/kg body weight.

Title:	Menargues et al. (1984): An evaluation of the mutagenic potential of cyanamide using the micronucleus test; Doc. No. 592-008; Department of Pharmacology and Toxicology, S.A. LASA Laboratories, Barcelona, Spain; published: Mutation Research, 136, 127-129
Guidelines:	Not indicated, however the method used complies to a great extent to OECD 474 (1983) and EEC B12.
Deviations:	None
GLP:	No, study is a publication in a scientific journal.
Acceptability:	The study is considered to be supplementary.

Materials and methods:

The test substance was identified as follows:

Cyanamide (Colme), oral solution

Batch: S-13

Purity: Not indicated

The ability of cyanamide (Colme) to cause chromosomal damage *in vivo* was investigated in the mouse micronucleus assay. Male and female Swiss mice were dosed orally by gavage with an aqueous solution of cyanamide (Colme). A dose rangefinding study was carried out and a LD_{50} value of 494 mg/kg was obtained. According to the results the doses of cyanamide (Colme) were selected representing ½ of the LD_{50} (247 mg/kg bw), 1/10 of the LD_{50} (49 mg/kg bw) and 1/50 (10 mg/kg bw). The vehicle control (distilled water) as well as the positive control cyclophosphamide were also tested.

Six mice/sex (treatment groups and positive control) and ten mice/sex (negative control) were exposed for 48 hours at all dose levels. Bone marrow smear slides were made and stained. 16 slides from 8 males and 8 females of the negative control group, and 10 slides from 5 males and 5 females for treatment and positive control group were chosen. 2000 erythrocytes, which included 1000 polychromatic and 1000 normochromatic cells were screened per slide for the presence of micronuclei. The ratio of poly- to normochromatic erythrocytes was determined to assess inhibition of erythropoiesis and all animals were examined after dosing and periodically throughout the duration of the study for toxic effects and/or mortalities.

Findings:

The stability of the test substance in distilled water was not investigated and the concentrations were not analytically verified. However, it was proven by Ivett (1987, Doc. No. 557-003, Annex IIA, 5.4/10) that cyanamide was stable in aqueous solutions.

The NCE/PCE ratios showed no significant changes, except for the highest dose of cyanamide (Colme; 247 mg/kg) which induced a significant decrease in the ratio indicating a haemolytic effect of the drug.

However, the test substance, cyanamide (Colme), induced no significant increases in micronucleated polychromatic and normochromatic erythrocytes over the levels observed in the negative controls. The positive control cyclophosphamide induced significant increases in micronucleated PCEs in both sexes.

Conclusion:

Cyanamide (Colme) did not induce micronuclei in polychromatic erythrocytes of mice when orally (gavage) treated up to 247 mg/kg body weight.

DNA damage and repair *in vivo*

Title:	Heidemann, A. (2003): Cyanamide - Summary and evaluation of mutagenicity testing; Doc. No. 581-008; SCC GmbH, Mikroforum Ring 1, 55234 Wendelsheim, Germany; unpublished
Guidelines:	n. a.: reference is an expert statement
GLP:	n. a.: reference is an expert statement

Executive summary of the expert statement: "It can be summarised that hydrogen cyanamide did not induce point mutations in bacterial and mammalian cells at relevant test concentrations. Furthermore, there was no indication that cyanamide can cause DNA damage and repair in primary rat hepatocytes *in vitro*. The weak positive result found in the mouse lymphoma test with LY5178 cells represents a clastogenic rather than a gene mutation effect. This is evidenced by negative results in the gene mutation HPRT test and in the positive results obtained in two *in vitro* studies assaying chromosomal aberrations. Most importantly, no evidence for a clastogenic potential was found in three *in vivo* genotoxicity studies (rat and mouse micronucleus tests) which demonstrated by showing toxic symptoms or changes in the NCE/PCE ratio, that cyanamide has reached the bone marrow cells. The rapid distribution of cyanamide within nearly all mammalian organs including bones is also confirmed by respective ADME studies.

From the described results and the available data base, an unscheduled DNA synthesis *in vivo* is not required as it has been shown that cyanamide did not induce gene mutations (neither in bacterial nor in mammalian cells) *in vitro*. Additionally, an *in vitro* UDS in primary rat hepatocytes revealed no genotoxic potential of the active substance.

In vivo studies in germ cells

The results of the *in vivo* studies in somatic cells demonstrated that cyanamide has no mutagenic or genotoxic potential for mammals. Therefore, there was no necessity to evaluate cyanamide in an *in vivo* study using germ cells.

4.9.2 Human information

No data submitted by the notifier.

4.9.3 Other relevant information

No data submitted by the notifier.

4.9.4 Summary and discussion of mutagenicity

The results of mutagenicity studies are summarised in Table 100. Cyanamide was evaluated for its mutagenic and genotoxic potential *in vitro* and *in vivo*.

Both Ames-Tests were negative indicating no mutagenic activity in bacterial cells. Two gene mutation assays using mammalian cells were carried out one using mouse lymphoma cells and the other V79-cells.

A weak positive result was obtained in the mouse lymphoma test at the thymidine kinase locus at the maximum concentrations (1600 and 1000 µg/mL, respectively), in experiment 1 and 2 without metabolic activation. However, the biological relevance of the observed slight increases is questionable as they were obtained at concentration above and at the level of 10 mM, the limit for mutagenicity tests carried out in cell cultures in order to avoid false positive results due to effects of osmolarity. Therefore the weak mutagenicity in this assay is questionable. Furthermore, no mutagenic effects were obtained at the HPRT-locus in V79 with the maximum concentrations of 1000 and 500 µg/mL without S9 mix and 30 and 250 µg/mL with S9 mix.

Two chromosome aberrations tests in CHO cells and in human lymphocytes showed distinct clastogenic effects of cyanamide. In the assay with CHO cells a significant dose-dependent increase in cells with aberrations was obtained with and without metabolic activation at the 20 hour preparation interval. A statistically significant increase in numerical chromosomal aberrations (exclusive gaps) was obtained at the maximum concentrations (33.3 µg/mL) without S9 mix and at 33.3 and 333.3 µg/mL in the presence of S9 mix in human lymphocytes. Both assays indicate a clastogenic response of cyanamide *in vitro*.

Three micronucleus assays were carried out in order to investigate the potential of cyanamide to cause chromosomal damage *in vivo*. One assay was performed with male and female Wistar rats, the remaining two with male and female ICR mice and Swiss mice, respectively. In the micronucleus assay carried out in the rat only one dose level of calcium cyanamide (153 mg/kg bw) administered via gavage was investigated. The dose was administered twice in an interval of 24 h. No increase in micronuclei was obtained. In the two assays carried out in mice the test substance (hydrogen cyanamide and cyanamide Colme, respectively) was administered via gavage and no induction of micronuclei was obtained up to the highest investigated dose (350 and 247 mg/kg bw, respectively).

DNA damage and repair was investigated *in vitro* in CHO cells (Sister Chromatid Exchange) and in the UDS test in primary rat hepatocytes. In both experiments there was no indication of DNA damage and repair caused by cyanamide. An *in vivo* UDS test was not considered necessary to perform as the weak positive and questionable response in the mouse lymphoma gene mutation test was attributed to clastogenic effects and not to point mutations. This assumption is supported by the negative result of the HPRT-Test in V79 cells.

The studies cover or exceed all endpoints required for mutagenicity and genotoxicity testing. It is concluded, that cyanamide has a clastogenic potential *in vitro*. However, the clastogenic effects observed *in vitro* could not be detected *in vivo*. Therefore, cyanamide is considered to be not clastogenic *in vivo*.

Table 100: Summary table of relevant in vitro and in vivo mutagenicity studies

Study type	Cells / species	Test conditions	Results	Reference
Ames test	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	+ S9: 20 - 2540 µg/plate - S9: 20 - 2540 µg/plate experiment 1 and 2	Negative	Jagannath and Myhr, C., 1987 Doc-No 557-001 TOX2004-369
Ames test	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538 *E. coli* strains GY 4015 and GY 5027	+ S9: 10 - 5000 µg/plate - S9: 20 - 5000 µg/plate	Negative	Cadena et al., 1984 Doc-No 592-007 TOX2004-370
HPRT test	Chinese hamster V79 cells	+ S9: 1.25 - 30.0 µg/plate - S9: 31.3 - 1000 µg/plate Experiment 1 + S9: 2.0 - 250 µg/mL S9: 15.6 - 500 µg/mL Experiment 2	Negative	Wollny and Arenz, 2000 Doc-No 550-001 TOX2001-428
Point mutation test at the Thymidin kinase locus	Mouse lymphoma cells L5178Y	+ S9: 5 - 35 µg/plate - S9: 50 - 1600 µg/plate Experiment 1 + S9: 5.0 – 22.5 µg/mL - S9: 50 – 1000 µg/mL Experiment 2	Negative below the regulatory limit concentration of 10 mM	Enninga, 1988 Doc-No 557-008 TOX2001-429
in vitro cytogenetic cells	Chinese hamster ovary cells (CHO)	+ S9: 438 - 1310 µg/mL 20 h preparation interval + S9: 321 and 428 µg/mL 10 h preparation interval - S9: 42.4 - 283 µg/mL 20 h preparation interval	Positive	Ivett, 1987 Doc-No 557-010 TOX2001-430
in vitro cytogenetic cells	Human lymphocytes	+ S9: 10 - 333 µg/mL - S9: 3.3 - 33.3 µg/mL	Positive	Enninga and van de Waart, 1988 Doc-No 557-007 TOX2001-431
in vitro sister chromatid exchange	CHO cells	+ S9: 10 - 500 µg/mL - S9: 10 - 500 µg/mL	Negative	De Raat, 1978 Doc-No 557-005 TOX2001-432
in vitro UDS test	primary rat hepatocytes	5.95 - 190 µg/mL	Negative	Cifone, 1987 Doc-No 557-002 TOX2004-375
in vivo micronucleus test	Wistar rats	153 mg/kg bw only one concentration test substance administered twice	Negative	Willems, 1979 Doc-No 557-006 TOX2001-433
in vivo micronucleus test	ICR mice	35 - 350 mg/kg bw treatment intervals 24, 48 and 72 hours	Negative	Ivett, 1987 Doc-No 557-003 TOX2004-378
in vivo micronucleus test	ICR mice	10 - 247 mg/kg bw treatment 48 hours	Negative	Menargues et al., 1984 Doc-No 592-008 TOX2004-382

4.9.5 Comparison with criteria

Following criteria for classification for gem cell mutagens are given in DSD and CLP regulation:

DSD	CLP regulation
Category 1 To place a substance in Category 1, positive evidence from human mutation epidemiology studies will be needed. Examples of such substances are not known to date. It is recognised that it is extremely difficult to obtain reliable information from studies on the incidence of mutations in human populations, or on possible increases in their frequencies. Category 2 To place a substance in Category 2, positive results are needed from assays showing (a) mutagenic effects, or (b) other cellular interactions relevant to mutagenicity, in germ cells of mammals in vivo, or (c) mutagenic effects in somatic cells of mammals in vivo in combination with clear evidence that the substance or a relevant metabolite reaches the germ cells. With respect to placement in Category 2, at present the following methods are appropriate: 2 (a) in vivo germ cell mutagenicity assays: - specific locus mutation test, - heritable translocation test, - dominant lethal mutation test. These assays actually demonstrate the appearance of affected progeny or a defect in the developing embryo. 2 (b) in vivo assays showing relevant interaction with germ cells (usually DNA): - assays for chromosomal abnormalities, as detected by cytogenetic analysis, including aneuploidy, caused by malsegregation of chromosomes, - test for sister chromatid exchanges (SCEs), - test for unscheduled DNA synthesis (UDS), - assay of (covalent) binding of mutagen to germ cell DNA, - assaying other kinds of DNA damage. These assays provide evidence of a more or less indirect nature. Positive results in these assays would normally be supported by positive results from in vivo somatic cell mutagenicity assays, in mammals or in mn (see under Category 3, preferably methods as under 3 (a)). 2 (c) in vivo assays showing mutagenic effects in somatic cells of mammals (see under 3 (a)), in combination with	The classification in **Category 1A** is based on positive evidence from human epidemiological studies. Substances to be regarded as if they induce heritable mutations in the germ cells of humans. The classification in **Category 1B** is based on: — positive result(s) from in vivo heritable germ cell mutagenicity tests in mammals; or — positive result(s) 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. It is possible to derive this supporting evidence from mutagenicity/genotoxicity tests in germ cells in vivo, or by demonstrating the ability of the substance or its metabolite(s) to interact with the genetic material of germ cells; or — positive results from tests showing mutagenic effects in the germ cells of humans, without demonstration of transmission to progeny; for example, an increase in the frequency of aneuploidy in sperm cells of exposed people. 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 cell genotoxicity tests which are supported by positive results from in vitro mutagenicity assays. Note: Substances which are positive in in vitro mammalian mutagenicity assays, and which also show chemical structure activity relationship to known germ cell mutagens, shall be considered for classification as Category 2 mutagens.

toxicokinetic methods, or other methodologies capable of demonstrating that the compound or a relevant metabolite reaches the germ cells. For 2 (b) and 2 (c), positive results from host-mediated assays or the demonstration of unequivocal effects in in vitro assays can be considered as supporting evidence. Category 3 To place a substance in Category 3, positive results are needed in assays showing (a) mutagenic effects or (b) other cellular interaction relevant to mutagenicity, in somatic cells in mammals in vivo. The latter especially would normally be supported by positive results from in vitro mutagenicity assays. For effects in somatic cells in vivo at present the following methods are appropriate: 3 (a) in vivo somatic cell mutagenicity assays: - bone marrow micronucleus test or metaphase analysis, - metaphase analysis of peripheral lymphocytes, - mouse coat colour spot test. 3 (b) in vivo somatic cell DNA interaction assays: - test for SCEs in somatic cells, - test for UDS in somatic cells, - assay for the (covalent) binding of mutagen to somatic cell DNA, - assay for DNA damage, e.g. by alkaline elution, in somatic cells. Substances showing positive results only in one or more in vitro mutagenicity assays should normally not be classified. Their further investigation using in vivo assays, however, is strongly indicated. In exceptional cases, e.g. for a substance showing pronounced responses in several in vitro assays, for which no relevant in vivo data are available, and which shows resemblance to known mutagens/carcinogens, classification in Category 3 could be considered.	

Comparison with criteria in DSD: No human data are available for cyanamide, hence a classification in category 1 is not possible. Neither in vivo heritable germ cell mutagenicity tests nor positive results from in vivo somatic cell mutagenicity tests in mammals are available, hence a classification in 2 is not possible. Despite the positive in vitro results (mutagenicity, clastogenicity), the respective in vivo studies showed a negative outcome, hence a classification in category 3 is considered not necessary. The criteria state: "Substances showing positive results only in one or more in vitro mutagenicity assays should normally not be classified."

Comparision with criteria in CLP regulation: No human data are available for cyanamide, hence a classification in category 1A is not possible. Neither vivo heritable germ cell mutagenicity tests nor

positive results from in vivo somatic cell mutagenicity tests in mammals are available, hence a classification in 1B is not possible. Despite the positive in vitro results (mutagenicity, clastogenicity), the respective in vivo studies showed a negative outcome, hence a classification in category 2 is considered not necessary.

4.9.6 Conclusions on classification and labelling

Based on the available studies and the comparison with the criteria a classification as germ cell mutagen is considered not necessary.

This proposal is in agreement with the outcome of the EFSA peer-review.

4.10 Carcinogenicity

4.10.1 Non-human information

4.10.1.1 Carcinogenicity: oral

Long-term toxicity in rats

Title:	Osheroff, M.R. (1991): Chronic toxicity Study in rats with aqueous hydrogen cyanamide; Doc. No. 537-001; Hazelton Laboratories America, Inc.; published: No.
Guidelines:	U.S. EPA 83-1 (1982); complies to a great extent to EEC B 30
Deviations:	None
GLP:	Yes; U.S. EPA
Acceptability:	The study is considered to be acceptable**.**

Materials and methods:

The test substance was identified as follows: Aqueous hydrogen cyanamide; Batch: 07-07-87; Purity: 50 % w/w, 53 % w/v active substance.

Aqueous hydrogen cyanamide was administered via gavage to 80 male and 80 female rats (Sprague Dawley, Crl:CD©BR) in this chronic toxicity study. The test substance was administered to three groups each containing 20 rats/sex/group. An additional group of 20 rats/sex received the vehicle (distilled water) and thus served as the concurrent control group. The dose levels were 0, 5, 15 and 60 mg/kg bw/day corresponding to 0, 2.5, 7.5 and 30 mg/kg bw/day active substance hydrogen cyanamide for the first 16 weeks. Due to the general health status of the animals the dose levels were adjusted beginning in week 17 to 0, 2, 5 and 15 mg/kg bw/day corresponding to 0, 1, 2.5 and 7.5 mg/kg bw/day active substance hydrogen cyanamide for Groups 1, 2, 3 and 4. The animals were killed after a 91 week administration period.

All animals were observed twice daily for mortality and moribundity and once daily for clinical signs. Individual physical examinations were recorded prior to treatment. Individual body weights were recorded prior to treatment, weekly for weeks 0 - 24, and every fourth week thereafter. Food

consumption measurements were recorded weekly for weeks 1 - 16 and every fourth week thereafter. Ophthalmoscopic examinations were performed pre-treatment and at termination using an indirect ophthalmoscope. Evaluation of clinical haematology, serum chemistry (including T3, T4 and TSH) and urinalysis were performed at weeks 14, 27, 52, 79 (except serum chemistry) and at termination on 10 rats/sex/group (adjusted for survival at termination). After 92 weeks of compound administration all remaining rats were sacrificed and subjected to complete gross examination. Organ weight evaluations and histomorphological examination were performed on selected organs.

Findings:

General observations:

Results of analytical chemistry analysis indicated that the test solutions were generally prepared within the acceptable range of target concentration. Stability evaluation of the low and high-dose solutions were established and showed stability over the entire range.

Adjusted percent survival among the male dosage groups was 47 %, 65 %, 70 % and 74 % for Groups 1, 2, 3 and 4, respectively. Adjusted percent survival in the females was 80 %, 65 %, 44 % and 88 % for Groups 1, 2, 3 and 4, respectively. In general, there was no pattern to the causes of death noted during this study and no cause of death could be attributed to treatment. The cause of death and/or main histopathological finding were of the nature typically noted at this laboratory in this age/strain of rat.

Treatment related clinical signs were most frequently observed in animals at the high dose group within the first 16 weeks of administration such as hunched posture, tremors and rough hair coat. Due to these observations, the dose levels have been reduced after 16 weeks of treatment. The occurrence of tremors and rough hair coat subsided a few weeks after the dose levels were lowered, whereas all other observed clinical signs were not considered treatment related since they were observed infrequently throughout the dose groups.

Statistical evaluation of mean body weight data revealed a significant depression in the absolute body weight value of the mid and high dose group in males and females at Weeks 4 and 16, the mid dose group males and high dose group males and females at Week 13, and the high dose group males and females at Weeks 52 and 91 (results see Table 101). Mean body weight gain values were significantly depressed for the mid dose group males at Weeks 0 - 13 and 0 - 16; high dose group males for Weeks 0 - 13, 0 - 16 and 0 - 52; mid dose group females for Weeks 0 - 16; and the high dose group females for Weeks 0 - 13, 0 - 16 and 0 - 52. The mean body weight gain value for the high dose group females was significantly increased for the interval covering Weeks 16 - 52 as compared to the control group female mean value. All results are summarised in Table 101 and Table 102.

Table 101: Chronic toxicity study in rats: body weights

	0 mg/kg bw/day	1 mg/kg bw/day**	2.5 mg/kg bw/day**	7.5 mg/kg bw/day**
Males:				
Start	231.1	226.7	226.7	226.2
Week 4	418.6	411.8	389.6*	314.3*
Week 13	598.0	575.6	509.5*	349.2*
Week 16	620.8	599.7	522.8*	345.9*
Week 52	770.2	767.0	707.4	539.6*
Week 91	787.4	760.1	747.6	534.4*
Females				
Start	176.2	174.7	175.0	177.9
Week 4	251.8	247.6	237.2*	214.5*
Week 13	303.1	300.0	290.2	231.2*
Week 16	314.6	302.7	294.0*	233.9*
Week 52	378.3	379.1	376.3	335.7*
Week 91	468.1	466.1	456.7	391.0*

* Significantly different from control by the Dunnets test, criteria, $p < 0.05$

** Dose levels during weeks 0 - 16 were 2.5, 7.5 and 30 mg/kg bw/day

Significant decreases in total food consumption values were observed at the high dose males during weeks 1 - 13, 1 - 16, 1 - 52, 16 - 52, 16 - 88 and 52 - 88. In the female animals the total food consumed in all dose groups is generally comparable to the food consumed by the control females with a slight but significant decrease in the high dose group females for weeks 1 - 13, 1 - 16 and 16 - 88.

At the termination of the study, no ophthalmological abnormalities related to the test compound occurred.

Some indications of recovery were noted in the mid and high dose group, in regard to body weight and food consumption (decreased values at the beginning of the study), subsequent to decreasing the dose levels at week 17.

Table 102: Chronic toxicity study in Rats: Body Weight Gain/Food Consumption

	0 mg/kg bw/day	**1 mg/kg bw/day****	**2.5 mg/kg bw/day****	**7.5 mg/kg bw/day****
Males				
Body weight gain, week 0 - 13 (% control)	100	95.1	77.1*	33.5*
Body weight gain, week 0 - 16 (% control)	100	95.7	76.0*	30.6*
Body weight gain, week 0 - 52 (% control)	100	100	89.3	58.1*
Body weight gain, week 16 - 52 (% control)	100	100	100	100
Body weight gain, week 16 - 91 (% control)	100	94.2	100	98.2
Body weight gain, week 52 - 91 (% control)	100	74.9	100	100
Food consumption, week 1 - 13 (% control)	100	99.2	95.4	73.9*
Food consumption, week 1 - 16 (% control)	100	99.6	94.6	73.3*
Food consumption, week 1 - 52 (% control)	100	100	98.0	81.6*
Food consumption, week 16 - 52 (% control)	100	100	100	92.0*
Food consumption, week 16 - 88 (% control)	100	100	99.1	89.9*
Food consumption, week 52 - 88 (% control)	100	100	99.7	90.0*
Females				
Body weight gain, week 0 - 13 (% control)	100	98.7	90.9	42.2*
Body weight gain, week 0 - 16 (% control)	100	92.6	86.0*	41.1*
Body weight gain, week 0 - 52 (% control)	100	100	99.8	78.7*
Body weight gain, week 16 - 52 (% control)	100	100	100	158*
Body weight gain, week 16 - 91 (% control)	100	100	100	100
Body weight gain, week 52 - 91 (% control)	100	95.6	93.6	61.1
Food consumption, week 1 - 13 (% control)	100	100	97.5	87.7*
Food consumption, week 1 - 16 (% control)	100	100	96.2	88.0*
Food consumption, week 1 - 52 (% control)	100	100	96.2	91.6
Food consumption, week 16 - 52 (% control)	100	97.9	97.1	94.6
Food consumption, week 16 - 88 (% control)	100	95.1	92.7	90.9*
Food consumption, week 52 - 88 (% control)	100	92.9	93.8	91.4

* Significantly different from control by the Dunnets test criteria, $p < 0.05$

** Dose levels during weeks 0 - 16 were 2.5, 7.5 and 30 mg/kg bw/day

Haematology and clinical chemistry:

Since the maximum-tolerated dose exceeded in the first 16 weeks of the study, the changes observed within the 16 weeks of the study are considered not to be related to an indication of direct toxicity but as an indirect result of the depressed food consumption.

Mean platelet counts were significantly decreased in the mid and high dose males at week 27 and in the high dose females at week 27.

Mean clinical chemistry data revealed significantly decreased mean serum glucose values in the high dose group males and females at week 27 and the high dose females at week 52. Mean total

cholesterol values were significantly increased in the low dose group males and females at week 27 only. The lack of a dose response raises considerable question regarding any potential treatment relationship of the total cholesterol increases. Significant decreases were noted in mean calcium values for the high dose males at weeks 27 and 92. Mean inorganic phosphorus values were significantly increased in the high dose males at weeks 27 and 92. Mean gamma glutamytransferase values were increased in the high dose males and females at week 14. The consistency of the decreases in triiodothyronine and thyroxine at week 92 in the high dose males suggests that there may be a relationship to treatment late in the study (see Table 103). The toxicological importance of the isolated significant decrease in thyroxine in the high dose males at week 14, and in triiodothyronine in the mid dose males and high dose females at week 92 remains unclear.

Various parameters showed a lack of dose response and an absence of statistical significance after decreasing the dose levels at week 16 and are therefore not considered to be toxicologically relevant.

Evaluation of the urinalysis data revealed an increase in incidence and grade for ketone bodies in the medium and high dose males and females at week 14, the high dose males and females at weeks 27, 52, 79 and the medium and high dose males and high dose females at week 92.

Table 103: Chronic toxicity study in rats: Mean triiodothyronine and thyroxin values (week 92)

Parameter	0 mg/kg	1 mg/kg	2.5 mg/kg	7.5 mg/kg
Triiodothyronine Week 92				
males				
(mg/100 mL) mean	89.8	66.9	60,2 *	44,9 *
S.D.	42.94	24.15	19.85	19.82
N	9	12	14	13
females				
(mg/100 mL) mean	81.0	71.8	67.8	61.7 *
S.D.	17.51	16.08	19.51	16.46
N	14	13	8	13
Thyroxin Week 92				
males				
(mg/100 mL) mean	3.3	3.4	3.3	2.3 *
S.D.	1.01	0 .46	1.04	0.84
N	9	11	14	13

* Significantly different from control value by Dunnets test criteria, $p < 0.05$.
mean = mean value
S.D. = standard deviation
N = number of animals

Gross pathology, organ weights and histopathology:

Gross macroscopic findings for the unscheduled deaths and in the terminally-sacrificed animals were observed for both males and females in all dose groups without apparent correlation to the dose and therefore, are not considered to be test substance related.

Absolute organ weights revealed significant decreases in mean terminal body weights in the high dose males and females and in the mean brain weights in high dose females. The mean organ-to-terminal body weight values revealed significant increases in mean brain, kidney, liver and testis with epididymides, as well as thyroid with parathyroid values in the high dose males and in the

mean thyroid with parathyroid in the high dose females. The majority of these differences, however, were directly related to the significantly depressed body weight in the high dose males and females including thyroid/parathyroid weights and therefore, are not considered to be compound-related changes.

Compound-related histomorphologic alterations consisted of reduced colloid, characterised by microfollicles, in the thyroid of the medium and high dose males and high dose females (results see Table 104 and Table 105). The increased incidence of reduced colloid was noted among unscheduled deaths and scheduled deaths in the medium and high dose males and high dose females. Other spontaneous disease lesions and incidental findings were generally of similar incidence and severity among the control and treated groups.

Table 104: Chronic toxicity study in rats: microscopic effects of the thyroid (unscheduled deaths)

Cyanamide (mg/kg bw/day):	**0**	**1****	**2.5****	**7.5****
Number of rats/group	11	6	6	4
Males:				
Thyroid				
Decreased Colloid	0	0	1 (0.5)	3 (1.5)
Hyperthrophy, Follicular Cell	3	0	0	1
"C" Cell Adenoma	0	0	0	0
Follicular Cell Adenoma	0	1	0	0
Number of rats/group	5	7	12	6
Females:				
Thyroid				
Decreased Colloid	0	0	2 (0.3)	5 (1.7)
Hyperthrophy, Follicular Cell	0	0	0	0
"C" Cell Adenoma	1	0	0	0
Follicular Cell Adenoma	0	0	0	0

** dose levels during weeks 0 - 16 were 2.5, 7.5 and 30 mg/kg bw/day
( ) Mean of graded findings in decreased colloid of the thyroid

Table 105: Chronic toxicity study in rats: microscopic effects of the thyroid (terminal sacrifice)

Cyanamide (mg/kg bw/day):	0	1**	2.5**	7.5**
Number of rats/group	9	12	14	14
Males:				
Thyroid				
Decreased Colloid	0 (0.0)	2 (0.3)	6 (0.7)	14 (2.1)
Hyperplasia, Follicular Cell	0	1	0	0
"C" Cell Adenoma	1	1	0	1
Follicular Cell Adenoma	0	1	0	0
Number of rats/group	15	13	8	14
Females:				
Thyroid				
Decreased Colloid	3 (0.3)	1 (0.1)	3 (0.6)	11 (1.5)
Hyperplasia, Follicular Cell	0	0	0	0
"C" Cell Adenoma	1	1	0	0
Follicular Cell Adenoma	0	0	0	0

** dose levels during weeks 0 - 16 were 2.5, 7.5 and 30 mg/kg bw/day
( ) Mean of graded findings in decreased colloid of the thyroid

Conclusion

Based on the data generated from this study it is concluded that a dose level of 30 mg/kg bw/day pure active substance cyanamide administered via gavage to male and female Sprague Dawley rats exceeded the maximum tolerated dose (MTD) based on the considerable depression in food consumption, body weights and clinical pathology indices. In the first 14 weeks effects in clinical pathology parameters were observed even at the lowest dose level. However, these effects subsided, subsequent to the lowering of the doses in this study. Based on the reversibility of theses effects noted at the week 14 interim clinical pathology evaluation in the low dose animals, the no-adverse-effect level (NOAEL) of aqueous hydrogen cyanamide is 1 mg/kg bw/day pure active substance hydrogen cyanamide which is based on histopathological effects in the thyroid (decreased colloid) obtained at the medium dose. The target organ in this study is the thyroid as indicated by histomorphological alterations.

Carcinogenicity in rats

Title: Ulland, et al. (1979): Bioassay of calcium cyanamide for possible carcinogenicity; Doc. No. 592-009; NCI Frederick Cancer Research Center, Maryland, USA; published: yes

Guidelines: Not indicated.

Deviations:
- 20 animals were used in the control group instead of at least 50.
- Two doses of the test item were used instead of three.
- In the first 13 weeks of the test period animals were weight only once per months, instead of once per week.
- The analytical determination of the diet was not performed.
- The food consumption was not determined.

- No blood smears were evaluated 12 and 18 months and prior to sacrifice.

GLP: No, study is a publication of the U.S. Department of Health, Education and Welfare.

Acceptability: The study is considered to be supplementary.

Materials and methods:

The test substance was identified as follows: Calcium cyanamide; Batch: not indicated; Purity: 63 % calcium cyanamide; Specification: not given.

Calcium cyanamide was administered in feed to 50 male and 50 female F344 (Fischer) rats per dose level in this oncogenicity study. In the concurrent control group only 20 males and 20 females were used. All surviving animals per sex and dose were sacrificed at 107 weeks. The dietary dose levels were 0, 100 and 200 ppm for males and 0, 100 and 400 ppm for females, respectively.

Feed consumption was not determined. The body weights were determined at least once per months, except for weeks 50 to 80 where no weights were recorded. The animals state of health was checked each day. Clinical examination and palpation for masses were performed each month.

Animals were assessed gross-pathologically followed by histopathological examinations.

Findings:

The stability of the test substance over the study period and the stability of the test substance in the food was not performed. Also the analytical determination of the homogeneity and the correctness of the dietary concentrations was not carried out. However, data from a 2-generation study in rats confirm the homogeneity of cyanamide in the diet (Koeter, 1986).

There were no test substance related mortalities in any of the treatment groups. Mean body weights of the high dose male and female rats were slightly lower than those of the corresponding controls, but are considered not to be compound related.

A variety of non-neoplastic lesions and disorders were encountered with regularity in both control and dosed groups of rats. Such lesions are considered as common in aged F344 rats and therefore not to be compound related.

The sites of neoplasms observed most frequently were the adrenal, pituitary, thyroid and testes. The pituitary neoplasms as well as adenomas (and hyperplasias) of the thyroid and interstitial-cell tumours of the testes occurred with comparable frequency in control and dosed rats. Therefore, they are not considered test substance-related. The incidence of adrenal neoplasms are summarised in the Table 106 below.

Table 106: Carcinogenicity study in rats: adrenal neoplasms*

	Cortical Adenoma	Phaeochromo-cytoma, benign	Phaeochromo-cytoma, malignant
Males			
Control	0/20 (0 %)	4/20 (20 %)	0/20 (0 %)
100 ppm	3/49 (6 %)	10/49 (20 %)	0/49 (0 %)
200 ppm	3/50 (6 %)	15/50 (30 %)	1/50 (2 %)
Females			
Control	3/19 (16 %)	0/19 (0 %)	0/19 (0 %)
100 ppm	1/50 (2 %)	4/50 (8 %)	0/50 (0 %)
400 ppm	7/50 (14 %)	6/50 (12 %)	1/50 (2 %)

* The one-tailed Fisher exact test and Cochran-Armitage test for linear trend in proportions, with continuity correction was used.

Table 107: Carcinogenicity study in rats: mammary gland neoplasms

Females	Adenocarcinoma	Adenocarcinoma or adenoma
Control	1/20 (5 %)	5/20
100 ppm	1/50 (2 %)	3/50
400 ppm	6/50 (12 %) Statistical trend	9/50

The number of cortical tumours in the dosed animals seemed to be balanced by similarly affected control rats. No statistical significant incidence was observed for this tumor. However, the incidence of phaeochromocytomas in the dosed females appears to be greater than the normal incidence and may be related to the administration of the test compound. Although the incidence of adrenal medullary tumours is high, statistical evaluation indicate neither a significant dose-related trend nor a significance by direct comparison of the dosed groups with the control. Therefore, the increased incidence is not to be considered related to the treatment of calcium cyanamide. The authors concluded that "In summary, no tumor at any site in the rats can clearly be associated with the administration of calcium cyanamide in this bioassay." and " [...] that under the conditions of this bioassay, the test formulation of calcium cyanamide was not carcinogenic for [...] F344 rats of either sex."

Conclusion

It is concluded that under the conditions of this bioassay, the test formulation of calcium cyanamide was not carcinogenic for F344 (Fisher) rats of either sex.

Carcinogenicity in mice

Title: Ulland, et al. (1979): Bioassay of calcium cyanamide for possible carcinogenicity; Doc. No. 592-009; NCI Frederick Cancer Research Center, Maryland, USA; published: Yes

Guidelines: Not indicated.

Deviations:
- 20 animals were used in the control group instead of at least 50.
- Two doses of the test item were used instead of three.
- In the first 13 weeks of the test period animals were weight only once per months, instead of once per week.

- The analytical determination of the diet was not performed.
- The food consumption was not determined.
- No blood smears were evaluated 12 and 18 months and prior to sacrifice.

GLP: No, study is a publication of the U.S. Department of Health, Education and Welfare.

Acceptability: The study is considered to be supplementary.

Materials and methods:

The test substance was identified as follows: Calcium cyanamide; Batch: Not indicated; Purity: 63 % calcium cyanamide; Specification: Not given.

Calcium cyanamide was administered in feed to 50 male and 50 female B6C3F1 mice per dose level in this oncogenicity study. In the concurrent control group only 20 males and 20 females were used. All surviving animals per sex and dose were sacrificed at 100 weeks. The dietary dose levels were 0, 500 and 2000 ppm for both males and females, respectively.

Feed consumption was not determined. The body weights were determined at least once per months. The animals' state of health was checked each day. Clinical examination and palpation for masses were performed each month.

Animals were assessed gross-pathologically followed by histopathological examinations.

Findings:

The stability of the test substance over the study period and the stability of the test substance in the food was not performed. Also, the analytical determination of the homogeneity and the correctness of the dietary concentrations were not carried out. However, data from a 2-generation study in rats confirm the homogeneity of cyanamide in the diet (Koeter, 1986, Doc-No. 553-001, Annex IIA, 5.6/02).

Significant test substance related mortalities were found in male mice but not in female animals as indicated by the Tarone test for positive dose-related trend in mortality (results see Table 108).

Table 108: Carcinogenicity study in mice: group survival (%)

Week of study	0 ppm	500 ppm	2000 ppm
Males			
100	100	90	76*
Females			
100	90	86	92

* statistically significant by Tarone test for dose-related trend (p = 0.005)

Mean body weights of the high dose male and female mice were slightly lower than those of the corresponding controls, except for the low dose females.

Several groups of neoplasms occurred. With the exception of the hemangiosarcomas and the malignant lymphomas, the remaining neoplasms occur with similar frequencies in control and dosed animals (results see Table 109 below).

Table 109: Carcinogenicity study in mice: Hemangiosarcomas and malignant lymphomas*

	Hemangiosarcomas	Malignant Lymphomas
Males		
Control	1/20 (5 %)	1/20 (5 %)
500 ppm	2/50 (4 %)	4/50 (8 %)
2000 ppm	10/50 (20 %)	3/50 (6 %)
Females		
Control	0/20 (0 %)	1/20 (5 %)
500 ppm	0/46 (0 %)	11/46 (24 %)
2000 ppm	1/50 (2 %)	18/50 (36 %)

* The one-tailed Fisher exact test and Cochran-Armitage test for linear trend in proportions, with continuity correction was used.

In male mice, the results of the Cochran-Armitage test indicate a dose-related trend in the incidence of hemangiosarcomas of all sites, but the results of the Fisher exact test revealed no significance for the findings. The current historical control records indicate an incidence of hemangiosarcomas in control groups of 13/323 (4 %), and the highest incidence ever seen in any control group was 2/19 (10 %). There is an indication of this neoplasm in the high-dose group. However, the absence of significant results in the Fisher exact test fails to confirm this association.

A dose-related trend in female mice was obtained in the incidence of lymphoma or leukaemia. The Fisher exact test establishes a significantly higher incidence in the high-dose group than in the control group. However, the incidence of the lymphomas or leukemias in historical-control female B6C3F1 mice was 67/324 (21 %), suggesting that the incidence of these tumours in the control group of the present bioassay is abnormally low. The authors ascertained that " [...] neither the incidences of hemangiosarcomas of the circulatory system in the male mice nor of lymphomas or leukemias in the female mice can clearly be related to administration of the test chemical." and concluded " [...] that under the conditions of this bioassay, the test formulation of calcium cyanamide was not carcinogenic for [...] B6C3F1 mice of either sex."

Conclusion

It is concluded that under the conditions of this bioassay, the test formulation of calcium cyanamide was not carcinogenic for B6C3F1 mice of either sex.

Carcinogenicity in mice

Title: Goodyer, M.J. (1990): Hydrogen cyanamide up to 104-week oral (drinking water) carcinogenicity study in the mouse; Doc. No. 555-001; Hazelton UK, Harrogate, England; published: no

Guidelines: OECD 451 (1981) and U.S. EPA 83-2 (1982); EEC B 32

Deviations: None

GLP: Yes, U.S. EPA

Acceptability: The study is considered to be acceptable.

Materials and methods:

The test substance was identified as follows:

Hydrogen cyanamide

Batch: lot no. 1: not stated, lot no. 2: 15 05 86, lot no. 3: 03 12 86, lot no. 4: 06 29 87, lot no.: 5 20 01 88

Purity: lot no. 1: 49 %, lot no. 2: 50 %, lot no. 3: 50.3 %, lot no. 4: 50 %, lot no. 5: 50 %

Hydrogen cyanamide was administered to 240 male and 240 female mice [Crl:CD-1 (ICR) BR] in this carcinogenicity study. 60 males and 60 females received the test substance in water at concentrations of 70, 200 or 600 ppm for 100 weeks (males) and 104 weeks (females), respectively. A further group of mice (60 males and 60 females) received drinking water and acted as the control group.

All animals were examined twice daily to detect dead or moribund animals. All animals were examined once daily for signs of ill health or overt toxicity and additionally, each animal was given a detailed clinical examination at weekly intervals.

Individual body weights were recorded before treatment on the first day of the study, at weekly intervals up to week 16, at four weekly intervals up to week 100 for males and week 104 for females and at sacrifice.

The food and water consumed by each cage of animals was determined at weekly intervals to week 16 and one week in every four thereafter.

Blood samples for total white cell counts were obtained from all surviving animals in weeks 52, all surviving males in week 100 and all surviving females in week 104.

A full internal and external examination was carried out at the end of administration in all animals.

Organ weights (brain, kidneys, liver and testes) from ten animals per sex per group were obtained.

Samples of all tissues from animals in the control and high dose groups, from animals that died or were killed during the study as well as gross lesions, tissue masses, lungs, livers, kidneys, thyroids and urinary bladders from all animals were fixed, sectioned, stained and evaluated.

The stability of the test substance over the study period and the stability of the test substance in drinking water were analytically confirmed. The test substance was not found in any control formulation during the study. The intended test substance concentrations in the drinking water were achieved throughout the study. The test substance intake is summarised in the Table 110.

Table 110: Carcinogenicity study in mice: Test substance intake

Dietary dose level (ppm)	Test substance intake males in mg/kg bw	Test substance intake females in mg/kg bw
0	0	0
70	8.4 - 12.9	11.0 - 16.4
200	24.3 - 34.7	26.8 - 43.5
600	59.1 - 92.2	77.9 - 124.2

Statistical evaluation of survival (Kaplan-Meier technique), body weight gains over the treatment period, food and water intake over selected intervals and all organ weight data (analysis of variance technique for normally distributed error (ANOVA) and t-tests) was carried out. The ovarian

granulosa-theca tumours were analysed statistically using the methods described in the IARC annex (Peto, R., 1980 Guidelines for simple sensitive significance tests for carcinogenic effects in long term animal experiments. Annex to IARC monographs on the evaluation of carcinogenic risk of chemicals to humans, supplement 2). The tumours were all classified as either fatal or non-fatal. The ad-hoc runs method was used to determine suitable time intervals for the analysis of non-fatal tumours. The incidence of fatal tumours was too low to perform statistical analysis, however where appropriate, combined fatal and non-fatal tumour analysis was performed. One directional tests for an increasing dose response among all groups were performed with pairwise one-directional tests for increased incidence between each treated group and its control group.

Findings:

About two-thirds of the animals died or were killed because of ill-health during the course of the study. However, the number of animals after a study period of 72 weeks (18 months) was sufficient (survival 73 % and more) and the recommendations of the OECD guideline 451 were fulfilled (survival more than 50 %). In males there was no treatment-related effect on the overall incidence of morbidity and mortality. Non-neoplastic conditions (amyloidosis and urogenital tract lesions) accounted for two-thirds of the death and are considered not to be treatment related.

In females, there was a slight excess of morbidity and mortality in the mid and high dose group (see Table 111). In addition, the pair-wise test of the high dose group against the control (estimated by Kaplan-Meier technique) was statistically significant ($p < 0.05$). Neoplasia accounted for over half of the morbidity and mortality in the females, but no single neoplastic or non-neoplastic condition accounted for the slightly higher incidences in these dose groups.

Table 111: Carcinogenicity study in mice: Group survival (%)

Week of study	0 ppm	70 ppm	200 ppm	600 ppm
Males				
72	83.3	81.7	83.3	73.3
100	36.7	38.3	43.3	36.7
Females				
72	88.3	83.3	81.7	75.0
104	40.0	33.3	23.3	23.3

No treatment-related changes in clinical conditions or behaviour were noted. The incidence of all types of palpable mass was low in the treated groups and comparable to that of the control groups.

Over the first week of treatment 16 males from the high dose group lost body weight, compared to two control males. In week 2, the weight loss continued for three males from group 4 and on of the control males. The remaining animals gaining or maintaining body weight. There was a statistically significant reduction in the mean body weight gain for treated male groups over the first 6 weeks, when compared to the control gain (results see Table 112). Subsequently, the body weight gain for the low and intermediate groups was comparable to or better than the control. Over the first week of the study, 12 females from the high dose group lost body weight, compared to seven control females. In week two, one animal from the high dose group and one animal form the control continued to loose weight, whereas the remaining animals gained weight. In females, the body weight gain over the first six weeks of the study was treatment-related reduced for the medium and high dose group, with statistical significant difference from the control. Subsequently, the weight gain for the intermediate and high dose groups was comparable to or better than the control.

Table 112: Carcinogenicity study in mice: Body weight gain

Group/sex	Mean body weight gain over weeks 0 - 6	Mean body weight gain over weeks 6 - 100/104+	Mean body weight gain over weeks 0 - 100/104+
Males			
0	6.6	5.8	12.4
70	5.5**	7.7	13.2
200	5.4**	6.0	11.4
600	4.8***	2.9*	7.7**
Females			
0	5.1	8.7	13.7
70	4.7	9.8	14.5
200	4.4*	9.3	13.7
600	3.8***	8.5	12.3

\+ males sacrificed week 101, females week 105
* statistically significant by t-test ($p < 0.05$
** statistically significant by t-test ($p < 0.01$)
*** statistically significant by t-test ($p < 0.001$)

The food consumption in the male treated groups was reduced over the majority of the study, but the reduction was only statistically significant from the control over the first six weeks of dosing at the intermediate and high dose groups (results see table below). In females the reduction of food intake was considered to be slight and the food consumption for all groups was considered to be satisfactory throughout the study (Table 113).

Table 113: Carcinogenicity study in mice: Group mean cage food consumption (g/animal/week over period)

Week of study	0 ppm	70 ppm	200 ppm	600 ppm
Males				
1 to 6	39.8	38.7	37.8*	38.1*
13 to 16	39.7	38.2	38.5	38.7
24 to 28	39.4	37.1	38.5	37.6
48 to 52	36.5	35.0	36.4	35.9
96 to 100	37.5	37.5	35.4	35.6
Females				
1 to 6	41.5	41.1	40.3	39.7
13 to 16	43.8	44.9	42.3	42.0
24 to 28	43.1	42.7	40.7*	40.3*
48 to 52	37.9	38.1	35.8	35.8
100 to 104	37.7	42.1	42.0	36.2

* statistically significant by t-test ($p < 0.05$)

There was a treatment-related statistically significant reduction in water consumption in the medium and high dose groups of males and females (results see Table 114). Over the periods weeks 1 to 6 and 13 to 16, there was a statistically significant reduction in water consumption when compared to the control consumption. In addition, water consumption was also reduced significantly for the high dose females over week 24 to 28, for medium group males over the period weeks 1 to 6 and for group 3 females over the periods weeks 1 to 6, 13 to 16 and 24 to 28.

Table 114: Carcinogenicity study in mice: Group mean cage water consumption (g/animal/week over period)

Week of study	0 ppm	70 ppm	200 ppm	600 ppm
Males				
1 to 6	39.6	38.7	34.1**	31.5**
13 to 16	38.1	37.3	33.5	29.6**
24 to 28	39.6	38.5	38.6	32.0
48 to 52	38.0	43.2	39.5	37.9
96 to 100	43.2	41.9	37.5	33.5
Females				
1 to 6	37.9	37.7	34.0***	29.7***
13 to 16	39.2	38.5	33.8**	30.2***
24 to 28	47.3	45.6	39.1***	37.7***
48 to 52	47.7	44.8	42.9	40.2
100 to 104	44.7	58.7	41.9	39.8

** statistically significant by t-test ($p < 0.01$)
*** statistically significant by t-test ($p < 0.001$)

No significant changes were found in the food conversion efficiency for any of the treated groups throughout the study.

No treatment-related changes were seen in the total and differential white blood cell counts measured during the study.

The mean absolute and relative brain weight (statistically significant) and the mean relative testes weight for the high dose males were increased when compared to the control. However, these organ changes were considered to be related to the reduction in body weight seen in the high dose males. All other organ weights for treated groups were comparable to the control.

No treatment-related histopathological effects were found in the low dose group. In the medium and high dose groups there was a dose-related chronic cystitis in the urinary bladder, accompanied in the high dose group by a marginal increase in the incidence and severity of atrophic basophilic tubules in the kidney (results see Table 115).

Table 115: Carcinogenicity study in mice: Incidences of microscopic effects

Hydrogen cyanamide (mg/kg/day):	**0**	**70**	**200**	**600**
Number of mice examined				
Males:				
Urinary bladder	**56**	**56**	**57**	**57**
Chronic cystitis				
Minimal	3	4	11	18
Slight	0	2	6	10
Moderate	2	1	0	11
Total	5	7	17	39
Kidney	**59**	**59**	**59**	**58**
Atrophic/basophilic tubules				
Minimal	22	19	25	16
Slight	4	9	4	12
Moderate	2	0	0	5
Total	28	28	29	36
Females:				
Urinary bladder	**59**	**54**	**55**	**56**
Chronic cystitis				
Minimal	6	8	18	16
Slight	0	0	5	16
Moderate	0	0	0	4
Total	6	8	23	36
Kidney	**60**	**60**	**59**	**58**
Atrophic/basophilic tubules				
Minimal	16	14	12	15
Slight	4	5	3	6
Moderate	0	0	0	3
Total	20	19	15	25

The tumour profile in the controls was generally consistent with that expected in ageing mice. The tumour in the low and intermediate groups was comparable to that seen in the control. In the high dose males there was a decrease in the incidence of liver tumours which was probably attributable to the reduced body weight gain of this group.

In females, there was an increased incidence of proliferative lesions in the stromal/luteal tissues of the ovary in the high dose group. The hyperplasias were predominantly of the luteal type, as were the granulosa-theca tumours. The 8 cases of granulosa-theca tumours in group 4 exceeded the range of 0 to 3 cases per group of 51 mice in 10 previous control groups. Statistically, there was a significant dose response trend in granulosa-theca tumours across the 4 groups ($p < 0.01$) regardless of whether the analysis included or excluded equivocal necrotic control case. Pairwise statistical comparison between groups 1 and 4 was significant ($p < 0.05$) if the necrotic control case was excluded but not significant ($p > 0.05$) if this case was included in the analysis (results see Table 116).

Table 116: Carcinogenicity study in mice: Incidences of hyperplastic and neoplastic lesions

Hydrogen cyanamide (mg/kg/day):	**0**	**70**	**200**	**600**
Number of mice examined				
Males:				
Liver	**59**	**57**	**57**	**58**
Adenoma ± Carcinoma	18	11	7	8
Females:				
Liver	**60**	**59**	**55**	**57**
Adenoma ± Carcinoma	0	0	1	1
Ovary	**60**	**59**	**60**	**58**
Stromal/Luteal hyperplasia	16	13	13	22
Granulosa-theca tumour	3*	1	6	8

* one lesion necrotic and diagnosis equivocal

Conclusion

About two-thirds of the animals died or were killed because of ill health not only in the treatment groups but as well in the controls. In females a slight increase in morbidity and mortality was obtained at 200 and 600 ppm. A statistically significant decrease in body weight gain was obtained in male animals at the high dose group. Dose related microscopic changes were seen at 200 and 600 ppm in the urinary bladder and at 600 ppm in the kidney in both male and female animals. No adverse effect was seen at the low dose (70 ppm) throughout the study and this was considered to be the NOAEL in terms of toxicity of hydrogen cyanamide referring to 4.2 mg/kg bw/day active substance cyanamide. The magnitude of the changes seen at the high dose level in body weight gain, survival, urinary bladder and the kidney demonstrated that the maximum tolerated dose (MTD) was exceeded at 600 ppm. The only treatment-related changes in the tumour profile were the reduction in the incidence of liver tumours in the high dose males and a slight increase in ovarian granulosa-theca tumours in high dose females. The reduction in liver tumours was probably attributable to the reduction in body weight gain seen. There were no changes in the tumour profile at 200 ppm referring to 12.2 to 17.4 mg/kg bw/day (for males) and 13.4 to 21.3 mg/kg bw/day (for females) pure active substance cyanamide, respectively.

A position paper on the carcinogenicity of cyanamide was prepared by Moeller & Hofer (2009) and submitted by the notifier. The relevant text passages of the position paper and the conclusion of the Rapporteur Member State (RMS) are presented in the following.

Three studies are available for the evaluation of the carcinogenic potential of cyanamide.

During the peer-review of the PPP-procedure, it was commented that in all of the three carcinogenicity studies, indications of tumor formation were observed. The notifier is of the opinion that only in the most recent, valid and fully acceptable study (Goodyer; 1990), an increased tumor incidence in female mice (granulosa theca tumors) was observed at the high dose (77.9-124.2 mg/kg bw/d, exceeding maximum tolerable dose, MTD).

Cyanamide was administered to mice at concentrations of 70 ppm (8.4-12.9 and 11.0-16.4 mg/kg bw/d males and females), 200 ppm (24.3-34.7 and 26.8-43.5 mg/kg bw/d males and females, respectively) and 600 ppm (59.1-92.2 and 77.9-124.2 mg/kg bw/d males and females until week 100 for males and week 104 for females (Goodyer; 1990).

No treatment-related histopathological effects were found in the low dose group. An increased incidence in granulosa theca tumors was observed for high dose females, i.e. 8 of 58 animals, compared to the control group showing 3 out of 60 animals with tumor.

In the intermediate and high dose groups, there was a dose-related chronic cystitis in the urinary bladder, accompanied in the high dose group by an increase in the incidence and severity of atrophic/basophilic tubules in the kidney. In addition, there was an adverse effect on body weight in the high dose males and females. Most importantly, cyanamide had impact on morbidity and mortality in the intermediate and high dose females. Reduction of survival by 58 % in the mid and high dose clearly indicates that the maximum tolerable dose (MTD) was exceeded. The MTD was exceeded already with the mid dose (23 % survival, no increased tumor incidence), and mortality in the high dose was not attributable to tumors. The applied cyanamide concentrations were already very high compared to those administered in the subchronic toxicity studies, i.e. 0.5-6 mg/kg bw/d in 90-d and 1-y studies and up to 45 mg/kg bw/d in reproductive toxicity studies. Above all, it can be assumed, that due to kidney toxicity, cyanamide was excreted less efficiently resulting in artificially higher systemic doses of cyanamide in the high dose group animals.

Dosing at levels above the MTD is known to have the potential to perturb biochemical pathways and can result in tumor formation by non-genotoxic mechanisms. Current thinking considers tumors at such dose levels irrelevant to human risk assessment if they occur at a high multiple of the anticipated human exposure and neither the tumors nor their non-neoplastic precursor lesions are observed at the MTD or below (Roth et al.; 2007).

Increased incidence of granulosa theca tumors in the high dose group, i.e. 600 ppm cyanamide corresponding to 77.9 to 124.2 mg/kg bw/d for females, which is a high multiple of the anticipated human exposure, is therefore considered to be a consequence of excessively high doses resulting in secondary tumor effects rather than effects attributable to the test substance. Irrespective of biological significance, pair-wise statistical comparison between the control und high dose group was not significant ($p < 0.05$), unless one of the three tumors in control animals was excluded as it was necrotic.

The authors concluded that significant tumorigenic activity of cyanamide is neither evidenced on biological grounds, nor on statistical grounds.

The carcinogenicity studies in rats and mice of Ulland (Angel et al.; 1979), published by the U.S. Department of Health, Education and Welfare, with rats and mice were considered to be supplementary by the RMS since these studies were performed prior to implementation of GLP and guidelines and therefore deviations from OECD 451 have to be mentioned. However, the method used complies with OECD guideline 451 to a great extent.

In both the rat and the mice study only 20 animals were used in the control group instead of at least 50 animals and only two doses of the test item were used instead of three. Furthermore, in the first 13 weeks of the test period animals were weight only once per month, instead of once per week and no blood smears were evaluated at 12 and 18 months and prior to sacrifice. No analytical determination of cyanamide in the diet was performed and food consumption was not determined.

The study part with B6C3F1 mice, groups of 50 animals of each sex received a commercial formulation containing 63 % calcium cyanamide in the diet for 100 weeks. The dietary doses were 500 or 2000 ppm for both males and females, respectively. The concurrent control groups consisted of 20 animals of each sex.

Survival was reduced significantly in a dose-related manner only for males with 76 % survival for the high dose compared to 100 % survival of the control, indicating that the MTD was exceeded.

An increased incidence of hemangiosarcoma was only reported for males of the high dose which exceeds MTD. The study author stated that the dose trend was statistically significant but the

differences in hemangiosarcoma incidence were not statistically significant. It is concluded that neither a biological nor statistical significant hemangio-sarcoma incidence was observed.

Table 117: Carcinogenicity study in mice: hemangio-sarcomas; Ulland (Angel *et al.*; 1979)

Groups	Hemangiosarcomas HCD: 13/323 max 2/19
Males	
Control	1/20 (5 %)
500 ppm	2/50 (4 %)
2000 ppm	10/50 (20 %)
Females	
Control	0/20 (0 %)
500 ppm	0/46 (0 %)
2000 ppm	1/50 (2 %)

Ulland (Angel et al.; 1979) investigated the potential carcinogenicity of calcium cyanamide in F344 rats with 20 control animals and 50 animals in each of both treatment groups (100, 200 ppm males; 100, 400 ppm females).

With the exception of pheochromocytomas most neoplasms occurred at comparable frequency in control and dosed rats. The incidence of benign pheochromocytomas in the dosed females, i.e. 4/50, 6/50 for low and high dose compared to 0/19 of control, appeared to be greater than the normal incidence. Based on the histopathologic examination, the study author considered calcium cyanamide not to be carcinogenic.

Based on statistics, the incidence of pheochromocytomas was not significant neither in the Cochran-Armitage test for dose-related trend nor by direct comparison of the dosed groups with the control.

The result of the Cochran-Armitage test for dose-related trend indicate borderline significance ($p = 0.042$) in the incidence of adenocarcinomas of the mammary gland in female rats, but the results of the Fisher exact test are not significant. When the incidence of either adenocarcinoma or adenoma of the mammary gland in female rats is analysed, neither the results of the Cochran-Armitage test nor the Fisher exact test attained statistical significance.

In a review of the Ulland (Angel et al.; 1979) carcinogenicity studies with rats and mice, made by the Data Evaluation/Risk Assessment Subgroup of the Clearinghouse on Environmental Carcinogens in December 13, 1978 (Angel et al.; 1979, page 111 - 112), this conclusion of the study author was confirmed. The reviewer agreed that the compound was not carcinogenic under the conditions of the test. He considered the inadequate numbers of matched controls as a shortcoming. He pointed out the higher total tumor incidence among high-dose treated male rats and suggested that it should be evaluated against historic controls. However, no data of historic controls are available. Assuming that the studies´ shortcomings did not affect the results of the studies, the reviewer said that calcium cyanamide would not appear to pose a risk of cancer to human beings.

The results for carcinogenicity in rats are further confirmed by the long-term study in rats of Osheroff (1991). Cyanamide was administered 91 weeks via gavage to Sprague-Dawley rats. Body weight (gain) was significantly reduced for the mid and high dose. The NOAEL of this study was 1 mg/kg bw/d based on histopathological effects in the thyroid obtained in the intermediate dose. No tumor formation was observed.

In summary, the authors concluded that cyanamide was not evidenced to pose a carcinogenic risk in studies with rats and mice. The conclusion of the authors is in line with the RMS.

Conclusion of the Rapporteur Member State (RMS): Three studies on carcinogenicity are available, two in mice and one with rats, and one long-term study with rats. In the chronic toxicity study with Sprague Dawley rats and in the carcinogenicity study with F344 (Fischer) rats no tumor at any site in the rats could be associated with the administration of the substance. In the carcinogenicity study with B6C3F1 mice calcium cyanamide was not carcinogenic under the conditions of this study. In the carcinogenicity study with [Crl:CD-1 (ICR) BR] mice at the high dose (600 ppm corresponding to 39.0 mg/kg bw/day hydrogen cyanamide) in females a slight increase in granulosa-theca tumours was found. The Maximum Tolerable Dose (MTD) was exceeded at this dose level. It is concluded that no treatment related changes in the tumour profile were found up to a dose of 200 ppm (12.1 mg/kg/ bw day hydrogen cyanamide). Cyanamide is not proposed to be classified as potential carcinogen.

4.10.1.2 Carcinogenicity: inhalation

No data submitted by the notifier.

4.10.1.3 Carcinogenicity: dermal

No data submitted by the notifier.

4.10.2 Human information

No data submitted by the notifier.

4.10.3 Other relevant information

Extract from the report of the PRAPeR experts' meeting:

"Background:

GR noted that in the carcinogenicity study in rats (Ulland, 1979), supplementary, that "the incidence of phaechromocytomas in the dosed females appears to be grater than the normal incidence and may be related to the administration of the test compound". In addition, a significant trend not reported in the DAR Dec. 2005 (P=0.042, calculated according to Cochran-Armitage test) for increased mammary gland adenocarcinomas in females was detected.

The applicant confirmed that the incidence of benign pheochromocytomas in the dosed females, i.e. 4/50, 6/50 for low and high dose compared to 0/19 of control, appeared to be greater than the normal incidence. However, based on statistics, the incidence of pheochromocytomas was not significant neither in the Cochran-Armitage test for dose-related trend, nor by direct comparison of the dosed groups with the control. Based on the histopathologic examination, the study author considered cyanamide not to be carcinogenic.

The result of the Cochran-Armitage test indicating a dose-related trend in the incidence of adenocarcinomas of the mammary gland in female rats is reported in the Additional Report p. 96 (see also Doc. 581-019). It is also detailed, that the analysis with the Fisher exact test was not significant, and further, that, if analysing the incidence of either adenocarcinoma or adenoma (25, 6 and 18%) of the mammary gland, neither the results of the Cochran-Armitage test nor the Fisher exact test attained statistical significance.

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Rat study (Ulland, 1979) carried out at the Nation Cancer Institute - mammary tumours are not statistically significant. Adenocarcinoma (5, 2,12%). Adenocarcinoma + adenoma (25,6,18) – not statistically significant but unequal group sizes were used so this significance is not accurate. No findings in the haemotopoetic system.

Background mean for phaechromocytomas in males was 13% and 16% for females (HCD not from NCI, but from NTP) indicating a high natural occurrence. Controls had no incidence, but there was a low number of controls (19). However, the controls used were representative.

The incidence of benign phaechromocytomas in females (0,8,12%) is more difficult to explain. Tumours would be expected in the control females, as these tumours are naturally occurring. In addition, the malignant phaechromocytomas do not seem to represent the progression of the benign tumours in this study.

Mouse study (Goodyer, 1990) - granulosa-theca cell tumours in females (3,1,6,8 incidences). HCD indicated a normal incidence of 0 - 3%. Stromal/luteal hyperplasia observed at the top dose. No statistical significance.

Mouse study (Ulland, 1979) – Increase in hemangiosarcomas in males and also an increase in malignant lymphomas in females (top dose showed a statistically significant increase). HCD for malignant lymphomas in females – mean 21%, whereas for hemangiosarcomas, range is 4-10% for males. No dose-response observed in females. The hemangiosarcomas are outside the HCD.

Both the rat and the second mouse study by Ulland have some limitations. Only two dose levels were tested and low number of controls used. Increase in mortality in top dose males in mouse study, however, 76% of animals survived until the end of the study.

Overall, genotoxicity tests were negative.

The inconsistency between the mouse studies may be a result of different strains used (also conducted in different laboratories and with different test materials; Ulland study conducted with calcium-cyanamide, Goodyer was with hydrogen-cyanamide). However, as there is no genotoxic mechanism expected, similar findings would be expected in both studies, even in the different strains.

The highest dose in Ulland mouse study was 2000 ppm (calcium cyanamide), in Goodyer study it was 600 ppm (hydrogen cyanamide). No comparability of actual cyanamide dose is available.

Based on the findings in the three carcinogenicity studies and the uncertainties over the endocrine effects of cyanamide, the majority of experts voted to propose classification of cyanamide with R40.”

4.10.4 Summary and discussion of carcinogenicity

Long-term dietary toxicity studies were conducted in rats and mice.

In the chronic toxicity study in Sprague Dawley rats (Osheroff, 1991) hydrogen cyanamide was administered via oral gavage for 91 weeks. In the high dose group males and females clinical observations demonstrated effects of general debilitation in the health. Due to these observations, the dose levels have been reduced after 16 weeks of treatment. Significant depressions in body weight and body weight gain values are obtained in intermediate and high dose males and females in the first weeks. Mean food consumption revealed significant depression in females and males of

the high dose group. Compound-related clinical pathology changes were found in males and females at the high dose group and at males in the intermediate dose group. Compound-related histopatholgical changes were found in the thyroid of the intermediate and high dose males and at the high dose females. No tumor at any site in the rats could be associated with the administration of the substance. The NOAEL of this study is 1 mg/kg/day active substance cyanamide based on the histopathological effects in the thyroid obtained in the intermediate dose. The target organ was the thyroid.

In a carcinogenicity study, groups of 50 male and female F344 (Fischer) rats (Ulland et al., 1979) were administered calcium cyanamide orally in the diet for 107 weeks (control group: 20 animals/sex). No tumour type in the rats could be associated with the administration of the substance.

In a carcinogenicity study, B6C3F1 mice (dose groups: 50 animals/sex, control group: 20 animals/sex) (Ulland et al., 1979) were administered calcium cyanamide orally in the diet for 100 weeks. Significant test substance-related mortalities were found in male mice. Mean body weights of the high dose males and females were slightly lower than those of the corresponding controls. No tumour type in the rats could be associated with the administration of the substance.

In a carcinogenicity study, [Crl:CD-1 (ICR) BR] mice (Goodyear, 1990) were administered hydrogen cyanamide via the drinking water in concentrations of 70, 200 and 600 ppm. A slight increase in morbidity and mortality in the intermediate and high dose females groups was obtained. In the first weeks of the study the body weight gain, the food and water consumption was reduced in the intermediate and high dose groups. Histopathological effects were obtained in the medium and high dose groups evidenced by a dose-related chronic cystitis in the urinary bladder and in the high dose group by atrophic basophilic tubules in the kidney. In females, there was an increased incidence of proliferative lesions in the stromal/luteal tissues of the ovary in the high dose group. The hyperplasias were predominantly of the luteal type, as were the granulosa-theca tumours. The 8 cases of granulosa-theca tumours in group 4 exceeded the range of 0 to 3 cases per group of 51 mice in 10 previous control groups. Statistically, there was a significant dose response trend in granulosa-theca tumours across the 4 groups ($p < 0.01$) regardless of whether the analysis included or excluded equivocal necrotic control case. Pairwise statistical comparison between groups 1 and 4 was significant ($p < 0.05$) if the necrotic control case was excluded but not significant ($p > 0.05$) if this case was included in the analysis. The Maximum Tolerable Dose (MTD) was exceeded at the high dose level. There were no treatment-related changes in the tumour profile at 200 ppm referring to approximately 12.2 mg/kg bw/day active substance cyanamide. The NOAEL was 70 ppm referring to approximately 4.2 mg/kg bw/day, based on increased mortality, reduction in body weight gain, food consumption and histopathological effects (cystitis (urinary bladder) and atrophic basophilic tubules in kidney) in the mid and high dose.

Table 118: Summary table of relevant carcinogenicity studies

Study type / species/ Dose levels	Comments	NOAEL	Reference
91-week gavage study in Sprague-Dawley rats with 0, 2.5, 7.5 and 30.0 for the first 16 weeks and 0, 1, 2.5 and 7.5 mg/kg bw/day pure active substance hydrogen cyanamide for the remaining time	Clinical observations at 7.5 mg/kg/day in males and females. Depression in the body weights, body weight gains and food consumption at the intermediate and high dose in the first weeks in males and females. Clinical pathology changes in males and females at the high dose group and in males at the intermediate dose group. Histopatho-logical findings in the thyroid in the intermediate and high dose males and high dose females. No such effects in the lowest dose group.	1 mg/kg/ bw/day of pure active substance hydrogen cyanamide	Osheroff, 1991 Doc-No 537-001
Carcinogenicity study in rats by dietary intake of 0, 100 and 200 ppm calcium cyanamide for males and 0, 100 and 400 ppm calcium cyanamide for females	Reduced body weights in the high dose of male and female rats.	Not determined	Ulland et al., 1979 Doc-No 592-009
Carcinogenicity study in mice by dietary intake of 0, 500 and 2000 ppm calcium cyanamide for males and females	Compound-related mortalities in high dose male mice. Reduced mean body weights in high dose males and female rats.	Not determined	Ulland et al., 1979 Doc-No 592-009
104-week oral (drinking water) carcinogenicity study in the mouse with doses of 0, 70, 200 and 600 ppm corresponding to approximately 0, 4.2, 12.1 and 25.5 mg/kg/ bw day pure active substance hydrogen cyanamide for males and 0, 6, 13.4 and 39.0 mg/kg bw/day pure active substance hydrogen cyanamide for females.	A slight increase in the morbidity and mortality in the medium and high dose group in females. Reduced food and water consumption in the medium and high dose groups of males and females. A chronic cystitis in the urinary bladder in the medium and high dose group was obtained accompanied by an increase in the incidence of atrophic basophilic tubules in the kidney in the male ani-mals. In the high dose females there was an increase in granulosa-theca tumours. This dose level is regarded to be beyond the MTD (see findings above). It is concluded that no treatment related changes in the tumour profile were found up to a dose of 200 ppm.	4.2 mg/kg bw/day pure active substance hydrogen cyanamide	Goodyear, 1990 Doc-No 555-001

4.10.5 Comparison with criteria

Table 119: Criteria for classification

DSD	CLP regulation
The placing of a substance into Category 1 is done on the basis of epidemiological data; placing into Categories 2 and 3 is based primarily on animal experiments. For classification as a Category 2 carcinogen either positive results in two animal species should be available or clear positive evidence in one species, together with supporting evidence such as genotoxicity data, metabolic or biochemical studies, induction of benign tumours, structural relationship with other known carcinogens, or data from epidemiological studies suggesting an association. Category 3 actually comprises 2 sub-categories: (a) substances which are well investigated but for which the evidence of a tumour-inducing effect is insufficient for classification in Category 2. Additional experiments would not be expected to yield further relevant information with respect to classification; (b) substances which are insufficiently investigated. The available date are inadequate, but they raise concern for man. This classification is provisional; further experiments are necessary before a final decision can be made. For a distinction between Categories 2 and 3 the arguments listed below are relevant which reduce the significance of experimental tumour induction in view of possible human exposure. These arguments, especially in combination, would lead in most cases to classification in Category 3, even though tumours have been induced in animals: - carcinogenic effects only at very high dose levels exceeding the 'maximal tolerated dose'. The maximal tolerated dose is characterised by toxic effects which, although not yet reducing lifespan, go along with physical changes such as about 10 % retardation in weight gain, - appearance of tumours, especially at high dose levels, only in particular organs of certain species known to be susceptible to a high spontaneous tumour formation, - appearance of tumours, only at the site of application, in very sensitive test systems (e.g., i.p. or s.c. application of certain locally active compounds), if the particular target is not relevant to man, - lack of genotoxicity in short-term tests in vivo and in vitro, - existence of a secondary mechanism of action with the implication of a practical threshold above a certain dose level (e.g., hormonal effects on target organs or on mechanisms of physiological regulation, chronic	A substance is classified in Category 1 (known or presumed human carcinogens) for carcinogenicity on the basis of epidemiological and/or animal data. A substance may be further distinguished as: Category 1A, known to have carcinogenic potential for humans, classification is largely based on human evidence, or Category 1B, presumed to have carcinogenic potential for humans, classification is largely based on animal evidence. The classification in Category 1A and 1B is based on strength of evidence together with additional considerations (see section 3.6.2.2). Such evidence may be derived from: — human studies that establish a causal relationship between human exposure to a substance and the development of cancer (known human carcinogen); or — animal experiments for which there is sufficient (1) evidence to demonstrate animal carcinogenicity (presumed human carcinogen). In addition, on a case-by-case basis, scientific judgement may warrant a decision of presumed human carcinogenicity derived from studies showing limited evidence of carcinogenicity in humans together with limited evidence of carcinogenicity in experimental animals. The placing of a substance in Category 2 (suspected human carcinogens) is done on the basis of evidence obtained from human and/or animal studies, but which is not sufficiently convincing to place the substance in Category 1A or 1B, based on strength of evidence together with additional considerations (see section 3.6.2.2). Such evidence may be derived either from limited (1) evidence of carcinogenicity in human studies or from limited evidence of carcinogenicity in animal studies. […] 3.6.2.2.3. Strength of evidence involves the enumeration of tumours in human and animal studies and determination of their level of statistical significance. Sufficient human evidence demonstrates causality between human exposure and the development of cancer, whereas sufficient evidence in animals shows a causal relationship between the substance and an increased incidence of tumours. Limited evidence in humans is demonstrated by a positive association between exposure and cancer, but a causal relationship cannot be stated.

stimulation of cell proliferation), - existence of a species - specific mechanism of tumour formation (e.g. by specific metabolic pathways) irrelevant for man. For a distinction between Category 3 and no classification arguments are relevant which exclude a concern for man: - a substance should not be classified in any of the categories if the mechanism of experimental tumour formation is clearly identified, with good evidence that this process cannot be extrapolated to man, - if the only available tumour data are liver tumours in certain sensitive strains of mice, without any other supplementary evidence, the substance may not be classified in any of the categories, - particular attention should be paid to cases where the only available tumour data are the occurrence of neoplasms at sites and in strains where they are well known to occur spontaneously with a high incidence.	Limited evidence in animals is provided when data suggest a carcinogenic effect, but are less than sufficient. The terms 'sufficient' and 'limited' have been used here as they have been defined by the International Agency for Research on Cancer (IARC) and read as follows: (a) Carcinogenicity in humans The evidence relevant to carcinogenicity from studies in humans is classified into one of the following categories: — sufficient evidence of carcinogenicity: a causal relationship has been established between exposure to the agent and human cancer. That is, a positive relationship has been observed between the exposure and cancer in studies in which chance, bias and confounding could be ruled out with reasonable confidence; — limited evidence of carcinogenicity: a positive association has been observed between exposure to the agent and cancer for which a causal interpretation is considered to be credible, but chance, bias or confounding could not be ruled out with reasonable confidence. (b) Carcinogenicity in experimental animals Carcinogenicity in experimental animals can be evaluated using conventional bioassays, bioassays that employ genetically modified animals, and other in-vivo bioassays that focus on one or more of the critical stages of carcinogenesis. In the absence of data from conventional long-term bioassays or from assays with neoplasia as the end-point, consistently positive results in several models that address several stages in the multistage process of carcinogenesis should be considered in evaluating the degree of evidence of carcinogenicity in experimental animals. The evidence relevant to carcinogenicity in experimental animals is classified into one of the following categories: — sufficient evidence of carcinogenicity: a causal relationship has been established between the agent and an increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in (a) two or more species of animals or (b) two or more independent studies in one species carried out at different times or in different laboratories or under different protocols. An increased incidence of tumours in both sexes of a single species in a well-conducted study, ideally conducted under Good Laboratory Practices, can also provide sufficient evidence. A single study in one species and sex might be considered to provide sufficient evidence of carcinogenicity when malignant neoplasms occur to an unusual degree with regard to incidence, site, type of tumour or age at onset, or when there are strong findings of tumours at multiple sites; — limited evidence of carcinogenicity: the data suggest a carcinogenic effect but are limited for making a definitive evaluation because, e.g. (a) the evidence of carcinogenicity is restricted to a single experiment; (b) there are unresolved questions regarding the adequacy of

	the design, conduct or interpretation of the studies; (c) the agent increases the incidence only of benign neoplasms or lesions of uncertain neoplastic potential; or (d) the evidence of carcinogenicity is restricted to studies that demonstrate only promoting activity in a narrow range of tissues or organs. 3.6.2.2.4. Additional considerations (as part of the weight of evidence approach (see 1.1.1)). Beyond the determination of the strength of evidence for carcinogenicity, a number of other factors need to be considered that influence the overall likelihood that a substance poses a carcinogenic hazard in humans. The full list of factors that influence this determination would be very lengthy, but some of the more important ones are considered here. 3.6.2.2.5. The factors can be viewed as either increasing or decreasing the level of concern for human carcinogenicity. The relative emphasis accorded to each factor depends upon the amount and coherence of evidence bearing on each. Generally there is a requirement for more complete information to decrease than to increase the level of concern. Additional considerations should be used in evaluating the tumour findings and the other factors in a case-by-case manner. 3.6.2.2.6. Some important factors which may be taken into consideration, when assessing the overall level of concern are: (a) tumour type and background incidence; (b) multi-site responses; (c) progression of lesions to malignancy; (d) reduced tumour latency; (e) whether responses are in single or both sexes; (f) whether responses are in a single species or several species; (g) structural similarity to a substance(s) for which there is good evidence of carcinogenicity; (h) routes of exposure; (i) comparison of absorption, distribution, metabolism and excretion between test animals and humans; (j) the possibility of a confounding effect of excessive toxicity at test doses; (k) mode of action and its relevance for humans, such as cytotoxicity with growth stimulation, mitogenesis, immunosuppression, mutagenicity. Mutagenicity: it is recognised that genetic events are central in the overall process of cancer development. Therefore evidence of mutagenic activity in vivo may

	indicate that a substance has a potential for carcinogenic effects.

There are no relevant data from epidemiological studies submitted by the notifier, hence no classification with Cat. 1 according to DSD regulation or with Cat. 1A according to CLP regulation is required.

Long-term dietary toxicity studies were conducted in rats and mice. In the chronic toxicity study in Sprague Dawley rats, there was no evidence of carcinogenic effects (Osheroff, 1991). In two studies (Ulland (1979) in mice and rats, the size of the dose groups and the control groups were different (50 vs. 20 animals/sex) making the statistical assessment difficult. No carcinogenic potential could be associated with the administration of the substance.

In a carcinogenicity study in [Crl:CD-1 (ICR) BR] mice with doses of 0, 70, 200 and 600 ppm (Goodyear, 1990), there was an increased incidence of granulosa theca tumors in the high dose group, i.e. 600 ppm cyanamide corresponding to 77.9 to 124.2 mg/kg bw/d for females, exceeding the Maximum Tolerable Dose (MTD), which is a high multiple of the anticipated human exposure.

Dosing at levels above the MTD is known to have the potential to perturb biochemical pathways and can result in tumor formation by non-genotoxic mechanisms. There was some evidence in molecular toxicology investigations (Borlak, J., 2009) that cyanamide downregulates hydroxysteroid dehydrogenases in liver cells and might interfere with steroid hormone production and metabolism (see 4.12.1.3 Specific investigations: other studies). Furthermore, there is evidence that the gene expression profile induced by cyanamide in human primary hepatocytes is distinctly different from those in dogs and rats (Borlak, J., 2009) (see 4.12.1.3 Specific investigations: other studies). Therefore, a species specific reaction at dose levels above the MTD in rats can not be excluded.

Overall, the tumor incidence is considered to be a consequence of excessively high doses rather than effects attributable to the test substance. Tumors at such dose levels should be regarded as irrelevant to human risk assessment if they occur at a high multiple of the anticipated human exposure and neither the tumors nor their non-neoplastic precursor lesions are observed at the MTD or below (Roth et al.; 2007). Irrespective of biological significance, pair-wise statistical comparison between the control und high dose group was not significant ($p < 0.05$), unless one of the three tumors in control animals was excluded as it was necrotic. In accordance with the authors of the study (Goodyear, 1990), the changes in tumor profile at this dose level are considered not sufficient for classification.

Taken together, oral administration that was used in the studies is a relevant exposure pathway. The compound was not genotoxic in vivo in the available studies. No consistent pattern of carcinogenicity regarding affected sex or species can be concluded from the available results of the submitted studies. Overall, dose levels employed were quite high, often associated with high toxicity. Where available, the observed incidences were near the historical control ranges.

No “sufficient evidence of carcinogenicity” can be demonstrated when considering the strengths and weaknesses of the available studies. Hence, no classification as Cat. 2 according to DSD or as Cat. 1B according to CLP regulation is proposed.

When balancing the factors for increasing or decreasing the level of concern, and deciding whether there is a “limited evidence of carcinogenicity”, it is proposed not no classify, due to the uncertainties in the available data package.

The PRAPeR 79 meeting of experts[1] agreed to propose classification of cyanamide with R40 (*c.f.*, section 4.10.3), however, based on the case presented above, this is not proposed by the dossier submitter.

4.10.6 Conclusions on classification and labelling

In summary, no classification for carcinogenic effects is proposed.

4.11 Toxicity for reproduction

4.11.1 Effects on fertility

4.11.1.1 Non-human information

Multi-generation studies:

Title: Obach, R. and Rives, A. (1985); Rives Ferriol, A. (1987): Colme® (Cyanamide oral solution) – Two generation reproduction-fertility study in rat Histopathological report of the two-generation reproduction study of Cyanamide in rat (doses of 7 and 2 mg/kg/day), Doc. No. 553-004, 553-005, 1857537, 1857538; Pharmacology and Toxicology Department, Sant Feliu de Llobregat (Bacrelona), Spain; published: yes

Guidelines: Not indicated, however the method used complies to a great extent to OECD 416 (1981) or EEC 34/35

Deviations: Treatment period F0 females: 15 days prior mating

Embryotoxicity part included

Sacrifice of one half of females (20 per group) on day 13 of pregnancy

Reproduction part:

F1 and F2 progeny: 4 pups/weanlings per litter used for behavioural and developmental parameters

GLP: No

Acceptability: The study is considered supplemental**.**

[1] European Food Safety Authority; Conclusion on the peer review of the pesticide risk assessment of the active substance cyanamide. EFSA Journal 2010;8(11):1873. [61 pp.] doi:10.2903/j.efsa.2010.1873. Available online: www.efsa.europa.eu/efsajournal.htm

Materials and methods:

The test substance was identified as follows:

Cyanamide as Colme® formulation (6 g active substance/100 mL)

Batch: S-13 and T-14

Purity: Not indicated

Appearance: Liquid

Specification: Not given

Control material: Bistilled water

Test animals

Species: Rat

Strain: CD-Sprague-Dawley

Age: Males: 8 weeks, females 15 weeks (each at initiation of dosing)

Weight at dosing: F0 males: 165 g (mean)

F0 females: 240 g (mean)

Source: Own breeding colony at Pharmacology and Toxicology Department, Sant Feliu de Llobregat (Bacrelona), Spain

Acclimation period: not indicated

Diet: Rodent Chow UAR A.04 (Panlab S.L.), ad libitum

Water: Tap water, ad libitum

Housing: Five males and five female per cage after randomisation

No further information for the further course of the study

Environmental conditions:

- Temperature: 20 ± 2 °C

- Humidity: 40 to 60 %

- Air changes: Not indicated

- Photoperiod: Alternating 12-hour light and dark cycles.

Sprague-Dawley CD rats received dose levels of 0 (control), 2, 7 and 25 mg/kg bw/day by gavage. Cyanamide was administered to groups of 20 males (data were presented for 24 males/group) and 40 females.

Eight weeks old male rats and 15 weeks old female rats were treated for 70 days and 15 days, respectively, until mating and throughout all subsequent phases of the study until sacrifice. After 15 days of administration, females were placed together with males (F0 generation). Twenty dams per

group were sacrificed on day 13 of pregnancy. The remainder of the pregnant rats continued on the compound and were allowed to litter normally. The number of pups per litter were adjusted to eight (four males and four females each, randomly on day 4 post partum. One male and one female per litter were selected at random to form the F1 generation. F1 males were treated from their weaning to the weaning of the F2 generation; however, the treatment schedule for the F1 females is unclear. They may have been untreated until 3 weeks before their scheduled mating based on the fact that, in contrast to the males, data on body weight, food and water consumption are only presented starting from this timepoint. The F0 parental generation was sacrificed at weaning of F1 progeny and F1 parental generation at weaning of F2 progeny.

Analyses of stability, homogeneity or correctness of the test solutions were not performed.

The following statistical methods were used:

- ANOVA (one/two ways analysis of variance) and Kruskal-Wallis test.
- Comparison between groups by T-test or Nemenyi Test according to parametric or non-parametric statistical analysis, in case of differences.
- Mann-Whitney U test as non-parametric comparison in case of different group sizes.
- Fertility rates were compared by means of contingency tables with Yates correction.

All parental animals (F0, F1) were observed daily for signs of toxicity or poor health. The occurrence of copulation was investigated by daily vaginal inspections for sperm during the mating period of three weeks. The presence of sperm defined day 0 of pregnancy. The 20 dams of the F0 generation sacrificed on day 13 of gestation were examined for number of embryos, presence of implantation sites and embryos undergoing resorption. Embryos and placentae were weighed. The remaining F0 dams were allowed to litter. The litters were examined for litter size, number of live and dead neonates, gross anomalies birth weight of pups. These F1 litters were examined daily for physical development and behaviour. A sample of 4 pups per litter (2 males and 2 females) was investigated on specific development and behaviour parameters (pinna detachment, fur growth, eye opening, incisor development, reflexes, testes descent, vaginal opening and open field behavior). The fertility and reproductive ability of the F1 generation was tested in the same way as in the F0 parents. The F2 generation animals were examined for postnatal development and behaviour just like the F1 generation.

The body weight of the F0 and F1 parental animals were determined in weekly intervals, with the exception of the F0 generation females, which were also weighed daily during pregnancy. The F1 and F2 litters were weighed at postnatal day 1, 4, 7, 14 and 21. Food and water consumption of the F0 and F1 parental animals was determined weekly.

All adult animals were subjected to gross pathology. Brain, pituitary gland, testes, epididymis, prostate, seminal vesicles, coagulating glands, ovaries and uterus were weighed after sacrifice of the F0 and F1 parental animals. Relative organ weights in this study have been calculated relative to brain, not to body weight. Histopathological examination of brain, pituitary gland and reproductive organs from male and female F0 and F1 generations of the control and the highest dosed groups was carried out. Testes were also examined histopathologically in the low and mid dose groups.

Findings:

Embryotoxicity test with sacrifice of F0 dams on day 13 of pregnancy:

No dam died during treatment period. There were no treatment-related clinical findings in the dams until sacrifice on day 13 of pregnancy. Body weight gains of high dose females prior to mating and during the first two weeks of pregnancy were lower than those of the other groups Food consumption in the high dose group was slightly decreased whereas water consumption was slightly increased.

Table 120: Body weights, food and water consumption of female rats in fertility/embryo toxicity study

Dose level (mg/kg bw/day)		**0**	**2**	**7**	**25**
Females on study		20	20	20	20
Prior to mating	Bw gain (g)	6.58	4.99	4.47	-0.43*
	Mean daily food intake (g/rat)	17.5	17.2	16.8	14.5
	Mean daily water intake (mL/rat)	25.8	26.7	24.5	27.8
Pregnancy D0-13	Bw gain (g)	23.0	19.96	19.06	15.34*
* statistically significant (p< 0.05)					

Litter parameters were affected at the high dose level of 25 mg/kg bw/day, showing reductions in the mean number of corpora lutea, implantation sites and live embryos and a correspronding increase in pre- and postimplantation losses. The weights of embryos or placentae showed no impairment.

Table 121: Litter parameters

Dose level (mg/kg bw/day)	**0**	**2**	**7**	**25**
Females on study (litters examined)	20	20	20	20
Corpora lutea (mean)	12.9	13.2	14.8	10.5*
Implantation sites (mean)	11.2	11.3	13.8	6.0*
Live embryos (mean)	10.2	10.1	12.5	4.5*
Resorptions (mean)	0.85	0.95	1.0	1.3*
Dead embryos (mean)	0.2	0.2	0.35	0.2
Mean embryo weight (mg)	60.8	66.06	63.15	62.67
Mean placental weight (mg)	88.9	91.1	85.2	91.5
Litters with preimplantation loss > 2	6	5	3	15*
Litters with postimplantation loss > 2	4	1	3	5
Litters with litter size < 3	3	3	0	10*
* statistically significant (p< 0.05)				

Effects on organ weights of the dams were seen at 25 mg/kg bw/day and the reduction of absolute and relative uterus weights was considered substance-related.

Table 122: Organ weights

Dose level (mg/kg bw/day)	0	2	7	25
Females on study	20	20	20	20
Dam brain weight absolute (g)	1.92	1.96	1.96	1.87
Dam pituitary weight absolute (mg)	13.75	13.05	13.55	11.4*
Dam pituitary weight relative (%)	0.007	0.007	0.007	0.006
Dam uterus weight absolute (g)	2.21	2.13	2.43	1.09*
Dam uterus weight relative (%)	1.00	1.08	1.24	0.59*
Dam ovaries weight absolute (mg)	115.85	124.2	124.0	107.5
Dam ovaries weight relative (%)	0.086	0.063	0.063	0.057
* statistically significant (p< 0.05)				

Two generation reproduction test:

No animals died during treatment period. The F0 male parental animals showed a slight decrease in body weight gain at 25 mg/kg bw/day in the premating period. Animal numbers in the high dose group of the F1 generation were too low for an adequate evaluation. Food and water consumption was not affected.

Table 123: Body weights during the premating period

Dose level (mg/kg bw/day)	0	2	7	25
F0 males				
Number	24	24	24	24
Body weight week 0 (g)	164.2	167.1	157.1	169.6
Body weight week 12 (g)	409.2	416.3	387.5	309.6*
F0 females				
Number	20	20	20	20
Body weight week 0 (g)	226.5	236.5	233.5	234.0
Body weight week 3 (g)	237.0	246.0	240.0	219.0*
F1 males				
Number	18	18	19	6
Body weight week 0 (g)	201.1	181.1	211.1	173.3
Body weight week 12 (g)	430.0	441.7	423.2	381.7
F1 females				
Number	20	12	17	6
Body weight week 0 (g)	171.0	175.8	169.4	160.0
Body weight week 3 (g)	262.0	275.0	268.2	260.0
* statistically significant by chi-square test (p< 0.001)				

Table 124: Food and water consumption during the premating period

Dose level (mg/kg bw/day)	0	2	7	25	0	2	7	25
	Food consumption (g/animal/day)				Water consumption (mL/animal/day)			
F0 males								
Number	24	24	24	24	24	24	24	24
Week 12	22.1	25.8	22.6	19.8	28.3	32.6	30.0	29.8
F0 females								
Number	20	20	20	20	20	20	20	20
Week 2	17.5	17.3	16.7	17.0	28.1	29.5	26.6	27.1
F1 males								
Number	18	18	19	6	18	18	19	6
Week 11	22.73	21.84	21.21	27.02	31.71	30.5	32.0	37.9
F1 females								
Number	20	12	17	6	20	12	17	6
Week 2	15.34	16.62	15.12	15.24	24.64	23.36	30.27	27.38

In the F0 males significant decreases were observed in relative epididymis and prostate weights at the highest dose group; relative prostate weights were also reduced in the mid dose group. No such effects were observed in the F1 generation. Relative testis weights were not influenced in any dose groups of the F0 and F1 generation. In both generations, bi- or uni-lateral testicular atrophy was observed in a few males. The percentage of affected animals was increased at the high dose; however, due to the lack of mating records it is unclear to what extent this testicular pathology contributed to the greatly reduced pregnancy rate of the F0 females in this group. The investigators consider the finding as possibly related to treatment but with a high degree of uncertainty. No relevant effects were noted on reproductive organs in females that became pregnant. None of the other organs examined (brain, pituitary, levator ani muscle) a treatment-related finding was recorded at any dose level.

Table 125: Reproductive organ weights and testicular histopathology

Dose level (mg/kg bw/day)	0	2	7	25	0	2	7	25
Males	F0				F1			
Animals evaluated	23	24	24	23	18	18	19	6
Testes absolute (g)	3.89	3.72	3.74	3.51	3.605	3.769	3.798	3.424
Testes relative (%)	1.94	1.79	1.81	1.81	1.83	1.87	1.95	1.84
Prostate gland absolute (mg)	892	968	745	582*	733	818	736	634
Prostate gland relative (%)	0.44	0.45	0.36*	0.31*	0.376	0.398	0.378	0.341
Epididymides absolute (g)	1.38	1.50	1.35	1.16*	1.310	1.329	1.349	1.097
Epididymides relative (%)	0.69	0.71	0.65	0.62*	0.668	0.654	0.693	0.59
Coagulation gland absolute (mg)	206	253	160	236	94.8	126.5	115.7	134.2
Coagulation gland relative (%)	0.095	0.119	0.077	0.125	0.048	0.062	0.059	0.073
Seminal vesicle absolute (g)	1.29	1.11	1.13	1.05	0.889	1.424	1.179	1.271
Seminal vesicle relative (%)	0.638	0.506*	0.569	0.528*	0.453	0.701	0.608	0.682
Testes wt < 3 g	0/23	1/24	2/24	4/23	1/18	1/18	0/19	1/6
Testicular atrophy bi-lateral	0/23	0/24	1/24	2/23	0/18	1/18	0/19	0/6
Testicular atrophy uni-lateral	0/23	0/24	1/24	2/23	0/18	0/18	0/19	1/6
Females	F0				F1			
Animals evaluated	20	20	20	9[#]	20	12	17	6
Uterus absolute (mg)	316	374	289	266*	407	406	380	390
Uterus relative (%)	0.167	0.193	0.154	0.148	0.218	0.213	0.198	0.202
Ovaries absolute (mg)	90.8	99.1	97.2	91.4	91.55	93.25	88.88	85.33
Ovaries relative (%)	0.048	0.058	0.052	0.051	0.049	0.049	0.048	0.045

* statistically significant by chi-square test ($p < 0.001$);
[#] mean uterus weight (abs/rel) for 11 non pregnant females: 540/0.276, no difference in ovary weight

Less than half of the F0 dams in the high dose group became pregnant and the body weight gains of those carrying a litter were decreased. The inconsistency with the embryotoxicity part of the study seems to be due to a selection of only pregnant females for cesarean section while non pregnant females remained in the littering group. When assessing both study parts together the pregnancy rate at the dose of 25 mg/kg bw/day is 29/40 (72.5 %), compared with 40/40 in the control group. No decrement in fertility was seen in the F1 parents but the number of animals available high dose group is clearly insufficient for a robust assessment.

In the high dose group decreases in the number of implantations and in the number of living neonates (only F1) were observed. This reduction in F1 litter size was accompanied by an increase in mean individual birth weights. Nevertheless, mean litter weight remained about 25 g below the control value and may thus explain the reduced maternal weight gain in the high dose group. No adverse effects on dam or litter parameters were found at lower doses. Adverse findings on litter parameters were restricted to the F0/F1 generation and could not be reproduced in the F1/F2 generation. This may be due to a chance effect, given the low number of animals tested, or it may indicate that only a subgroup of more resistant F1 conceptuses survived the intrauterine exposure to cyanamide.

Table 126: Reproductive performance and litter data

Dose level (mg/kg bw/day)	**0**	**2**	**7**	**25**	**0**	**2**	**7**	**25**
	F0				F1			
Females mated	20	20	20	20	20	12	17	6
Females pregnant	20	20	20	9	18	18	19	6
Pregnancy body weight gain (g)	106	130	124	63*	143	161	132	117*
Implantation sites (mean)	11.1	12.1	13.4	5.7*	12.5	13.7	13.0	11.7
Postimplantation loss (mean)	2.0	0.8	1.3	1.3	2.3	0.8	0.8	0.67
Stillbirths (mean)	0.3	0.3	1.0	0.1	0.15	0.25	0.06	0
Live pups (mean)	8.8	11.0	11.2	4.2*	10.1	12.6	12.2	11.0
Litter weight (mean, g)	56	73	69	31*	70	83	77	73
Pup birth weight (mean, g)	6.51	6.78	6.23	7.45*	7.03	6.61	6.48	6.77
Pup weight PND 14 (mean, g)	29.8	30.7	27.7	27.5	30.5	31.4	29.9	28.2
Pup weight PND 21 (mean, g)	45.7	46.8	42.7	43.9	48.5	47.4	45.3	44.0
* statistically significant by ANOVA followed by Duncan test/Kruskal-Wallis test followed by Man-Whitney U -test ($p < 0.05$)								

There was a slight effect on F1 and F2 pup body weight gain in the high dose group. However, the extent of the decrease in not considered adverse. Developmental and behavioural parameters exhibited only minor changes which are considered unrelated to treatment. A thourough evaluation of early postnatal development is not possible because relative age of the pups at testing can not be taken into account due to the lack of pregnancy duration data. Age at sexual maturation (vaginal opening) was not affected in female offspring. Data for males are not available as only testis descent but not preputial separation was examined. External and visceral anomalies were not observed at any dose level.

The study of Obach, R. and Rives, A. (1985) and Rives Ferriol, A. (1987) was reported as a publication in a scientific journal by Vallés, J. et al. (1987). A re-evaluation of reproductive toxicity of cyanamide under consideration of original study reports and relevant scientific literature was performed by Schilling (2009) and submitted by the notifier. The discussion and conclusion on the two generation reproduction-fertility study in rat (Obach and Rives, 1985; Rives-Ferriol, 1987) of this expert statement is presented in the following.

This study is considered as not acceptable for the use in risk assessment. It was not performed according to GLP and no analyses of test substance preparations were carried out. In addition, reporting, assessment and data presentation were not sufficient for a valid and complete interpretation of the reported findings.

The publication of Vallés et al. (1987) presents data that cannot be found in the original study by Obach and Rives (1985) or the related pathology report on effects of the low and mid dose level (Rives-Ferriol, 1987) on which the publication was based.

This study is considered supplementary but not suitable for risk assessment or for classification. One of the most important deficiencies was that the active ingredient content was cited as 60 mg/mL (i.e. about 6 %) but was reported as cyanamide at 0, 2, 7 and 25 mg/kg bw/day. The doses in terms of active ingredient used in this study cannot be retraced.

Group sizes in the results section differ from the data in the material and methods (reduced group sizes). The animal number of the raised F1 pups leading to the F1 generation parental animals is clearly too low for a reliable assessment of findings in all dose groups, especially in the high dose group with remaining 6 males/6 females. The same is true for the resulting low number in the F2 progeny, especially at the high dose of 25 mg/kg bw/day.

The replacement of treated females with untreated females due to reduced fertility was only stated in the publication, but is not documented in the original study report.

The systemic toxicity leads to a reduction of viability and probably caused a treatment related retardation of the sexual maturation of male animals. Hence resulting in a reduced number of pregnant females in the high dose group (25 mg/kg bw). Male fertility was reduced at the same dose level as systemic toxicity, therefore, it is considered as a secondary unspecific effect.

No reduction of relative testis weights (organ to brain weight ratios) was found for all dose groups of the F0 and F1 generation. Testicular atrophy (both bilateral and uni-lateral) was found in treated F0 males but the effect was not confirmed in F1 males. Therefore, the finding of testicular atrophy in F0 males cannot be considered as a sign for reproduction toxicity as the effect was neither increased nor at least reproduced in the F1 generation.

Under the conditions of this study the NOAELs for reproductive performance and fertility, parental toxicity as well as for developmental toxicity were 7 mg/kg bw/day. In conclusion, the author considers the study as not acceptable for the use in risk assessments.

Conclusion of the Rapporteur Member State (RMS):

The RMS agrees with the notifier that there are several discrepancies between the study report and the publication and within the study report itself. These relate almost exclusively to the number of animals used for specific tests. One important deficiency is that the number of surviving pups before culling on postnatal day 4 is not reported. Therefore, no additional data on early pup mortality are available from this study to compare to the study by Morseth (1990). Among the other

deficiencies noted in the evaluation submitted by the notifier the issue of dose preparation appears to be the most important. It is not described in this study report how dosing solutions were prepared from the 6 % cyanamide solution (Colme). However, in another study from this laboratory (Obach, R. et al., 1986) on pharmacokinetics of cyanamide in rats, the same batch (S-13) as in this study was used and it is stated there that the 6 % cyanamide solution was diluted further with distilled water to a concentration of 1.74 % parts per volume which was used for dosing the animals. The RMS therefore assumes that the dosage concentrations in this 2-generation study were prepared correctly and that doses of 2, 7 and 25 mg a.s. were administered to the animals on a per kg basis.

The treatment led to a decreased pregnancy rate in rats of the F0 generation at 25 mg/kg bw/day. Reproductive organ weights and histopathology of individual males cannot be used to elucidate this further since mating records were not included in the report. No clear effects on testis histopathology were noted. The few cases of testicular atrophy showed unilateral and bilateral expression with similar frequency and are not considered convincing evidence of a testicular toxicity. Female fertility was affected in the F0 generation at 25 mg/kg bw/day as indicated by a reduced number of corpora lutea and the decreased pregnancy rate. In addition, a high preimplantation loss and embryolethality were noted, leading to a decrease in live litter size. The NOAEL for parental, reproductive and offspring toxicity in this gavage study is 7 mg/kg bw/day.

Title: Koeter, H.W.M. et al. (1986): Oral two-generation reproduction study with an aqueous cyanamide solution (content 49 % w/w) in rats; Doc. No. 553-001; TNO, Zeist, The Netherlands; published: No

Guidelines: Not indicated, however the method used complies to a great extent to OECD 416

Deviations:
- No individual data given on precoital intervals, pregnancy duration, number of implantation sites, and external abnormalities in pups
- No data on pup growth between birth and litter size reduction on postnatal day 4

GLP: Yes; a GLP statement is included in the study report

Acceptability: The study is considered supplemental.

Materials and methods:

The test substance was identified as follows:

Cyanamide aqueous solution

Batch: Not indicated

Purity: 49 % cyanamide (w/w)

Appearance: Light yellow liquid

Specification: Not given

Cyanamide aqueous solution was administered to groups of 24 male and 24 female Wistar Cpb:WU rats (F0 parental generation) in the diet at concentrations of 0, 20, 60 and 180 ppm. Vitamin A was added to the diet at a concentration of 6339 IU/kg. The doses were selected upon the results of dietary studies in the same strain of rats (90-day toxicity study, Til, H.P. et al., 1975). The test substance preparations were analysed for stability and homogeneity. Concentrations were verified during the study period.

At the start of the study the age of the animals was about 6 weeks and the body weights ranged from 84.9 to 117.8 g in males and from 81.7 to 109.5 g in females. The rats were housed under conventional conditions in stainless steel cages, fitted with wire mesh floors and fronts, in a well-ventilated room at 22 ± 2 °C; humidity was at least 40 % and a 12 hour light-dark cycle was maintained. In the F0 generation 1 female was mated with 1 male for a period of maximum 3 weeks at least 70 days after the beginning of treatment to produce the F1a litter and subsequently re-mated to produce the F1b litter. The day of observation of sperm or the presence of a vaginal plug was considered day 0 of pregnancy. On day 4 after birth of the F1a generation, litters of more than eight pups were culled to 4 males and 4 females by random selection. At 21 days post partum the F1a litters were weaned and subjected to gross examination at autopsy. Two weeks after the weaning of the last F1a litters, the parents were mated again to produce the F1b litters. From the F1b pups, 24 males and 24 females/dose group were selected at weaning to produce the F2 generation (F2a and F2b litters). They received the test substance for at least 70 days before they were mated for the first time. The study terminated at weaning of the F2b litters.

The examination of parental animals included monitoring of clinical symptoms/mortalities, food consumption during the premating period and for females also during pregnancy, body weight development, reproductive performances, gross pathological inspection as well as histopathological examination of various tissues with special attention to the organs of the reproductive system and the thyroid gland. All pups were examined macroscopically at necropsy. In addition, the total number of pups born (dead and alive), the number of male and female pups and the number of pups showing abnormalities were determined. The pup weights were calculated by dividing the litter weight by the number of live pups.

Findings

It was shown that the test substance was not stable at room temperature for a period of 17 days. Therefore, fresh batches were prepared each week and stored at -20 °C until use. Correctness of dietary concentrations was proven by analytical determination at several intervals throughout the study. The test substance intake is given in Table 127.

Table 127: Mean test substance intake – 2-generation feeding study in Wistar rats (mg/kg bw/day)

Dose level (ppm)	0 (control)	20 ppm	60 ppm	180 ppm
F0 males	0	1.66	5.00	14.90
F0 females – pre-mating	0	1.85	5.64	16.79
F0 females – 1st pregnancy	0	1.49	4.50	13.91
F0 females – 2nd pregnancy	0	1.39	4.15	12.22
F1 males	0	1.49	4.78	13.90
F1 females – pre-mating	0	1.62	5.15	14.97
F1 females – 1st pregnancy	0	1.37	4.17	12.40
F1 females – 2nd pregnancy	0	1.28	3.88	11.54
Average (pre-mating F0/F1 animals)	**0**	**1.66**	**5.14**	**15.41**

Food intake was significantly decreased in the high dose group in both sexes during the pre-mating period. In the mid dose group during the first week (F0 generation) and during the first two weeks (F1 generation) a decrease in food consumption was also noted (Table 128). Marginal effects in the low-dose group are considered not to be toxicologically relevant.

In the F0 generation, body weights of both males and females during the pre-mating period were significantly lower in the high dose group when compared to the controls with first effects noticeable already at day 7. During the pre-mating period in the F1 generation decreased body weights were also observed in the high dose group (see Table 128). In both the F0 and the F1 generation, mean maternal body weights were significantly decreased in the high dose group when compared to the control during the first and second pregnancy as well as during lactation.

Table 128: Food intake and body weight development – 2-generation feeding study in Wistar rats

Dose level (mg/kg bw/day)	0	1.66	5.14	15.41
Food consumption (% of control)				
F0 males – pre-mating	100	102	98	91*
F0 females				
– pre-mating	100	94	96	89*
– 1st pregnancy	100	98	97	92*
– 2nd pregnancy	100	96	96	88*
F1 males – pre-mating	100	100	97	86*
F1 females				
– pre-mating	100	99	101	91*
– 1st pregnancy	100	98	97	87*
– 2nd pregnancy	100	101	99	89*
Body weight gain (g)				
F0 males – pre-mating	296.5	289.5	273.4	235.7*
F0 females				
– pre-mating	127.6	119.8	119.5	102.7*
– 1st pregnancy	119.5	120.5	116.2	110.6
– 2nd pregnancy	121.2	118.9	116.0	110.5
– 1st lactation	30.6	27.6	30.3	36.3
– 2nd lactation	13.7	17.1	25.5	28.3
F1 males – pre-mating	284.2	289.6	274.2	241.6*
F1 females				
– pre-mating	132.4	133.0	136.8	122.7*
– 1st pregnancy	111.1	114.2	107.9	91.7
– 2nd pregnancy	110.0	117.5	106.3	98.5
– 1st lactation	20.2	26.1	21.9	13.4
– 2nd lactation	17.2	11.3	15.7	26.7

* statistically significant by Dunett test ($p < 0.05$)

Mating and fertility were unaffected by dietary exposure to cyanamide including the highest dose level. No meaningful differences were observed in the F0 and F1 generations in the number of successful matings, the number of pregnancies carried to term and the duration of gestation. However, both the F0 and the F1 females in the high dose group had decreased mean litter sizes at birth during their first pregnancies and the pup weights at birth or at weaning were reduced in first

and second high dose litters. A slight postnatal growth reduction was also observed at the intermediate dose level. In addition, high dose offspring viability indices appeared reduced, especially during the neonatal period until postnatal day 4 (see Table 129).

The gross examination of the pups in the first and second litter of the F0 and F1 generation did not reveal any compound-related changes. No compound-related macroscopic changes were seen in pups killed on postnatal day 4 or in pups that died during lactation.

Table 129: Fertility and litter data of F0 and F1 generation – 2-generation feeding study in Wistar rats

Dose level (mg/kg bw/day)	0	1.66	5.14	15.41
F0 - 1st pregnancy				
Cohabitated pairs	24	24	24	24
Mated females	24	24	24	24
Pregnant females	24	22	23	23
Mean precoital time (days)	3.50	3.75	3.42	2.71
Mean duration of pregnancy (days)	21.9	21.7	21.6	21.7
Live litters	24	22	23	23
Live litter size at birth	11.3	11.0	11.2	10.1
Live litter size on postnatal day 4	11.0	11.0	10.0	9.3*
Pup viability index postnatal day 4	97	100	90	92
Weaning index (of pups alive on day 4)	97	100	98	96
Pup weight at birth (g)	6.0	6.2	5.7	5.7
Pup weight at weaning (g)	43.1	44.2	42.8	40.2
F0 - 2nd pregnancy				
Cohabitated pairs	24	24	24	24
Mated females	24	24	24	24
Pregnant females	22	18	21	22
Mean precoital time (days)	2.58	2.04	2.50	2.46
Mean duration of pregnancy (days)	21.8	21.7	21.6	21.6
Live litters	22	18	21	22
Live litter size at birth	10.4	9.9	10.2	10.5
Live litter size on postnatal day 4	10.0	9.7	9.2	9.7
Pup viability index postnatal day 4	93	97	90	92
Weaning index (of pups alive on day 4)	95	99	83	94
Pup weight at birth (g)	5.8	6.0	5.6	5.6
Pup weight at weaning (g)	48.3	48.9	44.1*	42.4**

Dose level (mg/kg bw/day)	0	1.66	5.14	15.41
F1 - 1st pregnancy				
Cohabitated pairs	24	24	24	24
Mated females	24	24	24	24
Pregnant females	21	22	24	22
Mean precoital time (days)	3.25	2.79	3.25	2.25
Mean duration of pregnancy (days)	22.0	21.9	21.8	21.7
Live litters	21	22	24	22
Live litter size at birth	9.8	10.0	9.6	8.2*
Live litter size on postnatal day 4	9.7	10.0	9.4	7.7**
Pup viability index postnatal day 4	99	100	98	93
Weaning index (of pups alive on day 4)	100	100	99	99
Pup weight at birth (g)	6.4	6.4	6.0	5.5**

Dose level (mg/kg bw/day)	0	1.66	5.14	15.41
Pup weight at weaning (g)	47.7	46.6	44.9	39.9**
F1 - 2nd pregnancy				
Cohabitated pairs	24	24	24	24
Mated females	24	23	24	24
Pregnant females	21	22	24	20
Mean precoital time (days)	1.96	2.96	4.21	2.67
Mean duration of pregnancy (days)	22.0	22.0	21.8	21.8
Live litters	21	22	24	20
Live litter size at birth	10.2	10.1	9.5	9.7
Live litter size on postnatal day 4	10.2	9.9	9.5	8.1*
Pup viability index postnatal day 4	100	98	100	80**
Weaning index (of pups alive on day 4)	99	94	100	92
Pup weight at birth (g)	6.1	6.2	6.1	5.6*
Pup weight at weaning (g)	46.6	46.6	48.9	42.2

* $p < 0.05$; ** $p < 0.01$

Relative thyroid weights of the F0 and the F1 parental animals were significantly increased at the high dose level. Absolute thyroid weights were also slightly above the values measured in the other dose groups and the controls. No relevant effects were observed regarding liver and testis weights in any of the generations. The results are summarised in Table 130.

Table 130: Mean absolute and relative organ weights of parental F0 and F1 generation – 2-generation feeding study in Wistar rats

Dose level (mg/kg bw/day)	0 (control)		1.66		5.14		15.41	
F0	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Liver (g)	15.56	9.22	17.34*	8.92	16.67	9.32	15.26	8.44**
Thyroid (g)	0.026	0.026	0.028	0.026	0.029	0.026	0.031*	0.028
Testes (g)	3.62	-	3.61	-	3.56	-	3.65	-
Liver (g/kg bw)	33.3	32.9	34.7	32.4	33.9	33.8	34.3	32.9
Thyroid (g/kg bw)	0.057	0.093	0.057	0.096	0.060	0.093	0.071**	0.110**
Testes (g/kg bw)	7.84	-	7.31	-	7.31	-	8.24	-
F1	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Liver (g)	16.25	9.34	17.04	9.35	16.52	9.73	15.87	8.59
Thyroid (g)	0.024	0.025	0.025	0.025	0.026	0.025	0.028	0.027
Testes (g)	3.72	-	3.73	-	3.66	-	3.65	-
Liver (g/kg bw)	32.1	33.2	32.5	33.0	33.2	34.2	35.6**	33.1
Thyroid (g/kg bw)	0.048	0.089	0.048	0.089	0.053	0.089	0.063**	0.106*
Testes (g/kg bw)	7.53	-	7.22	-	7.33	-	8.16	-

* statistically significant by Dunett test ($p < 0.05$)
** statistically significant by Dunett test ($p < 0.01$)

Gross examination of the F1 generation parental rats revealed small testicles and/or soft testicular tissue in several animals of the high dose group and in a few rats of the lower dose groups but not in the control (see

Table 131). In the F0 generation no such effects were observed.

Table 131: Gross pathological effects F1 generation – 2-generation feeding study in Wistar rats

Dose level (mg/kg bw/day)	0 (control)	1.66	5.14	15.41
Lesions/No. of animals	**24**	**24**	**24**	**24**
Testes [(a)]				
• translucent appearance	0	1	0	0
• watery tissue	0	0	0	2
• bi-lateral small	0	0	1	1
• unilateral small	0	1	0	3
• bi-lateral atrophy	0	1	0	0
• unilateral absent	0	0	0	1

(a) number of organs with macroscopic changes

Treatment-related histopathological changes in the thyroids (increased number of small follicles lined by cuboidal epithelium and reduction of colloid) were observed in male and female F0 and F1 animals in the high dose group. In the three treatment groups, test substance-related histopathological changes were observed for the testes (atrophic seminiferous tubules, complete unilateral or bilateral athrophy, interstitial cell proliferation, reduced amounts of spermatozoa in the epididymis) in the F1 generation. The effect became more severe in the intermediate and high dose groups as indicated by the increased number of affected tubules per testes. No such effects were observed in the control group. The results are summarised in Table 132.

Table 132: Histopathological effects F0 and F1 generation – 2-generation feeding study in Wistar rats

Dose level (mg/kg bw/day)	0 (control)		1.66		5.14		15.41	
	Males	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
No. of animals	**24**	**24**	**24**	**24**	**24**	**24**	**24**	**24**
Thyroid - activated F0 generation	1	2	2	3	3	3	8*	15**
Thyroid - activated F1 generation	5	1	5	1	4	1	18**	6*
Testes – tubular atrophy F1 generation	0	-	2*	-	6*	-	5*	-
Testes – interstitial cell proliferation F1 generation	0	-	0	-	2	-	6*	-

* statistically significant by Dunett test (* $p < 0.05$; ** $p < 0.01$)

The result of the peer review on the two-generation study performed by Koëter et al. (1986) is presented in the following (Weber & Creasy, 2009).

Individual animal lesions found during the peer review could not be correlated with the individual lesions recorded in the original report due to the fact that only hand-written summary tables are presented in the original report. However, the slides were labelled with their group number as well as animal number and so it was possible to compare the peer review diagnoses with the original diagnoses on a group level.

There were no testicular findings in any F0 animals. Table 133 presents the outcome of the re-evaluation of the F1 generation.

Individual animal data of the slide evaluation are presented in the peer review report. Figures are given as examples demonstrating slide quality and are representative for recorded lesions.

Table 133: Summarised data of re-evaluation results of testes from rats of TNO Study No. V 86.465/241475 (F1 generation)

Lesion	Group A (Control)	Group B (0.81 mg/kg)	Group C (2.52 mg/kg)	Group D (7.55 mg/kg)
No. rats examined	24	24	24	24
Spermatid retention/degeneration	0	1	0	0
Tubular dilatation	0	3*	3	0
Tubular degeneration/depletion	0	5	7	7

* 1 case with tubular degeneration/depletion

The diagnostic criteria for the terms used by the peer reviewer are defined as:

- Spermatid retention/degeneration: retention and phagocytosis of step 19 spermatids by Sertoli cell and degeneration of elongating spermatids (steps 12-19)
- Tubular dilatation: dilated tubular lumen with intact spermatogenesis but reduced epithelial height
- Tubular degeneration/depletion: partial or complete loss of spermatogenic cells often leaving tubules lined only by Sertoli cells.

There were no findings in the testes of control F1 animals but tubular dilatation and tubular degeneration/depletion were present in rats from the cyanamide treated groups. The absence of any similar findings in the F0 generation suggests the findings in the F1 animals are developmental in origin. Both findings in F1 animals, tubular dilation and tubular degeneration/depletion were sometimes unilateral and sometimes bilateral. The incidences did not follow a dose response.

A re-evaluation of reproductive toxicity of cyanamide under consideration of original study reports and relevant scientific literature was performed by Schilling (2009) and was submitted by the notifier. The discussion and conclusion on the two-generation reproduction study in rats performed by by Koëter et al. (1986) is presented in the following.

This study is considered as not acceptable for the use in risk assessment although it was performed according to GLP and complied to a great extent to the OECD 416 guideline requirements at that time. There was a lack of individual as well as historical control data and information on stability and homogeneity of the dose preparations. In addition, especially the reporting, assessment, interpretation and data presentation in the study report was considered as very limited. Therefore it is not possible to definitively relate any of the effects to the test item.

In fact this study was rejected by the Department of Pesticide Regulation of California, USA on December 3, 1993 (Nelson, 1993) due to the lack of individual data, inadequate description of the test material, and a limited number of dietary analyses with a problem of stability of the active substance and resulting in an inaccuracy in the calculation of dosages.

Therefore, the dietary levels of 0, 20, 60 and 180 ppm should be expressed as dose level ranges only (0, 0.53-1.39, 1.68-4.25, 5.01-12.14 mg/kg bw/day active substance, respectively). In the following, mean values of approximately 0, 0.81, 2.52, 7.55 mg/kg bw/day active substance, respectively, are used.

Dietary administration is not considered reliable compared to an application by gavage. Substance intake via the diet varies during certain phases as can be seen in the respective table for calculation and presentation of the mean substance intake.

The historical control data for tubular atrophy in the testes were considered as not sufficient since beside low study/animal numbers, primarily data of F3 animals were provided. Furthermore, it was noted that the supplier of the rats as well as the rat strain was changed (Woutersen & Bruijntjes, 2005).

A recently performed histopathological peer review (Weber & Creasy, 2009) emphasised that individual animal lesions found during the peer review could not be correlated with the individual lesions recorded in the original report due to the fact, that only hand-written summary tables are presented in the original report. However, the slides were labelled with their group number as well as animal number and so it was possible to compare the peer review diagnoses with the original diagnoses on a group level.

The peer review pathologists concluded that testicular changes were present in this study, where the test item was administered at much lower concentrations of 0, 0.81, 2.52 und 7.55 mg/kg (groups 1, 2, 3 and 4, respectively) in the diet. In this study, there were no lesions in the F0-Generation but there was a not dose-related incidence of tubular degeneration/atrophy and of tubular dilation in the testes of F1 males. The results of this study are not considered reliable on the basis that there was no dietary analysis of the test compound. Thus, the peer review pathologists came to the same conclusion as the Department of Pesticide Regulation of California, USA on December 3, 1993 (Nelson, 1993). The study is rejected and should not be used to assess the toxic potential of cyanamide. However, the oral gavage study (Morseth, 1990), which was conducted to acceptable standards (in accordance with FIFRA guidelines) should be used. The study demonstrated a lack of testicular toxicity in the F0 and F1 generations of a standard 2 generation rat reproductive toxicity study.

The dietary administration of cyanamide to Wistar rats in this study over two generations with two littering periods per generation had no effect on mating and fertility with the exception of a reduced litter size in the 1st F1 pregnancy at the high dose level. Among the various testicular findings recorded, only the increased incidence of F1 males with interstitial cell proliferation in the testes at the high dose level may be considered as substance-related effect and an indication of slightly impairment of fertility. With regard to possible effects caused by treatment one has also to consider that doses of 0, 0.81, 2.52, 7.55 mg/kg bw/day active substance in this study compared to of the previous study dose levels of 0, 1.25, 3.75 and 15.0 mg/kg bw/day pure active substance until termination, i.e. dose level at least twice as high, definitively no effects on the testicular tubules were noted at any dose level. Considering the lowest dose level of this study of 0.81 mg/kg bw/day active substance and the highest dose level of the previous study of 15.0 mg/kg bw/day, i.e. about 19fold difference, it is more than unlikely that substance-related testicular findings may have occurred even if one considers the difference in administration route or possible strain differences. The same holds true, if one considers that in the long-term study in Sprague-Dawley rats receiving dose levels of 0, 2.5, 7.5 and 30 mg/kg bw/day active substance for 16 weeks followed by 0, 1.25, 7.5 3.75 and 15.0 mg/kg bw/day active substance until termination, again no effects on the testes were noted at any dose level.

Further, observable differences between the treated F0/F1 parental animals and the concurrent controls were regarded to be incidental in nature and not of toxicological or biological concern.

Signs of parental toxicity in both generations (F0 and F1) were confined to the rats of the high dose groups. Systemic toxic effects consisted of reduced food consumption, lower body weights, and

retarded body weight gains. In addition, the thyroid gland was identified as the target organ due to increased weights and histopathological findings at this dose level.

It is concluded that substance induced signs of developmental toxicity were only observed in the progeny of the F1 parental generation in form of initially affected postnatal survival (F2 pups 2nd pregnancy) and reduced birth weights and retarded body weight gain until weaning in both litters in the high dose groups. In none of the F1 or F2 pups of each the 1st and 2nd pregnancy substance-induced morphological alterations were noted.

Consequently, the NOAELs for reproductive performance and fertility, parental toxicity as well as for developmental toxicity can be considered as 60 ppm (corresponding to 2.52 mg/kg bw/day active substance) under the conditions of this study.

Conclusion of the Rapporteur Member State (RMS):

The study is supplemental for the assessment of reproductive and developmental endpoints that are influenced by the direct exposure of the F0 and the F1 generation to cyanamide. It is the only study in which offspring are known to be exposed during the lactation period. With respect to the study deficiencies claimed by the notifier, the RMS with access to the individual data annexes from a national registration procedure has come to a different perception on the quality of report and study. Individual data are available for the relevant endpoints, including body weights, food consumption, litter data and toxicological findings. Food intake per kg body weight can be calculated for individual animals and dose groups for various phases of the study as accurately as this is possible for any other dietary study. As food consumption was not measured during lactation (as is the case in many dietary 2-generation studies) the intake of the dams during this period must be approximated by doubling the value calculated for pregnant females. The information given in the study report on the mean and variability of substance concentrations in 10 analysed samples taken from all dose levels at various time points (5-10 %) allows the conclusion that the animals were exposed to the nominal concentrations in the diet throughout the study. The reported frequency of diet preparation (weekly), diet storage (at -20 °C), and frequency of feed changes (every 2-3 days) indicates that the problem of test substance instability in feed was recognised and solved before the study was initiated. Therefore, it is possible to relate the observed effects to the exposure to the test substance and there is no greater uncertainty about the actual dose levels than there is normally in dietary studies of this kind. The only shortcoming is that the sponsor SKW Trostberg AG apparently failed to supply the batch identification to the contract laboratory that conducted the study. However, the description of the test substance as an aqueous solution of cyanamide (49 % w/w) corresponds to various batches with an active substance content of 520 g/L used in a number of other toxicity studies with cyanamide. A rejection of the dietary study as proposed by the notifier is not warranted.

In this 2-generation study in Wistar rats with dietary administration of cyanamide mating and fertility were found unaffected with the exception of a reduced litter size in the 1st pregnancies of high dose F0 and F1 dams (180 ppm, equivalent to 7.55 mg/kg bw/day active substance). Pup weights at birth and at weaning were decreased in each of the F1 and F2 litters of the high dose groups, even in 1st litters that had the nutritional advantage of lower litter sizes pre-culling. Neonatal viability was slightly reduced at the high dose only (viability indices of 80-93 % compared with 93-100 % in controls).

No testicular findings were recorded in the F0 males at any dose and in F1 males of the control group. There was an increased incidence of F1 males with interstitial cell proliferation at the high dose level and tubular atrophy was described in all treated groups. However, only a subset (25 %)

of the animals at risk for testicular lesions has been examined in this study, which may have been a reason for the lack of a dose-response. Therefore, the testicular findings were not considered to be a reliable basis for dietary or dermal risk assessment.

The histopathology findings have been confirmed by a recently performed peer review (Weber & Creasy, 2009). It is unclear why the peer reviewers were unable to correlate their findings with the individual lesions recorded in the original report as the individual data appendix of the study report by Koëter et al. contains a printout of the testicular lesions by animal number and dose group. When comparing these tables with the re-evaluation data, the RMS noted a very good agreement with respect to individual animals affected and severity of the changes.

Other signs of systemic toxicity were confined to the rats of the high dose groups in both generations (F0 and F1) and consisted of reduced food consumption, lower body weights, and retarded body weight gains. In addition, the thyroid gland was identified as a target organ based on increased weights and histopathological findings at this dose level.

The NOAELs for parental toxicity and reproductive performance are at 60 ppm (corresponding to 2.5 mg/kg bw/day active substance), while the NOAEL for developmental toxicity is 20 ppm (corresponding to 1.3 mg/kg bw/day active substance based on food intake of male offspring in the week after weaning as a surrogate for intake during late lactation phase) based on decreased pup weight at 60 ppm and above.

Title: Morseth, S.L. (1990): Two-Generation Reproduction Study in rats with aqueous hydrogen Cyanamide (50 % w/w); Doc. No. 543-001; Hazelton Laboratories America, Inc., Leesburg, USA; published: No

Guidelines: U.S. EPA 83-4 (1982), OECD 416 (1981)

Deviations: None

GLP: Yes; U.S. EPA, a GLP statement is included in the report

Acceptability: The study is considered acceptable**.**

Materials and methods:

The test substance was identified as follows:

Aqueous hydrogen cyanamide

Batch: 07/07/87

Purity: 50 % (w/w)

Appearance: Clear colourless liquid

Specification: See volume 4 of the Draft Assessment Report (confidential)

The test substance was analysed for purity, homogeneity and stability by the sponsor. Test substance preparations were examined for stability and homogeneity. Concentrations were verified during the study period.

Aqueous hydrogen cyanamide was administered to groups of 26 male and 26 female 7-week-old Crl:CD BR Sprague-Dawley rats (F0 parental generation) by gavage with concentrations of the active substance corresponding to 0, 2.5, 7.5 and 30.0 mg/kg bw/day for the first 12 weeks. From the beginning of week 12 (two weeks before pairing) the doses were lowered because of the severely decreased body weight gain of the high dose animals. The amended dose levels of 0, 1.25, 3.75 and 15.0 mg/kg bw/day were used throughout the remainder of the study. The vitamin A content of the feed has not been reported.

F0 males were dosed once daily for approximately 14 weeks prior to mating, and dosing continued until termination. F0 females were dosed once daily for approximately 14 weeks prior to mating and throughout the mating, pregnancy, lactation and post lactation periods. 1 female was mated with 1 male overnight for a period of up to 3 weeks to produce the F1 litter. From the F1 pups 26 males and 26 females/dose group were selected as F1 parental generation to produce the F2 generation following treatment with dose levels of 0, 1.25, 3.75 and 15.0 mg/kg bw/day for at least 14 weeks.

The examination of parental animals included monitoring for clinical symptoms/mortalities, food consumption, body weight development, mating and reproductive performances. Pathological examination was performed by gross inspection as well as by histopathological examination with special attention to the organs of the reproductive system. Pups were sexed, weighed and monitored with respect to their viability and growth. All pups were examined macroscopically at necropsy (external and organ findings), stillborn pups and pups that died intercurrently were additionally examined for any skeletal findings. Litter size reduction in litters with more than 8 pups was performed on postnatal day 4. Culled pups were sacrificed by intraperitoneal injection of sodium pentobarbital and examined for visceral abnormalities.

Results:

It was shown that the test substance was stable when stored refrigerated and its homogeneity was proven. Correctness of concentrations was proven analytically.

Signs of toxic effect in the high dose F0 males became apparent after 8 weeks of treatment and included rough hair coats and thin appearance. In addition, high dose males and females had substantially lower mean body weight gains compared to the control group. An effect on body weight was also evident in mid dose males and females during the pre-mating phase. Lower mean food consumption tended to parallel the reduction of body weight gain. Males of the F1 generation which was exposed to the reduced dose levels throughout exhibited lower food consumption and smaller body weight increments during the pre-mating phase at the dose of 15 mg/kg bw/day (see Table 134). A slight effect on body weight gain was seen in the mid dose males and high dose females but it was neither as consistent nor as severe. Clinical observations of the F1 animals did not reveal any evidence of a treatment-related effect. During pregnancy, body weight gain values in F1 generation in the high dose group were below the corresponding control mean values but were generally above the control values during lactation. Food consumption of the F1 animals showed lower values in the high dose group compared to the controls during pre-mating and pregnancy phases but not in the lactation period.

Table 134: Food intake and body weight development – 2-generation gavage study in Sprague-Dawley rats

Dose level (mg/kg bw/day)	**0**	**2.5 / 1.25**	**7.5 / 3.75**	**30 /15**
Food consumption (% of control)				
F0 males – pre-mating	**100**	**103**	**99**	**80***
F0 females				
– pre-mating	100	102	96	84*
– pregnancy	100	102	95	92*
– lactation	100	95	94	84*
F1 males – pre-mating	100	97	96	90*
F1 females				
– pre-mating	100	104	100	93*
– pregnancy	100	108	101	80*
– lactation	100	100	103	93*
Body weight gain (g)				
F0 males – pre-mating	287	273	252	130*
F0 females				
– pre-mating	135	133	119	70*
– pregnancy	130	143	139	111
– lactation	-1.4	0.6	3.0	25
F1 males – pre-mating	486	465	459	397*
F1 females				
– pre-mating	231	243	236	208*
– pregnancy	119	129	126	94
– lactation	1.4	-7.5	6.6	27

* statistically significant by Dunett test ($p < 0.05$)

Mating ability was unimpaired by treatment in the F0 and the F1 generation. Most females mated during the first oestrus and the distribution of matings that occurred at second oestrus or later did not indicate a test substance-related effect. Low fertility and gestation indices were obtained in the F0 animals at the high dose group. Although this may be related to the reduced state of health of these animals the finding was also present, although less notable, in the F1 generation. No substance-induced morphological abnormalities were found in the offspring. Neonatal survival (day 0 - 4) of the F1 and the F2 pups, however, was significantly lower in all treated groups compared to the control group. The investigators do not consider this to be treatment-related because of considerable variability in the historical data base. However, the historical data have not been provided for comparison and the reviewer considers the concurrent control to be more representative for the actual study conditions than historical control ranges.

F1 neonates had reduced birth weights in the mid and high dose group. Although the finding in the intermediate dose could be related to the higher mean live litter size the comparison with the low dose indicates that a test substance effect on foetal growth was present. Weaning weights were also lower in the mid and high dose groups. For the F2 pups at the high dose level a slight effect on birth weight was observed and the weights attained at weaning were marginally lower than in the control. Data are summarised in Table 135.

Table 135: Fertility and litter data of F0 and F1 generation – 2-generation gavage study in Sprague-Dawley rats

F0				
Dose level (mg/kg bw/day)	**0**	**2.5 / 1.25**	**7.5 / 3.75**	**30 /15**
Cohabitated pairs	26	26	26	26
Mated females	26	22	24	23
Not pregnant	6	6	6	11*
Pregnant females	20	20	20	15*
Precoital interval > 4 days (%)	70	86	73	75
Mean duration of pregnancy (days)	22.1	22.1	21.9	22.1
Total prenatal litter loss	0	0	0	3
Live litters	20	20	20	12
Live litter size at birth	12.85	14.75	14.40	10.67
Live litter size on postnatal day 4	13.00	13.26	12.90	8.33*
Pup viability index postnatal day 4	92	83**	88**	84**
Litters with pup mortality > 2 (day 1 - 4)	0	6 (30 %)	6 (30 %)	3 (25 %)
Weaning index (of pups alive on day 4)	92	85	87	88
Male pup weight at birth (g)	6.75	6.12	5.95	6.08
Female pup weight at birth (g)	6.40	6.2	5.7	5.7*
Male pup weight at weaning (g)	51.1	47.6	43.3*	41.5*
Female pup weight at weaning (g)	49.4	47.7	40.2**	41.5*

F1				
Dose level (mg/kg bw/day)	**0**	**1.25**	**3.75**	**15**
Cohabitated pairs	26	26	26	26
Mated females	25	24	26	23
Not pregnant	2	5	3	7
Pregnant females	24	21	23	19
Precoital interval > 4 days (%)	92	88	85	72
Mean duration of pregnancy (days)	21.8	22.0	21.9	22.0
Total prenatal litter loss	0	0	2	2
Live litters	23	20	21	17
Live litter size at birth	12.65	13.15	14.71*	11.53
Live litter size on postnatal day 4	11.65	11.20	12.75	9.24
Pup viability index postnatal day 4	93	87*	82**	81**
Litters with pup mortality > 2 (day 1 - 4)	2 (9 %)	3 (15 %)	7 (33 %)	4 (24 %)
Weaning index (of pups alive on day 4)	87	81	87	97
Male pup weight at birth (g)	6.47	6.58	6.48	6.25
Female pup weight at birth (g)	6.06	6.14	6.11	5.91
Male pup weight at weaning (g)	44.8	43.1	43.8	42.6
Female pup weight at weaning (g)	43.3	42.7	41.2	39.9

* $p < 0.05$; ** $p < 0.01$

Gross pathological and histopathological data from F0 and F1 adults did not reveal any significant treatment-related changes. The results for relevant male parameters are summarised in Table 136.

Table 136: Histopathological findings in testes of F0 and F1 males – 2-generation gavage study in Sprague-Dawley rats

Dose level (mg/kg bw/day)	0 (control)		1.25		3.75		15.0	
	F0	F1	F0	F1	F0	F1	F0	F1
No. of animals	25	26	0	0	1	0	26	26
Tubular degeneration	0	3	-	-	1	-	2	3
Hypospermia	0	1	-	-	1	-	0	1
Interstitial cell hyperplasia	0	0	-	-	-	-	0	1

In order to elucidate the question whether the fertility effects correlate with histological findings in testes and/or epididymides and/or prostate, an assessment of individual animal data of high dose group males (F0) was prepared by the notifier (Table 137): Males with histological findings in testes and/or epididymides and/or prostate did not show a different reproductive performance/success compared to animals without such indication of effects. According to the notifier, there is no indication for a causal link between effects on male reproductive organs and the observed reproductive toxicity.

Table 137: Assessment of individual animal data of high dose group males (F0)

No histopathological findings in testes, epididymides, prostate	Histopathological findings in testes, epididymides, prostate	
4	11	Successful mating
4	4	No successful mating

The result of the histopathology peer review on the Two-Generation study of Morseth et al. (1990) is presented in the following (Weber & Creasy (2009).

The animal allocation could not be followed by comparing slides and report. Following review of the testes from F0 animals, there were 2 animals showing tubular degeneration, and within the F1 cohort, 6 animals displayed this finding. That is consistent with the originally described findings in the control and high dose groups (F0: 0 vs. 2 animals affected out of 24/group, F1: 3 vs. 3 animals affected out of 24/group). These results do not suggest any effect of cyanamide on the testis.

Conclusion of the Rapporteur Member State (RMS):

This study is considered valid for the assessment of reproductive and developmental endpoints that are influenced by the direct exposure of the F0 and the F1 generation to cyanamide. Due to the unknown exposure scenario for pups during lactation the study may be less useful to assess effects on the developmental period between birth and weaning.

Cyanamide was systemically toxic when administered orally administered by gavage to Sprague-Dawley rats at doses of 15 or 30 mg/kg bw/day. The slightly lower weight gain (6 %) of the F1 parental males at the mid dose level of 3.75 mg/kg bw/day is not considered adverse. Reproductive performance and fertility was affected in F0 and F1 parental animals at the top dose group, resulting in a low fertility index in both generations and in an increase of total litter resorptions in the F0 generation. Gross pathology and histopathology revealed no adverse treatment-related effects and the histopathology assessment was confirmed by a recently performed peer review (Weber & Creasy, 2009).

Substance-induced developmental toxicity was observed for high dose F1 and F2 pups in form of lower litter size, birth weight and body weight gain. Based on the percentage of total pups affected, a reduced survival during early postnatal life was seen for F1 and F2 neonates in all dose groups although a clear dose-relationship was only observed in the F2. A litter-based assessment showed

for both generations that the number and percentage of litters affected by mortality of two or more neonates increased in all cyanamide dose groups as compared to the respective control groups (F1: 2/20, 7/20, 7/20, 5/12; F2: 2/23, 5/20, 7/21, 6/16). In none of the F1 or F2 pups substance-induced morphological alterations were noted by external and visceral inspection.

Table 138: Pup deaths postnatal day 0-4

Dose level (mg/kg bw/day)	**0**	**1.25**	**3.75**	**15**	**0**	**1.25**	**3.75**	**15**	**0**	**1.25**	**3.75**	**15**
	F1				**F2**				**F1+F2 combined**			
% of all liveborn pups	3.9	14.6	10.4	21.9	7.9	7.2	17.5	18.9	6.0	11.1	14.1	20.1
% of litters (mean)	7.6	17.4	11.6	15.8	7.0	13.1	17.8	18.0	7.3	15.3	14.8	17.1
Litters with >1 pup dead (N and %)	2/20 (10)	7/20 (35)	7/20 (35)	5/12 (41.7)	2/23 (8.7)	5/20 (25)	7/21 (33.3)	6/16 (37.5)	4/43 (9.3)	12/40 (30)	14/41 (34.1)	11/28 (39.3)

The parental NOAEL and the NOAEL for reproduction were 3.75 mg/kg bw/day, while the overall NOAEL for developmental toxicity was <1.25 mg/kg bw/day based on increased pup mortality.

4.11.1.2 Human information

Medical surveillance on manufactoring plant personnel, which also included special investigations of functional disorders regarding the testes and the thyroid gland and potential sensitising properties, did not reveal any signs of diseases or health impairments caused by cyanamide.

In a medical examination it was investigated if there are effects of cyanamide exposure on the testes and the thyroid gland. According to this investigation disturbances of the gonadal function and the thyroid function can be excluded (Mertschenk et al, 1993).

For further details see section 4.12.1.6 “Human information”

Cyanamide in alcohol therapy

Calcium cyanamide has been worldwide intensively used as drug to deter drinking in alcoholics. Intolerance reactions towards alcohol occur in man after daily cyanamide doses higher than 20 mg. In general daily doses of more than 0.4 – 1 mg/kg bw cyanamide have been used in the alcohol aversion therapy. The duration of the treatment ranges from a few months to a few years. In some cases patients have taken cyanamide for more than 10 years. No signs of reproductive disorders have been observed.

For further details see section 4.12.1.6 “Human information”

4.11.2 Developmental toxicity

4.11.2.1 Non-human information

Developmental toxicity study in rats

Title: Morseth, S.L. (1989): Rat teratology study with aqueous hydrogen cyanamide; Doc. No. 551-002; Hazelton Laboratories America Inc., Leesburg, USA; published: No.

Guidelines: U.S. EPA 83-3 (1982)

Deviations: None

GLP: Yes; a GLP statement is included in the final report.

Acceptability: The study is considered acceptable**.**

Materials and methods:

The test substance was identified as follows:

Aqueous hydrogen cyanamide

Batch: 07/07/87

Purity: 53 % w/v

Appearance: Clear, colourless liquid

Specification: See document J

Aqueous hydrogen cyanamide was examined for potential maternal and embryotoxic effects in rats. A total of 100 female Crl:CD BR Sprague-Dawley rats were mated and randomly assigned to one of 4 test groups (25 animals per test group). The animals received the compound in distilled water by gavage from day 6 through 15 of gestation. The dose levels were 0 (sham treated control), 10, 30 and 90 mg/kg bw/day, corresponding to 0, 5, 15 and 45 mg/kg bw/day of pure active substance at a dose volume of 10 mL/kg. All animals were observed twice daily for mortality and moribundity as well as for clinical symptoms. Body weight and food consumption were recorded at appropriate intervals. On day 20 all surviving animals were sacrificed for Caesarean section. Foetuses were weighed, sexed and examined for external, skeletal or soft tissue anomalies and developmental variations.

Table 139: Dosing scheme of study

Group	Number of females	Dosage concen-tration (mg/mL)	Dosage volume mL/kg/day	Dosage level mg/kg bw/day	Dose of active substance mg/kg bw/day
1*	25	0	10	0	0
2	25	100	10	10	5
3	25	300	10	30	15
4	25	900	10	90	45

* treated with the vehicle (distilled water) only

Findings:

The stability of the test substance preparation and accuracy of dose levels applied were proven.

All animals survived until the scheduled sacrifice. Following the first two applications, some of the mid and high dose group animals were hypoactive. After application of the third dose this effect was not seen anymore.

A dose-related effect was obtained in mean body weight gain and mean food consumption. Mean absolute body weight values were significantly lower in the mid and high dose group compared to the corresponding control. The mean maternal body weight change was significantly lower than the corresponding values for the control group for all treated groups during gestation day 6 - 8, 6 - 16 and 6 - 20. Net maternal body weight gains on day 20 were significantly lower relative to the control in the mid and high dose group. This is paralleled by the reduced food consumption in these groups. No compound-related gross-pathology findings were observed. The mean gravid uterine weight value for high dose animals was significantly decreased, reflecting the lower foetal weights in this group.

At caesarean section the pregnancy rate was 100 % for all groups. One high dose female lost its entire litter (14 embryos) due to resorption. When this single litter loss is disregarded the number of embryofoetal resorptions was not different between the dose groups. Mean number of corpora lutea and implantations, preimplantation loss were similar for all groups. The percentage of early, late and total resorptions, live foetuses and male foetuses were similar in the control and compound-treated groups. Maternal and litter results are summarised in Table 140.

Table 140: Maternal and litter data – embryotoxicity study in rats

Dose level (mg/kg bw/day)	0	5	15	45
Females on study	25	25	25	25
Females with hypoactivity	0	0	8	8
Pregnant dams	25	25	25	25
Dams with total litter loss	0	0	0	1
Body weight gain day 6 - 16 (g)	50.2	40.9*	31.9*	6.2*
Body weight gain day 6 - 20 (g)	105.8	94.2*	81.4*	51.0*
Food consumption day 6 - 16 (% of control)	100	96	89*	76*
Food consumption day 16 - 20 (% of control)	100	96	92*	77*
Carcass weight (g)	287.0	280.6	265.2*	243.9*
Net weight change from day 0 (g)	53.1	47.8	37.9*	15.5*
Gravid uterus weight (g)	77.5	75.3	69.0	61.4*
Corpora lutea (mean)	17.4	17.3	16.9	16.9
Implantation sites (mean)	14.7	14.5	13.9	14.2
Live litter size (mean)	14.2	14.1	13.3	13.8
Viable foetuses (%)	100	100	100	96
Dead foetuses (%)	0	0	0	4
Early resorptions (total)	12	9	11	26
Late resorptions (total)	1	0	3	0
Post implantation loss (mean %)	3.6	2.3	3.7	7.3

Adjusted and unadjusted mean foetal body weight values at the high dose group were significantly lower than the corresponding control values. The slight reduction in mean foetal weight at 15 mg/kg bw/day is considered to be a substance-related effect because of the lower mean litter size in this group which would be expected to be associated with an increase in foetal weight had the development been unaffected. No external abnormalities of the foetuses were observed. Visceral evaluation revealed a significantly increased incidence of diaphragmatic hernia in the high dose group. This is considered to be related to the inhibitory effect of cyanamide on aldehyde

dehydrogenases, in this case retinal dehydrogenase 2 (Raldh2) which is the major retinoic acid-synthesising enzyme. Deficiencies in retinoic acid signalling are associated with diaphragmatic hernias in rodents and possibly humans (Greer et al., 2003). In addition, skeletal malformations, mainly of the vertebrae, were noted in a few foetuses of this group. Variations related to a less advanced state of general ossification and to possible interference with the process of rib ossification were present to a greater extent in the high dose group and correspond to the reduction in foetal weight. These variations included unossified hyoid body, incomplete ossification of the skull, bipartite vertebral centra, incomplete ossification of vertebral arches, less than four caudal vertebrae ossified, unossified sternebrae, incomplete ossification of the sternebrae, 14th rudimentary ribs, wavy or bent ribs and unossified pubes.

The results are summarised in Table 141.

Table 141: Foetal effects – embryotoxicity study in rats

Dose level (mg/kg bw/day)	0	5	15	45
Number of litters (foetuses) evaluated	25 (355)	25 (353)	25 (333)	24 (330)
Mean foetal weight (g)	3.26	3.19	3.13	2.84*
Foetal abnormalities: litter incidence (foetal incidence)				
Diaphragma hernia	0	0	0	5 (7)*
25 presacral vertebrae	0	0	0	1 (1)
27 presacral vertebrae	0	0	0	1 (1)
Vertebral centrum bipartite	2 (2)	4 (4)	4 (4)	8 (12)*
Hemicentrum	0	0	0	3 (3)
Less than four sacral vertebrae ossified	0	0	0	2 (3)
Sacral vertebra absent	0	0	0	1 (1)
Wavy/bent ribs	2 (3)	2 (2)	2 (2)	11 (21)*
Thickened ribs	1 (2)	2 (3)	0	7 (9)
Sternebrae 1 - 4 unossified	2 (2)	3 (3)	4 (5)	8 (19)*
Sternebrae 1 - 4 incompletely ossified	1 (1)	2 (2)	7 (11)	11 (16)*

* statistically significant by Dunnett´s test ($p < 0.05$)

Conclusion:

The maternal NOAEL in rats is below 5 mg/kg bw/day pure active substance cyanamide. This is based on a 20 % reduction in mean body weight gain during the treatment period which resulted in a net weight increase at caesarean section which was 10 % lower than that of the control group.

The NOAEL for embryo-/foetotoxicity is 5 mg/kg bw/day of pure active substance cyanamide based on the reduced foetal weights in the presence of a lower mean litter size. Malformations (diaphragmatic hernia and vertebral malformations) were observed at 45 mg/kg bw/day, a dose with considerable maternal toxicity.

Developmental toxicity study in rabbits

Title: Koeter, H.W.M. (1989): Oral embryotoxicity/teratogenicity study with an aqueous cyanamide solution (content 49 %) in New Zealand White Rabbits; Doc. No. 551-001; TNO-CIVO Toxicology and Nutrition Institute, Zeist, The Netherlands; published: No.

Guidelines: Not indicated, however the method described complies to a great extent to OECD 414

Deviations: None

GLP: Yes; a GLP statement is included in the final report

Acceptability: The study is considered acceptable**.**

Materials and methods:

The test substance was identified as follows:

Cyanamide

Batch: 250185

Purity: 49 %

Appearance: Light yellow liquid

Specification: See document J

Cyanamide was examined for potential maternal and embryotoxic effects in rabbits. 24 to 25 artificially inseminated New Zealand White rabbits per test group received the compound in distilled water via intraoesophagic intubation from day 6 through 19 of pregnancy. The dose levels were 0 (sham treated control), 4, 12 and 36 mg/kg bw/day at a dose volume of 2 mL/kg, corresponding to 0, 2, 6 and 18 mg/kg bw/day pure active substance cyanamide. The animals were observed for clinical symptoms and body weight was recorded at appropriate intervals. On gestation day 29 all surviving animals were sacrificed for caesarean section. All foetuses were weighted, sexed and examined for external, skeletal and soft tissue anomalies and developmental variations.

Table 142: Dosing scheme of the study

Group	Number of females	Dosage concen-tration (mg/mL)	Dosage volume mL/kg/day	Dosage level mg/kg bw/day	Dose of active substance mg/kg bw/day
1*	25	0	2	0	0
2	24	2	2	4	2
3	24	6	2	12	6
4	24	18	2	36	18

* treated with the vehicle (distilled water) only

Findings:

The homogeneity of cyanamide in aqueous medium has been demonstrated (Osheroff, M.R., 1991, Doc-No. 537-002). No data on stability of the test substance preparation and accuracy of dose levels applied are given in the report.

One control group female died after a premature delivery. Premature birth also occurred in 2 does at 6 mg/kg bw/day. One high dose female died and another had a total litter resorption. These effects were not considered to be substance related due to their low incidence and lack of dose-relationship. During the study no abnormalities in condition or behaviour were observed that could be ascribed to treatment in maternal animals. During the treatment period, a moderate weight loss was observed in the high dose group compared to the corresponding control group. No significant differences were

observed in food consumption. At autopsy no treatment-related gross observations were obtained. Both the fertility and gestation indices were within the normal range.

There were no treatment-related changes in the number of corpora lutea, number of implantation sites and pre-implantation loss. In the high dose group incidences of early resorptions and dead foetuses, and consequently the post-implantation loss, were slightly increased. Due to this increase in embryo-/foetolethality, the mean number of live foetuses was slightly lower in this group. No such effects were observed at lower doses. The maternal and litter results are summarised in Table 143.

Table 143: Maternal and litter data – embryotoxicity study in rabbits

Dose level (mg/kg bw/day)	0	2	6	18
Females on study	25	24	24	24
Females that died	1	0	0	1
Pregnant females	23	20	23	20
Does with total litter loss or preterm birth	1	0	2	1
Does with live litters at caesarean section	22	20	21	18
Body weight gain day 6 - 19 (g)	108.7	119.5	85.7	-10.3
Body weight gain day 0 - 29 (g)	268.0	315.5	218.3	77.1
Food consumption day 6 - 19 (% of control)	100	106	108	93
Food consumption day 19 - 29 (% of control)	100	108	102	102
Gravid uterus weight (g)	482	436	429	407
Corpora lutea (mean)	11.1	9.9	9.4**	10.1
Implantation sites (mean)	8.9	8.4	7.7	8.3
Live litter size (mean)	8.2	7.2	7.1	6.8
Viable foetuses (%)	98	95	98	93
Dead foetuses (%)	2	5	2	7
Early resorptions (total)	8	9	7	17
Late resorptions (total)	4	6	5	3
Litters with > 2 resorptions or dead foetuses	1	2	1	5
Post implantation loss (mean %)	8.0	13.9	10.0	20.2

A tendency to a somewhat lower mean foetus weight was observed at 18 mg/kg bw/day which in conjunction with the somewhat reduced mean litter size indicates embryo-/foetal growth retardation at this dose. Macroscopic examination of foetuses also revealed a relatively high number of small foetuses.

An increased number of minor eye anomalies occurred at the mid and high dose group. This increase in the occurence of retinal folds is considered to be a consequence of the inhibitory effect of the test substance on aldehyde dehydrogenases. In the developing eye, this could affect retinal dehydrogenases (possibly Raldh2) which are necessary for the local formation of retinoic acid needed for retina invagination (Mic et al., 2004). Since only 50 % of the foetuses in each litter have been evaluated for eye anomalies there is considerable uncertainty about the NOAEL for this effect and about the real incidence in the treated groups. The more frequent observation of litters with two or more affected foetuses and the concomittantly increased severity from predominantly unilateral to almost equal numbers of bilateral defects are taken to indicate that the dose of 6 mg/kg bw/day is a LOAEL. An increased incidence of several minor anomalies (small meningial haemorrhages and/or haemorrhages of the olfactory bulb as well as the incidence of haemorrhagic gallbladder appendices) were significantly increased at the highest dose level. In addition, focal disintegration of the liver structure was observed in the top-dose group only.

Minor skeletal anomalies and skeletal variants were observed in several control foetuses as well as in the different test groups. However, no indication of any treatment-related effects was observed. The foetal results are summarised in Table 144and Table 145.

Table 144: Foetal effects – embryotoxicity study in rabbits

Dose level (mg/kg bw/day)	0	2	6	18
Number of litters (foetuses) evaluated	22 (181)	20 (144)	21 (149)	18 (130)
Mean foetal weight (g)	40.4	40.7	42.3	38.8
Foetal abnormalities: litter incidence (foetal incidence)				
Placental focal fibrosis	0	4 (5)	3 (7)	3 (3)
Small foetus	4 (8)	3 (3)	3 (4)	4 (15)
Diaphragma hernia	0	1 (1)	0	0
Retinal folds (unilateral)	7 (7)	6 (7)	6 (7)	7 (10)
Retinal folds (bilateral)	1 (1)	2 (2)	5 (7)	4 (7)
Retinal folds (uni- or bilateral)	8 (8)	8 (9)	9 (14)	9 (17)*
Meningial/olfactory bulb haemorrhage	5 (5)	5 (7)	7 (8)	6 (10)*
Gallbladder haemorrhagic appendix	8 (11)	8 (13)	8 (13)	8 (17)*
Liver focal desintegration	0	0	0	3 (6)*

* statistically significant by Dunnett´s test ($p < 0.05$)

Table 145: Eye abnormalities – embryotoxicity study in rabbits

Dose level (mg/kg bw/day)	0	2	6	18
Number of litters (foetuses) evaluated	22 (94)	20 (77)	21 (79)	18 (70)
Number of litters with				
0 affected foetuses	14	12	12	9
1 affected foetus	8	7	6	5
2 affected foetuses	0	1	1	1
3 affected foetuses	0	0	2	2
4 affected foetuses	0	0	0	1

A re-evaluation of reproductive toxicity of cyanamide under consideration of original study reports and relevant scientific literature was performed by Schilling (2009) and submitted by the notifier. The discussion and conclusion on the developmental toxicity studies in rabbits by Koëter and Marwijk (1989) of this expert statement is presented in the following.

This study is assessed as appropriate and valid since it complied to internationally accepted testing guidelines at that time and was performed according to GLP. The reporting and data presentation in the study report was considered appropriate. Conclusion on maternal findings is considered appropriate since concurrent control values and historical control data (Woutersen & Bruijntjes, 2005, Doc. No. 533-004, IIA 5.6/07, TOX2007-437) were taken into consideration. However, the evaluation of fetal findings is considered as somehow superficial since especially fetal morphology findings were evaluated disregarding historical control data, possible preparation artefacts (e.g. as it is indicated for eye anomalies, especially for retina folds) or missing dose-dependency. In addition, the terminology used and the classification of fetal morphological findings do not correspond to the current terminology (e.g., the cited minor anomalies in soft tissue or skeleton are currently classified as “unclassified observations” or variations).

Cyanamide was administered by gavage to artificially inseminated New Zealand White rabbits from implantation at GD 6 through gestational day 19 at dose levels of 0, 2, 6 and 18 mg/kg bw/day pure active substance.

Signs of maternal toxicity occurred only at the high dose level in form of losses in body weight and adjusted body weight during the period of substance application. In addition, the total litter loss at this dose level, due to resorption, could be also an indication for maternal stress. At the high dose level an influence on gestational parameters consisted of an increase in early resorption leading to a higher post-implantation loss.

Prenatal developmental toxicity was noted at the high dose level by increases in dead and smaller fetuses, lower number of pups and fetal body weight as well as due to an increase in some soft tissue findings currently classified as unclassified observations.

Among the soft tissue findings, minor eye anomalies in form of retina folds were observed in all groups including the control group. However, an increased incidence was only recorded, if one considers the bi-lateral occurrence at the mid and high dose level. However, in respect to the uni-lateral or combined uni- and bilateral incidences of all groups including the control group, no clear dose-related effect could be noted due to the scattered occurrence. A possibility for the predominant "occurrence" of retinal folds only in treated rabbits could be explained by a biased investigator as the soft tissue examinations were not performed "blinded" (this means without knowing the dosing of the respective groups). Moreover, in the additionally supplied historical control data it is cited that this effect was probably not scored in former times.

In addition, not only according to the IFTS (International Federation of Teratology Societies) (http://www.ifts-atlas.org/ifts/search-index.html) but especially experienced laboratories and investigators assess this finding currently as a processing artefact. For example, in their survey on spontaneous incidences of endpoints from prenatal developmental toxicity studies in rabbits, Viertel and Trieb (2003) omitted retina folds because some of them were subsequently found to be artefacts due to Bouin's fixation.

A most recent publication (French et al., 2008) pointed out that in the rabbit fetus, "slight retinal folding" is commonly observed. Therefore, they used magnetic resonance imaging (MRI) to assess rabbit retinal architecture in fresh specimens, which was then reassessed following Bouin's fluid fixation. No retinal folding was detected in fresh specimens but it was observed in a majority of fetuses post-fixation. The use of Davidson's fixative followed by Bouin's fluid showed a markedly lower incidence of "slight retinal folding". Finally, the authors concluded that slight retinal folds in the rabbit fetus are likely artifactual and can be reduced using Davidson's fixation prior to Bouin's.

It can be concluded that retina folds are not treatment related, but processing artefacts as Bouin´s fixation was used. Generally retina folds can not be clearly differentiated from processing artifacts. This is supported by a relatively high incidence in the control group for uni-lateral and combined incidences, and a lack of dose response-relationship for the unilateral and combined incidences.

A treatment related effect on the fetal skeleton was not observed and there was no indication for teratogenicity up to and including the high dose level.

The author concluded that the NOAEL for maternal toxicity and prenatal developmental toxicity is 6 mg/kg bw pure active substance.

Conclusion of the Rapporteur Member State (RMS):

In rabbits dosed with 0, 2, 6 and 18 mg/kg bw/day cyanamide caused maternal toxicity in the high dose group. Body weight loss and a decrease in body weight gain during the period of substance application was observed. One female had a total litter resorption. A reduction in gravid uterine weight was noted as a consequence of lower live litter sizes and marginally reduced foetal weights. On the foetal level this prenatal developmental toxicity in the high dose group presented as embryo-

and foetolethality and a higher prevalence of small foetuses. A number of soft tissue changes have been reported. Among these findings, minor eye anomalies (retina folds) were observed in all groups including the control group. A dose-related increase in severity was suggested by the distribution of unilateral and bilateral observations. However, a recently published study comparing the retinal structure of control rabbit foetuses by magnetic resonance imaging of the fresh specimen with their state after Bouin's fixation provides evidence that slight retinal folds are rather a processing artefact than a treatment-related effect (French, J. et al., Retinal folding in the term rabbit fetus - Developmental abnormality or fixation artifact? Reproductive Toxicology 26, 262-266, 2008). Taking this new information into account, no indication for teratogenicity was detected in rabbits up to and including the high dose level. The NOAEL for maternal toxicity and prenatal developmental toxicity in rabbits is 6 mg/kg bw pure active substance.

4.11.2.2 Human information

Medical surveillance on manufactoring plant personnel which also included special investigations of functional disorders regarding the testes and the thyroid gland and potential sensitising properties, did not reveal any signs of diseases or health impairments caused by cyanamide.

In a human study it was investigated if there are effects of cyanamide exposure on the testes and the thyroid gland. According to this investigation, disturbances of the gonadal function and the thyroid function can be excluded (Mertschenk et al, 1993).

For further details see section 4.12.1.6 “Human information”

Cyanamide in alcohol therapy

Calcium cyanamide has been worldwide intensively used as drug to deter drinking in alcoholics. Intolerance reactions towards alcohol occur in man after daily cyanamide doses higher than 20 mg. In general daily doses of more than 0.4 – 1 mg/kg bw cyanamide have been used in the alcohol aversion therapy. The duration of the treatment ranges from a few months to a few years. In some cases patients have taken cyanamide for more than 10 years. No signs of reproductive disorders have been observed.

For further details see section 4.12.1.6 “Human information”

4.11.3 Other relevant information

4.11.3.1 Proposed mechanism of action:

Mechanistic considerations were discussed by the notifier as follows (Schilling, 2009):

The evaluation below is related to questions raised in respect to a possible mechanism of action comparable to nitrofen exposure, influence of ALDH1 family enzymes or impact on vitamin A metabolism.

Cyanamide is known to inhibit aldehyde dehydrogenase (ALDH) and is therefore used as a drug to deter alcoholism. ALDH is not a single enzyme but comprises an enzyme family with several tissue and substrate-specific isoenzymes.

Additional references were cited and provided within the DAR to support the hypothesis that some reproductive and developmental effects may be a consequence of an interference of cyanamide with vitamin A homoeostasis and metabolism since the metabolite retinoid acid (RA) is known to be required for spermatogenesis, favorable female reproduction and for embryonic differentiation and development. It was proposed in the DAR that the effects observed when exposing rats and rabbits to cyanamide could be a consequence of the inhibition of (one of the) RA producing enzyme(s) leading to a deficiency in locally required RA-signalling.

In the DAR it is referred to publications concerning the model substance nitrofen in respect to the induction of congenital diaphragmatic hernia due to impaired vitamin A metabolism and others, investigating functions as well as alterations in specific enzyme systems and their expression during vitamin A metabolism important for spermatogenesis, female fertility and ocular development (Akmal et al., 1997; Allan & Greer, 1997; Babiuk et al. 2004; Greer et al., 2003; Maly et al., 2003; Mey et al. , 2003; Mic et al. 2004; Thebaud et al., 1999; Vermot et al, 2000).

In the DAR it is mentioned that Nitrofen and three other teratogens which induce diaphragma defects in rodents have been shown to inhibit Raldh2 *in vitro* with a good correlation between teratogenic potency and inhibitory activity (Mey et al., 2003). Based on this it was proposed for cyanamide to follow the same mode of action.

Among ALDH1 three retinaldehyde dehydrogenase genes (Raldh1/ALDH1A1, Raldh2/ALDH1A2, and Raldh3/ALDH1A3) are expressed in unique spatiotemporal patterns.

Molotkov and Duester (2003) revealed genetic evidence that retinaldehyde dehydrogenase Raldh1 (Aldh1a1) functions downstream of alcohol dehydrogenase Adh1 in metabolism of retinol to retinoic acid. Previous genetic studies have revealed that alcohol dehydrogenase Adh1 is required for efficient clearance of excess retinol to prevent toxicity, thus demonstrating that the mechanism involves oxidation of excess retinol to retinoic acid (RA). Whereas Adh1 plays a dominant role in the first step of the clearance pathway (oxidation of retinol to retinaldehyde), it is unknown what controls the second step (oxidation of retinaldehyde to RA). Genetic evidence was presented that aldehyde dehydrogenase Aldh1a1, also known as retinaldehyde dehydrogenase Raldh1, plays a dominant role in the second step of retinol clearance in adult mice. Thus, it can be concluded that the cytosolic ALDH1 is of key importance for the metabolism of retinol.

There are 2 major ALDH isozymes in the liver: cytosolic ALDH1 and mitochondrial ALDH2.

ALDH2 plays no key role in the metabolism of retinol according to Wang et al. (2002).

Investigations by Marchner and Tottmar (1978) showed that the mitochondrial ALDH2 (low Km ALDH) was clearly inhibited even with low cyanamide concentrations. However, cyanamide, in concentrations usually applied during the alcohol-aversion therapy, had practically no influence on ALDH1 (high Km ALDH). Consequently there is still no evidence that cyanamide impacts retinol metabolism.

Thus, it is concluded that the nitrofen associated mechanism does not apply for cyanamide.

The role of Vitamin A in mammalian reproduction and embryonic development was comprehensively reviewed by Clagett-Dame and DeLuca (2002). In this review it was clearly demonstrated that a variety of congenital defects belong to the so-called “vitamin A-deficiency syndrome”. The main effects of this syndrome consist of ocular abnormalities, the most obvious effect being open instead of closed eyes upon gross examination. In addition, embryonic defects occur in the genitourinary tract, kidneys, diaphragma (herniated), lung (agenesis or rudimentary), aortic arch and heart.

None of the above described effects indicative for vitamin A deficiency were observed in reproductive toxicity studies (Morseth, 1990, Koëter, 1986) or the fetuses of the prenatal developmental toxicity studies (Morseth, 1989, Koëter and Marwijk, 1989), with the exception of diaphragma hernia in rat fetuses at an excessive maternal toxic dose level.

The notifier (Schilling, 2009) considered inappropriate to assume a specific mode of action only based on the occurrence of solitary effects, especially, when different species are involved and there is no clear treatment-relationship.

Based on differential ALDH isoenzyme inhibition of cyanamide compared to nitrofen and also based on different effect pattern of cyanamide compared to Vitamin A deficiency syndrome the notifier (Schilling, 2009) finally concluded that the mechanism of action proposed in the DAR is not scientifically sound.

Conclusion:

Both the notifier and the RMS agree that cytosolic ALDH1 isozymes (Raldh1/ALDH1A1, Raldh2/ALDH1A2, Raldh3/ALDH1A3) are important for the synthesis of retinoic acid (RA) from retinaldehyde. While Raldh1 (Aldh1a1) appears to be a major enzyme for this function in adult liver of mice (Molotkov and Duester, 2003), Raldh2 (ALDH1A2) is reported to be the major ALDH1 enzyme in the embryo (Clagett-Dame and DeLuca, 2002). Raldh2$^{-/-}$ mice die in utero before embryonic day 10.5 if not rescued by administration of large amounts of RA to the mother, and the inhibition of this enzyme by nitrofen and other substances that induce diaphragmatic hernia has been proposed as the teratogenic mode of action for these compounds (Thebaud et al., 1999; Babiuk et al., 2004; Greer et al., 2003; Mey et al., 2003). Nitrofen, 4-biphenyl carboxylic acid, bisdiamine, SB- 210661 and cyanamide all induce a specific type of diaphragmatic hernia (posterolateral, Bochdalek-type) in rodents.

The notifier argues that this mechanism does not apply to the teratogenic action of cyanamide in rats because the substance had practically no influence on ALDH1 activity in concentrations that totally inhibit the low Km mitochondrial ALDH2 enzyme (which is not involved in RA production) so that there is no evidence that cyanamide impacts retinol metabolism. However, although rat ALDH1 has a higher Km than ALDH2 (15 µM vs. 0.2 µM; Klyosov et al., 1996; Possible role of liver cytosolic and mitochondrial aldehyde dehydrogenases in acetaldehyde metabolism. Biochemistry 35, 445-4456), it is not a high Km enzyme, having a Km in the micromolar and not millimolar range. As low Km enzymes ALDH1 and ALDH2 are sensitive to the inhibitory action of cyanamide (Watabiki et al., 2004; Comparative intralobular distribution of low Km aldehyde dehydrogenase activities in rat and guinea pig livers. Acta Histochemica et Cytochemica 37, 281-285). Moreover, according to the publication by Marchner and Tottmar (1978), even high Km enzymes (~ 1 mM) are inhibited significantly (50 %) in rat liver *in vivo* by a cyanamide dose of 5 mg/kg bw, the lowest dose used in the rat developmental toxicity study (Morseth, 1989). Thus, it is concluded that cyanamide exposure may lead to a depletion of RA in embryonal tissues through inhibition of Raldh2 and to malformations in highly RA-dependent tissues like the developing diaphragm.

A peer review of the rat teratology study of Morseth (1989) was performed by Harris (2012) and submitted by the notifier. The result of the peer review is presented in the following.

Harris reviewed the rat teratology study (Morseth, S.L. (1989): Rat teratology study with aqueous hydrogen cyanamide; reported in chapter 4.11.2.1) and concluded that the mode of action of the test substance cyanamide is not inhibition of retinoic acid because the diaphragmatic hernias occurred

without any associated defects. He believes that this finding was mistakenly classified since the laboratory staff was not well-trained and the Bouin's fixative lead to shrinkage of the tissue. He proposed to conduct a new study according to OECD TG 414. The results of the peer review are discussed in detail in chapter 4.11.5 Comparison with criteria.

4.11.3.2 Medical data and information

Medical surveillance on manufacturing plant personnel

No signs of diseases or health impairments caused by cyanamide were found during medical surveillance on manufactoring plant personnel. Medical examinations also included special investigations of functional disorders regarding the testes and the thyroid gland and potential sensitising properties.

Employees of the calcium cyanamide production plant of Degussa AG (formerly SKW Trostberg AG) are under constant medical supervision regarding worker safety. Special emphasis during these preventive examinations was given to the question of potential sensitising properties of hydrogen cyanamide.

The company physician of Degussa AG (formerly SKW Trostberg AG) could not diagnose striking diseases of workers handling calcium cyanamide or calcium cyanamide containing products. However, the contact with cyanamide leaded to hypersensitivity to alcohol, an effect which is used in alcoholism therapy (Gfaller, 1976).

In a human study it was investigated if there are effects of cyanamide exposure on the testes and the thyroid gland. Blood samples were collected from 21 exposed and 9 unexposed persons and examined for follicle stimulating hormone (FSH), luteinising hormone (LH) and testosterone. In addition, total T3 (TT3) levels, total T4 (TT4), thyroxine binding globulin (TBG) and thyrotropin (TSH) were determined to investigate the thyroid function. For a rough estimation of the degree of exposure towards cyanamide, urine samples were collected from the participants and examined for acetylcyanamide (syn.: N- acetylcyanamide), the main urinary metabolite of cyanamide. A clearly recognisable exposure could be demonstrated in the workers employed in the calcium cyanamide production units. The comparison of the persons exposed and unexposed to cyanamide revealed normal hormone levels in both groups. According to this investigation, disturbances of the gonadal function and the thyroid function can be excluded (Mertschenk et al, 1993).

Cyanamide in alcohol therapy

Calcium cyanamide has been worldwide intensively used as drug to deter drinking in alcoholics. The consumption of alcoholic beverages after intake of cyanamide leads to intolerances. This is probably due to an inhibition of acetaldehyde dehydrogenase, thus leading to a retardation in ethanol breakdown which stops on the stage of acetaldehyde accumulating in the blood. Intolerance reactions towards alcohol occur in man after daily cyanamide doses higher than 20 mg. In general daily doses of more than 0.4 – 1 mg/kg bw cyanamide have been used in the alcohol aversion therapy. The duration of the treatment ranges from a few months to a few years. In some cases patients have taken cyanamide for more than 10 years. No signs of reproductive disorders have been observed.

4.11.4 Summary and discussion of reproductive toxicity

Multigeneration toxicity studies in rats:

Table 146: Summary table of relevant reproductive toxicity studies

Method	Remarks	Results	Reference
2-generation reproduction study in Sprague-Dawley rats with 0, 2, 7 and 25.0 mg/kg bw/day cyanamide administered via gavage.	Testicular atrophy in F0 and F1 generation, reduced fertility rates, reduced number of implantations and live births at 25 mg/kg bw/day.	NOAEL: 7 mg/kg bw/day for parental animals, offspring and reproduction parameters	Vallés et al., 1987 Doc-No 592-010
2-generation reproduction study in Wistar rats with 0, 20, 60 and 180 ppm, referring to 1.3, 2.5 and 7.55 mg/kg bw/day pure active substance cyanamide	Testicular changes in F1 generation at ≥ 1.3 mg/kg bw/day. Decreased pup weights at 7.55 mg/kg bw/day in second litter of the F0 generation. Decreases in mean litter size at birth and in neonatal viability at 7.55 mg/kg bw/day.	Reproduction/ fertility NOAEL: 2.5 mg/kg bw/day Offspring NOAEL: 1.3 mg/kg/ bw/day Parental NOAEL: 2.5 mg/kg bw/day	Koeter et al., 1986 Doc-No 553-001
2-generation study in Crl:CD BR rats with aqueous cyanamide 0, 2.5, 7.5 and 30 mg/kg bw/day for the first 12 weeks and 0, 1.25, 3.75 and 15 mg/kg bw/day pure active substance cyanamide for the remaining time, administered by gavage	Reduction of body weight and food consumption at 7.5 and 15 mg/kg bw/day. Low fertility index in F0 and F1 animals at 7.5 and 15 mg/kg bw/day, respectively. Increased total litter resorption at 15 mg/kg bw/day. Decreased pup survival at ≥ 1.25 mg/kg bw/day.	Offspring NOAEL: < 1.25 mg/kg bw/day Reproduction/ fertility NOAEL: 3.75 mg/kg bw/day Parental NOAEL: 3.75 mg/kg bw/day	Morseth, 1990 Doc-No 543-001

Three studies on reproduction toxicity in rats are reported, two with substance administration by gavage and one where the test substance was mixed with the feed.

In the most recent study (Morseth et al., 1990) reproductive performance and fertility was affected in F0 and F1 parental animals at the top dose group, resulting in a low fertility index in both generations and in an increase of total litter resorptions in the F0 generation.

In a further study (Obach and Rives, 1985; Rives-Ferriol, 1987), at the high dose level (25 mg/kg bw/d) only, reduced male and female fertility was observed. Unilateral or bilateral testicular atrophy was reported for a few high dose F0 males (4/23).

In a dietary 2-generation reproduction toxicity study (Koëter et al., 1986) cyanamide mating and fertility were found unaffected with the exception of a reduced litter size in the 1st pregnancies of high dose F0 and F1 dams. There was an increased incidence of F1 males with interstitial cell proliferation at the high dose level and tubular atrophy was described in all treated groups. However, only a subset (25 %) of the animals at risk for testicular lesions has been examined in this study and there was a lack of a dose-response of the observed changes. Therefore, the testicular findings were not considered to be a reliable basis for risk assessment.

Testicular findings were observed in repeated dose toxicity studies in dogs, too.

Table 147: Summary table of relevant repeated dose toxicity studies in dogs with testes findings

Method	Remarks	Results	Reference
90-day gavage study in beagle dogs* with 0.6, 2 and 6 mg/kg bw/day pure active substance cyanamide * 4 - 5 month old at study begin	T3 and T4 decrease at 2 and 6 mg/kg bw; histopathological findings in testes and epididymidis regarding spermatogenesis most pronounced at 6 mg/kg bw/day accompanied by reduced testes weights; anaemia at 6 mg/kg bw in both sexes	NOAEL (RMS): 0.6 mg/kg bw/day of cyanamide NOAEL (PRAPeR 79): < 0.6 mg/kg bw/day of cyanamide	Til et al., 1982 Doc-No 533-002 TOX2001-425
Supplementary (90 day) oral toxicity study in dogs** (mature males only) with 0, 0.6 and 6.0 mg/kg bw/day pure active substance cyanamide ** 12 – 15 months at study begin	Test substance related effects in male dogs at 6.0 mg/kg bw/day (retarded body weight gain, reduced food consumption as well as evidence on testicular damage)	NOAEL: 0.6 mg/kg bw/day of cyanamide	Til, H. P. et Beems, R., 1986 Doc. No. 533-003 Z32048 / Z32032
52-weeks oral gavage study in Beagle dogs*** with 0, 0.2, 1.0 and 5.0 mg/kg bw/day pure active substance cyanamide *** 6 - 8 months at study begin	Test substance related effects in female and male dogs at 5.0 mg/kg bw/day (e.g. anaemia, several parameter of serum-chemistry, T4 decrease, histopathological changes in liver, spleen, gallbladder, thymus and testes/ epididymidis)	NOAEL: 1 mg/kg bw/day of cyanamide	Osheroff, 1989 Doc-No 537-002 TOX2004-368

In a 90-day oral study of Til et al. (1982) histopathological findings in testes and epididymidis regarding spermatogenesis accompanied by reduced testes weights were most pronounced at the highest dose group at 6 mg/kg bw/day in beagle dogs, about 4 months old at study begin. In 2 animals of the high dose group testes atrophy and absent spermatogenesis accompanied by reduced testes weights was observed, whereas no such findings were observed in the 4 control animals. Slight changes in testes and epididymides found in the lower dose groups are regarded as findings unrelated to treatment of which degree and incidence disappear within the background.

In a supplementary (90 day) oral toxicity study with dogs (Til, H. P. et Beems, R., 1986) one of four mature male beagle dogs (12 – 15 months at study begin) from the high dose group (6 mg/kg/day) had tubular degeneration/depletion in the testes, sloughed germ cells/debris in the epididymis and reduced sperm in the epididymis. Although these changes could be incidental, their possible relationship to cyanamide administration cannot be excluded.

In a 1-year study by Osheroff, M.R. (1989) testicular findings (moderate degeneration of seminiferous tubules in the testes) in one dog (6 - 8 months old at study begin) at 5 mg/kg bw/day, which could not be excluded with certainty as treatment-related.

Developmental toxicity study in rats and rabbits:

Table 148: Summary table of relevant reproductive toxicity studies

Method	Remarks	Results	Reference
Teratology Study in Crl:CD BR rats with 0, 5, 15 and 45 mg/kg bw/day pure active substance cyanamide	Compound-related decrease in body weight gain at ≥ 5 mg/kg bw/day. Mean food consumption reduced by 8-11 % in the mid dose group and by 23-24 % in the high dose group during gestation days 6-20. Corrected body weight gain was reduced by 29 % and 71 % in the mid and high dose group, respectively. Hypoactivity in 8 dams of each dose group during the first two exposure days. Decrease of gravid uterine weight value at 45 mg/kg bw/day. Lower mean foetal body weight values at 15 mg/kg bw/day. Visceral and skeletal malformations observed at the high dose.	Maternal NOAEL: < 5 mg/kg bw/day Embryo-/foetal NOAEL: 5 mg/kg bw/day	Morseth, 1989 Doc-No 551-002
Embryotoxicity/ teratogenicity study in New Zealand rabbits with 0, 2, 6, 18 mg/kg bw/day pure active substance cyanamide.	Body weight loss during treatment at 18 mg/kg bw/day. Increase in post-implantation loss and slightly decreased foetal weight at 18 mg/kg bw/day.	Maternal NOAEL: 6 mg/kg bw/day Embryo-/foetal NOAEL: 6 mg/kg bw/day	Koeter, 1989 Doc-No 551-001

The prenatal developmental toxicity of cyanamide was investigated in rats and rabbits complying to international test guidelines and GLP.

In rats (Morseth, 1989) signs of prenatal developmental toxicity consisted of reduced foetal weights in the high dose. Foetal weights were also lower in the mid dose group. This is considered relevant because of the lower mean litter size in this group. This would be expected to be associated with an increase in mean foetal weight had the development been unaffected. Foetal morphology was affected at the high dose level presenting an increased incidence of diaphragmatic hernia, isolated cases of skeletal malformations and a variety of skeletal variations.

In rabbits (Koëter and Marwijk (1989) dosed with 0, 2, 6 and 18 mg/kg bw/day no indication for teratogenicity was detected in rabbits up to and including the high dose level.

Table 149: Summary table of relevant reproductive toxicity studies

Method	Remarks	Results	Reference
2-generation reproduction study in Sprague-Dawley rats with 0, 2, 7 and 25.0 mg/kg bw/day cyanamide administered via gavage.	Testicular atrophy in F0 and F1 generation, reduced fertility rates, reduced number of implantations and live births at 25 mg/kg bw/day.	NOAEL: 7 mg/kg bw/day for parental animals, offspring and reproduction parameters	Vallés et al., 1987 Doc-No 592-010
2-generation reproduction study in Wistar rats with 0, 20, 60 and 180 ppm, referring to 1.3, 2.5 and 7.55 mg/kg bw/day pure active substance cyanamide	Testicular changes in F1 generation at ≥ 1.3 mg/kg bw/day. Decreased pup weights at 7.55 mg/kg bw/day in second litter of the F0 generation. Decreases in mean litter size at birth and in neonatal viability at 7.55 mg/kg bw/day.	Reproduction/ fertility NOAEL: 2.5 mg/kg bw/day Offspring NOAEL: 1.3 mg/kg/ bw/day Parental NOAEL: 2.5 mg/kg bw/day	Koeter et al., 1986 Doc-No 553-001
2-generation study in Crl:CD BR rats with aqueous cyanamide 0, 2.5, 7.5 and 30 mg/kg bw/day for the first 12 weeks and 0, 1.25, 3.75 and 15 mg/kg bw/day pure active substance cyanamide for the remaining time, administered by gavage	Reduction of body weight and food consumption at 7.5 and 15 mg/kg bw/day. Low fertility index in F0 and F1 animals at 7.5 and 15 mg/kg bw/day, respectively. Increased total litter resorption at 15 mg/kg bw/day. Decreased pup survival at ≥ 1.25 mg/kg bw/day.	Parental NOAEL: 3.75 mg/kg bw/day Offspring NOAEL: < 1.25 mg/kg bw/day Reproduction/ fertility NOAEL: 3.75 mg/kg bw/day	Morseth, 1990 Doc-No 543-001
Teratology Study in Crl:CD BR rats with 0, 5, 15 and 45 mg/kg bw/day pure active substance cyanamide	Compound-related decrease in body weight gain at ≥ 5 mg/kg bw/day. Decrease of gravid uterine weight value at 45 mg/kg bw/day. Lower mean foetal body weight values at 15 mg/kg bw/day. Visceral and skeletal malformations observed at the high dose.	Maternal NOAEL: < 5 mg/kg bw/day Embryo-/foetal NOAEL: 5 mg/kg bw/day	Morseth, 1989 Doc-No 551-002
Embryotoxicity/ teratogenicity study in New Zealand rabbits with 0, 2, 6, 18 mg/kg bw/day pure active substance cyanamide.	Body weight loss during treatment at 18 mg/kg bw/day. Increase in post-implantation loss and slightly decreased foetal weight at 18 mg/kg bw/day.	Maternal NOAEL: 6 mg/kg bw/day Embryo-/foetal NOAEL: 6 mg/kg bw/day	Koeter, 1989 Doc-No 551-001

4.11.5 Comparison with criteria

Table 150: Toxiclogical results concerning adverse effects on sexual function and fertility

Toxicological result	DSD criteria	CLP criteria
2-generation reproduction study in Sprague-Dawley rats with 0, 2, 7 and 25.0 mg/kg bw/day cyanamide administered via gavage (Vallés et al., 1987, Doc-No 592-010): - **Impaired fertility** in F0 and F1 generation at parental toxicity (25 mg/kg bw/day) - **Testicular atrophy** in F0 and F1 generation, reduced number of implantations and live births at parental toxicity (25 mg/kg bw/day).	<u>Category 1</u>: Substances known to impair fertility in humans <u>Category 2</u> Substances which should be regarded as if they impair fertility in humans - clear evidence in animal studies of impaired fertility in the absence of toxic effects, or, - evidence of impaired fertility occurring at around the same dose levels as other toxic effects but which is not a secondary nonspecific consequence of the other toxic effects - other relevant information <u>Category 3</u> Substances which cause concern for human fertility - results in appropriate animal studies which provide sufficient evidence to cause a strong suspicion of impaired fertility in the absence of toxic effects, or evidence of impaired fertility occuring at around the same dose levels as other toxic effects, but which is not a secondary non-specific consequence of the other toxic effects, but where the evidence is insufficient to place the substance in Category 2, - other relevant information	<u>Category 1A</u>: Known human reproductive toxicant <u>Category 1B</u>: Presumed human reproductive toxicant largely based on data from animal studies - clear evidence of an adverse effect on sexual function and fertility in the absence of other toxic effects, or - the adverse effect on reproduction is considered not to be a secondary non-specific consequence of other toxic effects <u>Category 2</u>: Suspected human reproductive toxicant - some evidence from humans or experimental animals, possibly supplemented with other information, of an adverse effect on sexual function and fertility and - and where the evidence is not sufficiently convincing to place the substance in Category 1 (deficiencies in the study). - the adverse effect on reproduction is considered not to be a secondary non-specific consequence of the other toxic effects
2-generation reproduction study in Wistar rats with 0, 20, 60 and 180 ppm, referring to 1.3, 2.5 and 7.55 mg/kg bw/day pure active substance cyanamide (Koeter et al., 1986, Doc-No 553-001): - **Decreases in mean litter size** at birth at a parental toxic dose (7.55 mg/kg bw/day) - Adverse **testicular changes** in F1 generation (tubular dilation and tubular degeneration/depletion) at ≥ 1.3 mg/kg bw/day.		
2-generation study in Crl:CD BR rats with aqueous cyanamide 0, 2.5, 7.5 and 30 mg/kg bw/day for the first 12 weeks and 0, 1.25, 3.75 and 15 mg/kg bw/day pure active substance cyanamide for the remaining time, administered by gavage (Morseth, 1990, Doc-No 543-001): - **Low fertility index** in F0 and F1 animals at parental toxic doses (7.5 and 15 mg/kg bw/day, respectively). - Increased total litter resorption at 15 mg/kg bw/day.		
90-day gavage study in beagle dogs (4 - 5 month old at study begin) with 0.6, 2 and 6 mg/kg bw/day pure active substance cyanamide (Til et al., 1982, Doc-No 533-002, TOX2001-425): - Adverse histopathological findings in **testes and epididymidis** regarding		

spermatogenesis most pronounced at 6 mg/kg bw/day accompanied by reduced testes weights (NOAEL (RMS): 0.6 mg/kg bw/day of cyanamide)		
Supplementary (90 day) oral toxicity study in dogs (mature males only, 12 – 15 months at study begin) with 0, 0.6 and 6.0 mg/kg bw/day pure active substance cyanamide (Til, H. P. et Beems, R., 1986, Doc. No. 533-003, Z32048 / Z32032): - Adverse **testicular damage** (tubular degeneration/depletion in the testes, sloughed germ cells/debris in the epididymis and reduced sperm in the epididymis) at 6.0 mg/kg bw/day (NOAEL: 0.6 mg/kg bw/day of cyanamide)		
52-weeks oral gavage study in Beagle dogs (6 - 8 months at study begin) with 0, 0.2, 1.0 and 5.0 mg/kg bw/day pure active substance cyanamide (Osheroff, 1989, Doc-No 537-002, TOX2004-368): - Adverse **changes in testes/ epididymidis** at 5.0 mg/kg bw/day (NOAEL: 1 mg/kg bw/day)		

Reduced fertility was observed in two 2-generation reproduction studies in Sprague-Dawley rats (Vallés et al., 1987) and in Crl:CD BR rats (Morseth, 1990). In these studies cyanamide was administered via gavage. In a third 2-generation study in Wistar rats with dietary administration of cyanamide a reduced litter size in the 1st pregnancies of high dose F0 and F1 dams was observed (Koeter et al., 1986). Reduced fertility was observed in the presence of parental toxicity in all three 2-generation reproduction studies in rats.

Histological findings in testes accompanied by other toxic effects were observed in dogs. The testes findings were most pronounced at the highest dose group in young dogs. It can be assumed that findings in testes of young dogs are related to imature age of the animals or occur spontaneously. However, all available studies have minor to massive limitations. There are indications that Cyanamide might cause some reproductive effects at clear systemic toxic doses. No consistent correlation of the different effects like fertility or testes changes in rats were noted. There is no causal link between findings in testes and variations in fertility indices because those effects do not occur simultaneously in any of the studies.

There are no epidemiological data to evaluate effects on fertility, hence cyanamide can not be placed in Category 1 (DSD) or category 1A (CLP).

Overall, in several studies there were single indications for adverse effects, however neither a consistent pattern could be observed, nor could the findings be reproduced in the respective other studies. Additionally, some methodological limitations in the studies need to be taken into account (discussed at the respective studies). Overall, the dossier submitter sees “some evidence” but not a “clear evidence” for adverse effects on reproduction and therefore, proposes a classification with category 3 (R62, DSD) and category 2 (H361f, CLP regulation).

Table 151: Toxicological results concerning adverse effects on development

Toxicological result	DSD criteria	CLP criteria
Teratology Study in Crl:CD BR rats with 0, 5, 15 and 45 mg/kg bw/day pure active substance cyanamide; (Morseth, 1989, Doc-No 551-002) Compound-related decrease in body weight gain at ≥ 5 mg/kg bw/day. Mean food consumption reduced by 8-11 % in the mid dose group and by 23-24 % in the high dose group during gestation days 6-20. Corrected body weight gain was reduced by 29 % and 71 % in the mid and high dose group, respectively. Hypoactivity in 8 dams of each dose group during the first two exposure days. Decrease of gravid uterine weight value at 45 mg/kg bw/day. Lower mean foetal body weight values at 15 mg/kg bw/day. Visceral and skeletal malformations observed at the high dose. Maternal NOAEL: < 5 mg/kg bw/day Embryo-/foetal NOAEL: 5 mg/kg bw/day	<u>Category 1</u>: Substances known to cause developmental toxicity in humans <u>Category 2</u> Substances which should be regarded as if they cause developmental toxicity to humans - clear results in appropriate animal studies in the absence of marked maternal toxicity, or, - at around the same dose levels as other toxic effects but which is not a secondary nonspecific consequence of the other toxic effects - other relevant information <u>Category 3</u> Substances which cause concern for humans owing to possible developmental toxic effects - results in appropriate animal studies which provide sufficient evidence to cause a strong suspicion of developmental toxicity in the absence of signs of marked maternal toxicity, or at around the same dose levels as other toxic effects, but which are not a secondary non-specific consequence of the other toxic effects, but where the evidence is insufficient to place the substance in Category 2, - other relevant information	<u>Category 1A</u>: Known human reproductive toxicant <u>Category 1B</u>: Presumed human reproductive toxicant largely based on data from animal studies - clear evidence of an adverse effect on development in the absence of other toxic effects, or - the adverse effect on reproduction is considered not to be a secondary non-specific consequence of other toxic effects <u>Category 2</u>: Suspected human reproductive toxicant - some evidence from humans or experimental animals, possibly supplemented with other information, of an adverse effect on development and - the evidence is not sufficiently convincing to place the substance in Category 1 (deficiencies in the study). - the adverse effect on reproduction is considered not to be a secondary non-specific consequence of the other toxic effects
Embryotoxicity/ teratogenicity study in New Zealand rabbits with 0, 2, 6, 18 mg/kg bw/day pure active substance cyanamide. (Til, H. P. et Beems, R., 1986, Doc. No. 533-003, Z32048 / Z32032) Body weight loss during treatment at 18 mg/kg bw/day. Increase in post-implantation loss and slightly decreased foetal weight at 18 mg/kg bw/day. Maternal NOAEL: 6 mg/kg bw/day Embryo-/foetal NOAEL: 6 mg/kg bw/day		

There are no appropriate epidemiological studies demonstrating developmental effects of cyanamide in humans. Classification for a Category 1A according Regulation (EC) No 1272/2008 is not warranted. Likewise, Category 1 according to DSD is not warranted.

The prenatal developmental toxicity of cyanamide was investigated in rats and rabbits complying to international test guidelines and GLP.

According Regulation (EC) No 1272/2008 major manifestations of developmental toxicity include death of the developing organism, structural abnormality, altered growth, and functional deficiency.

In rats (Morseth, 1989), signs of prenatal developmental toxicity consisted of reduced foetal weights in the mid and high dose group. Foetal morphology was affected at the high dose level of 45 mg/kg bw/day presenting an increased incidence of diaphragmatic hernia, isolated cases of skeletal malformations and a variety of skeletal variations. In rabbits (Koëter and Marwijk 1989) slightly decreased foetal weight at the highest dose group was observed. No indication for teratogenicity was detected up to and including the high dose level.

In the study of Morseth (1989), diaphragmatic hernias occurred with an incidence rate of about 4.3 % (7/163 pups, in 5 out of 24 litters) in the rat offspring of the high dose group (45 mg/kg bw/d). Maternal toxicity in the high and medium dose group (15 and 45 mg/kg bw/d) was considered significant. There were no deaths, but hypoactivity was seen in 8 dams of each dose group, during the first two exposure days, and the corrected body weight gain was reduced by 29 % and 71 %, respectively, compared to the control. Mean food consumption was reduced by 8-11 % in the mid dose group and by 23-24 % in the high dose group during gestation days 6-20.

In addition, there was clear evidence from other studies that repeated daily doses of 4.5-40 mg/kg bw/day caused significant systemic toxicity in rats. At these dose levels, consistent and marked effects on thyroid morphology (increased number of small follicles, reduction of colloid content) were observed in the 28-day study (Osheroff, 1988), in the 90-day study (Til et al., 1975) and in the two-generation reproduction study (Koëter et al., 1986). Furthermore, repeated daily doses of 45 mg/kg bw/day are expected to produce marked systemic toxicity in rats since this dose level is only about 3-fold lower than the doses causing mortality following a single oral administration (Engel, 1973; Daamen, 1994).

Diaphragmatic hernias are considered as malformation.

There is evidence that cyanamide might induce fetal diaphragmatic hernias via the following mechanism of action: 1) Cyanamide enters the maternal blood circulation and is metabolised to the active metabolite nitroxyl (HNO). 2) HNO enters the fetal blood circulation and reaches the tissue of the developing diaphragm. 3) HNO inhibits retinaldehyde dehydrogenase genes (Raldh2/ALDH1A2) of the diaphragmatic tissue, thereby reducing the concentration of retinoic acid in the tissue. 4) Decrease of retinoic acid during the retinoic acid-dependent diaphragm formation disrupts normal tissue development and leads to diaphragmatic hernias.

Metabolic activity leading to malformations (e.g. conversion of cyanamide to nitroxyl postulated to be responsible for the aldehyde dehydrogenase (ALDH)-inhibitory properties of cyanamide) significantly takes place in the maternal liver (see 4.12.1.5 Species-specific differences in cyanamide-induced toxicities). The foetal liver does not have acquired sufficient metabolic capacity to bioactivate cyanamide. Thus, a specific maternally-mediated mechanism leading to malformations can be demonstrated.

The peer review of the study of Morseth (1989) conducted by Harris (2012) showed some limitations. He discussed the fetal dissection method and favoured the fresh microdissection of non-fixed fetuses instead of using Bouin’s fixative. Fresh fetal dissection is the general procedure for rabbit fetuses but not for rats which are rarely dissected in a fresh state in regulatory toxicity studies. Harris further argued that the diaphragmatic hernias seen in this study were artefacts

generated during dissection by a scissor. Scissors are not used in Wilson sectioning, fixed whole fetuses are cut with a razor blade at defined sites. If the diaphragmatic hernias were only artefacts of the dissection technique they should occur more often. However they are rare malformations and if they occur they are associated with certain substances and not with the dissection technique. In addition, if the diaphragmatic hernias were artefacts, they should have be seen also in the control and lower dose groups, but in the study of Morseth (1989) these findings were seen in high dose fetuses only. Harris further argued that the laborator staff was not well trained. The study director and the laboratory conducted developmental toxicity studies for several years before the cyanamide study was performed. Thus, the laboratory is very experienced in this field of toxicity testing.

The peer reviewer expected associated defects in addition to the diaphragmatic hernias since nitrofen given to rats in early gestation can induce a variety of malformations together with diaphragmatic hernias. Nitrofen is the reference substance for the induction of diaphragmatic hernias and doses are chosen to induce these malformations in 100% of the fetuses. Cyanamide was not tested in a comparable dosing regimen. Beside inhibition of retinaldehyde dehydrogenase genes (Raldh2), nitrofen has more action sites which may account for malformations of other fetal structures, in addition to the diaphragm.

In conclusion, the RMS cannot support the view of the peer reviewer and still has the opinion that a maternally-mediated mechanism leading to the diaphragmatic hernias. A new study, as proposed by Harris (2012) does not appear to be required from the regulatory point of view.

.According Guidance to Regulation (EC) No 1227/2008 on Classification, Labeling and Packing of substances and mixtures (3.7.2.2.1 Classification in the presence of parental toxicity; 14 May 2009 – IHCP, Dg Joint Research Centre, European Commission) “Classification shall not automatically be discounted for substances that produce developmental toxicity only in association with maternal toxicity, even if a specific maternally-mediated mechanism has been demonstrated. In such a case, classification in Category 2 may be considered more appropriate than Category 1. …”.

Since developmental effects relevant for classification (i.e. diaphragmatic hernia) were observed only in one species at a high dose level associated with significant maternal toxicity or marked systemic toxicity, classification in Category 2 (CLP) and in Category 3 (DSD) is considered appropriate.

4.11.6 Conclusions on classification and labelling

Reduced fertility in rats and adverse testes effects in dogs have been demonstrated most pronounced only at high doses. All available studies have minor to massive limitations. No consistent pattern of the different effects like fertility or testes changes in rats is noted. There is no causal link between findings in testes and variations in fertility indices. For this reason concerning adverse effects on sexual function and fertility classification in Category 3 (R62) according to DSD criteria and Category 2 (H361f) according to CLP criteria, is proposed.

In rats and rabbits developmental toxicity was observed at maternal toxic doses. Abnormality (teratogenic effects) produced by a specific maternally-mediated mechanism was observed in rats only at the highest dose level of 45 mg/kg bw/day pure active substance cyanamide in association with maternal toxicity. For this reason classification concerning effects on development in Category 3 (R63, DSD criteria) and Category 2 (H361d, CLP criteria) is considered appropriate.

The proposed classification and labelling is in accordance with the proposal of the PRAPeR 79 meeting of experts[1] (R62-R63).

4.12 Other effects

4.12.1 Non-human information

4.12.1.1 Neurotoxicity

Special neurotoxicity studies were not made available by the notifier.

4.12.1.2 Immunotoxicity

Special immunotoxicity studies were not made available by the notifier.

4.12.1.3 Specific investigations: other studies

4.12.1.4 Supplementary studies on the active substance

Title:	Daamen, P.A.M. (1994): Effect of acetylcysteine on the acute oral toxicity with cyanamide in the rat; Doc. No. 521-003; Notox, Netherlands; Project No 101699; published: no
Guidelines:	None, but study follows the recommendations of OECD 401 and was conducted to elucidate if acetylcysteine might be a potential antidote.
Deviations:	Not applicable
GLP:	Yes, a respective QAU statement is presented in the report
Acceptability:	The study is considered to be supplementary.

Materials and methods:

Cyanamide was identified as follows:

Batch: 08/11/93

Purity: Content of active substance: 50 % (w/w); 53 % (w/v)

Appearance: Aqueous solution, slightly coloured

Specification: See document J

[1] European Food Safety Authority; Conclusion on the peer review of the pesticide risk assessment of the active substance cyanamide. EFSA Journal 2010;8(11):1873. [61 pp.] doi:10.2903/j.efsa.2010.1873. Available online: www.efsa.europa.eu/efsajournal.htm

Acetylcysteine was identified as follows:

Batch: 93E21

Purity: Content: 300 mg acetylcysteine per ampoule (3 mL)

Appearance: Colourless solution

The objective of the study was to assess the effect of acetylcysteine on the acute toxicity of cyanamide when administered to rats in a single oral dose. This study should provide information on the antidote potential of acetylcystein, injected intravenously at a fixed time after a single oral dose of cyanamide of 230 mg/kg bw (corresponding to LD_{50}). One group of animals was orally dosed with cyanamide only, while a second group was orally dosed with cyanamide (at the same dose) followed by an intravenous injection of acetylcysteine (300 mg/kg bw) 10 to 15 minutes later.

Each group consisted of five male and five female rats which had been fasted overnight prior to dosing. Animals were subjected to daily observations and weekly determination of body weight. Macroscopic examination was performed on the day of being found dead or at the end of the experimental period (15 days). The test material was prepared immediately prior to dosing.

Findings:

Table 152: Acute oral toxicity of cyanamide and effect of acetylcysteine

Males		**Females**	
Group	Mortality***	Group	Mortality***
1*	2/5	1*	2/5
2**	4/5	2**	2/5

* 230 mg/kg bw cyanamide

** 230 mg/kg bw cyanamide by gavage and 300 mg/kg bw acetylcyteine, intravenously injected 12 to 17 minutes after administration of oral dose

*** deaths occurred within 25 hours of dosing on day 9

The incidence of mortality among the sexes combined from the oral dosing group and the oral/intravenous dosing group was 4 and 6, respectively. Clinical signs noted among the animals with cyanamide only included lethargy, tremors, hunched posture, uncoordinated movements, laboured respiration and piloerection. All surviving males had recovered on days 9, 11 or 14, the surviving females had not recovered by termination of the study. They still showed hunched posture on day 15.

No or low body weight gain was noted by the surviving animals over the first week of study period. All surviving animals showed improved body weight gain over the second week of the study.

Macroscopic post mortem examination of the animals that died or were killed in extremis during the study revealed enlarged stomach, thickened limit ridge in stomach, accentuated lobular liver pattern, kidney with irregular surface cortex, foci in cortex and grey-white appearance of cortex and medulla in size reduced spleen, haemorrhages in lungs and thymus, red foci in lungs, watery contents in thoracic cavity.

Macroscopic post mortem examination of the surviving animals at termination, revealed grey/white irregular surface of medulla of kidneys, granulated surface of cortex of kidneys; grey/white and red foci in lungs; thymus and testes reduced in size.

Expected mortality was noted in the oral dosing group (group 1), but a higher number of males died in the oral/intravenous combined group (group 2). No differences in clinical signs and macroscopic abnormalities were observed between the two groups.

Conclusion:

Based on these results, it can be concluded, that intravenous injection of acetylcysteine (300 mg/kg bw) after oral dosing of cyanamide resulted in a higher mortality of male rats. Mortality in fermale rats was comparable with the mortality rate in rats dosed with cyanamide alone.

Title:	Obach, R. et al. (1985): Lack of hepatotoxicity after long-term administration of cyanamide in rats: A histological and biochemical study; Doc. No. 592-048; Research Department of S.A. Lasa Laboratories; Barcelona, Spain; published
Guidelines:	None, study was conducted to elucidate the hepatoxicity after long-term administration to cyanamide in rats.
Deviations:	Not applicable
GLP:	No; study is a publication
Acceptability:	The study is considered to be supplementary.

Materials and methods:

The test substance was identified as follows:

Cyanamide solution (COLME)

Batch: S 13 and T-14

Purity: Solution contains 60 mg/mL cyanamide

Appearance: Aqueous solution

Six month oral administration

The study was performed using Sprague-Dawley rats (20 males and 20 females per group). The animals received daily 0, 2, 7 and 25 mg/kg bw of cyanamide solution (COLME) by gavage. At the end of the treatment, a blood sample of each rat was taken and the animals were sacrificed. Immediately thereafter the liver was fixed for further histological examinations. The following clinical chemical parameters were analysed in blood: alkaline phosphatase, bilirubin, cholesterol, aspartate aminotransferase, alanine aminotransferase.

Twelve month intraperitoneal administration

The study was performed using Wistar and Sprague-Dawley rats. They received 0, 8 and 16 mg/kg bw of cyanamide intraperitoneally as aqueous solution (COLME). After 1, 3, 6, 9 and 12 months of treatment, five rats per dose were sacrificed for liver histopathological examination. At the sacrifice, blood samples were taken from the animals sacrificed after 12 months to investigate the following clinical chemical parameters: alkaline phosphatase, aspartate aminotransferase; alanine aminotransferase, cholesterol and bilirubin.

Findings:

No principle difference was found in the study part with oral and intraperitoneal administration.

The hepatic section showed small size foci of parenchymal inflammatory non-specific infiltrates of mononuclear cells, which were present in comparable degree and prevalence in all treated and control groups. There were also changes of minute vacuolation within hepatocytes, which are probably glycogenic in nature. These findings were observed in all groups and were more prominent in females, the high dose group (25 mg/kg bw). There were no differences in this parameter when comparing low (2 mg/kg bw) and mid (7 mg/kg bw) dose treated animals to the controls. No hepatocyte inclusion bodies were detected in any of the orally treated animals or in those which received cyanamide intraperitoneally.

Some chemical parameter values (bilirubin, alkaline phosphatase and alanine aminotransferase) were found to be increased when compared to control, but only at very high doses (7 - 25 mg/kg bw). The authors reported that these increased parameters in rats are still within the range of historical control data for such studies. Furthermore no histological correlation has been found in the liver. It was concluded that investigations of clinical chemical parameters did not show significant impairment of hepatic function in treated animals.

Discussing the results of the study the authors emphasised that the doses used in the study are much higher than those used in human therapy where 0.5 - 1 mg/kg bw are the therapeutic range. With regard to inclusion bodies which have been observed in alcoholic patients under aversion therapy with cyanamide the authors concluded that the absence of alcohol consumption cannot be absolutely assured. It was supposed that the results of this study in rats without ethanol influence indicates a possible role of acetaldehyde in the development of the inclusion bodies found in man.

Conclusion:

The histological examination of liver samples from the rats treated with cyanamide did not indicate hepatic injury or inclusion bodies, which would confer a conspicuous ground glass appearance to hepatocytes. Furthermore, clinical chemical values did not show significant impairment of hepatic function in treated animals.

4.12.1.5 Species-specific differences in cyanamide-induced toxicities

Title:	Borlak, J. (2009): Molecular toxicology investigations to better understand the species-specific differences in cyanamide-induced toxicities; Doc. No. 513-001; Fraunhofer Institute of Toxicology and Experimental Medicine (ITEM); Germany; Project No 19G07022; 1890552, published: no
Guidelines:	EMEA guidelines for the detection of early signals of drug-induced hepatotoxicity in non-clinical studies (June, 2006, DRAFT).
Deviations:	Not applicable
GLP:	Yes, partially
Acceptability:	The study is considered to be supplementary.

Materials and methods:

Cyanamide F1000 was identified as follows:

Batch: N24/02, 710001, 710101

Purity: 99.8 %, 99.7 %, 99.3 %

Appearance: Colourless crystals

Stability in culture medium was demonstrated up to a storage time of seven days at 37 %. Cyanamide in water is demonstrated to be stable for a minimum of 6 month.

Vehicle:

water for cyanamide

Solvent DMSO for N-acetyl transferase (NAT) and ALDH inhibitors and substrates

Positive control: p-aminobenzoic acid (PABA), sulfamethazine (SMZ)

Test system: Primary hepatocytes; cytosolic and microsomal fractions of primary hepatocytes; recombinant NAT enzymes, canine peripheral blood mononuclear cells (PBMCs)

Species: Human, rat, dog

Strain: Sprague Dawley rat, Beagle dog

four individual human (3 males, 1 female), rat (4 males) and canine (2 males, 2 females) donors;

three individual human (3 females), rat (3 males), canine (3 males) donors;

3 canine donor blood pools of 4 individual donors each

In a mechanistic study, primary hepatocytes of four individual human, rat and canine donors were incubated for 96 hours with cyanamide at a concentration of 10 µg/mL for human donors and 5 µg/mL for rat and canine donors. Impact of cyanamide on albumin, ATP and urea concentration, LDH activity (membrane integrity), CYP450 activity and 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) concentration (cell vitality) was investigated. N-acetylation of cyanamide was assessed via analysis of N-acetylcyanamide in culture medium, in cytosolic and microsomal cell fractions of hepatocytes of 3 additional individual human, rat and canine donors, and also in incubation experiments with human recombinant NAT-1 and NAT-2 enzymes. The influence of competitive NAT-1 substrate (PABA), NAT-2 substrate (SMZ), and different inhibitors for NAT and ALDH on cell vitality and N-acetylation was also investigated. Gene expression analysis of the transcriptome was performed with hepatocytes of four individual donors of each species in order to detect species specific impact on mRNA transcription and thus elucidate mechanistic aspects of cyanamide toxicity. Finally, intracellular ROS level and catalase activity was assessed in canine PBMCs.

The following methods were applied. ATP was determined in a cell pellet fraction using the ATP dependency of light-emitting luciferase-catalysed oxidation of luciferin with the ATP Bioluminescence Assay Kit HS II (Roche Diagnostics). Lactate dehydrogenase (LDH) activity was measured using a colorimetric assay (Cytotoxicity Detection Kit (Roche Diagnostics)). Albumin synthesis was determined in the culture medium with an enzyme-linked immuno-sorbent assay

(ELISA) using peroxidase-conjugated goat anti human albumin (MP Biomedicals). Cell vitality was measured by means of the MTT assay based on cleavage of the tetrazolium salt MTT by metabolically active cells. Urea synthesis was measured in the medium with an enzymatic test using urease and glutamate dehydrogenase. CYP450 isozyme activity was assessed via metabolism of exogenously added testosterone. Intracellular level of reactive oxygen species (ROS) was assessed by staining with dichlorofluorescin diacetate and detection via Flow cytometry. Catalase activity was measured with the Amplex® Red Catalase Assay Kit (Invitrogen, Germany) based on the catalase mediated reaction to produce water and oxygen from H_2O_2. The role of NAT-1 and NAT-2 enzymes in the acetylation of cyanamide was investigated by use of human recombinant NAT-1 and NAT-2 enzymes. Time-dependent incubations with cyanamide (5 µg/mL) were performed at an enzyme concentration of 5 µg/mL under experimental conditions based on manufacturer's instructions (BD, Biosciences, Germany).

Conversion of cyanamide to N-acetylcyanamide in cytosolic and microsomal fractions of liver tissue was investigated in a concentration- and time-dependent fashion using samples from three individual human, canine, and rat donors. Cross-validation of an HPLC-MS-based method to detect N-acetylcyanamide in supplemented William's E medium was performed. The reference method for the detection of N-acetylcyanamide was provided by the sponsor. An HPLC-UV-based method was implemented to detect and quantify p-aminobenzoic acid (PABA) and sulfamethazine (SMZ), as well as their acetylated derivatives

Transcriptome analysis was done according to the manufacturer’s recommendation (Affymetrix Gene Chip* Expression Analysis Technical Manual, Santa Clara, CA, USA), using the GeneChip® Rat Genome 230 2.0 array (more than 31,000 probe sets, analysing over 30,000 transcripts and variants from over 28,000 well-substantiated rat genes), the Human Genome U133 Plus 2.0 array (over 47,000 transcripts from 38,500 well-characterised human genes), and the GeneChip® Canine Genome 2.0 (18,000 C. familiaris mRNA/EST-based transcripts and over 20,000 non-redundant predicted genes). The study focused on genes that were concordantly regulated by cyanamide in different donors. Gene expression results were validated by real time PCR. Further investigations of data obtained by microarray analysis were done by using the several bioinformatic tools including Pronciplew component analysis, Cluster analysis and pathway analysis.

Findings:

Biochemical data:

Treatment of cell cultures with cyanamide at modest cytotoxic concentrations induced a mild response in the regulation of gene expression. Whole genome analyses evidenced regulation of genes, which are involved in a number of processes such as liver specific-functions and protein synthesis. This was in accordance with clinical chemistry parameters, such as urea and albumin production, which slightly decreased with cyanamide treatment.

One major finding of this study was the species-specific difference in the metabolic clearance, i.e. acetylation of cyanamide (normalised to protein content) in dog liver cytosols, which was found to be significantly lower when compared to human and rat cytosols. The magnitude of N-acetylation in liver cytosolic fractions of the three different species was: human > rat > dog. The low acetylation capacity of microsomal and cytosolic fractions of canine liver tissue is in accordance with literature reports, where it was frequently questioned whether canine cells bear any cytosolic NAT activity. If at all, only minor acetylation activity was demonstrated in canine cells, which was mainly limited to the mitochondrial compartment. In the light of the generally distinct and less effective xenobiotic metabolism of the canine liver a reduced rate of cyanamide elimination via N-

acetylcyanamide could possibly contribute to an accumulation of cyanamide in dog liver and in other target organs of toxicity.

N-acetylcyanamide is reported to be the major urinary metabolite of cyanamide. According to the findings of this study, human liver tissue (microsomal and cytosolic compartments), when compared with liver cell fractions obtained from rat and canine donors, seem to be more competent in detoxification of cyanamide.

Prolonged treatment of hepatocyte cultures of all three species over 96 hours resulted in an induction of cyanamide acetylation, which can be concluded from elevated levels of N-acetylcyanamide in cell cultures media. Interestingly, this response was most pronounced in canine hepatocyte cultures and resulted in the finding that, on treatment day 5, N-acetylcyanamide levels measured in medium of canine hepatocytes cultures was comparable to those in human hepatocytes. The comparison of time-dependent change in acetylation rates shows an induction and thus a shift in acetylation capacity among the species after 5 days. Canine hepatocytes behave different, i.e. showing stronger acetylation over time, whereas humans show greatest acetylation rates on day 1.

Incubation assays indicated that NAT-1 and NAT-2 proteins are not responsible for the conversion of cyanamide to N-acetylcyanamide. This was indirectly supported by the finding that combinational treatments with NAT-1/2 inhibitor quercetin and competitive substrates for NAT-1 (PABA) and NAT-2 (SMZ) did not influence the production of N-acetylcyanamide in cultures of primary hepatocyte cultures.

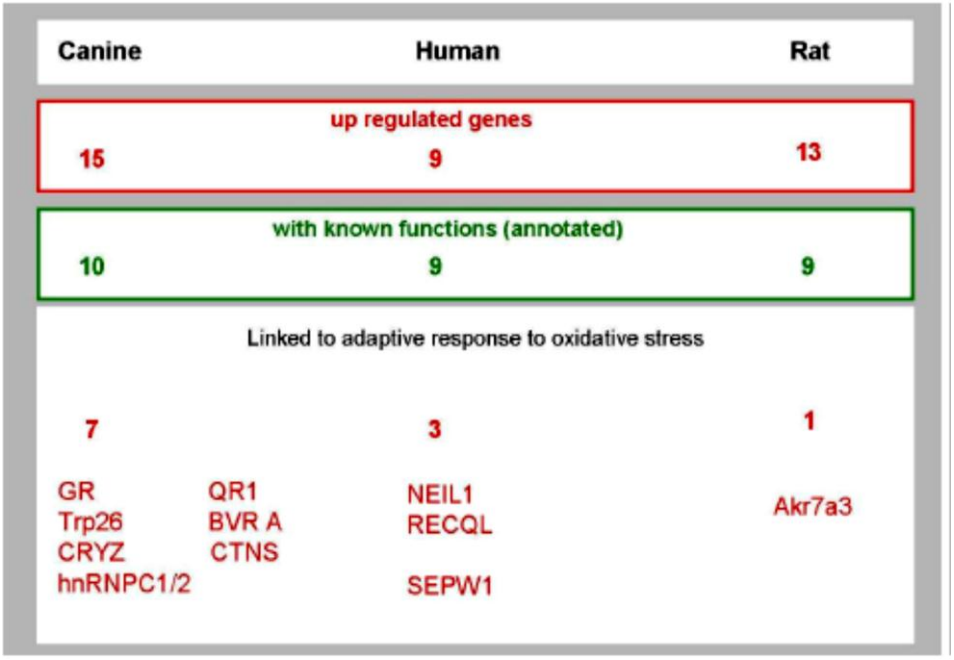
Gene expression and pathway analysis:

Concentrations of 5 and 10 µg/mL of cyanamide applied to the hepatocytes in this study were above the C_{max} values of 0.18 ± 0.03, 0.91 ± 0.11, and 1.65 ± 0.27 µg/mL achieved in the plasma of healthy volunteers who had received cyanamide orally at doses of 0.3, 1.0, and 1.5 mg/kg, respectively, based on the dose-range used for treatment of alcoholism (usually from 30 to 150 milligram per person/day; Yamauchi et al., 2000), with the maximal dose ever reported being 200 mg (which corresponds to 3 mg/kg bw/d, body weight 70 kg). Therefore, the concentration used in this study can be considered adequate to investigate the impact of cyanamide on gene expression, particularly to humans. Functional analyses of gene expression changes produced by cyanamide in hepatocytes of the three species may provide indications about a possible capability to induce adverse effects *in vivo* and possible species-specific differences.

Cyanamide induced a weak regulation pattern in cultures of primary hepatocytes of all three species. Only slight to moderate changes in gene expression levels (maximum fold changes 1.7-2.5 for upregulated genes and (-2.9) - (-4.0) for downregulated genes) were observed with a relatively small number of regulated genes. The total number of genes similarly regulated in the different donors in response to cyanamide treatment was in the same order of magnitude for all three species, i.e. 45, 64, and 90 down- and upregulated genes for rat, human, and dog. Despite the smaller number of regulated genes in human hepatocytes 24 genes exhibited expression changes equal to or more than 2-fold was as compared to the dog (9 genes) or the rat (8 genes), likely because of the 2-fold higher concentration of cyanamide used for the treatment of human hepatocytes. A list of the significantly regulated genes can be found at the end of this chapter.

Cyanamide exposure resulted in activation of genes involved in antioxidant response (Figure 2). This was especially evident for canine hepatocytes where most of the upregulated genes with known functions (7 from 10) were related to oxidative stress.

Figure 2: Cyanamide exposure activated in hepatocytes genes involved in adaptive response to oxidative stress



Only in dog hepatocytes cyanamide exposure led to downregulation of the apoptosis inhibitor c-Flip, for which expression is known to decrease in response to hydroxyperoxide. In human hepatocytes, 3 of 9 genes induced by cyanamide were involved in DNA repair and antioxidant defense. In rat hepatocytes, from 9 annotated genes induced by cyanamide the gene coding for Akr7a3 (aldo-keto reductase family 7, member A3 or aflatoxin aldehyde reductase) may represent a protective response to oxidative stress. Therefore, the activation of the genes discussed above indicates that oxidative stress is induced by cyanamide in canine and human hepatocytes. This induction may relate to cyanamide's metabolism and to mechanisms of action as a dormancy-breaking agent in plants and as an alcohol-deterrent in humans.

The major metabolic pathway in mammals is N-acetylation, which yields the urinary metabolite N-acetylcyanamide and is responsible for cyanamide detoxification. A second minor pathway involves oxidation of cyanamide to a reactive metabolite, postulated to be responsible for the aldehyde dehydrogenase (ALDH)-inhibitory properties of cyanamide exploited in the therapy of chronic alcoholism. In addition to ALDH, cyanamide also inhibited hepatic catalase, the enzyme responsible for the minor metabolic pathway, *in vivo*. It was postulated that catalase converts cyanamide to the instable N-hydroxycyanamide which would produce cyanide and nitroxyl by decomposition. Nitroxyl (HN=O) was suggested to react with sulfhydryl groups of the ALDH and to be responsible for its reversible and irreversible inhibition. Production of cyanide in the catalase-mediated oxidation of cyanamide was confirmed *in vitro* in the rat but not *in vivo* in humans. Cyanide may be responsible for inhibition of catalase and induction of oxidative stress as it can inhibit antioxidant enzymes and initiate generation of peroxides and hydroxyl radicals. In canine PBMCs a markedly reduced clearance of exogenous H_2O_2 in response to the inhibition of catalase activity by cyanamide at 10 µg/mL resulted in significantly elevated intracellular ROS levels. Cyanamide treatment alone did not significantly increase intracellular ROS, indicating that either the induction of the antioxidant response or the remaining activity of the catalase are sufficient to maintain low ROS levels in hepatocyte cultures. Canine erythrocytes have been demonstrated to display lower levels of catalase activity compared to human erythrocytes. Similar findings have

been reported for other tissues, such as liver and kidney and the dog may therefore be particularly sensitive to the toxic effects of impaired ROS detoxification. The mechanism by which cyanamide exerts its dormancy-breaking effect in plants is not clear, but it has been shown to inactivate catalase in grape buds shortly after its application, leading to the accumulation of hydrogen peroxide and the development of oxidative stress. Expression changes of known stress response genes in grape and kiwifruit buds supported the notion that oxidative stress may be involved in the signaling cascade leading to dormancy release. A role of Ca^{2+} signaling in this process is suggested. Several genes linked to calcium signaling were regulated by cyanamide also in human hepatocytes; however, genes that were upregulated in plants, SNF-like protein kinase (SNF1LK) and ATPase, Ca^{2+} transporting, plasma membrane 4 (ATP2B4), were repressed in the hepatocytes. Nevertheless, this indicates that similar pathways may be affected by cyanamide in plant and animal cells.

Gene expression of ALDH was not altered in cyanamide-treated hepatocytes, but exposure of human hepatocytes resulted in repression of class I alcohol dehydrogenases, i.e., subunits of alcohol dehydrogenase 1 ADH1A (= ADH1), ADH1B (= ADH2), and ADH1C (= ADH3), responsible for catalysing oxidation of primary and secondary alcohols to aldehydes and ketones. Alcohol dehydrogenases (ADH) and aldehyde dehydrogenases (ALDH) catalyse successive stages in the metabolism of various alcohols of both exogenous and endogenous origin. ADH1 and ADH2 are the major medium-chain dehydrogenase/reductase (MDR) enzymes in liver retinol metabolism, while ADH3 (less active) and ADH4 (most active) participate in retinoic acid (RA) generation in tissues. Other repressed genes, known to be also transcriptionally regulated by RA stereoisomers, were phosphoenolpyruvate carboxykinase 1 (soluble) PCK1, a key enzyme in gluconeogenesis, and epoxygenase CYP2C8, which is involved in the metabolism of xenobiotics, RA and arachidonic acid and affects numerous cell processes.

Furthermore, decreased expression of the nuclear receptor family member NR0B2 (small heterodimer partner) was observed. NR0B2 functions as a transcriptional repressor of other nuclear receptors (i.e. retinoid acid receptors, retinoic X receptors, constitutive androstane receptor, glucocorticoid receptor, thyroid hormone receptor among others) and seems to fulfill a central role in the modulation of nuclear receptor signalling pathways. NR0B2 is expressed in a wide spectrum of tissues and its roles in the liver, pancreas, and adipose tissue have been characterised. In rat hepatocytes, reduced expression of similar to retinoid binding protein 7 (predicted) was observed. RBP7 is a cellular retinol-binding protein coding for one of the cellular proteins interacting selectively with retinoids including retinol and retinal and structurally similar natural derivatives or synthetic compounds that need not have vitamin A activity.

Genes whose expression was significantly regulated and predominantly repressed by cyanamide in human and rat hepatocytes comprise diverse groups of genes with functions in lipid, protein, and carbohydrate metabolisms, transport, nucleosome assembly, regulation of transcription, cell growth and apoptosis, and xenobiotics metabolism. Although the genes that were sensitive to cyanamide in human and rat hepatocytes were not necessarily identical similar pathways appeared to be affected, e.g. linoleic acid metabolism, xenobiotic metabolism by CYP enzymes, arachidonic acid metabolism, monoterpenoid biosynthesis and limonene and pinene degradation. In contrast, the regulation of gene expression induced by cyanamide in canine hepatocytes was very different from those in human and rat cells. In dog hepatocytes, cyanamide treatment led to repression of genes involved in proliferation and survival, involved in bile acid homeostasis, or immune responses, pathways which were not observed in the other two species.

In human hepatocytes, cyanamide downregulated the nuclear receptors PPARGC1A and RORA, involved in the regulation of cell metabolism, and in accordance with this reduced the transcriptional levels of a number of genes involved in lipid, carbohydrate, and protein metabolism. Several genes involved in calcium ion homeostasis and the 11β- and 17β-hydroxysteroid

dehydrogenases were also affected by cyanamide. The latter enzyme was also downregulated in rat hepatocytes. Both enzymes are involved in the metabolism of steroid hormones.

Conclusion:

Primary hepatocytes from humans, dogs and rats showed slight signs of cytotoxicity and a reduction of cell viability by 30 % at most when treated *in vitro* with cyanamide for 96 hours at concentrations of 5-10 µg/mL. Cell toxicity was assayed by LDH leakage, ATP levels, urea and albumin production. Oxidative stress resulting from the inhibition of catalase appears to contribute to the cytotoxicity. Cyanamide metabolism by N-acetylation was shown in the cell cultures and was inducible over the incubation time at different rates for the three species. Human hepatocytes exhibited a greater capacity for acetylation and thus detoxification that rat and dog liver cells. Production of N-acetylcyanamide was highest in cytosolic fractions. Human liver cell fractions showed the highest acetylation rate of cyanamide, but also concentration-dependent saturation within the investigated concentration range. The capacity of N-acetylation activity towards cyanamide in microsomal and cytosolic cell fractions followed the order human > rat > dog.

Cyanamide induced a number of gene expression changes in primary hepatocyte cultures from of all three species. Maximum fold changes for different mRNAs were 1.7 to 2.5 for up regulated genes and -2.9 to -4 for downregulated genes in a relatively small number of regulated genes. The number of genes with statistically significant altered gene expression can be grouped in a descending order: dog (15 up-, 75 downregulated) > human (9 up-, 55 downregulated) > rat (13 up-, 32 downregulated). Upregulated genes were mostly involved in anti-oxidant defense, especially in dogs. The gene expression profile induced by cyanamide in canine hepatocytes was distinctly different from those in humans and rats; not a single pathway was commonly regulated when compared to human hepatocytes. In contrast, five commonly affected pathways were identified in rat and human hepatocytes. In addition, there is some evidence that cyanamide downregulates hydroxysteroid dehydrogenases in liver cells and might interfere with steroid hormone production and metabolism. Caveats associated with this gene expression study relate to a lack of information on posttranscriptional and translational regulation, because mRNA and not protein levels were measured, and to the possible difference of gene regulation in other non-hepatic target tissues.

4.12.1.6 Human information

Medical surveillance on manufacturing plant personnel

Employees of the calcium cyanamide production plant of Degussa AG (formerly SKW Trostberg AG) are under constant medical supervision regarding worker safety. Special emphasis during these preventive examinations was given to the question of potential sensitising properties of hydrogen cyanamide. In the following several findings in humans (workers in production and farmers) are summarised.

The company physician of Degussa AG (formerly SKW Trostberg AG) could not diagnose striking diseases of workers handling calcium cyanamide or calcium cyanamide containing products. However, the contact with cyanamide leaded to hypersensitivity to alcohol, an effect which is used in alcoholism therapy (Gfaller, 1976).

It is reported that a certain effect of $CaCN_2$ is the reaction of intolerance after alcohol intake, which expresses as a so-called burning, a flush with redness and feeling of heat of the head, the neck and the upper part of the body often combined with tachycardia and dyspnea. Further health impairments, sometimes with fatal end, were reported repeatedly in the older literature. Convincing

evidences for the causal connections between the exposure to $CaCN_2$ and these damages are missed. They exclusively concerned farmers, but not workers in the production of $CaCN_2$. Except of the damages of the skin also diseases of the respiratory and gastrointestinal tract, the kidneys, the nervous and circulatory system as well as favouring of infectious complications and goitrogenic effects were in discussion. Furthermore, it was suggested that $CaCN_2$ causes a lack of reduced glutathione in the organism (Schiele et al., 1981).

An occupationally medical field study was performed on 65 workers of a calcium cyanamide factory with long- term exposure to $CaCN_2$. The maximal $CaCN_2$ concentration measured at the working places was 8.3 mg/m^3. No signs of diseases or health impairments which are possibly caused by $CaCN_2$ were found with the medical examination in the above mentioned organs and organ systems. There also was no decrease of the glutathione content of the total blood detectable. With an alcohol load of 22 voluntary workers 1 till 7 hours after the working shift in 6 cases modest and in 7 cases weak flush reactions were observed (Schiele et al, 1981).

Animal studies in rats and dogs revealed signs of functional disorders regarding the testes and the thyroid gland. These findings resulted in the question whether such effects are relevant for male industrial workers exposed to cyanamide. Blood samples were collected from 21 exposed and 9 unexposed persons and examined for follicle stimulating hormone (FSH), luteinising hormone (LH) and testosterone. In addition, total T3 (TT3) levels, total T4 (TT4), thyroxine binding globulin (TBG) and thyrotropin (TSH) were determined to investigate the thyroid function. For a rough estimation of the degree of exposure towards cyanamide, urine samples were collected from the participants and examined for acetylcyanamide (syn.: N- acetylcyanamide), the main urinary metabolite of cyanamide. A clearly recognisable exposure could be demonstrated in the workers employed in the calcium cyanamide production units. The comparison of the persons exposed and unexposed to cyanamide revealed normal hormone levels in both groups. According to this investigation disturbances of the gonadal function and the thyroid function can be excluded (Mertschenk et al, 1993).

32 persons engaged in the calcium cyanamide and hydrogen cyanamide production units of Degussa AG (formerly SKW Trostberg AG) participated in an extensive preventive medical examination. Special emphasis was put on the question of potential sensitising properties of hydrogen cyanamide. For each test person a comprehensive medical report was made including allergological findings from epicutaneous testing for hypersensitisation towards hydrogen cyanamide. The exposure studies showed that the test persons were exposed to hydrogen cyanamide to various extents (0.2 - 139.7 mg per two hands). These data naturally only indicate a momentary concentration. However, they show that for an average working period of 15.11 years in the calcium cyanamide production unit a permanent exposure did occur. The results from all these examinations did not show any case of confirmed or suspected allergy towards hydrogen cyanamide. It was concluded that the practical use of hydrogen cyanamide does not involve any allergic risk (Gloxhuber et al, 1989).

Two groups (I and II) of approximately 30 persons employed in the calcium cyanamide production units and associated areas of Degussa AG (formerly SKW Trostberg AG) participated in extensive preventive medical examinations. Particular emphasis was placed on the question of potential sensitising properties of cyanamide. For each test person from group I a comprehensive medical report was prepared, which included allergological findings from epicutaneous testing for hypersensitisation to cyanamide. In view of the fact that N-acetylcyanamide is the main urinary metabolite of cyanamide in man, which is detectable in urine, the test persons from group II were examined with respect to the excretion of N-acetylcyanamide prior to and after the work period. These persons also participated in medical and clinical examinations. The examinations of both groups did not give any indication of an increasing number of certain diseases. No case of

confirmed or suspected allergy to cyanamide was found. The present results of these occupational medical examinations confirm the findings of a previous field study with calcium cyanamide performed in 1981 (Mertschenk et al, 1991).

At Degussa AG (formerly SKW Trostberg AG), every day approximately 170 workers have direct contact to hydrogen cyanamide and its calcium salt. This number does not include persons (approximately 50) which have occasionally, but intensively contact with these products during repair and maintenance, e.g. mechanics etc.

From medical records of Degussa AG (formerly SKW Trostberg AG) workers since 1945 and on the basis of the observations of the company doctor since 1981, is confirmed that it has not been necessary up to now to transfer any workers from the hydrogen cyanamide and/or calcium cyanamide production plants to another working place due to allergic reactions or acute/chronic dermatitis (Bornemann, 1988).

Direct observation, e. g., clinical cases and poisoning incidents

It is reported that calcium cyanamide is intensively used as drug to deter drinking in alcoholics. The consumption of alcoholic beverages after intake of cyanamide or calcium cyanamide leads to intolerances. These are put down to an inhibition of acetaldehyde dehydrogenase thus leading to a retardation in ethanol breakdown which stops on the stage of acetaldehyde. Vasomotor effects such as dilatation of the skin vessels especially in the upper part of the thorax, in rarer cases also dizziness, dyspnea and tachycardia are typical for this phenomenon. The acetaldehyde accumulation in blood is probably one reason for this symptomatology. Gloxhuber (1989) reported that the intolerance reactions towards alcohol occur in man after daily cyanamide doses higher than 20 mg. In general doses of more than 50 mg of cyanamide or citrated calcium cyanamide are used in the alcohol aversion therapy. There are thousands of patients, which were treated with cyanamide or calcium cyanamide. Several clinical observations in alcohol treatment with cyanamide or calcium cyanamide were summarised. Assuming that no alcohol was consumed during the treatment, the observed toxic reactions can be regarded as drug side effects of cyanamide and/or calcium cyanamide. The observed drug side-effects with normal doses were generally slight and non-specific: symptoms like stomach distress, sore eyes, dizziness and itching that normally disappeared after a short period, even when the therapy was continued (Gloxhuber, 1989).

It is reported that a 29 year old man became ill, while spraying kiwi trees with cyanamide. The applicator was wearing a Tyvek suit, rubber boots, rubber gloves and a half-face respirator. He started spraying in the morning. He quit for lunch and removed his protective ensemble without decontamination. After lunch he started to stand up and became so dizzy that he collapsed, vomited twice and was taken to the hospital.

This worker experienced hypotension, vertigo, nausea, puffiness of the face and hypokalemia without exposure to alcohol while applying hydrogen cyanamide. He was wearing a full protective ensemble and there was no patient history or objective signs of alcohol use. The hypotension was without tachycardia and lasted four days after the exposure. The employee did not decontaminate before removing his protective ensemble for lunch but he was slightly ill in the morning. His illness accelerated after lunch. While the employee denied consuming any alcoholic beverages or having any illness prior to working the day of the exposure, the employer developed the information that the employee attended a party the night before, was complaining of a cold when he went to work that day and had a canister fall off his respirator the morning of the illness. Typically, illness with cyanamide has been associated with Antabuse reaction when used with alcohol (Lessenger, 1998).

A publication on hydrogen cyanamide-related illnesses reported in Italy during 2002-2004 was submitted by the notifier and summarised in the following (Anonymous; 2005). Furthermore, consideration of a warning phrase on the label is addressed by the notifier. The relevant text passages are presented in the following.

After Dormex®, a pesticide containing hydrogen cyanamide (49 % by weight), was introduced in Italy in 2000, a total of 23 cases of acute illness associated with exposure to this chemical were identified in early 2001. This led to a temporary suspension of sales and usage of Dormex on February 2002, and strengthening of protective measures, as specified on the pesticide label when sales were resumed on June 2003.

A total of 28 hydrogen cyanamide-related illnesses were identified during 2002-2004. Five cases were identified before the Dormex suspension was enacted, nine cases were identified during the suspension (whether workers used chemical purchased before the suspension or if an illegal purchase had occurred during the suspension is unknown), and 14 cases were identified after the suspension was lifted. All of the cases occurred in males; median age was 41 years (range: 25-65 years). All cases occurred from late December through early March of each year, which is the only period when Dormex is used in Italy. A total of 25 of the 28 cases occurred in persons who were exposed during application of Dormex. Another person was exposed while handling a Dormex packet found in the field and whose contents inadvertently spilled on the person, and one person was exposed from unintentional ingestion after the product was poured from its original container into a drinking bottle. For one case, no information on activity at time of exposure was available. Among the 25 cases involving exposure during application, 20 (80 %) occurred in persons who were exposed while using a backpack sprayer, two (8 %) while sitting in an open tractor cab, and one (4 %) while crossing a treated field; information was not available for two cases (8 %). Among the 21 cases with information available on use of personal protective equipment (PPE), only one involved a person who wore complete PPE (e.g., air-purifying respirator, goggles/face shield, chemical-resistant gloves, protective suit, and footwear). The majority (14 [66 %]) of these persons used incomplete PPE (e.g., five reported using an air-purifying respirator but no other PPE), and six used no PPE. No deaths and no illnesses of high severity were identified. Eleven (40 %) cases were classified as of moderate severity and 17 as low severity. Among the 14 cases that occurred after the Dormex suspension was lifted, seven (50 %) were of moderate severity. The latency between exposure and onset of adverse effects ranged from 30 minutes to 30 hours. In 13 cases, signs or symptoms appeared immediately after alcohol consumption. Skin-related signs and symptoms occurred in most cases.

It should be pointed out that among the 21 cases involving exposure during application and with information available on use of PPE, 20 persons did not wear personal protective equipment in accordance with label recommendations. Furthermore, it has to be considered that a warning phrase regarding the adverse effects of recently consumed alcohol in combination with application of Dormex has already been included on the label for the national registrations in Spain, Italy, Greece, France and Portugal.

Conclusion of the Rapporteur Member State (RMS): A warning phrase on the label as proposed during the peer-review in the PPP-procedure regarding the adverse effects of recently consumed alcohol in combination with application of cyanamide is supported.

Observations on exposure of the general population and epidemiological studies if appropriate

Reports of dermal sensitisation

Marconi et al. (1960) compared the side effects of disulfiram and calcium carbimide (calcium cyanamide) with a placebo in the treatment of alcoholism in 23 patients. They observed allergic reactions of the skin in some cases, however there was no difference between the 3 groups and they concluded that there are no hints regarding a sensitising potential of cyanamide (Marconi et al., 1960).

A 32-year old man had been working for a year and a half in a psychiatric ward as an assistant handling numerous medicines, mostly in the form of capsules and pills and sometimes as solutions. Four months ago, the pulps of his thumb, index and middle fingers on the right hand, showed an erythemato-squamous dermatitis which was very itchy and later developed fissuring. He did not respond to topical applications and antihistamines. He noted, however, that the dermatitis sometimes improved spontaneously when he was away from work.

During a normal working day he was in direct contact with about 40 medicines, such as tranquillisers, neuroleptics, vitamins, antialcoholic drugs, etc. Specific tests were performed with the medicines he normally handled; he gave a +++ positive reaction to Colme at 10 and 50 %. The reactions were still visible after 30 days as a bluish-black coloration. Thirty controls were negative. Colme contains 6 g per 100 mL of cyanamide in a stabilising vehicle of sorbic acid, sedium acetate and distilled water. Patch tests with the substances showed positive reactions only to cyanamide at 0.1, 0.5, 1 and 5 % in water at 48 and 96 h. All control tests were negative. The patient henceforth avoided all contact with Colme and his dermatitis resolved completely (Conde-Dalazar et al., 1981).

A research chemist developed a dermatitis of the upper lips around the nose, eyelids, ears, backs of hands, upper arms and sides of thighs. He had been working with paraformaldehyde, nitrogen and carbodimide (cyanamide) in DMSO. Another man in the same laboratory also had acute dermatitis of the face.

Patch tests with carbodimide in concentrations of 0.01, 1.0 and 5.0 % and applied for 48 hours were positive at 48 and 96 hours. The 5.0 per cent concentration appeared to be toxic. Six control subjects showed a toxic reaction to the 5.0 per cent dilution. At least two developed a local flaro at 14 days. One was re-tested and found to be sensitive to the 0.01 dilution (Calnan, 1970).

A 35-year old woman had a personal and family history of atopy and a previous nickel contact dermatitis. She had had a vesicular dermatitis with itching and sealing on her fingers for 2 month. A few days before consultation, she developed redness and swelling on the back of her left hand, marked oedema of the up of her nose and small papules on her palate and lips. She had been giving Colme solution to her husband for 4 month, as treatment for alcoholism. Colme contains 6 g per 100 mL of cyanamide (carbodiimide) in a stabilising vehicle of sorbit acid, sodium acetate and distilled water. She was tested with sorbic acid 5.7 per acetic acid 3 % aqueous dilution and Colme at 1 and 10 % aqueous dilution. Patch tests showed positive reactions to nickel sulphate (++) and 1 and 10 % Colme (++) at 48 and 96 h. On the day before her acute reaction, her husband changed the drug for water. Then in order to detect the difference she dropped some Colme on the back of her left hand to smell and taste it. This explains the localisation of her lesions. After 4 weeks the patient was completely cured because she had absolutely no contact with Colme. A minimum increase of pigmentation with a slight greyish colour, but not the bluish-black coloration was observed at the site of the Colme patch (de Corres and Lejarazu, 1982).

Three cases of allergic contact dermatitis from various forms of cyanamide have been described during the use of its as its calcium salt in a drug (Colme) for treating alcoholism.

A 35-year-old nurse had a 3-month history of erythematovesicular dermatitis on her left hand. She presented with severe oedema that limited the movement of her fingers. Erythematoviolaceous lesions, with some haemorrhagic blisters, were found. In addition, there was slight erythema on her right hand. She handled numerous drugs in her work, but suspected that Colme was the offender.

A 32-years-old assistant in a geriatric hospital, with a previous history of dyshidrotic eczema, presented with a 5-day history of erythemato-edematous lesions and isolated bullae on the hands, with pain and burning. He gave many drugs to alcoholic in patients and suspected that his dermatitis flared when he handled Colme.

A 54-year-old nun, who was an assistant in a geriatric hospital, presented with eczema of her fingers, dorsa of hands and face. Bullous lesions were found on her fingers. She had 4 crops of lesions during 1 ½ years. She believed her dermatitis to be due to the Colme that she gave to alcoholic inpatients. As in Case Nos. 1 and 2, lesions healed when she was away from work and was treated with topical corticosteroids (Bujan et al., 1994).

Conclusion:

On the basis of the above human data and based on the information from the cyanamide production Degussa AG (formerly SKW Trostberg AG), that no cases of confirmed or suspected allergy towards cyanamide occurred even though these persons were heavily exposed, there is very limited evidence that cyanamide causes skin sensitisation in humans.

Cyanamide in alcohol therapy

Calcium cyanamide was first tested for the treatment of alcoholism in 1956 (Armstrong and Knerr, 1956; cited in Frankos, 1987). It is reported that it is an effective deterrent to alcohol consumption because it blocks metabolism of acetaldehyde (an ethanol metabolite) by inhibition of hepatic aldehyde dehydrogenase. As aldehyde builds up in the bloodstream, a variety of unpleasant symptoms including facial flushing, tachycardia, dyspnea, hypotension, headache, nausea and vomiting occur in the affected individual (Brein et al, 1980; cited in Frankos, 1987). Thus an alcoholic, who is on a regular regime of calcium cyanamide, has a powerful incentive to abstain (Frankos, 1987).

It is reported that a large numbers of people have apparently taken therapeutic doses (50 mg every 12 to 24 hours) for years without an obvious ill effect and in one reported case a man ingested 34 tablets at one time without benefit of medical attention for approximately 10 hours. In spite of this he showed a normal pulse, blood pressure and respiratory rate. The only side effects noted were nausea, anorexia and mild drowsiness which lasted 2 days (Foster, 1959; cited in Frankos, 1987).

Vazquez et al. (1983) reported on the hepatic lesions produced in 17 alcoholic patients who had received cyanamide or disulfiram as aversion therapy. The characteristic lesion consists of cytoplasmic inclusions. They are found predominately in periportal hepatocytes. They appear to be persistent but are lost after death of the inclusion-bearing liver-cell when both the inclusion body and the dead hepatocyte are removed by macrophages. As well as the inclusion bodies, portal or periportal inflammation and necrosis of isolated liver-cells are seen. In one case, in which two biopsies were performed, cirrhosis developed while the patient was on cyanamide (Vazquez et al., 1983).

Vazquez et al. (1980) described the distinctive inclusions found on liver-biopsy specimens from three chronic alcoholics treated with cyanamide. Subsequent they studied four new alcoholics treated with cyanamide. In patients taking cyanamide, hepatic involvement correlates well with duration of treatment. Moreover, every patient taking cyanamide presents the characteristic hepatic

alteration. The authors concluded that these predictable lesions are developed in alcoholics taking this drug (Vazquez et al., 1980).

In a retrospective review of 2400 consecutive liver biopsy specimens, 60 cases with ground glass hepatocytes were identified, 41 specimens gave a positive reaction to orcein stain and 19 a negative staining. These 19 specimens were obtained from chronic alcoholics who had been admitted to a detoxification program that used aversive drugs and who were hepatitis B surface antigen negative. The use of cyanamide (Colme) could be documented in 11 instances. In addition to ground glass hepatocytes, which were periodic acid- Schiff positive and had a periportal or paraseptal distribution, these liver specimens showed a variety of hepatic lesions: cirrhosis in five cases, portal and periportal inflammation in six, triaditis in five, portal fibrosis in two and minimal changes in one. Patients with shorter courses of cyanamide were those who had less severe histologic lesions. In three patients who had a liver biopsy carried out before the cyanamide treatment ground glass hepatocytes were not found. The authors concluded that the data indicate that ground glass hepatocytes that stain with periodic acid- Schiff may develop after cyanamide treatment. They are associated with structural hepatic damage of varied severity in patients submitted to a long- term treatment (Bruguera et al., 1986).

A case of toxic liver disease induced by cyanamide in a patient in treatment with this drug during 18 months, was presented. The authors reviewed the different liver cell alterations produced by a long-term treatment with cyanamide, which produces fibrosis and portal inflammation, as well as polished cells with different characteristic features. These alterations forced the establishing of close controls of patients in treatment with this type of anti-alcoholism drug, as well as the reduction of the duration of therapy, this questioning the efficacy of the treatment of chronic alcoholism with this aversive drug (Llorente et al., 1989).

This is the first study that indicates the histopathological progression of the liver disease that was caused by the combination of both chronic alcohol use and cyanamide. Two sequential liver biopsy specimens were obtained on 29 alcoholics who relapsed with varying histories of cyanamide treatment. Cyanamide induced ground glass inclusions (GGIs) in the hepatocytes were observed. Two groups were identified, depending on whether GGIs proliferated or regressed, which was, in turn, found contingent on the duration of cyanamide treatment and the drug- free period. Group 1 included 14 cases in which GGIs either emerged only second biopsy specimen or else were increased in the second specimen as compared in the initial specimen. Group 2 composed of 15 cases in which GGIs were either not observed in either specimen or decreased in the second specimen as compared in the initial specimen. Acidophilic bodies were sequentially increased in five cases (36 %) of group 1 and in none of group 2. The severity of portal inflammation worsened in 10 cases (71 %) of group 1 but in 2 cases (13 %) of group 2, although the changes in fibrotic process did not differ between two groups. These differences could not be explained on the basis of the daily ethanol consumption and the length of relapses of the two groups. Thus, when cyanamide-treated alcoholics relapsed, the combined effect of cyanamide and alcohol produced the development of acidophilic bodies and portal inflammation along with the emergence of GGIs (Yokoyama et al., 1995).

In some countries, cyanamide is used as an alcohol intake inhibitor with few harmful side effects. So far, 3 cases of allergic contact dermatitis and only 1 of lichenoid drug eruption due to cyanamide have been reported.

The clinical manifestations, course, histopathologic and immunohistochemical findings of 7 cases of cyanamide-induced drug eruption are described. Of the 7 patients, 6 developed exfoliative dermatitis and one a lichen-planus-like eruption. Three patients with exfoliative dermatitis showed high fever, severe itching, anorexia, insomnia and general malaise, suggesting the diagnosis of

hypersensitivity reaction. In all cases mononuclear cell infiltration in the upper dermis with epidermotropism and dyskeratosis of the epidermal cells were observed histopathologically. The infiltrating cells consisted mainly of T_4-positive lymphocytes (Kawana, 1997).

A 34-year-old housewife with alcohol dependence vomited severely, lost consciousness and died after she took more than 20 mL of 1 %- calcium cyanamide and alcoholic beverage containing about 129 g of ethyl alcohol. An autopsy was performed around 16 h after death. The body weighed 55.5 kg and moderate lung oedema was found. Ethanol concentrations were 4.24 mg/g in the left heart blood, 4.39 mg/g in the right heart blood, and 21.55 mg/g in the stomach contents. Cyanamide concentrations were 0.63 μg/g in the left heart blood 0.20 μ/g in the right heart blood, and 0.22 μg/g in the stomach contents. The cause of death was determined to be acute ethanol intoxication with alcohol-cyanamide reaction (Kojima et al., 1997).

Mukasa et al. (1964) reported that cyanamide has sure and prompt anti-alcoholic effects. It has a safe and almost no harmful side-effects and can be used in a simple manner. Furthermore, the use of a small dose (10 to 60 mg) of cyanamide prevents morbid alcoholism and permits the adjustment of the patient's alcohol tolerance by an appropriate dosage. The appropriate dosage of cyanamide differs with individuals and can not be fixed categorically. Roughly, however, the dose can be fixed at best at 10 to 60 mg. Since this medicine is effective, for at least 12 hours, starting 10 minutes after administration of the alcoholic it is enough to use it only once a day. Experience shows, that the period of treatment should be at least five months (Mukasa et al., 1964).

It is reported that cyanamide inhibits aldehyde dehydrogenase (ALDH), the enzyme which catalyses the oxidation of acetaldehyde to acetic acid. When calcium cyanamide-treated alcoholics consume ethanol, their blood acetaldehyde levels raise, resulting in some or all of the following effects: tachycardia, tachypnea, dyspnea, flushing, hypotension, headache, nausea and vomiting. There are areas of concern associated with anti-alcohol drug therapy. Medical complications may arise when ethanol is ingested by individuals pre-treated with calcium cyanamide. It is concluded that there is evidence of toxicity associated with the repeated administration of these drugs and also of acetaldehyde-induced hepatoxicity and cardiotoxicity in experimental animals. It is regarded as possible that alcoholics who receive the drug and who continue to drink may experience some degree of acetaldehyde-induced cardiotoxicity and/or hepatoxicity. In the view of reports of appreciable anti-thyroid activity in animals and liver damage in alcoholics, the authors recommend that calcium cyanamide should be used with careful medical supervision (Peachy et al., 1981).

It is reported that the initial clinical trials with calcium cyanamide indicated that alcoholics experienced fewer side effects and milder drug-ethanol reactions than those treated with disulfiram.

It is reported that calcium cyanamide exerts significant antithyroid activity in animals and is used with caution in alcoholics with thyroid disease. Calcium cyanamide was recommended in place of disulfiram in alcoholics treated with severe liver dysfunction. The significance of inclusion bodies with calcium cyanamide is not clear but these changes in the smooth endoplasmatic reticulum could be due to the effects of one or all of the following: excessive ethanol use, raised blood acetaldehyde levels or the alcohol deterrent drug with which the patient was treated.

Calcium cyanamide was administered orally in 50 mg doses taken twice daily to provide continuous anti-alcohol protection. It is reported that for those individuals who only drink at certain times of the day, one dosage of calcium cyanamide taken up to 12 hours before the usual drinking period will provide adequate protection against drinking during that interval (Peachy, 1981).

The disposition kinetics of ethanol and its toxic metabolite acetaldehyde were investigated in 10 healthy male volunteers (non-smokers with moderate drinking habits) who ingested 0.25 g/kg ethanol after an overnight fast. This dose of ethanol was given 2 hours after they swallowed 50 mg

calcium carbimide or placebo. The distribution volume of ethanol was 0.64 L/kg after calcium carbimide and 0.68 L/kg after placebo. The elimination of ethanol from the body was 1.9 mmol/kg after calcium cyanamide and 2.11 mmol/kg after placebo. The area under the ethanol concentration increased after calcium cyanamide treatment implying a change in clearance. The disposition of acetaldehyde was markedly different in subjects pre-treated with calcium cyanamide. The peak blood concentrations ranged from 40 - 242 µmol/L compared with 1.7 - 6.5 µmol/L after placebo. The apparent half-life of acetaldehyde after inhibition of acetaldehyde dehydrogenase was 23 min on average with a range of 18 - 31 min. The area under the acetaldehyde-time curve was increased significantly after calcium cyanamide pre-treatment. The calcium cyanamide ethanol interaction caused intense facial flushing in all subjects beginning 20 - 30 min after drinking. The reaction was most intense at about 30 - 60 min after drinking and was almost gone after 120 min. The most intense flushing coincided with the peak concentrations of ethanol and acetaldehyde in blood and breath. Some of the volunteers even complained of difficulties in taking deep inhalations and felt faint on standing up. There were marked increases in heart rate and decrease in diastolic blood pressure at or near time of most intense flush. Three hours after start of drinking some of the subjects were pale in the face and complained of headache. Despite abnormally high concentrations of acetaldehyde in blood and breath after pre-treatment with calcium cyanamide, the elimination kinetics of ethanol were not markedly changed from the placebo control trial. The authors concluded that these results do not support a significant role of acetaldehyde in regulating *in vivo* oxidation of ethanol in humans (Jones and Hillbom, 1988).

Peachy et al. (1980) reported that oral administration of ethanol up to 24 hours following calcium cyanamide (0.7 mg/kg) produced an increase in acetaldehyde level, tachycardia and increased pulse pressure due to decrease in diastolic blood pressure with reports of palpitations, shortness of breath and facial warmth by most subjects. Positive linear correlation exists between acetaldehyde level and physiological changes and acetaldehyde level and symptom responses, but there was appreciable individual variability in these responses. The magnitude and duration of chemical and physiological changes were greatest for the 4 hour calcium cyanamide pre-treatment interval and the 0.5 g/kg ethanol dose. For the 12 hour calcium cyanamide pre-treatment interval symptoms were reported for the times of increased acetaldehyde level and as the calcium cyanamide pre-treatment interval was shortened, symptom were intense but more variable. Symptom response ceased before the acetaldehyde level decreased before the acetaldehyde level decreased below 4.0 g/mL and the heart rate declined below 100 beats/min. Symptom responses were significant compared to control for the 5.0 g/kg ethanol dose only. Increase in heart rate and increase in pulse pressure were highly correlated with acetaldehyde level (Peachy et al., 1980).

Peachy et al. (1981) reported 3 cases to illustrate cardiovascular responses during carbimide-ethanol reactions.

A 46 year old male alcoholic, consented to take part in a cyanamide (citrated calcium cyanamide) ethanol study after 14 days of heavy drinking. Initially, he reported no history of angina, hypertension or syphilis, but after the study he volunteered additional medical history of bradycardia. On admission, his cardiovascular status, liver and renal function were all normal. Fifteen days after admission, he received 0.4 mg/kg ethanol orally over 15 minutes 1 hour after a standard breakfast and 10 hour after one dose of 0.7 mg/kg carbimide. During the experimental study, the blood acetaldehyde level and heart rate increased over 60 minutes, while blood pressure was relatively unchanged. Sixteen days after the first experiment he participated in an experiment in which he received a 0.4 mg/kg ethanol drink 12 hours after 0.7 mg/kg calcium cyanamide. The blood ethanol and acetaldehyde levels were similar to the changes in the first experiment. The heart rate and the pulse pressure changes observed over the first hour were the same as the changes that occurred in the first experiment and by 75 minutes he appeared to have recovered. However, 15

minutes later, he reported nausea, began retching and within a few seconds, he appeared pale and sweaty and complained of light-headedness. Immediately thereafter, he lost consciousness. Radial pulse was not palpable, heart sounds were absent and there was no systolic blood pressure. There was no evidence of myocardial activity.

A 39 year old male alcoholic with a 15 year history of alcohol abuse consented to take part in a cyanamide-ethanol reactions experiment. He received 0.4 mg/kg ethanol orally taken over 15 minutes and 1 hour after a standard breakfast and 10 hour after a single dose of 0.7 mg/kg carbimide. A moderately intense cyanamide-ethanol reaction occurred and at 30 minutes the volunteer described a floating sensation. At this time blood pressure decreased and heart pressure was increased.

A 37 year old male alcoholic with a 12 year history of drinking participated in the cyanamide-ethanol-reaction study in which he received 0.5 mg/kg ethanol, 4 hour after 0.7 mg cyanamide. The blood and ethanol levels rose and diastolic and systolic blood pressure fell. The volunteer experienced shortness of breath during the reaction and complained of exhaustion during and for several hours after the experiment (Peachy et al., 1981).

It is reported that cyanamide, as aversive drug widely used in Japan, develops ground glass inclusion bodies in the hepatocytes at high incidences, which may be associated with portal inflammation and fibrosis. When cyanamide-treated alcoholics relapse drinking, the combined effect of cyanamide and alcohol produce more severe portal inflammation along with the emergence of ground glass inclusions. Disulfiram also causes hepatitis, but there have been no comparative studies of effects of cyanamide and disulfiram on liver function (Tamai et al., 2000).

The authors reviewed the laboratory data of 408 alcoholics admitted for a 3 month course of alcohol detoxification and rehabilitation. Patients tested negative for hepatitis virus markers and were diagnosed as not having cirrhosis. Among the subjects 222 patients received cyanamide treatment (a daily dose of 70 mg) without a history of disulfiram treatment, and 186 received disulfiram (a daily dose of 200 mg) without a history of cyanamide treatment. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels obtained at 0, 4, 8 and 12 weeks of administration of each aversive drug were compared between the two alcoholic groups.

Elevation of serum transaminases (AST > ALT) probably due to alcoholic liver disease quickly fell after abstinence. In patients who took cyanamide, the ALT levels were significantly higher at 4 and 12 weeks than in those who took disulfiram. Re-elevations of ALT after alcohol detoxification were more frequently observed in those who took cyanamide than in those who took disulfiram (19.4 % vs. 5.9 %, $p < 0.001$). The re-elevations of ALT were slight to moderate, being more than 3-fold in three (1.4 %) patients who took cyanamide and four (2.2 %) who took disulfiram. The re-elevations occurred more frequently in those with a history of cyanamide treatment before the present treatment than in those who took cyanamide for the first time (31.1 % vs. 16.4 %, $p < 0.05$).

Cyanamide, compared with disulfiram, was more frequently associated with elevations of ALT that persisted after abstinence (Tamai et al., 2000).

The authors present the clinical and histological findings in a series of 42 liver biopsies from 39 chronic alcoholics treated with cyanamide as aversion therapy. All biopsies displayed characteristic cytoplasmic inclusions in the liver-cells. Fibrosis and disruption of the parenchymal- connective tissue interface were observed in all cases (Morena et al., 1984).

A 53-year old male alcoholic received cyanamide treatment for 4.5 months and completely abstained without cyanamide treatment for 6 years. A liver biopsy shortly after abstinence showed extensive pericellular fibrosis, but a biopsy after 6 years showed very mild fibrosis.

A 43-year old male alcoholic remained completely abstinent with cyanamide treatment for 5 years and complained of general fatigue. His serum transaminases were slightly elevated and hepatic hyperechogenicity was observed on ultrasonography. Only mild pericellular fibrosis was present in the liver biopsy specimen obtained shortly after abstinence, but after 5 years the second liver biopsy showed that thin septum-like fibrosis that formed portal-to-portal and portal-to-central linkage had developed and ground glass hepatocytes had emerged extensively.

A 29-year old female alcoholic complained of general fatigue and a slight fever after 1.5 years of abstinence with cyanamide treatment. Slight elevation of serum transaminases and hepatic hyperechogenicity were observed. The liver biopsy showed extensive ground glass hepatocytes and thin septum-like fibrosis that formed portal-to-portal linkage.

A 61-years old male alcoholic who remained completely abstinent while taking cyanamide for 3 years showed slight elevation of serum transaminases. Liver biopsy showed extensive ground glass hepatocytes and extension of thin septum-like fibers from portal tract to the lobule. Ultrasonography revealed hepatic hyperechogenicity (Suzuki et al., 1994).

Thomssen and Reinicke (1981) described a case of a 37 year old female with a long history of alcoholism. She was admitted to hospital complaining of epigastric distress and anorexia. For 2 years she received a daily oral dose of 0.25 g of Dipsan (cyanamide). During the treatment liver biopsies were carried out, showing histopathologic liver changes (ground glass inclusions in hepatocytes) during treatment with cyanamide and with total disappearance without sequelae after cessation of the treatment (Thomssen and Reinicke, 1981).

It is reported that cyanamide (COLME-drops) is a registered medicament in Austria used for treating chronic alcoholism (Anonymous, 1992; Anonymous, 1992).

1 mL (= 20 drops) of this aqueous solution contains 60 mg cyanamide.

According to the report (Mader, 2002) by Prof. Mader (Anton-Proksch-Institute Vienna; Therapy Centre for Alcohol and Medication Addicts), on his experience with the use of COLME-drops for the treatment of alcohol abuse, the medical treatment of alcohol abuse starts with a dose of 20 drops twice a day. This corresponds to 120 mg cyanamide/person/day. After a few weeks, the daily dosage is reduced to 15 - 20 drops, corresponding to 45 - 60 mg cyanamide/day. The duration of the treatment ranges from a few months to a few years. Patients, who in some cases have taken this medicament for more than 10 years, have received a dosage of 10 drops per day, corresponding to 30 mg cyanamide/day. For a person of 70 kg, a daily dose of 30 - 120 mg cyanamide corresponds to an oral intake of 0.4 - 1.7 mg cyanamide/kg bw/day. According to the report of Prof. Mader, no essential problems have been noted in the course of therapeutic experiences with COLME during the last 20 years (per year about 100 patients suffering from alcohol abuse have been treated with cyanamide) underlining the good compatibility of the administered doses of cyanamide.

Conclusion:

It is reported that calcium cyanamide is used as an effective deterrent to alcohol consumption because it blocks metabolism of acetaldehyde, an ethanol metabolite, by inhibition of hepatic aldehyde dehydrogenase. Cyanamide exposure and alcohol consumption induces several clinical symptoms, e.g. facial flushing, tachycardia, dyspnea, hypotensia, headache, nausea and vomiting.

According to personal information from the notifier, cyanamide is/was authorised as an alcohol deterrent in Spain, Russia, Ukrania and Japan. Approx. 720.000 cases have been treated. During routine pharmacovigilance, no effects on testes have been reported, however a specific investigation

regarding testes effects was not conducted. These information were given only orally and were not checked by the dossier submitter.

Periodic safety reports by Colme and others

Periodic safety reports by Colme were submitted by the notifier. The results are summarised in the following.

Colme, an oral solution containing cyanamide, is currently authorised to be marketed in Spain as an alcohol-aversive agent in the treatment of alcohol dependency, which contains cyanamide as an active ingredient. The duration of treatment is approximately 100 days. The recommended daily dose during the duration of treatment is 20 drops per day. One drop contains 3 mg cyanamide. In consequence, each patient was exposed to 60 mg/day during the duration of treatment (equivalent to 0.85 - 1 mg/kg bw/day, assuming a body weight of 60 - 70 kg). The periodic safety reports (PSR) summarise the experiences with the therapeutical use of Colme in Spain in the periods from 1971-2001 (Anonymous, 2001) and 2001-2006 (Anonymous, 2006), and Domingo (2007). They are described in Müller et al. (2007). The number of patients treated with Colme was 402,263 and 320,000 in the 10-year period from 1996 to 2000 and from 2001 to 2005, respectively (total 722,263).

1971-2001:

The first periodic safety report (PSR) contains safety information on cyanamide (Colme) from the date of marketing authorisation, 28 October 1971, to 30 June 2001. Between 1996 and 2000 around 80,400 patients were treated annually in Spain.

The adverse reactions, given in this PSR, were notified to the company directly by health professionals or through the Divison of Pharmacoepidemiology and Pharmacovigilance of the Spanish Drug Agency. Additionally, cases were taken from scientific literature. In all of these cases, Colme was, beside others, one of the drugs considered to be the possible cause of the adverse reactions.

Based on the data from notifications and from literature, the adverse reactions of cyanamide (Colme) can be classified into four main categories:

- haematological changes
- changes and findings in the hepatic histology
- allergic skin reaction
- cardiovascular changes

The haematological changes consist of seven cases of aplastic anaemia or agranulocytosis associated with cyanamide. In some cases the findings were associated with allergic contact dermatitis. Despite the severity of the symptoms, all of these cases recovered without sequelae.

The changes in the hepatic histology were noted in alcoholic patients treated with cyanamide at high doses and were reversible when the medication was withdrawn. Despite these changes appearing to be specific to cyanamide, the probability that the accumulation of acetaldehyde and alcohol's own hepatic toxic activity, have also contributed to these findings is high.

Allergic skin reactions occurred in nine cases, mostly through accidental contact of the solution with the skin in people handling Colme. Skin reactions of the same kind were observed in staff handling Colme on the production site and in the manufacturer's control laboratory.

One case of AV block (conduction block between arteria and ventricles) in the group of cardiovascular changes was described in a patient receiving treatment with Colme and an antidepressant of the selective serotonin re-uptake inhibitor class. Details and the outcome of this case are unknown.

2001-2006:

The second periodic safety report for Colme includes overall seven cases of adverse reactions, notified to the company between 1 July 2001 and 30 June 2006. During this time period approximately 64,000 patients were treated every year in Spain with Colme.

The adverse reactions noted, affected the following body systems:

- skin and subcutaneous tissue
- blood and lymphatic
- hepatobiliary

Concerning disorders in the blood and lymphatic system, three cases of agranulocytosis or medullary aplasia were recorded. All these reactions occurred in patients, treated with Colme and with other drugs for which this type of haematological reactions has been described. It is assumed, that a possible interaction between the drugs was the origin of the adverse reactions.

In the category of hepatobiliary disorders one case of Hepatitis was notified. The patient received treatment with two further drugs for a long unspecified period of time. The notifier considered all the drugs that the patient was receiving to be the cause of the reaction. For one of these drugs, hepatitis is indicated as a possible adverse effect, but not for Colme.

One case of overdose, a suicide attempt with cyanamide, was described in literature.

4.12.2 Summary and discussion

Cyanamide exposure (ingestion or inhaltion) alone when handled improperly or more pronounced in combination with alcohol consumption induces vasomotoric reactions, known as "Cyanamide Flush"; including several clinical symptoms, e.g. facial flushing, tachycardia, dyspnea, hypotensia, headache, nausea, vomiting, tightness in the chest and sensation of coldness in the extremities. In general these symptoms disappear with no residual effects on general health without specific treatment. In the cases of exposure to larger quantities (gram range/day) severe irrritating properties of hydrogen cyanamide to the mucous membranes were also observed. Additional effects such as trembling, convulsion, salivation, danger of aspiration, pains behind the sternum and in the epigastrum, unconsciousness and final exits can occur. After dermal exposure severe irritation can occur. In the case of poisoning a symptomatic therapy is recommended. No specific antidote is known. The main metabolite of cyanamide, N-acetylcyanamide can be measured in urine samples from humans and is suitable for biomonotoring purposes.

Calcium cyanamide has been worldwide intensively used as drug to deter drinking in alcoholics. The consumption of alcoholic beverages after intake of cyanamide leads to intolerances. This is probably due to an inhibition of acetaldehyde dehydrogenase thus leading to a retardation in ethanol breakdown which stops on the stage of acetaldehyde accumulating in the blood. Intolerance reactions towards alcohol occur in man after daily cyanamide doses higher than 20 mg. In general daily doses of more than 0.4 – 1 mg/kg bw cyanamide have been used in the alcohol aversion

therapy. The duration of the treatment ranges from a few months to a few years. In some cases patients have taken cyanamide for more than 10 years.

Several Spanish publications reported of abnormal liver histology produced in alcoholic patients who had received cyanamide as aversion therapy. These generally describe “ground glass hepatocytes”, which are not found in normal livers. Two of the reports also suggest that calcium cyanamide may cause actual liver disease. The significance of these cytoplasmic inclusion bodies has not been clarified. It is supposed that it could be due to the effects of excessive ethanol use, raised blood acetaldehyde levels and/or the alcohol deterrent drug. No signs of diseases or health impairments caused by cyanamide were found during medical surveillance on manufactoring plant personnel. Medical examinations also included special investigations of functional disorders regarding the testes and the thyroid gland and potential sensitising properties.

5 ENVIRONMENTAL HAZARD ASSESSMENT

Cyanamide is currently not legally classified related to the environmental hazards. The aquatic effect studies that are relevant for classification are presented in the following.

For the assessment of the aquatic environment all available studies were checked.

5.1 Degradation

5.1.1 Stability

Table 153: Hydrolytic degradation of Cyanamide in sterile buffers at 50/65 °C and 80 °C

Method/ Guideline	pH	Temperature [°C]	Initial TS concentration, C_0 [µg/ml]	Reaction rate constant, K_h [1/h]	Half-life, DT_{50} [h]	Coefficient of correlation, r_2	Reference
BBA Merkblatt Nr. 55 I; EPA-FIFRA 161-1	5	50	100	0.00062	1100	0.77	Eskötter, H. (1990), Doc No 711-001; Doc IIIA, Section A7.1.1.1.1/01
		80		0.01141	60.7	0.98	
	7	65		0.00123	563	0.97	
		80		0.00472	147	0.99	
	9	50		0.00229	302	0.96	
		80		0.0966	7.2	0.99	

Table 154: Hydrolytic degradation of Cyanamide in sterile buffers at 22 °C and 25 °C and 12 °C, calculated on the basis of eq. 25 of the TGD and the Arrhenius equation

pH	Half-life, DT_{50} [days]				Rate constant k [h^{-1}]	
	22 °C	25 °C	12 °C(*)	12 °C(**)	12 °C(*)	12 °C(**)
5	1200	830	958.2	4458	3.01×10^{-5}	6.48×10^{-6}
7	2300	1630	1628.2	8444	1.77×10^{-5}	3.42×10^{-6}
9	810	490	263.1	4456	1.1×10^{-4}	6.48×10^{-6}

(*) calculated using equation (25), TGD Part-II (EC, 2003)
(**) calculated by CA using the Arrhenius equation

The hydrolysis of Cyanamid was studied as a function of pH-value. Because at 25° C no hydrolytic degradation was observed, the test was performed at elevated temperatures. The half-life time for 22°C and for 25°C was recalculated from the experimental data using the Arrhenius equation. For the temperature correction to the average outdoor temperature in the EU of 285.15 K equation (25) in the TGD Part II (EC, 2003) was applied. Because the application of this equation can only provide approximated results and the respective experimental data were available, the temperature correction was performed by CA applying the Arrhenius equation. Despite the different calculation approaches, the results of these calculations show that Cyanamide is hydrolytic stable at all investigated pH-values.

Table 155: Photolysis in water

Method/ Guideline	Initial TS concentration	Total recovery of test substance [% of appl. a.s.]	Photo-lysis rate constant (k^c_p)	Direct photo-lysis sun-light rate constant (k_{pE})	Reaction quantum yield (Φ^c_E)	Half-life ($t_{1/2E}$) (days)	Reference
EPA Pesticide Assessment Guidelines, Subdivision N, Section 161-2, Photodegradation studies in water, October 1982	20 mg/L	Mass balance: 98.3 (light exposed) (mean) 96.1 (dark control) (mean)	Not reported	Not reported	Not determined, since the adsorption coefficient at 290 nm is below 10 in the UV-VIS spectra	Exposed: pH 5: 28.9 pH 7: 38.5 Non exposed: pH 5: 116 pH 7: 139	Schmidt, JM (1991), Doc No 712-001, Doc IIIA, Section A7.1.1.1.2/ 01

Under laboratory conditions (Xenon lamp 290 - 400 nm), Cyanamide was moderately degraded. Photolytic half-lives were calculated to be 28.9 d and 38.5 d in the light exposed samples at pH 5 and pH 7, respectively. Urea was detected as major degradation product in the light-exposed samples at maximum concentrations of 12.2 % of IMD (initial measured dose) at pH 7 and 42.4 % of IMD at pH 5. Urea was also detected in the pH 5 dark control samples at concentrations up to 8.18 % of IMD. Quantum yield was not determined in the presented study, since the adsorption coefficient at 290 nm is below 10 in UV-VIS spectra. Therefore, it can be concluded that in contrary to the laboratory conditions natural irradiation in Central Europe does not cause relevant direct photolytic degradation.

Table 156: Phototransformation in air

Guideline / Test method	Time-dependent OH radical concentration [OH radicals cm^{-3}]	Overall reaction rate constant k [cm^3 $molecule^{-1} \times s^{-1}$]	Half-life [h]	Chemical lifetime [h]	Reference
No Guideline available Estimation method by AOPWIN version 1.90	24-hours-day-time concentration of 0.5×10^6	0.0000×10^{-12}	The molecule does not react with OH-radicals	-	Peter, S (2003), Doc No 743-003; Doc IIIA, Section A7.3.1/01

Degradation of organic compounds in the atmosphere is mainly based on the reaction with hydroxyl radical. For this reaction the rate constant can be estimated by AOP. Together with information on the hydroxyl radical concentration in the atmosphere an estimate of the atmospheric half life is possible.

As a standard, an atmospheric hydroxyl radical concentration of 5 x 105 radicals/cm³ is assumed. According to the Atkinson calculation, Cyanamide is stable in the atmosphere. It is, however, questionable whether the Atkinson calculation allows for an adequate estimation of the photochemical degradation of Cyanamide. It has to be considered that Cyanamide is a substance that is chemically far away from typical organic molecules like phenols or halogen-hydrocarbons, for which the model seems to be better suited.

5.1.2 Biodegradation

5.1.2.1 Screening tests

Table 157: Ready biodegradability

Method/ Guideline	Test type	Test parameter	Inoculum			Additional substrate	Test substance Concentration	Degradation		Reference
			Type	Concentration	Adaptation			Incubation period	Degree [%]	
OECD 301 E	ready	Dissolved organic carbon	Activated sludge	2 mg suspended solids/l	No	No	29.4 and 58.8 mg a.s./l	28 d	0	van der Hoek, E; Hanstveit, AO (1988), Doc No 713-001; Doc IIIA, 7.1.1.2.1/01
Similar to OECD 301 B, modified test concentrations, additional test media	ready	CO_2 evolution	Activated sludge	30 mg dry weight/l and 100 mg dry weight/l	No	No, but in the second study part nitrogen free test medium was used, thus, Cyanamide served as the only nitrogen source	2 and 30 mg a.s./L, test concentrations related to DOC/l out of acceptable range	56 d, also measured after 28 days	Mean of two replicates19% (28 days)	Matla, YA, Hanstveit, AO (1990), Doc No 713-002; Doc IIIA, 7.1.1.2.1/02

The biodegradability of Cyanamide was investigated in a test on ready biodegradability according to OECD guideline 301 E. In this test Cyanamide was shown to be not readily biodegradable with 0% degradation within 28 days. A second study on ready biodegradability by measuring the CO_2 evolution was performed but could not be validated due to considerable deviations from OECD guideline 301 B concerning the concentration of Cyanamide applied. However, the results of this study confirm with 19% degradation in 28 days the characterisation of Cyanamide as not readily biodegradable.

5.1.3 Summary and discussion of degradation

Cyanamide has shown a biodegradation of 0% in 28 days in a test according to OECD guideline 301 E and has therefore to be regarded as not readily biodegradable.

5.2 Environmental distribution

5.2.1 Adsorption/Desorption

Table 158: Results of Adsorption/desorption screening test

Method/ Guideline/ Tested soils	Classification	Adsorbed a.s. [%]	K_d[1] (ml/g)	K_{OC} (ml/g)	Reference
OECD 106					Rüdel, H. 1990
Hörstel (8.97 mg/L)	Acidic sand	> 2	0.092	6.81	
Hörstel (0.89 mg/L)	Acidic sand	> 2	0.059	4.35	
Jülich	Loamy silt	> 2	0.060	6.34	
Borstel Ap	Slightly acidic silty sand	> 2	0	0	
Arithmetic mean				4.38	

[1] K_d = Distribution coefficient

The adsorption/desorption characteristics of Cyanamide on three soils were investigated. In all samples the adsorption of Cyanamide to soil was low. After 48 hour, the adsorption was less than 2 % for all soils and both concentrations. Linear distribution coefficients (K_d) were calculated from the mean ratios of adsorbed test substance to test substance in the aqueous phase. For the soil Hörstel adsorption coefficients, normalised to organic carbon K_{oc}, of approximately 6.81 mL/g and 4.35 mL/g were calculated for the high (8.97 mg/L) and low (0.89 mg/L) test concentrations, respectively. For the soil Jülich the K_{oc} value was calculated to be 6.34 mL/g, whereas with the soil Borstel Ap no adsorption was detectable. In compliance with the guideline, the desorption part of the study was not performed, because significant adsorption (approximately 25 % or less) had not occurred. The derived K_{oc} –values indicate, that Cyanamide will not be adsorbed in soils and point towards a high mobility potential of Cyanamide in soil.

5.3 Aquatic Bioaccumulation

Table 159: Summary of relevant information on aquatic bioaccumulation

Method	Remarks	Results	Reference
Standard equation (74), TGD on Risk Assessment (2003), Part II, chapter 3.8.3.2	- Log K_{OW} (measured) = -0.72	BCF = 0.049	-

5.3.1 Aquatic bioaccumulation

5.3.1.1 Bioaccumulation estimation

Table 160: Estimations on aquatic bioconcentration

Basis for estimation	log K_{OW} (measured)	Estimated BCF for fish (freshwater) on wet weight basis	Estimated BCF for fish eating bird/predator	Reference
Standard equation (74), TGD on Risk Assessment (2003), Part II, chapter 3.8.3.2	-0.72	0.049	-	-

An approximate estimation of the bioconcentration factor BCF_{fish} on basis of log K_{ow} = -0.72 was performed using the standard equation (74) given in the EU Technical Guidance Document (TGD) on Risk Assessment (2003), Part II, 3.8.3.2:

Log BCF_{fish} = 0.85 · log K_{ow} - 0.70

Log BCF_{fish} = 0.85 · (-0.72) - 0.70

Log BCF_{fish} = -1.312

BCF_{fish} = 0.049 (on wet weight basis)

Furthermore, no other indicators point to an intrinsic potential for bioconcentration; the surface tension, for instance, is 65.2 mN/m and thus lies above the trigger value of ≤ 50 mN/m.

5.3.1.2 Measured bioaccumulation data

In consequence of the low estimated BCF_{fish} an experimental study with fish was not required.

5.3.2 Summary and discussion of aquatic bioaccumulation

The aquatic bioaccumulation potential of Cyanamide can be classified as low.

5.4 Aquatic toxicity

Table 161: Summary of relevant information on aquatic toxicity

Method	Remarks	Results	Reference
Acute toxicity test to *Daphnia magna*	OECD 202	EC_{50} = 3.2 mg a.s./L	Adema, M.M. (1983)
Long term toxicity test with *Daphnia magna* (reproduction)	U.S. EPA-FIFRA, 40 CFR, Section 158.490, Guideline 72-4 (b).	NOEC = 0.05 mg a.s./L	Murrell, H.R. and Leak, T. (1995)

5.4.1 Fish

Short-term toxicity to fish

Table 162: Acute toxicity of Cyanamide to fish

Guideline/ Test method	Species	Endpoint / Type of test	Exposure		Results [mg a.s./L]			Remarks	Reference
			design	duration	LC0	LC50	LC100		
EPA 660/3-75-009 (1975) equivalent to OECD 203	*L. macro-chirus* (Bluegill Sunfish)	mortality	static	96 h	NOEC < 30.6	43.1	-	results based on nominal conc.	McAllister, W.A. et al. (1985) Doc. No. 821-002

Acute toxicity of Cyanamide to fish was investigated in a study which can be considered equivalent to OECD Guideline 203. *L. macrochirus* were exposed to a 49% aqueous solution of Cyanamide under static conditions for 96 h. No analytical measurement was conducted, but the test is nevertheless regarded as valid as Cyanamide is assumed to be stable in the test system. This was shown in a toxicity test with green algae (see below, Seyfried, B. (2000)) that was also performed under static conditions. Analytical measurement of the test substance concentration in this test shows mean measured concentrations between 85 and 80 % of the nominal concentration. A 96h-LC_{50} of 88 mg of a 49 % (w/w) aqueous solution of Cyanamide/L was determined, this corresponds to 43.1 mg a.s./L.

Long-term toxicity to fish

Table 163: Prolonged toxicity of Cyanamide to fish

Guideline/ Test method	Species	Endpoint / Type of test	Exposure		Results [mg a.s./L]			Remarks	Reference
			design	duration	LC0	LC50	LC100		
OECD 204, U.S. EPA-FIFRA, Guideline 72-1.	*O. mykiss (*Rainbow Trout)	mortality	flow-through	21 days	NOEC 3.7	11.8	-	results based on mean measured conc.	Bowman, J. and Herzig, R. (1990) Doc. No. 826-001

The prolonged toxicity to fish was investigated according to OECD Guideline 204 with *O. mykiss* under flow-through conditions for 21days. A total of 20 trout per concentration were exposed to water control and nominal test substance concentrations of 1.9, 3.8, 7.5, 15.0 and 30.0 mg of a 49 % (w/w) aqueous solution of Cyanamide/L. Mean measured concentrations were 99 ± 2.2 % of nominal concentrations. The 21day-LC_{50} was 24 mg of a 49 % (w/w) aqueous solution of Cyanamide/L, this corresponds to 11.8 mg a.s./L.

5.4.2 Aquatic invertebrates

Short-term toxicity to aquatic invertebrates

Table 164: Acute toxicity of Cyanamide to invertebrates

Guideline/ Test method	Species	Endpoint /Type of test	Exposure		Results [mg a.s./L]			Remarks	Reference
			design	duration	EC0	EC50	EC100		
OECD 202 (1981)	*D. magna* (water flea)	immobilisation	static	48 h	NOEC 1.6	3.2	-	results based on nominal conc.	Adema, M.M. (1983) Doc. No. 822-001

The acute toxicity of a 49 % aqueous solution of Cyanamide (Alzodef) to *Daphnia magna* was determined in an unaerated, static, 48-hour test according to OECD Guideline 202. Treatments consisted of a diluted water control, and nominal concentrations of 1.8, 3.2, 5.6, 10.0, 18.0, 32.0,

56.0, 100.0, 180.0 and 320.0 mg of a 49 % aqueous solution of Cyanamide/L. Every treatment contained four replicates with each five daphnids. In this test no analytical measurement was conducted, but as Cyanamide is assumed to be stable through the whole test duration (see above), the test is considered as valid and the 48h-EC_{50} is 6.5 mg of a 49 % (w/w) aqueous solution of Cyanamide/L, this corresponds to 3.2 mg a.s./L.

Long-term toxicity to aquatic invertebrates

Table 165: Long-term toxicity of Cyanamide to invertebrates

Guideline/ Test method	Species	Endpoint / Type of test	Exposure		Results [mg a.s./L]			Remarks	Reference
			design	duration	NOEC	EC50	LC100		
U.S. EPA-FIFRA, 40 CFR, Section 158.490, Guideline 72-4 (b)	*Daphnia magna* (water flea)	survivalgrowth **reproduction**	flow-through	21 d	- < 0.0115 **0.05***	> 0.2 - -	- - -	results based on mean measured conc.	Murrell, H.R. and Leak, T. (1995)

* The evaluation of the applicant resulted in different effect values. However, there was a mistake in the raw data.

The effects of a 50 % (w/w) aqueous solution of Cyanamide on the growth and reproduction of *Daphnia magna* were assessed in an unaerated, flow-through, 21-day test. Treatments consisted of a water control and nominal concentrations of 0.025, 0.05, 0.1, 0.2 and 0.4 mg of a 50 % (w/w) aqueous solution of Cyanamide/L, forty daphnids (4 replicates containing 10 daphnids) were exposed to each test level. Mean measured concentrations ranged from 92 to 105 % of nominal concentrations. In the test medium the test item was sufficiently stable during the test period of 21 days. Nevertheless, the results are based on mean measured concentrations.

Survival of *Daphnia magna* was not significantly affected in any test level when compared to the control. The 21-d-EC_{50} based on immobility was > 0.4 mg of a 50 % (w/w) aqueous solution of Cyanamide/L, this corresponds to 0.2 mg a.s./L. Also the mean weights were not significantly different from the control at any test concentration. The time to first brood was 7 days for the control and all test concentrations.

For the endpoint number of offsprings the 0.21 and 0.41 mg of a 50 % (w/w) aqueous solution of Cyanamide/L - test levels were significantly different when compared to the control and therefore the NOEC reproduction is 0.1 mg of a 50 % (w/w) aqueous solution of Cyanamide/L, this corresponds to 0.05 mg a.s./L. The parameter length was significantly affected in all concentrations compared to control and therefore NOEC length is < 0.023 of a 50 % (w/w) aqueous solution of Cyanamide/L, this corresponds to < 0.0115 mg a.s./L. As the mean weights were not significantly different to the control at any test concentration and as reproduction is regarded as most relevant parameter for the survival of a population, the appropriate effect value for assessment is the NOEC for reproduction of 0.05 mg a.s./L.

5.4.3 Algae and aquatic plants

Algae

Table 166: Growth inhibition of Cyanamide on algae

Guideline/ Test method	Species	Endpoint / Type of test	Exposure		Results [mg a.s./L]			Remarks	Reference
			design	duration	NOErC	EbC50[1]	ErC50[2]		
OECD 201 (1984)	*Pseudokirchneriella subcapitata* (freshwater microalgae)	growth inhibition	static	72 h	2.6	6.5	14.7	results based on nominal conc.	Seyfried, B. (2000) Doc. No. 823-003

1 calculated from the area under the growth curve; 2 calculated from growth rate

Pseudokirchneriella subcapitata (formerly: *Selenastrum capricornutum*) was exposed for 96 h under static conditions to a 51.1% aqueous solution of Cyanamide in three replicates and six controls. The mean measured concentrations ranged from 85 to 90 % of nominal concentrations, confirming the stability of Cyanamide in the test system, therefore effect values are based on nominal concentrations of the test item. Between 72 and 96 h there was no exponential growth of the algae, therefore effect values related to the 72-h-growth period are regarded relevant. The 72 h effect values contained in the table above were calculated by the CA. The relevant endpoint for the assessment is the growth rate with an ErC_{50} of 32.5 mg of a 51.1% (w/w) aqueous solution of Cyanamide/L, this corresponds to 14.7 mg a.s./L. The NOErC amounts 5.0 mg of a 51.1% (w/w) aqueous solution of Cyanamide/L, this corresponds to 2.6 mg a.s./L.

There is another growth inhibition test with *Anabaena flos-aquae* (Hertl, J. (2000)) available with a lower ErC50 (0.65 mg a.s./L) than the ErC50 from the test with *Pseudokirchneriella subcapitata*. However, the test with *A. Flos-aquae* does not fulfil validity criteria according to OECD 201 (i.e. exponential growth in the control) and so the lower effect value could not be used for classification and labelling purposes. The test validation with justifications for invalidity is presented in the IUCLID data set (Hertl, J. (2000) Doc.No. 823-004).

Other aquatic organisms (including sediment)

Not relevant for this type of dossier.

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

5.1 → Degradation: not readily biodegradable; hydrolytic stable; no photolytic potential in water and air

5.2 → Environmental distribution: high mobility potential in soil

5.3 → Aquatic Bioaccumulation: log Kow < 4 (low bioaccumulation potential)

5.4 → Aquatic Toxicity: not acute toxic (EC/LC_{50} > 1 mg/L), but toxic to aquatic life with long lasting effects (NOEC < 0.1 mg/L)

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

The effect level for acute category 1 with $EC_{50} \leq 1$ mg a.s./L was not reached for Cyanamide. The lowest acute value with an EC_{50} of 3.2 mg a.s./L is from an acute toxicity test with *Daphnia magna.*

In a long-term toxicity study with *Daphnia magna* a NOEC for reproduction of 0.05 mg a.s./L was determined, which triggers the environmental classification for chronic toxicity for not rapidly degradable substances.

According to CLP-Regulation the substance is proposed to be classified as Aquatic Chronic 1 (H410).

M-Factor: The chronic M-Factor is 1 based on the NOEC from test with daphnids of 0.05 mg a.s./L for a not ready degradable substance (i.e. $0.01 < NOEC \leq 0.1$ mg/L).

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