Acrylamide

Health-based calculated occupational cancer risk values

Gezondheidsraad **Voorzitter**
Health Council of the Netherlands

Aan de Staatssecretaris van Sociale Zaken en Werkgelegenheid

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Mijnheer de staatssecretaris,

Bij brief van 3 december 1993, nr DGV/BMO/U-932542, verzocht de staatssecretaris van Welzijn, Volksgezondheid en Cultuur namens de minister van Sociale Zaken en Werkgelegenheid de Gezondheidsraad om gezondheidskundige advieswaarden af te leiden ten behoeve van de bescherming van beroepsmatig aan stoffen blootgestelde personen. Tevens vroeg de staatssecretaris om in het geval van genotoxische kankerverwekkende stoffen luchtconcentratieniveaus af te leiden die samenhangen met een extra kans op overlijden aan kanker van 4 per 1 000 én 4 per 100 000 werknemers door beroepsmatige blootstelling gedurende een 40-jarig arbeidsleven.

In dat kader bied ik u hierbij een advies aan over de luchtconcentratieniveaus van de kankerverwekkende stof acrylamide. Dit advies is opgesteld door de Commissie WGD van de Gezondheidsraad en beoordeeld door de Beraadsgroep Gezondheid en Omgeving. Daarbij wil ik u laten weten dat de Commissie WGD in dit advies tevens een huidnotatie heeft aanbevolen. Verder brengt de commissie de neurotoxiciteit van acrylamide onder de aandacht. Zij verwacht echter niet dat neurotoxiciteit optreedt bij de afgeleide luchtconcentratieniveaus.

Ik heb dit advies vandaag ter kennisname toegezonden aan de minister van Volksgezondheid, Welzijn en Sport, de minister van Sociale Zaken en Werkgelegenheid en de staatssecretaris van Volkshuisvesting, Ruimtelijke Ordening en Milieu.

Hoogachtend,

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Acrylamide

Health-based calculated occupational cancer risk values

Dutch Expert Committee on Occupational Standards
a committee of the Health Council of the Netherlands

to:

the State Secretary of Social Affairs and Employment

No. 2006/05OSH, The Hague, June 28, 2006

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Contents

Samenvatting *9*

Executive summary *11*

1 Scope *13*
1.1 Background *13*
1.2 Committee and procedure *14*
1.3 Data *14*

2 Acrylamide *15*
2.1 General information *15*
2.2 Carcinogenicity studies *16*
2.3 Selection of the suitable study for estimating occupational cancer risk *22*
2.4 Lifetime low-dose exposure: carcinogenic activity in experimental animals *23*
2.5 Health risk in humans *24*
2.6 Calculation of the HBC-OCRv *24*
2.7 Existing occupational exposure limits *25*
2.8 Toxicity profile *26*

3 Skin notation *31*
3.1 Observations in humans *31*
3.2 Animal data *32*
3.3 Recommendation *33*

References *35*

Annexes *41*
A Request for advice *43*
B The committee *45*
C Comments on the public review draft *49*
D Neurotoxicity *51*
E Conclusions of the Committee on the Evaluation of the Carcinogenicity of Chemical Substances *55*
F Data on mutagenicity and genotoxicity *57*

Samenvatting

Op verzoek van de Minister van Sociale Zaken en Werkgelegenheid berekent de Commissie WGD van de Gezondheidsraad de concentratieniveaus in de lucht (HBC-OCRV's*) die samenhangen met een extra kans op overlijden aan kanker van 4 per 1 000 en 4 per 100 000 door beroepsmatige blootstelling aan stoffen, die door de Europese Unie of door de Commissie WGD als genotoxisch kankerverwekkend zijn aangemerkt. In dit rapport maakt zij zo'n berekening voor de door de EU aangemerkte kankerverwekkende stof acrylamide. Voor de berekening heeft de commissie gebruik gemaakt van de methode die beschreven is in het rapport 'Berekening van het risico op kanker'.[45]

Naar schatting van de commissie is de concentratie van acrylamide in de lucht, die samenhangt met een extra kans op overlijden aan kanker van

- 4 per 1 000 ($4x10^{-3}$), bij 40 jaar beroepsmatige blootstelling, gelijk aan 160 $\mu g/m^3$;
- 4 per 100 000 ($4x10^{-5}$), bij 40 jaar beroepsmatige blootstelling, gelijk aan 1,6 $\mu g/m^3$.

Verder beveelt de commissie voor acrylamide een huidnotatie aan.

* HBC-OCRV's: health-based calculated occupational cancer risk values.

Executive summary

At the request of the Minister of Social Affairs and Employment, the Dutch Expert Committee on Occupational Standards (DECOS), a committee of the Health Council of the Netherlands, derives so-called health-based calculated – occupational cancer risk values (HBC-OCRVs) associated with excess mortality levels of 4 per 1,000 and 4 per 100,000 as a result of working life exposure to substances, that have been classified by the European Union or the DECOS as genotoxic carcinogen. In this report the committee presents such estimates for acrylamide. For the estimation, the committee used the method described in the report 'Calculating cancer risk due to occupational exposure to genotoxic carcinogens'.[45]

The committee estimated that the concentration of acrylamide in the air, which corresponds to an excess cancer mortality of

- 4 per 1,000 ($4x10^{-3}$), for 40 years of occupational exposure, equals to 160 $\mu g/m^3$;
- 4 per 100,000 ($4x10^{-5}$), for 40 years of occupational exposure, equals to 1.6 $\mu g/m^3$.

Furthermore, the committee recommends a skin notation for acrylamide.

Chapter 1

Scope

1.1 Background

In the Netherlands, occupational exposure limits for chemical substances are set using a three-step procedure. In the first step, a scientific evaluation of the data on the toxicity of the substance is made by the Dutch Expert Committee on Occupational Standards (DECOS), a committee of the Health Council of the Netherlands, at request of the Minister of Social Affairs and Employment (annex A). This evaluation should lead to a health-based recommended exposure limit for the concentration of the substance in air. Such an exposure limit cannot be derived if the toxic action cannot be evaluated using a threshold model, as is the case for substances with genotoxic carcinogenic properties. In that case, an exposure-response relationship is recommended for use in regulatory standard setting, *i.e.,* the calculation of so-called health-based calculated occupational cancer risk values (HBC-OCRVs). The committee calculates HBC-OCRVs for compounds, which are classified as genotoxic carcinogens by the European Union or by the committee.

For the establishment of the HBC-OCRVs, the committee generally uses a linear extrapolation method, as described in the committee's report 'Calculating cancer risk due to occupational exposure to genotoxic carcinogens'.[45] The linear model to calculate occupational cancer risk is used as a default method, unless scientific data would indicate that using this model is not appropriate.

In the next phase of the three-step procedure, the Social and Economic Council advises the Minister of Social Affairs and Employment on the feasibility of using the HBC-OCRVs as regulatory occupational exposure limits. In the final step of the procedure, the Minister sets the official occupational exposure limits.

1.2 Committee and procedure

This document contains the derivation of HBC-OCRVs by the committee for acrylamide. The members of the committee are listed in annex B. The first draft of this report was prepared by Ms MI Willems of the TNO Nutrition and Food Research, Zeist, The Netherlands, for the Ministry of Social Affairs and Employment.

In 2004, the President of the Health Council released a draft of the report for public review. The individuals and organisations that commented on the draft are listed in annex C. The committee has taken these comments into account in deciding on the final version of the report.

1.3 Data

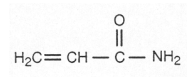
The evaluation of the carcinogenicity and other toxic effects of acrylamide has been based on reviews by IARC.[48,49] Where relevant, the original publications were reviewed and evaluated as indicated in the text. In addition, literature has been retrieved from the online databases Chemical Abstracts, Toxline, and Medline, covering the period 1966 to February 2006.

Chapter 2

Acrylamide

2.1 General information

Acrylamide does not exist as a natural product. Occupational exposure to acrylamide may occur during acrylamide production and use. Its identification, physical and chemical properties are shown below.[21,49,73]

Chemical name	:	acrylamide
CAS registry number	:	79-06-1
EEC no.	:	201-173-7
IUPAC name	:	2-propenamide
Synonyms	:	acrylic acid amide, acrylic amide, ethylene carboxamide, 2-propeneamide, prop-2-enamide, propenoic acid amide, vinyl amide
Description	:	odourless white crystalline solid which sublimes slowly at room temperature
Use	:	intermediate in polymer manufacture, especially polyacrylamides which are used in the treatment of municipal drinking water and wastewater; flocculant; in the pulp and paper, textile, and paints and coatings industries
Molecular formula	:	C_3H_5NO
Structure	:	
Molecular weight	:	71.08
Boiling point	:	125 °C at 25 mm Hg (3.3 kPa)
Melting point	:	84.5 °C
Relative density (air = 1)	:	2.46

Vapour pressure	:	0.9 Pa at 25 °C
Water solubility	:	2,155 g/L at 30 °C
Partition coefficient	:	-0.67 to -1.65 (log $P_{o/w}$)
Solubility in organic solvents	:	soluble in polar solvents such as acetone (631 g/L), methanol (1,550 g/L) and ethanol (862 g/L). Almost insoluble in non-polar solvents such as carbon tetrachloride
EC classification[a]	:	Carc. Cat. 2; R45, may cause cancer Mut. Cat.2; R46, may cause heritable genetic damage Repr cat 3; R62, T; R25-48/23/24/25, Xn ; R21/21, Xi ; R36/38, R43

[a] According to the 28th ATP of Council Directive 67/548/EEC of 5 August 2001.

2.2 Carcinogenicity studies

2.2.1 Overall conclusion

In 1994, IARC concluded that for acrylamide there was inadequate evidence in humans and sufficient evidence in experimental animals that the compound was carcinogenic.[49] Therefore, it classified the compound as probably carcinogenic to humans (Group 2A).

In addition, the European Union classified acrylamide in carcinogenic category 2, and in mutagenic category 2.

Recently, DECOS evaluated the carcinogenicity and genotoxicity of acrylamide.[46] The committee concluded that acrylamide should be regarded as carcinogenic to humans (comparable with EU category 2), and that it is genotoxic.

2.2.2 Human data

Since high concentrations of acrylamide may be formed in certain types of food prepared or cooked at high temperatures (*i.e.*, potato chips, French fries, coffee), there is concern about cancer risk from consuming these 'acrylamide-rich' food products.[32,65] However, in two recently published hospital- and population-based case-control studies, no association was found between consumption of acrylamide-rich food products and cancer risk.[61,71] In the meantime, the Norwegian Food Agency[28] estimated a mean daily oral intake of acrylamide to 0.49 μg/kg bw/day in males and 0.46 μg/kg bw/day in females, which should result in a lifetime cancer risk of 0.6×10^{-3}.

Regarding occupational exposure, IARC[49] and several other international authorities[39,64] reported on two cohort mortality studies (Sobel *et al*. 1986[77], and Collins *et al*. 1989[19]), which had been conducted among workers exposed to

acrylamide. A summary of the studies is given below. However, these studies have limited power to detect small increases in tumour incidence.

In the study by Sobel *et al*. (1986)[77], 371 employees participated, who worked in the acrylamide and polyacrylamide producing and processing area of one chemical plant in the USA, and began employment between 1955 and 1979. From 1957 to 1970, the acrylamide exposure ranged from 0.1 to 0.6 mg/m^3 in air (8-h TWA); after 1970, personal exposures were below 0.1 mg/m^3 for all job classifications. The investigators did not find an increased tumour and mortality rate. However, this outcome may be due to the small cohort size, and the short exposure and latency period.

In the other study, performed by Collins *et al*. (1989)[19], the cancer mortality in four plants, one in the Netherlands and three in the USA, was investigated in order to examine cancer risk by occupational exposure to acrylamide. The study comprised 2,293 workers who were potentially exposed to the substance between 1925 and 1983. For these workers, exposure to acrylamide was defined as a cumulative exposure greater than 0.001 mg/m^3-year, which is approximately equivalent to a one-day average concentration of 0.3 mg/m^3. Furthermore, to reflect current and proposed regulated levels of exposure, four categories of cumulative exposure were defined: <0.001 mg/m^3-years; 0.001-0.030 mg/m^3-years; 0.030-3.0 mg/m^3-years; and, > 3.0 mg/m^3-years. No excess of total cancer was observed among the exposed workers. There was a slight increase in cancer of the respiratory system, the pancreas and Hodgkin's disease, but these were not statistically significant. In addition, no trend in cancer mortality was found with increasing cumulative exposure.

Ten years later, an update of the study by Collins *et al*.[19] was published by Marsh *et al*. (1999).[58] In that follow up, only the three American plants were included. The update comprised 8,508 workers, of which 3,282 deaths were identified in the period 1925-1994; death certificates were obtained for 3,111 deaths. Although excess overall mortality risk for several cancer sites was found (*e.g*., the brain and central nervous system; the thyroid gland; the testis and other genital organs; cancer of the respiratory system), none of these risks were statistically significant or associated with exposure to acrylamide. Additionally, exploratory exposure-response analysis of rectal, oesophageal, pancreatic, and kidney cancer revealed increased standard mortality ratios, but little evidence of an exposure-response relationship. Overall, the findings of the update are in line with the findings in the study by Collins *et al*.[19]

2.2.3 *Animal data*

Carcinogenicity studies on animals were limited to oral exposure.

Johnson *et al*. (1986)[51] investigated the chronic toxicity and oncogenic potential of acrylamide in rats. Male and female Fischer 344 rats (90 rats/sex/group) were given drinking water containing 0, 0.01, 0.1, 0.5 or 2.0 mg/kg bw per day for 2 years. Ten rats/sex/group were randomly selected for examination after 6, 12 and 18 months on study. On all animals a complete gross pathologic examination was performed. Furthermore, organs obtained from necropsy were examined on microscopic pathologic lesions.

From the 21st month and onward, the mortality was increased in rats receiving 2.0 mg/kg bw/day, especially in females. At the end of the study, the increased mortality was statistically significant for both sexes. Furthermore, some rats from all dose groups showed excessive lacrimation and enlarged salivary glands on study day 210 for a period of up to 10 days. This was consistent with sialodacryadenitis virus (SDA) infection. However, the viral infection did not significantly affect the body weight, survival or tumour incidences.

Overall, ingestion of acrylamide increased the incidences of a variety of tumours in both male and female rats (Table 2.1). Most of these tumours developed in organs that are hormonally controlled (thyroid, uterus, mammary). The increases of these types of tumours were statistically significant in rats receiving the highest dose (2.0 mg/kg bw/day). Furthermore, scrotal mesotheliomas were also significantly increased at 0.5 mg/kg bw (18%, compared to concurrent (5%) and historical control (3.1%; range 2.0-6.0%) values), whereas other types of tumours were not. The authors consider these scrotum mesotheliomas as malignant, which is consistent with the potential biological behaviour of these tumours in Fisher 344 rats. Furthermore, the authors noted that scrotal mesotheliomas are very rare in humans. In contrast, rats (Fisher 344) appear unique in their ability to give spontaneous scrotal mesotheliomas.

A comparable study was performed by Friedman *et al.* (1995).[37] They used the same strain of rats, Fischer 344, and administered acrylamide in the drinking water at doses of 0, 0.1, 0.5 or 2.0 mg/kg bw to males, and 0, 1.0 or 3.0 mg/kg bw to females for two years. On all animals, complete necropsy and gross pathology examinations were performed.

Concerning mortality in males, the mortality was low through week 60, but increased from week 68-72 till the end of the study (75% at the end compared to 44% and 53% in two control groups). Over the first 23 months, the female rats

did not show any difference in mortality among the groups, but this increased slightly in the last month in the high dosed group. At termination, the mortality data were 28%, 40%, 35% and 49% for the two control groups, the low dosed and the high dosed animals, respectively.

Table 2.1 Number of tumour-bearing animals in rats given acrylamide in the drinking water for 2 years (Johnson *et al*. 86).[51]

Tissue/diagnosis[a]	dose (mg/kg bw per day)				
	0	0.01	0.1	0.5	2.0
Males					
CNS tumours of glial origin or glial proliferation suggestive of early tumour	5/60	2/60	0/60	3/60	8/60
thyroid gland, follicular cell adenomas	1/60	0/58	2/59	1/59	7/59*
testes, mesotheliomas of the tunica albuginea, malignant, with or without metastasis	3/60	0/60	7/60	11/60*	10/60*
adrenal gland, phaeochromocytomas, benign, primary[b]	3/60	7/59	7/60	5/60	10/60*
Females					
mammary tumours	10/60	11/60	9/60	19/58	23/61*
CNS tumours of glial origin or glial proliferation suggestive of early tumour	1/60	2/59	1/60	1/60	9/61*
thyroid gland, follicular adenomas or adenocarcinomas	1/58	0/59	1/59	1/58	5/60*
oral cavity, squamous papillomas	0/60	3/60	2/60	1/60	7/61*
uterus, adenocarcinomas	1/60	2/60	1/60	0/59	5/60*
clitoral gland, adenomas[c]	0/2	1/3	3/4	2/4	5/5*
pituitary adenomas[b]	25/59	30/60	32/60	27/60	32/60*

[a] Selection of microscopic observations with the most pronounced effects. [b] The historical incidence of adrenal gland phaeochromocytomas in males was 8.7% (range, 1.2-14.0%); that of pituitary adenomas in females was 38.1% (range, 28.2-46.9%).
[c] Only clitoral glands with gross lesions were examined histologically.
* $p = 0.05$; pair-wise Mantel-Haenszel comparison with the control group adjusted for mortality.

A summary of the lesions found in the animals is given in Table 2.2. As in the study by Johnson *et al*. (1986)[51], increased numbers of scrotal mesotheliomas were observed, which became statistically significant at the highest dose (2.0 mg/kg bw/day).

Using light and electron microscopy these tunica vaginalis mesotheliomas were considered to be of benign origin.[20] The committee noted that, although there is a morphological spectrum of mesothelial lesions, in general all mesotheliomas are considered to be malignant.[14]

Table 2.2 Selected tumour incidences in F344 rats, given acrylamide in the drinking water for 2 years (Friedman *et al*. 1995).[37]

	males					females			
dose (mg/kg bw per day)	0	0	0.1	0.5	2.0	0	0	1.0	3.0
Initial no. of animals per group	102	102	204	102	75	50	50	100	100
CNS*[a] *(glial origin)									
-brain, no. examined	*102*	*102*	*98*	*50*	*75*	*50*	*50*	*100*	*100*
astrocytomas	1	0	0	0	2	0	0	2	2
oligodendrogliomas	0	1	1	1	0	0	0	0	0
-spinal cord, no. examined	*82*	*90*	*68*	*37*	*51*	*45*	*44*	*21*	*90*
astrocytomas	0	0	1	0	1	0	0	0	1
total rats with CNS tumours	1	1	2	1	3	0	0	2	3
***thyroid gland**, no. examined*	*100*	*102*	*203*	*101*	*75*	*50*	*50*	*100*	*100*
follicular cell adenomas	2	1	9	5	12*	0	0	7	15
follicular cell adenomas, multiple	0	0	0	0	3	0	0	0	1
follicular cell carcinomas	1	2	3	0	3	1	1	3	7
total rats with follicular cell neoplasms	3	3	12	5	17	1	1	10	23*
***testes**, no. examined*	*102*	*102*	*204*	*102*	*75*				
testes, mesotheliomas of the tunica albuginea, considered as malignant	4	4	9	8	13*				
***mammary gland**, no. examined*						*46*	*50*	*94*	*95*
fibroadenomas						5	4	20*	26*
adenocarcinomas						2	0	2	4
hyperplasia						26	27	33	47
total rats with mammary gland neoplasms						7	4	21*	30*

[a] The brain (three levels) and the spinal cord (three levels) were examined in all control and high dose male rats and in all low and mid dose male rats that were found dead or sacrificed moribund.
* Statistically significant, $p < 0.001$ (Peto *et al*., 1980).

In male animals, tumours in the central nervous system of glial origin and in the thyroid glands were found. Concerning the thyroid glands, in particular the follicular cell adenomas were significantly increased in rats given 2.0 mg/kg bw/day.

Concerning female animals, only a few developed central nervous system tumours of glial origin. However, they showed a dose-dependent increase in follicular cell neoplasia of the thyroid gland, which was statistically significant at a dose of 3.0 mg/bw/day. Furthermore, the number of animals with mammary gland neoplasms was significantly increased at 1.0 and 3.0 mg/kg bw/day com-

pared to the two control groups. The latter was mainly due to the high incidence of mammary fibroadenomas.

Using the data of Johnson *et al*. (1986)[51] and Friedman *et al*. (1995)[37], the Crump Group compared T25 values (for tunica vaginalis tumours), which were estimated by two different methods: the 'traditional' single point estimate method, and the benchmark dose (BMD) method.[53,80] The T25 is the chronic daily dose in mg/kg bw which gives in 25% of the animals tumours at a specific tissue site, after correction for spontaneous incidence, within the standard lifespan of that species. The authors preferred the use of the BMD method above the single point estimate method, because the BMD method resulted in less variation of the T25 values between the two studies. Since risk estimation in this report is based on No-Observed Adverse Effect Levels (NOAELs) rather than T25 values, and at such low 'NOAEL' levels the variation between the two studies is smaller than at T25, the committee believes that using a linear 'one point estimate' model is still justified.

Tumour initiation and promotion studies

Bull and his colleagues (1984)[15,16] performed several initiation and promotion experiments in various strains of mice (Sencar, ICR-Swiss or A/J). Acrylamide was applied by various exposure routes (oral, topical or intraperitoneal), in combination with topical application of 12-*O*-tetradecanoylphorbol (TPA), a known skin tumour promotor (maximum dose of acrylamide applied, 50 mg/kg bw/application; repeated three to six times over two to eight weeks). The endpoints studied were skin tumour development, and in some studies also lung tumour development.

Acrylamide did not initiate skin tumours without TPA, but it did in combination with TPA in a dose-dependent manner (data not shown in this report). Lung tumours were found in A/J and ICR-Swiss mice after intraperitoneal injection of acrylamide and after oral exposure (by gavage), respectively, all without TPA treatment (data not shown).

The committee noted the unusual experimental design and concluded that several findings were contradictory.

2.3 Selection of the suitable study for estimating occupational cancer risk

Overall, the committee is of the opinion that the epidemiological studies are insufficient for assessing cancer risk, because of the limited power of these studies to detect small increases in tumour incidences and mortalities.

Regarding animal studies, of interest are the two adequately designed studies in rats, which were exposed to acrylamide through drinking water for two years (Friedman *et al*. 1995[37], Johnson *et al*. 1986[51]). The experimental design was almost the same: the same rat strains were used (Fischer 344); in both studies animals were exposed to acrylamide through drinking water; the doses between the studies were comparable (range between 0 and 3.0 mg/kg bw/day); both studies lasted 2 years; and, on all animals complete necropsy and gross pathology examinations were performed. One difference is that during the experiment some animals in the Johnson-study showed indications of a temporal SDA infection. However, most likely this may not have influenced to outcome. Another difference is that in the Friedman-study more animals per group were used than in the Johnson-study.

Overall, rats exposed to acrylamide showed clear increases in tumours in several organs (*e.g*., mesotheliomas in the tunica vaginalis of the testes, mammary gland fibroadenomas, thyroid follicular cell adenomas (both sexes)); some of these increases were statistically significant in the Johnson-study, but not in the Friedman-study (*e.g*., CNS tumours of glial origin); and, other tumours were only found in the Johnson-study, but not in the Friedman-study (*e.g*., uterine adenocarcinomas, mammary gland adenocarcinomas (female rats), oral papillomas (female rats), pheochromocytomas (males rats)).

Regarding the type and location of tumours, a majority shows a possible relationship with disturbed endocrine function (*e.g*., the thyroid, the mammary glands, the testes). This may point to an endocrine, nongenotoxic mechanism. Such a mechanism for the development of mesotheliomas found in the tunica vaginalis of the scrotum was suggested by Friedman *et al*.[37] and discussed in a report prepared for AMPA (Acrylamide Monomer Producers Association).[53] In this theory a connection between prolactin and testosterone levels, Leydig cell tumours and tunica vaginalis tumours is proposed for Fisher344 rats. However, the investigators were not able to prove their theory, one of the reasons being the very high background rate of Leydig cell tumours (90 to 97%) in the Johnson- and the Friedman-study (only reported by Friedman *et al*.[37]; no raw data presented). However, given that acrylamide is clearly genotoxic[49,68,75], the commit-

tee cannot exclude that these tunica vaginalis mesotheliomas have arisen following direct damage to the hormone-producing organ. Therefore, the committee based its cancer risk assessment on the genotoxic potency of acrylamide.

In conclusion, both the Johnson- and the Friedman-study are suitable for estimating occupational cancer risk. Of particular interest are the tunica vaginalis mesotheliomas found in the scrotum of male rats in both studies. These type of tumours were significantly increased at a dose as low as 0.5 mg/kg bw in the Johnson-study. Also in the Friedman-study they were increased at this dose, but not to a statistical significant degree.

Tunica vaginalis mesotheliomas do occur in humans, although very rare. Furthermore, in general they are considered to be malignant because of their biological behaviour. Therefore, the committee considers this type of tumours relevant. Furthermore, since no other types of tumours were found to be significantly increased at 0.5 mg/kg bw, the committee preferred to estimate its cancer risk values on these mesotheliomas.

Given the similarities of the Johnson- and the Friedman-study, the committee considered combining the data. However, the committee concluded that combining these studies introduces more uncertainties due to differences in study design. Consequently, since combining the results of both studies does not have added value, the Johnson study was used for estimating cancer risk values. The incidence of tunica vaginalis mesotheliomas in male rats given 0.5 mg acrylamide per kg bw per day was 11/60 versus 3/60 in controls (Johnson *et al*. 1986).[51]

2.4 Lifetime low-dose exposure: carcinogenic activity in experimental animals

The committee generally uses a linear extrapolation model for estimating cancer risk values, unless concentration-response data indicate that other models better estimate these values. In the case of acrylamide, the committee has discussed the use of a benchmark dose model. However, based on benchmark dose data presented in the evaluation report of the Joint FAO/WHO Expert Committee on Food Additives[33], the committee is of the opinion that this approach has no additional benefit and would result in comparable outcomes as the linear method. Therefore, the committee used the linear method for estimating cancer risk values.

Under lifespan conditions, the calculated incidence of tumour-bearing animals per mg/kg bw/day is calculated as follows*:

$$I_{dose} = \frac{I_e - I_c}{D \times (X_{po}/L) \times (X_{pe}/L) \times (\text{days per week}/7)}$$

$$I_{dose} = \frac{11/60 - 3/60}{0.5 \times (104x(7/1{,}000)) \times (104x(7/1{,}000)) \times 7/7}$$

$$I_{dose} = 0.5 \text{ [mg/kg bw/day]}$$

2.5 Health risk in humans

To estimate the additional lifetime risk of cancer in humans under lifespan conditions on the basis of results in animal experiments, it is assumed that no difference exists between experimental animals and man with respect to toxicokinetics, mechanism of tumour induction, target susceptibility, etc., unless specific information is available which justifies a different approach. Furthermore, it is assumed that the average man lives 75 years, weighs 70 kg, and is exposed 24 hours per day, 7 days per week, 52 weeks per year for lifetime.

2.6 Calculation of the HBC-OCRV

To estimate the potential additional lifetime risk of cancer in humans under workplace exposure conditions, it is assumed that the average man lives 75 years, is exposed 8 hours per day, 5 days per week, 48 weeks per year for 40 years, and inhales 10 m^3 per 8-hour-working day. Using as starting point the estimated incidence of 0.5 per mg/kg bw/day, the additional lifetime cancer risk per mg/m^3 under occupational exposure conditions, the HBC-OCRV, amounts to

$$\text{HBC-OCRV} = I_{dose} \times (40/75 \text{ years}) \times (48/52 \text{ weeks}) \times (5/7 \text{ days}) \times (10\ m^3) \times (70 \text{ kg bw})^{-1}$$

$$\text{HBC-OCRV} = 0.5 \times 0.53 \times 0.92 \times 0.71 \times 10 \times 70^{-1}$$

$$\text{HBC-OCRV} = 2.5 \times 10^{-2} \text{ [mg/m}^3\text{]}^{-1}$$

Based on the HBC-OCRV of 2.5×10^{-2} per mg/m^3, the additional lifetime cancer risk for acrylamide amounts to:

* I is estimated tumour incidence; I_e and I_c are tumour incidences in exposed and control animals, respectively; D is the daily dose (mg/kg bw); X_{po} and X_{pe} are exposure and experimental period, respectively; L is the standard lifespan for the animal species in question (L rats is assumed to be 1,000 days).

- 4×10^{-3}, for 40 years of exposure to 160 µg/m^3;
- 4×10^{-5}, for 40 years of exposure to 1.6 µg/m^3.

2.7 Existing occupational exposure limits

2.7.1 Occupational Exposure Limits

Several countries have established an occupational exposure limit (8-h TWA) for acrylamide. These include: the United Kingdom (0.3 mg/m^3, Maximum Exposure Limit[43]; Sweden and Denmark (0.03 mg/m^3)[6,63]; and, the United States of America (0.03 mg/m^3, ACGIH and NIOSH; 0.3 mg/m^3, OSHA)[5]. Furthermore, Germany has set Technical Exposure Limits: 0.06 mg/m^3 for use of solid acrylamide; and, 0.03 mg/m^3 for other acrylamides.[24] No exposure limits were set in the Netherlands and the European Union.[47,60]

Additionally, in a few countries a skin notation is added, indicating that skin absorption of acrylamide may considerably contribute to the body burden (*e.g.*, the United Kingdom[43], Germany[24], Sweden[63], Denmark[6], ACGIH and OSHA[5]).

2.7.2 Cancer classification and cancer risk values

As in the Netherlands, several other countries classified acrylamide as a carcinogen. For instance: Germany classified the compound as a category 2 carcinogen[24]; the United Kingdom listed it as may cause cancer (risk phrase R45)[43]; Sweden and Denmark marked the compound as carcinogenic[6,63]; and, ACGIH (the USA) classified the compound as an A3 carcinogen (*i.e.*, confirmed animal carcinogen with unknown relevance to humans).[5]

In 2005, a summary report was released by a Joint Expert Committee of the World Health Organization (WHO) and the UN Food and Agriculture Organization (FAO).[33] This committee concluded on the basis of tests in animals that cancer was the most important toxic effect of acrylamide, and indicated that risk assessment should be based on the genotoxicity and carcinogenicity of the compound. In addition, in the risk assessment report of the European Union, it was stated that "the lead effects of acrylamide are genotoxicity and the potential for carcinogenicity. For both mutagenicity and carcinogenicity it is not possible to identify a threshold level of exposure below which there would be no risk to human health and it is not possible to derive a toxicologically valid margin of safety".[31]

Furthermore, the US Environmental Protection Agency and the Norwegian Food Agency presented quantitative cancer risk assessments. These assessments

were based on the chronic carcinogenicity study by Johnson *et al*. (1986).[51] The assessments were somewhat different since they were based on different mathematical models. Using a linear multistage procedure and pooled benign and malignant tumour incidence data, the Environmental Protection Agency[29] calculated that 40 years of occupational exposure to concentrations of 0.03 mg/m^3 should result in an excess cancer risk of $2x10^{-3}$. In addition, using data on mammary tumours, and linear extrapolation; the tumour incidence indicators T25 and LED10; and, a scaling factor per kg bw in the extrapolation from rats to humans, the Norwegian Food Agency[28] calculated that a lifetime cancer hazard after lifelong exposure to 1 g/kg bw/day was on average $1.3x10^{-3}$ (LED10, 1.0×10^{-3}; T25, 1.6×10^{-3}).

2.8 Toxicity profile

It is beyond the scope of this risk assessment to evaluate in detail the non-carcinogenic effects of acrylamide. Therefore, for more details the reader is referred to evaluations and reviews published by other international authorities and reviewers, such as IARC[48,49], the American National Toxicity Program (NTP-CERHR)[65], the European risk assessment report on acrylamide[31], the German Chemical Society[39], NIOH and NIOSH [64], Shipp *et al*. (2002)[76], and Dearfield *et al*. (1988).[21] In the sections below, only a brief summary is given.

2.8.1 Human data

On repeated exposure, damage to both the central and peripheral nervous system has been described in occupationally exposed humans.[49] For instance, Hagmar *et al.* (2001)[42] reported on work-related peripheral nervous symtpoms, such as tingling or numbness in the hands or feet, in a group of tunnelworkers. These symptoms were due to a combination of dermal and inhalation exposure. Workplace studies indicate that workers exposed to 0.3 mg/m^3 or higher have increased prevalence of symptoms related to peripheral neuropathy compared to workers exposed to lower levels.[31] However, in the European Risk Assessment Report (2002)[31] it was stated that no adequate human information was available to establish a dose-response relationship between inhalatory exposure and neurotoxicity, because of insufficient information on control groups, and the considerable contribution of dermal exposure.

Upon contact with the skin and eyes, acrylamide may cause skin and eye irritation[23]. Also, one case of acute contact dermatitis is reported.

No human data were available to IARC or the committee on the reproductive and prenatal effects, or to genetic and related effects.

2.8.2 Animal data

A number of repeated dose studies have been performed in animals. The animals were mainly exposed to acrylamide via the food or drinking water. No data on inhalation exposure are available to the committee. The major effects of acrylamide concern neuropathy, cancer, and developmental and reproductive effects.[39,49,51,64]

Toxicokinetics and mode of action [21,22,39]

Acrylamide is highly water soluble. Therefore, after oral, dermal and inhalation exposure it is rapidly absorbed and distributed throughout the body.

In rats and mice, acrylamide is metabolized primarily via glutathione conjugates to mercapturic acids. By this way it is detoxified and quickly released from the body via the urine. Furthermore, the compound has been shown to be metabolized *in vitro* and *in vivo* in mice and rats (and in humans) to the reactive epoxide, glycidamide.[49] Both acrylamide and its epoxide form haemoglobin adducts *in vivo*.

One of the major non-carcinogenic effects of acrylamide are neurological effects. Although numerous studies have attempted to elucidate the mechanism of neurotoxicity, it is not yet completely clarified.

Acute toxicity

For the rat, guinea pig and rabbit, the LD_{50} after oral administration lies between 150-230 mg/kg bw. Furthermore, an LD_{50} value of 400 mg/kg bw was found after dermal application to the rat.[23,39,51]

Acrylamide is a slight skin and eye irritant. The findings in rabbits disappeared after 24 hours.[39]

Neurotoxicity

Concerning neurotoxicity, acrylamide induces peripheral neuropathy (central-peripheral distal axonopathy) in a variety of animals after subacute, subchronic and chronic administration. These effects may disappear after stopping exposure. Slight neurotoxic effects were found at doses as low as on average 1.0 mg/kg

bw/day in rats receiving acrylamide in the drinking water for a longer period (Burek *et al*. 1980).[17] In addition, based on data presented by Johnson *et al*. (1986)[51]and Friedman *et al.* (1995)[37] (2-year rat carcinogenicity studies), the European Union set a NOAEL for acrylamide neurotoxicity of 0.5 mg/kg bw/day, and a LOAEL of 2.0 mg/kg bw/day.[31]

An extensive description of the three studies mentioned in the previous paragraph is given in annex D of this report. Based on these studies, the committee considers 0.5 mg/kg bw/day as the NOAEL, which is in agreement with the conclusion of the European Union. Using this as starting point, a health-based occupational exposure limit of 490 μg/m^3 (8-h TWA) would be recommended.

Developmental and reproductive toxicity

In 2002, the Health Council of the Netherlands published a report on the reproductive and developmental toxicity of acrylamide.[44] In summary, "acrylamide has adverse effects on male and female reproduction capacity (*e.g*., reduced sperm count and motility, reduced number of pregnancies) in animals, but these effects were predominantly found at levels at which neurotoxic effects were observed. The effects on fertility cannot be explained from the neurotoxic effects and both effects may share a common molecular mechanism (via motorproteins). Therefore the compound is considered as a substance that should be regarded as if it impair fertility in humans (category 2 plus labelling with R60)". The Health Council did not recommend classifying acrylamide with respect to developmental effects, and effects during lactation, because of a lack of appropriate data.

Mutagenicity and genotoxicity

The genotoxic properties of acrylamide have been examined thoroughly by the Committee on the Evaluation of the Carcinogenicity of Chemical Substances of the Health Council. The conclusions and the genotoxicity data on which this conclusion is based are given in annex E and F, respectively. DECOS adopts the conclusions of this committee that acrylamide is a clastogen and a weak genotoxic carcinogen with a stochastic mechanism of action. In particular mutagenicity data obtained from Big Blue mouse cells, *in vitro* and *in vivo*, showed that acrylamide is genotoxic; not only the amount of DNA-adducts was significantly increased, but also the mutation spectra differed from those of the background mutations.

2.8.3 *In conclusion*

Based on the data available on the non-carcinogenic toxicity of acrylamide, the committee concludes that a health-based recommended occupational exposure limit for acrylamide in the air based on neurotoxicity, is higher than the concentration level (160 $\mu g/m^3$) associated with the referential lifetime cancer risk level of $4x10^{-3}$.

Chapter 3

Skin notation

The committee assessed whether for acrylamide a skin notation is needed. The purpose of a skin notation is to indicate the need to prevent skin contamination when systemic effects may result from percutaneous absorption of a substance as a gas, a solid, or a liquid. Below a summary is given of the available studies on skin absorption of acrylamide in humans and animals.

3.1 Observations in humans

Fennell *et al*. (2005)[34] studied the metabolism of acrylamide in humans. To the skin of the volar forearm a 50% solution of $^{13}C_3$ acrylamide was applied (24 cm^2; three daily doses of 3 mg/kg bw). After drying the site was covered with a gauze for 24 hours. For the analysis of hemoglobin adducts, blood samples were collected 24 hours after the first administration, and 48, 72, and 96 hours later. A total of six male volunteers participated, of which one did not receive acrylamide.

Of the total dose applied (calculation after analysis: 2.48 mg/kg bw) about 65 to 71% was recovered from dermal dam and wash solutions. Therefore, the mean absorbed dose was 0.86 $\pm$ 0.14 mg/kg bw/day. The authors indicate that this value 'probably represents the maximum that could be absorbed'. Comparing hemoglobin adducts after dermal and oral administration, the authors observed that only 17% (*N*-(2-carbomoylehtyl)valine adduct) to 25.3% (*N*-(2-carbamoyl-

2-hydroxyethyl)valine adduct) of the acrylamide penetrated the skin and was systemically available.

3.2 Animal data

Sumner *et al*. (2003)[78] studied the metabolism of acrylamide administered dermally, and measured the hemoglobin adducts produced. Radiolabeled acrylamide (162 mg/kg bw; dissolved in water) was topically applied to the dorsal skin (2.5 cm^2) of four male F344 rats for 24 hours. During these 24 hours urine and feces were collected. No skin irritation was observed during the treatment. After the treatment the animals were sacrificed to collect blood and various organs and tissues. The amount of applied dose that was absorbed ranged between 14 and 30%. Of the absorbed dose 36% was recovered in the urine, 0.5% in the feces, and 53% in body tissues.

For comparison, the portion of the inhaled dose (2.9 ppm for 6 hours) that was excreted in the urine was 31% of the total absorbed dose; in feces this was 3.1%, and in body tissues 56%. The total [^{14}C]-acrylamide recovered from rats was 89 ± 8.9 μmol/kg bw).

Dearfield *et al*. (1988)[21] evaluated the literature of metabolism of acrylamide. They reported that acrylamide is completely absorbed after oral administration (Miller *et al.* 1982)[59] in rats, but after dermal administration approximately 25% of the applied dose (2 or 50 mg/kg bw) was absorbed in the first 24 hours of exposure (Ramsey *et al.* 1984; source Dearfield *et al*. 1988).[21] Over 60% of the applied dose was excreted in the urine within 24 hours. In addition, Frantz *et al.* (1985)[36] reported that 26% of a 0.5% aqueous solution was absorbed through the skin of rats in 24 hours and that, after the skin was washed, an additional 35% remained in the skin.

Ten years later Frantz *et al*. (1995; source Ship 2002)[76] again reported on the dermal absorption of acrylamide. Three male F344 rats received a single dermal application of 2 mg/kg bw [^{14}C]-acrylamide on the dorsal side of the back (area: 16-20 cm^2). Blood samples were collected at several time intervals up to 24 hours following application. Also, at 12-hour intervals urine and feces, and expired $^{14}CO_2$ were collected. After 24-hours of exposure approximately 30% of the applied radioactivity was absorbed systemically and was recovered in the tissues and excreta (14% in urine; 7% in carcass; 4% in expired $^{14}CO_2$ and skin; less than 1% in feces). Of the total amount of administered dose 21% was considered unabsorbed.

3.3 Recommendation

ECETOC (1998)[30] recommends a skin notation when exposure of 2,000 cm^2 of skin (*i.e*., hands plus forearms) to acrylamide during one hour leads to an additional uptake of 10% of the maximum allowed uptake by inhalation (HBR-OEL or HBC-OCRV).

The committee based the calculation on human data presented by Fennell *et al*. (2005)[34]. The amount of acrylamide that was absorbed during 24 hours of exposure was 0.86 mg/kg bw. The authors estimated that about 17% of the absorbed amount reached the systemic circulation (hemoglobin adduct measurements), that is 0.15 mg/kg bw. The average body weight of the six volunteers was 85.8 ± 9 kg, and the exposure surface on the volar arms was 24 cm^2. The uptake of acrylamide on 2,000 cm^2 per hour corresponds to

$$\frac{0.15\ [mg/kg\ bw/24h] \times 85.8\ [kg\ bw] \times 2{,}000\ [cm^2]}{24\ [hr] \times 24\ [cm^2]} = 45\ mg$$

Assuming furthermore that a volume of 10 m^3 is inhaled in 8 hours and that a fraction f (by default assumed to be 0.5) of the atmospheric acrylamide is absorbed by the lungs, the uptake of acrylamide by inhalation is

0.16 [mg/m^3; HBC-OCRV, 4×10^{-3}] x 10 [m^3] x 0.5= 0.8 mg

0.0016 [mg/m^3; HBC-OCRV, 4×10^{-5}] x 10 [m^3] x 0.5= 0.008 mg

The calculations show that the one-hour additional dermal uptake considerably exceeds the 10% of the uptake by inhalation of the HBC-OCRVs. Therefore, the committee recommends a skin notation for acrylamide.

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A Request for advice

B The committee

C Comments on the public review draft

D Neurotoxicity

E Conclusions of the Committee on the Evaluation of the Carcinogenicity of Chemical Substances

F Data on mutagenicity and genotoxicity

Annexes

Annex **A**

Request for advice

In a letter dated October 11, 1993, ref DGA/G/TOS/93/07732A, to, the State Secretary of Welfare, Health and Cultural Affairs, the Minister of Social Affairs and Employment wrote:

Some time ago a policy proposal has been formulated, as part of the simplification of the governmental advisory structure, to improve the integration of the development of recommendations for health based occupation standards and the development of comparable standards for the general population. A consequence of this policy proposal is the initiative to transfer the activities of the Dutch Expert Committee on Occupational Standards (DECOS) to the Health Council. DECOS has been established by ministerial decree of 2 June 1976. Its primary task is to recommend health based occupational exposure limits as the first step in the process of establishing Maximal Accepted Concentrations (MAC-values) for substances at the work place.

In an addendum, the Minister detailed his request to the Health Council as follows:

The Health Council should advice the Minister of Social Affairs and Employment on the hygienic aspects of his policy to protect workers against exposure to chemicals. Primarily, the Council should report on health based recommended exposure limits as a basis for (regulatory) exposure limits for air quality at the work place. This implies:

- A scientific evaluation of all relevant data on the health effects of exposure to substances using a criteria-document that will be made available to the Health Council as part of a specific request

for advice. If possible this evaluation should lead to a health based recommended exposure limit, or, in the case of genotoxic carcinogens, a 'exposure versus tumour incidence range' and a calculated concentration in air corresponding with reference tumour incidences of 10^{-4} and 10^{-6} per year.

- The evaluation of documents review the basis of occupational exposure limits that have been recently established in other countries.
- Recommending classifications for substances as part of the occupational hygiene policy of the government. In any case this regards the list of carcinogenic substances, for which the classification criteria of the Directive of the European Communities of 27 June 1967 (67/548/EEG) are used.
- Reporting on other subjects that will be specified at a later date.

In his letter of 14 December 1993, ref U 6102/WP/MK/459, to the Minister of Social Affairs and Employment the President of the Health Council agreed to establish DECOS as a Committee of the Health Council. The membership of the Committee is given in annex B.

Annex **B**

The committee

- GJ Mulder, *chairman*
  emeritus professor of toxicology, Leiden
- RB Beems
  toxicologic pathologist; National Institute of Public Health and the Environment, Bilthoven
- LJNGM Bloemen
  epidemiologist; Exponent Inc., Terneuzen
- PJ Boogaard
  toxicologist; SHELL International BV, The Hague
- PJ Borm
  toxicologist; Centre of Expertise in Life Sciences, Hogeschool Zuyd, Heerlen
- JJAM Brokamp, *advisor*
  Social and Economic Council, The Hague
- DJJ Heederik
  professor of risk assessment in occupational epidemiology; IRAS, University of Utrecht, Utrecht
- TM Pal
  occupational physician; Dutch Centre for Occupational Diseases, Amsterdam
- IM Rietjens
  professor of toxicology; Wageningen University, Wageningen.

- T Smid
  occupational hygienist/epidemiologist; KLM Health Safety & Environment, Schiphol; and, professor of working conditions, Free University, Amsterdam
- GMH Swaen
  epidemiologist; Dow Chemical Company, the Netherlands
- RA Woutersen
  toxicologic pathologist; TNO Quality of Life, Zeist
- P Wulp
  occupational physician; Labour Inspectorate, Groningen
- ASAM van der Burght, *scientific secretary*
  Health Council of the Netherlands, The Hague
- JM Rijnkels, *scientific secretary*
  Health Council of the Netherlands, The Hague

With respect to the pathology data, the committee consulted an additional expert, prof dr V Feron, formely working at TNO Quality of Life. In addition, DECOS consulted the Committee on the Evaluation of the Carcinogenicity of Chemical Substances of the Health Council for the genotoxicity data. The committee consisted of:

- prof dr ir AA van Zeeland, *chairman*;
  professor of Molecular Radiation Dosimetry and Radiation Mutagenesis, Leiden University Medical Center, Leiden
- dr PJ Boogaard;
  toxicologist; Shell International BV, The Hague
- Ms HC Dreef-Van der Meulen;
  former toxicological pathologist, Organon, Oss
- prof dr LALM Kiemeney; professor in cancer epidemiology, Radboud University Nijmegen, Nijmegen
- prof dr H van Loveren;
  professor of immunotoxicology, University of Maastricht, Maastricht; and, National Institute of Public Health and the Environment, Bilthoven
- prof dr GJ Mulder;
  professor of toxicology, Leiden/Amsterdam Center for Drug Research, Leiden
- Ms dr MJM Nivard;
  molecular biologist and genetic toxicologist, Leiden University Medical Center, Leiden

- dr H Te Riele;
  molecular biologist, the Netherlands Cancer Institute,
  Amsterdam
- dr H Roelfzema, *advisor*;
  Ministry of Health, Welfare and Sport, The Hague
- prof dr W Slob;
  professor of quantitative risk assessment, University of
  Utrecht, Utrecht; and, Institute of Public Health and the Environment,
  Bilthoven
- dr RA Woutersen;
  toxicological pathologist, TNO Quality of Life, Zeist
- prof dr EJJ van Zoelen;
  professor of cell biology, Radboud University Nijmegen, Nijmegen
- dr GMH Swaen;
  epidemiologist, Dow Benelux BV, Terneuzen
- Ms dr A van der Burght, *secretary*;
  The Health Council, The Hague
- Ms dr JM Rijnkels, *secretary*;
  The Health Council, The Hague

The first draft of the present advisory report was prepared by Ms MI Willems, from the Department of Occupational Toxicology of the TNO Nutrition and Food Research, by contract with the Ministry of Social Affairs and Employment.

Secretarial assistance was provided by Ms M Javanmardi.
Lay-out: Ms M Javanmardi.

The Health Council and interests

Members of Health Council Committees are appointed in a personal capacity because of their special expertise in the matters to be addressed. Nonetheless, it is precisely because of this expertise that they may also have interests. This in itself does not necessarily present an obstacle for membership of a Health Council Committee. Transparency regarding possible conflicts of interest is nonetheless important, both for the President and members of a Committee and for the President of the Health Council. On being invited to join a Committee, members are asked to submit a form detailing the functions they hold and any other material and immaterial interests which could be relevant for the Committee's work. It is the responsibility of the President of the Health Council to assess whether

the interests indicated constitute grounds for non-appointment. An advisorship will then sometimes make it possible to exploit the expertise of the specialist involved. During the establishment meeting the declarations issued are discussed, so that all members of the Committee are aware of each other's possible interests.

Annex C

Comments on the public review draft

A draft of the present report was released in 2004 for public review. The following organisations and persons have commented on the draft document:

- T Scheffers, the Netherlands;
- Dr P Ungeheuer, Polyelectrolyte Producers Group, Germany;
- Dr E González-Fernández, Ministerio de Trabajo y Asuntos Sociales, Spain;
- Dr D Zumwalde, National Institute for Occupational Safety and Health, the USA.

Annex **D**

Neurotoxicity

Animal data on the neurotoxicity of acrylamide is limited to oral exposure.

In a well-designed study by Burek *et al*. (1980)[17] male and female Fischer 344 rats were given daily 0, 0.05, 0.2, 1, 5, and 20 mg acrylamide per kg body weight for up to 93 days. The acrylamide was administered in the drinking water. At each dose level there were 20 male and 10 female animals. Ten of the twenty males were kept alive for a further 144 days of recovery; the other animals were killed at 93 days. Also extra males were included for interim kills and ultrastructural (electron microscope) studies (n=3 in the 0.05, 0.2, 1 and 5 mg/kg bw groups; n=5 in the 20 mg/kg bw group).

During the exposure period control animals and animals in the highest dose group were weekly tested on the onset of neuropathy using the landing foot-spread (hind limb spraying) method. As soon as the high-dose animals (20 mg/kg bw) scored positive also the animals of the lower-dose group (5 mg/kg bw) were tested weekly. Since the animals of the latter group did not respond, animals in the other groups were not tested. Once the test was positive animals were not tested anymore.

Directly after ending the exposure a complete necropsy examination was performed on all animals, except one, which died before termination of experiment. The same examination was performed on 4 animals per group after 144 days of recovery. Tissues, specifically those from the central and peripheral nervous system were prepared for light microscopic examination. Sciated nerves from the

extra males were prepared for electron microscopic examination at different time points during and after exposure (n=3/time point).

The mean body weights of the animals at 20 mg/kg bw were statistically significantly decreased compared to control animals and the lower dosed groups. This significant decrease was observed within two (males) to three (females) weeks after starting exposure. Females at the highest-dose group also showed statistically significantly lower intake of water consumption compared to the other groups and the control animals. Males at the highest dose group were less clearly affected. The body weights and water intake of animals in the lower dose groups did not significantly differ from those of the control animals.

Measurements using the landing food spread method revealed that only animals at 20 mg/kg bw showed functional neuropathy (statistically significant after 22 days of exposure). In this group, the effects progressed during exposure, so that by the end all rats dragged their back lags. No treatment-related clinical effects were observed in the lower-dose groups.

Neurologic treatment-related histopathological alterations were primarily found in the peripheral nerves (*i.e.*, sciatic nerves) and spinal cord of the 20 mg/kg bw group, and in the peripheral nerves of the 5 mg/kg bw group. These included both axon and myelin degeneration. Such lesions were observed in all male and female rats given 20 mg/kg bw and in most male and female rats given 5 mg/kg bw. In the latter group the lesions were less severe and varied from equivocal to very slight in part of the animals. No peripheral nerve lesions were observed in rats at the 0.05, 0.2 and 1 mg/kg bw dose groups.

Sciatic nerve tissues were prepared for electron microscopy for all dose groups at day 90 only (n=3/group). At earlier time points only tissue was taken from the 20 mg/kg bw dose group. The main ultrastructural alterations observed were axonal degeneration and axonal loss (*e.g.,* axolemma invaginations of Schwann cells). These kinds of lesions were most severe in the 20 mg/kg group (55% of the examined fields showed alterations), but also in the 5 mg/kg bw group and to a lower extent in the 1 mg/kg bw lesions were present (in 34% and 25% of the examined fields, respectively). No treatment-related ultrastructural alterations were observed in the 0.2 and 0.05 mg/kg bw groups.

In male animals drinking daily 20 mg/kg bw acrylamide, which were allowed to recover, clear improvements and total recovery of neuropathy were reported. Also their body weights returned to control values. In addition, the investigators observed a clear partial reversion of lesions found in the sciatic and brachial nerves that, according to them, would have been completely reversed if the recovery period after exposure was longer than 144 days. Also ultrastructural analyses revealed regeneration and remyelination of the axons of Schwann cells.

For the 5 and 1 mg/kg bw groups the regeneration and remyelination was complete after 111 days and 25 days of recovery, respectively. At 144 days of recovery, in the 20 mg/kg bw group lesions were still present, but these lesions were less severe compared to the last day of exposure (recovery day 1), and recovery appeared to progress.

The investigators also reported on clinical chemistry analyses and histopathologic effects in other mayor organs, including: decreased relative and absolute weights of the thymus and testis (20 mg/kg bw); and histopathologic tissue alterations in the liver, testis (atrophy), and skeletal muscles (see original paper for complete reporting). In addition, these lesions partially or completely reversed during the recovery period.

In the two-year drinking water studies by Johnson *et al.* (1986)[51] and Friedman *et al*. (1995)[37] also neuropathy during exposure and histologic examination of the peripheral nerves was performed. In the Johnson-study male and female Fischer 344 rats were given in the drinking water a dose of 0.01, 0.1, 0.5 and 2 mg/kg bw acrylamide, daily for two years; in the Friedman-study the doses applied were 0, 0.1, 0.5 and 2 mg/kg bw for males only, and 0, 1 and 3 mg/kg bw for female rats. More details on the experimental designs can be found in section 2.2.3 in this report.

In both studies no treatment-related clinical signs of neurotoxicity or neurologic dysfunction were observed.

In the Johnson-study, microscopic observations revealed degeneration of the tibial nerve in all groups, including control animals. The degeneration of the tibial nerves included focal swelling of individual nerve fibers with fragmentation of the myelin and axon. The degree of degeneration in rats dosed with 0.01, 0.1 and 0.5 mg/kg bw for 2 years did not differ from those observed in control animals. However, in animals dosed with 2 mg/kg bw the tibial nerve degeneration was increased in incidence and severity compared to control animals (statistically significant in females only; authors noted the low incidence, necessitating pooling of data with next lower severity). No treatment-related effects in other examined peripheral nerves were observed.

Similar lesions, including those reported by Burek *et al*. (1980)[17], were described by Friedman *et al*. (1995) for the sciatic nerve. In the highest dosed groups (2 mg/kg bw, males; 3 mg/kg bw, females) the number of animals with sciatic nerve degeneration was increased compared to control animals and the lower dosed groups. However, the increase did not reach statistical significance.

A summary of the neurotoxicity data is given below.

Dose applied mg/kg bw/day	Exposure duration	Peripheral nerve degeneration			Remarks
		tissue	light microscope	electron microscope	
0.01	2 years	tibial	-	n.d.	Johnson *et al.*[51]
0.05	13 weeks	sciatic	-	-	Burek *et al.*[17]
0.1	2 years	tibial	-	n.d.	Johnson *et al.*
	2 years	sciatic	-	n.d.	Friedman *et al.*[37]
0.2	13 weeks	sciatic	-	-	Burek *et al.*
0.5	2 years	tibial	-	n.d.	Johnson *et al.*
	2 years	sciatic	-	n.d.	Friedman *et al.*
1.0	13 weeks	sciatic	-	+	Burek *et al.*; complete reversion within 25 days of recovery.
	2 years	sciatic	-	n.d.	Friedman *et al.*
2.0	2 years	tibial	+	n.d.	Johnson *et al.*; statistically significant in females only; data pooled due to low incidence.
	2 years	sciatic	+	n.d.	Friedman *et al.*; not statistical significant.
3.0	2 years	sciatic	+	n.d.	Friedman *et al.*; not statistical significant.
5.0	13 weeks	sciatic	+	+	Burek *et al.*; complete reversion within 111 days of recovery.
20.0	13 weeks	sciatic	+	+	Burek *et al.*: almost complete reversion within 144 days of recovery.

-, no changes in pathology; +, significant changes in pathology; n.d., not determined.

The lowest exposure at which peripheral nerve degeneration was observed was at 1.0 mg/kg bw/day (Burek *et al*. 1980)[17]. At this level significant axonal degeneration and axonal loss in sciatic nerve tissues were observed using an electron microscope. The committee considers these types of lesions as relevant for humans. At 0.5 mg/kg bw/day no examinations using the electron microscope were made, but light mircroscopic examination revealed no abnormalities in the peripheral nerves. This means that the committee considers 0.5 mg/kg bw/day as a no-observed adverse exposure level (NOAEL).

In deriving a health-based recommended occupational exposure level (HBROEL), the committee takes inter- and intraspecies variation (factor 10) into account. Also, the NOAEL is corrected for the duration of exposure (7 days/5 days) and human body weight (70 kilo). This results in an internal dose of 4.9 mg, which corresponds to 490 $\mu g/m^3$ (concentration in the air is the internal dose divided by respiratory volume during 8 hours of work (10 m^3) and the percentage that is systemically available after oral intake (100% (value 1), worst case scenario)).

Annex **E**

Conclusions of the Committee on the Evaluation of the Carcinogenicity of Chemical Substances

A committee of the Health Council,
December 6, 2005

Genotoxicity of acrylamide

Acrylamide induced DNA-adducts in various organs after *in vivo* administration, and mutations in Big Blue mouse cells *in vitro* as well as *in vivo*. The spectra of these mutations were different compared to background mutation spectra. Furthermore, acrylamide induced significant DNA and chromosome damage in various test systems (*i.e.*, Comet assays, micronuclei, sister chromatid exchanges, chromosomal aberrations, heritable translocations, dominant lethal mutations). On the other hand it scored negative in various bacterial mutation assays, and in *in vitro* hprt-tests. Low activities were found in *in vivo* mouse and *Drosophila* spot tests.

Most likely, the genotoxicity of acrylamide is due to its metabolite glycidamide. The latter is formed by epoxidation of acrylamide by the cytochrome P450-2E1 enzyme. The efficiency to metabolize acrylamide into glycidamide is high in mice, whereas data in the literature suggest lower efficiency levels in humans; efficiency levels in rats are in between those of humans and mice.

The committee considers acrylamide as a genotoxic agent with clear clastogenic properties through formation of its glycidamide metabolite. This conclusion is in agreement with the earlier conclusion of DECOS. However, it may be

concluded that the genotoxicity of acrylamide is weak, based on the following observations: i) acrylamide is not mutagenic in standardized bacterial mutation assays, nor in *hprt*-tests, and ii) although acrylamide is mutagenic in the *in vitro* mutation test using Big Blue mouse cells, the activity was weak, but distinguishable, and less than that of glycidamide. Big Blue mice are mice to which a transgenic reporter gene (lacI or CII) of bacterial origin has been incorporated. It is a newly developed sensitive *in vitro/in vivo* assay to detect DNA mutations.

Is acrylamide a stochastic genotoxic agent?

Acrylamide and glycidamide significantly increased the number of mutations in Big Blue mouse cells (*in vitro* and *in vivo*); both acrylamide and its metabolite predominantly caused in these mice G:C → T:A transversions and –1/+1 frameshifts in cII genes. The spectrum of these mutations differed from those obtained from controls (unexposed). This finding strongly points to a stochastic mode of action. Furthermore, *in vitro* and *in vivo* data from different sources suggest that both acrylamide and glycidamide cause specific DNA-adducts, although acrylamide to a lesser extent than glycidamide.

In conclusion, the committee is of the opinion that acrylamide is a stochastic genotoxic agent. The committee, furthermore, agrees with DECOS to use a non-threshold model in deriving cancer risk values.

Annex **F**

Data on mutagenicity and genotoxicity

DNA adducts

exposure conditions	results	remarks / conclusions	reference
In vitro. Glycidamide was reacted with cytidine and thymidine in aqueous-buffered solutions. Adducts were isolated and characterized by reversed phase HPLC and NMR spectroscopy.	Reaction with thymidine yielded one adduct: N3-GA-dThd. Reaction with cytidine yielded three adducts: N3-GA-Cyd-1, N3-GA-Cyd-2, and N3-GA-Urd. The cytidine adducts were produced in approximately 10 times lower yields the thymidine adducts.		Backman *et al*. 2004[7]
In vitro tests using normal human bronchial epithelial cells, and Big Blue mouse embryonic fibroblasts carrying a λ phage cII transgene. AA and glycidamide was added in serum-free medium for 4 hours. For mutation analysis cells were maintained for an additional 8 days in medium without AA. Concentrations applied: AA, 32 nM, 320 nM, 3.2 μM, 32 μM, 320 μM, 3.2 mM, 16 mM, 160 mM and 320 mM; glycidamide, 50 nM, 500 nM, 5 μM, 50 μM, 500 μM, 5 mM and 10 mM.	DNA adducts (terminal transferase-dependent PCR; human cells, p53; fibroblasts cII transgene). DNA adducts were more easily detected in cII than in p53 genes. In both cell types AA and glycidamide induced DNA adducts. Formation of AA-adducts reached a plateau in micromolar range, whereas glycidamide -adducts increased dose -dependently. cII mutation spectrum. AA and glycidamide induced a statistically significantly different mutation spectrum compared to control-treated cells, with an excess of G→ C transversions and A → G transitions (p=0.001).	The authors concluded that AA and glycidamide were able to form promutagenic DNA adducts.	Besaratinia and Pfeifer 2004[12]

In vitro tests using Big Blue mouse embryonic fibroblasts carrying a λ phage cII transgene. AA was added in serum-free medium for 4 hours. For mutation analysis cells were maintained for an additional 8 days in medium without AA. Concentrations applied: 32 nM, 320 nM, 3.2 μM, 32 μM, 320 μM, 3.2 mM, 16 mM, 160 mM and 320 mM.	DNA adducts (terminal transferase-dependent PCR). Preferential adduct formation was observed at specific nucleotide positions along the cII gene. Formation of DNA adducts at most nucleotide positions was dose-dependent. DNA mutation spectrum. AA treated cells showed a statistically significantly different mutation spectrum compared to control-treated cells, with an excess of G→ C transversions and A → G transitions (p=0.024).	The authors concluded that AA was able to form promutagenic DNA adducts.	Besaratinia and Pfeifer 2003[11]
In vivo DNA adducts in Balb/c mice and Sprague-Dawley rats (all males). Animals received a single intraperitoneal injection of ^{14}C-labeled AA (mice, 53 mg/kg bw, n=3; rats, 46 mg/kg bw, n=3). Six and 19 hours later they were sacrificed, and various organs were collected (mice: the liver, kidneys, and brain; rats: the liver, lung, kidney, spleen, brain and testis).	The major adduct formed was N7-GA-Gua. The level adducts was similar in different organs of the two rodent species.	Authors suggest that "the results support the notion that glycidamide is relatively evenly distributed among tissues and that the organ-specificity in AA carcinogenesis cannot be explained by a selective accumulation of the DNA-reactive metabolite in target organs".	Segerbäck *et al*. 1995[75]
In vivo analysis of AA and glycidamide DNA adducts in isolated DNA in the liver, lungs and kidneys of male adult and neonatal C3H/HeN-MTV mice. Single i.p. injection of 50 mg/kg bw (AA or GA), and 0, 1, and 10 mg AA/kg bw. Animals were sacrificed 6 hours after injection.	*In vitro* characterization of glycidamide adducts yielded N7-GA-Gua (highest level), N3-GA-Ade (lowest level) and N1-GA-dA adducts. *In vivo* exposure yielded also N7-GA-Gua and N3-GA-Ade adducts in adult (2000 adducts/10^{-8} nucleotides, and 20 adducts/10^{-8} nucleotides, respectively) as well as in three-day-old mice. The amount of adducts was lower after AA treatment than after glycidamide treatment. Adducts were furthermore found in all examined organs.		Gamboa da Costa *et al*. 2003[38]
In vivo DNA adduct analysis using male Sprague Dawley rats (4/group). Single oral (gavage) dose of AA dissolved in water at 18, or 54 mg/kg bw. Positive and negative controls included. Animals were killed at various time points. Organs selected were the brain, liver, and testes. Also blood was collected.	N3-GA-Ade DNA adducts were found in much less quantity than N7-GA-Gua adducts. Adducts were found in the brain, liver, and at lower levels in the testes. N7-GA-Gua adducts ranged between 100 and 700 adducts/10^{-8} nucleotides, whereas N3-GA-Ade ranged roughly between 1 and 9 adducts/10^{-8} nucleotides.		Maniére *et al*. 2005[56]

In vivo DNA adducts in various organs using Fischer 344 rats and B6C3F$_1$ mice (n=3-4/group). Single *intraperitoneal injection* of 50 mg AA/kg bw, or 61 mg glycidamide/kg bw. Rodents were sacrificed 6 hours after injection. Repeated dose: rodents received 0.14 mM AA in *drinking water* (corresponds to 1 mg/kg bw) for 50 days.	Mouse and rat single dose study. N7-GA-Gua and N3-GA-Ade DNA adducts were found in all tissues examined (the liver, kidneys (mice only), lungs (mice only), leucocytes, thyroid (rats only), brain (rats only), and testis) after AA and glycidamide treatment (levels between 1,000 and 2,000 adducts/10^8 nucleotides in mice; and up to 3,000 adducts/10^8 nucleotides in rats). Glycidamide produced more DNA adducts than AA. Mouse and rat repeated dose study. A regular increase in N7-GA-Gua adducts in the livers of the animals was observed. In rats this reached a steady-state level after about 14 days (up to 400 adducts/10^8 nucleotides in mice; up to 160 adducts/10^8 nucleotides in male rats) .	Authors conclude that "these results provide strong support for a genotoxic mechanism of AA carcinogenicity in rodents". See also two separate studies below.	Doerge *et al.* 2005[25]
In vivo DNA adduct analysis in the liver of male and female B6C3F1 mice. Animals were given AA (n=6) by *gavage* (0.1 mg/kg bw) or an equivalent amount of glycidamide (n=6). Eight hours after treatment, the animals were killed. Negative controls were included.	N7-GA-Gua adducts were found in the liver after AA and glycidamide treatment: - Control: ≈ 1 adduct/10^8 nucleotides; - AA: ≈ 4.5 adducts/10^8 nucleotides; - glycidamide: ≈ 7 adducts/10^8 nucleotides.	See also studies below and above.	Doerge *et al.* 2005[26]
In vivo DNA adduct analysis in the liver of male and female F344 rats. Animals were given AA (n=6-7) by *gavage* (0.1 mg/kg bw) or an equivalent amount of glycidamide (n=6-7). Ten hours after treatment, the animals were killed. Negative controls were included.	N7-GA-Gua adducts were found in the liver after AA and glycidamide treatment: - Control: < 1 adduct/10^8 nucleotides; - AA: ≈ 3 (male) and ≈ 8 (female) adducts/10^8 nucleotides; - glycidamide: ≈ 5.5 (male) and ≈ 7 adducts/10^8 nucleotides.	See also two separate studies above.	Doerge *et al.* 2005[27]

Gene Mutations

exposure conditions	Results	remarks / conclusions	reference
In vitro mutagenicity testing using *Klebsiella pneumoniae*.	Glycidamide scored negative. Highest concentration tested was 50 mM/L.	No effects.	Voogd *et al*. 1981[82]
1) Ames test using TA98, TA100, TA1535, TA1537. AA up to 50 mg/plate 2) *E.coli* microsome test (WP2 *uvrA⁻*). AA up to 50 mg/plate. 3) Hprt-test in V79H3 cells. AA up to 7 mM for 24 hours.	AA scored negative in the mutagenicity tests.	Authors conclude that "AA thus seems to be a typical clastogenic rodent carcinogen without any gene mutation potential". See also *in vitro* studies in the same publication concerning DNA damage, cell transformation and chromosomal aberrations.	Tsuda *et al.* 1993[79]
In vitro Ames test using TA98, TA100, TA1535 and TA1537 strains. Doses of AA applied: 625, 1250, 2500 and 5000 µg/plate. Controls included in the test.	With S9 mix. AA scored positive in TA 100 (2500 and 5000 µg/plate; $p<0.01$) and TA98 (2500 µg/plate; $p<0.05$) Without S9 mix. AA scored positive in TA98 at 2500 µg/plate ($p<0.05$) and at 5000 µg/plate ($p<0.01$). In all other situations AA scored negative.	Mixed results.	Yang *et al*. 2005[84]
In vitro mutagenicity testing. Tests included: *Salmonella*/ microsome test; hprt mutation test in L5178Y mouse lymphoma cells; chromosomal aberration and SCEs in V79 cells; mouse bone marrow micronucleus test; the sex-linked recessive lethal (SLRL), somatic mutation, and mutation and recombination assays in *Drosophila melanogaster*.	Acrylamide showed genotoxic activity in most tests, except the *Salmonella*/ microsome test, and the *Drosophila* SLRL test.	No mutagenicity.	Knaap *et al.* 1988[52]

In vitro hprt-test using V79 cells. AA and glycidamide were dissolved in DMSO and medium. Incubation time 24 hours. Mutations were expressed during a 4-5 day period. Mutant frequency and cytotoxicity were determined in triplicate; cloning efficiency in duplicate. MNNG served as a positive control. Doses applied: AA, 100, 1,000, 5,000 and 10,000 μM; glycidamide, 400, 800, 1,200, and 2,000 μM; MNNG, 5 μM. DMSO 0.1% served as negative control.	Background hprt mutation frequency was les than 6 mutants per 10^6 cells). Acrylamide: No cytotoxicity below 5,000 μM. Also no significant hprt mutations observed (less than 10 mutants/10^6 cells). Glycidamide: Significant cytotoxicity of 800 μM and higher (50% at 2,000 μM). Hprt mutations increased significantly with increasing dose of 800 μM and higher (800 μM, 14±8 mutants/ 10^6 cells). MNNG induced approximately 75 mutants/10^6 cells at 10 M.	Acrylamide did not show genotoxic activity. Glycidamide exerts a rather moderate genotoxic activity.	Baum *et al*. 2005[10]
In vitro hprt-test using Chinese hamster ovary cells (wild-type and cells deficient in DNA repair). Cells were exposed to glycidamide (up to 4 mMh) for one hour.	Cell survival was lower in deficient cells compared to wild-type cells (0.6 mMh versus 3 mMh, respectively). The hprt mutagenicity frequency could only assessed in the wild-type cells due to the high cytotoxicity in the deficient cells. Mutagenicity was found to be at the border of detection, but nevertheless statistically significant.	Low activity.	Johansson *et al*. 2005[50]
In vitro tests using Big Blue mouse embryonic fibroblasts carrying a λ phage cII transgene. AA was added in serum-free medium for 4 hours. For mutation analysis cells were maintained for an additional 8 days in medium without AA. Concentrations applied: 32 nM, 320 nM, 3.2 μM, 32 μM, 320 μM, 3.2 mM, 16 mM, 160 mM and 320 mM.	Cells showed cytotoxicity at 3.2 mM and higher. cII Mutation induction and frequency (frequency of number of mutant cII plaques per total number of plaques, plus confirmation by DNA sequence analysis). Induction was initiated at 3.2 μM ($11.2x10^{-5}$, 95%CI 6.2 to $16.2x10^{-5}$) and increased dose-dependently thereafter. In cells with millimolar doses, the cII mutation frequency was lower and similar to that of untreated cells. The jackpot spontaneous mutations and AA-induced single base substitutions accounted for 21% and 17% of the cII mutations. For nonjackpot mutations these were 73 and 81%, respectively.	Acrylamide was a weak but distinguishable mutagen in this test system. See also publication below for data on glycidamide.	Besaratinia and Pfeifer 2003[11]

In vitro tests using normal human bronchial epithelial cells, and Big Blue mouse embryonic fibroblasts carrying a λ phage cII transgene. Glycidamide was added in serum-free medium for 4 hours. For mutation analysis cells were maintained for an additional 8 days in medium without glycidamide. Concentrations applied: 50 nM, 500 nM, 5 μM, 50 μM, 500 μM, 5 mM and 10 mM.	Human and mouse cells treated with glycidamide showed cytotoxicity at 5 mM and higher. cII Mutation induction and frequency (frequency of number of mutant cII plaques per total number of plaques, plus confirmation by DNA sequence analysis). Glycidamide increased dose-dependently the frequency of cII mutations compared to control (21×10^{-5} (500 μM) and 6×10^{-5} (control).	Glycidamide was more mutagenic that AA at any given dose. The authors conclude that "the mutagenicity of AA in human and mouse cells is based on the capacity of its epoxide metabolite glycidamide to form DNA adducts". See data above for data on acrylamide.	Besaratinia and Pfeifer 2004[12]
In vivo genotoxicity testing using male and female Big Blue mice. Animals were given 1, 100 or 500 mg AA or equimolar doses of glycidamide per Liter drinking water, for 3 to 4 weeks. Twentyfour hours after finishing treatment micronucleated reticulocytes in peripheral blood was assessed. In addition, three weeks after finishing treatment, lymphocyte hrpt and liver cII mutagenesis assays were performed by sequence analysis.	AA and glycidamide increased the lymphocyte hrpt mutant frequency; the highest dose produced a 16 to 25 fold increase compared to controls ($p<0.01$; in female controls, $1.5 \pm 0.3 \times 10^{-6}$; in male controls, $2.2 \pm 0.5 \times 10^{-6}$). AA and glycidamide also increased the liver cII mutant frequency; the highest dose of AA or glycidamide produced a 2 to 2.5 fold increase compared to control values ($p<0.05$; in controls, $26.5 \pm 3.1 \times 10^{-6}$ and $28.4 \pm 4.5 \times 10^{-6}$). In males, the frequency of micronucleated reticulocytes in peripheral blood was significantly increased 1.7 – 3.3 fold at the highest dose of AA or glycidamide compared to controls ($p<0.05$; control male frequency, 0.28%).	AA and glycidamide produced similar mutation spectra, and these were significantly different from that of control mutant ($p<0.01$). The pre-dominant mutations in cII genes were G:C → T:A transversions and –1/+1 frameshifts in a homopolymeric run of Gs. The authors concluded that both AA and glycidamide are genotoxic in mice.	Manjanatha *et al*. 2005 ; data from abstract[57]
In vivo recombination-based transgenic mouse assay for efficient detection of germ-line gene-conversion events. Mice had two mutually defective reporter (*lacZ*) genes under the regulatory control of a spermatogenesis-specific promotor. Male mice were given i.p. injections of 50 mg AA/kg bw for 5 consecutive days. Positive (chlorambucil) and negative controls were included. The animals were killed 21 to 23 days after the last injection.	Conversion events were visualized by histochemical staining or flow-cytometry analysis of transgenic spermatids. AA did not produce converted spermatoids.	No effects.	Murti *et al*. 1994[62]

In vivo standard mouse spot test using T and HT stocks. Pregnant females were given i.p. injections of 0, 50 and 75 mg AA/kg bw, either once at day 12 of pregnancy, or three times on days 10, 11, and 12. The offspring was tested for coat-colour spots of genetic relevance (indicating somatic mutations).	Number of litters (weaning) ranged between 27 and 52. Compared to controls, AA did significantly increase the number of relevant spots after a single injection ($p<0.04$) and after repeated injections ($p<0.001$) at both concentrations.	Positive effect in spot test.	Neuhäuser-Klaus and Schmahl 1989[66]
In vivo wing spot test using *Drosophila melanogaster*. Two strains with recessive wing markers were used: the *multiple wing hair* strain (*y:mwh jv*), and the *flare* strain (*flr^3/ In(3LR)TM3, Ser*) (50 larvae/ vial). Larvae were fed medium for the rest of their development until pupation. Concentrations of AA in medium: 0, 1.0, 1.5, and 2.0 mM/2.5 mL medium.	AA induced a statistically significant, but small, increase in the frequency of wing spots (range number of wings, 48 – 70; range frequency single small spots, 0.15 – 0.34; range frequency large single spots, 0.02 – 0.14; range frequency twin spots, 0.03 – 0.06; range frequency total spots, 0.23 – 0.50).	Small increase in wing spot test.	Batiste-Alentorn *et al*. 1995[9]
In vivo somatic white-ivory system using *Drosophila melanogaster*. Strain used: males, *y^{2}Dp (1:1:1:1)w^i*, and females *(C(1)DX, yf)* on X-chromosome. The larvae after mating (200 larvae/vial) were fed medium for the rest of their development to adults. Dose of AA in medium: 0, 30 and 50 mM.	Males were examined for eye-color mosaicism, and the no. of pigmented ommatidia was determined. - Mortality observed (%): 36, 53, 53.2; - Mortality induced (%): -, 26.56, 26.88; - No. of spots: 9/2,260, 7/1,598, 11/2,070; - Frequency of spots (%): 0.40, 0.44, 0.53; - No. of males with eye colour mosaicism: 9, 7, 11. Three values correspond to 0, 30 and 50 mM, respectively	The number and frequency of spots was very low. No dose-response relationship was obtained.	Batiste-Alentorn *et al*. 1994[8]
In vivo sex-linked recessive lethal (SLRL) mutations in postmeiotic and meiotic germ cells of male *Drosophilas* (Canton-S stock). AA tested at 0 and 50 μM by feeding, and 0 and 2,500 μM by injection.	AA scored negative in both exposure routes.	No effects.	Foureman *et al*. 1994[35]
In vivo (white/white+)(w/w$^+$) eye mosaic assay. *Drosophila* larvae were exposed to a range of AA concentrations (0-80 mM).	AA had a relatively low DNA reactivity.	Low activity.	Vogel and Nivard 1993[81]

DNA and chromosome damage

exposure conditions	results	remarks / conclusions	reference
In vitro Comet assay using human blood lymphocytes (three male donors). AA was added to the heparinized blood samples at final concentrations of 1,000 to 6,000 μM, and glycidamide between 100 and 3,000 μM. Exposure time was 1, 2, or 4 hours. Bleomycin served as positive control.	Acrylamide was inactive at all concentrations (tail intensity below 3 %). Glycidamide induced concentration dependent increases of DNA damage (significant at 300 μM and upwards) after 4 hours of incubation. Acrylamide (10,000 μM) and glycidamide (300 μM) mixture did not induce more DNA damage than glycidamide alone.	Acrylamide did not show genotoxic activity. Glycidamide exerts a rather moderate genotoxic activity.	Baum *et al*. 2005[10]
In vitro Comet assay using normal human lymphocytes. AA was added to the suspensions of the lymphocytes for 1 hour. Final concentrations of AA ranged between 0.1 and 50 μM. All experiments included positive (10 μM hydrogen peroxide) and negative (medium) controls.	Cytotoxicity. AA induced a concentration-dependent decrease in the viability of the cells (50 μM, viability was 87.5%). DNA damage (Comet assay). At 0.5 μM a significant increase in DNA damage (double strand breaks) was observed (pH 9). No single and double strand breaks were observed at pH 12.1. The DNA damage was repaired within two hours after stopping exposure. Caspase-3 activity (enzyme responsible for breakdown of the cell during apoptosis). The enzyme was activated after incubation with 50μM AA (p<0.001).	According to the authors, the results suggest "that AA or its metabolite may be genotoxic".	Blastiak *et al*. 2004[13]
In vitro Comet assay in V79 cells, Caco-2 (expresses substantial CYP2E1 activity), and primary rat hepatocytes. Cells were exposed to AA (up to 6 mM) and glycidamide (up to 600 μM) for 24 hours. Controls were included.	Acrylamide did induce significant DNA strand breaks in V79 and Caco-2 cells at the highest concentration (6 mM), but not at lower concentrations. No effects were observed in primary hepatocytes. Glycidamide did induce DNA strand breaks at concentrations of 30 M (V79 after 6 hour exposure), 300 μM (Caco-2 after 3 hours), and 60 μM (hepatocytes after 3 hours) and upwards.	According to the authors Caco-2 cells express CYP2E1 activity. The enzyme activity in primary hepatocytes declines to about 60% in 26 hours after isolation. V79 cells do not express CYP2E1 activity. Depletion of intracellular GSH by a chemical (BSO) increased the amount of DNA strand breaks after AA and glycidamide exposure in V79 cells.	Puppel *et al*. 2005[72]

In vivo Comet assay using male Sprague Dawley rats (4-5/group). Single oral (gavage) dose of AA dissolved in water at 18, 36, or 54 mg/kg bw. Positive and negative controls included. Animals were killed at various time points. Organs selected were the brain, liver, bone marrow, adrenals and testes. Also blood was collected.	24 Hours after dosing. The olive tail moment was statistically significantly increased: at 36 and 54 mg/kg bw in leucocytes; and, at 54 mg/kg bw in the brain. The percentage DNA in the tail was statistically significantly increased: at 54 mg/kg bw in leucocytes; at 36 and 54 mg/kg bw in the brain; and, at 54 mg/kg bw in the testes. In other organs no significant increases in DNA damage was observed. Two and 5 hours after dosing (54 mg/kg bw). Two hours after dosing a significant increase in percent DNA in the tail was observed in the testes and adrenals; after five hours significant increases were observed in the leucocytes, bone marrow, liver and adrenals.	AA induced micronuclei in various organ tissues.	Maniére *et al*. 2005[56]
In vitro micronuclei (MN) induction using human heparinized blood lymphocytes (fifteen unrelated donors). AA and glycidamide was added for 71 hours. Concentrations used: AA, 500 – 5,000 μM; glycidamide, 50 – 1,000 μM; bleomycine (positive control), 4 μM.	Baseline MN frequencies ranged from 0 to 6‰. Acrylamide induced at 5,000 μM a two-fold increased in MN frequency compared to negative control (average values, 4.1±2.7 cells; range 1-10‰) in 7 of 15 donors. The increase was not statistically significant. Glycidamide did not significantly increase MN frequency (average value at 1,000 μM, 6.1±4.2 cells, range 1-13‰).	Acrylamide and glycidamide did not show micronuclei induction at the concentrations used.	Baum *et al*. 2005[10]
In vivo micronuclei frequency testing and Comet assay using female wild type and CYP2E1-null mice. AA was injected i.p. once daily for 5 consecutive days (0, 25 and 50 mg/kg bw). One day later the animals were sacrificed.	Micronuclei frequency in erythrocytes. A significant dose-related increase in frequency was observed in wild-type erythrocytes, but not in CYP2E1-null cells. DNA damage by Comet assay in leucocytes, the liver and the lung. A significant dose-related increase in DNA damage was observed in wild-type erythrocytes, but not in CYP2E1- null cells for all organs tested.	Authors suggest that "genetic damage in somatic cells of mice treated with AA is dependent upon metabolism of the parent compound by CYP2E1". Earlier, the same authors found comparable results in germ cells.	Ghanayem *et al*. 2005[40]

In vivo micronuclei induction in spermatids and peripheral blood reticulocytes of male BALB/c mice (n=4-5/group). Animals received a single i.p. injection of 50 or 100 mg AA/kg bw. Spermatids were collected 2, 14, and 16 days after exposure; blood cells 1, 2, and 3 days after exposure. Positive (mitomycine C) and internal controls were included.	Spermatids. Cells in the Golgi phase did no show significant increase in percentage of nucleated cells. Cells in the Cap phase showed an increase in nucleated cells after 14 days (50 mg/kg bw) and 16 days (50 and 100 mg/kg bw) ($p<0.05$). Also repeated exposure (4x 50 mg/kg bw) increased the percentage of nucleated spermatids ($p<0.001$). Peripheral blood reticulocytes. The percentage of micronucleated cells was increased after exposure to 50 mg/kg bw (tested after 1 and 2 days) and 100 mg/kg bw (tested after 2 days) compared to controls ($p<0.05$).	Authors also tested for SCE induction in spermatids: a single dose of 50 and 100 mg/kg induced SCEs ($p<0.001$, compared to control).	Russo *et al.* 1994[74]
In vivo micronucleus test. Male CBA mice; a single i.p. injection of AA (dissolved in 10 µL PBS/g mouse); positive control, 1 mg/kg bw colchicine; blood samples taken 42 hours after injection. Experiment 1 (n=2/group): 22 different doses (range 0 – 100 mg/kg bw) Experiment 2 (n=5/group): seven different doses (0, 1, 3, 6, 12, 24, and 30 mg/kg bw)	Mean frequency of micronucleated polychromatic erythrocytes in peripheral blood (per mille). Experiment 1: range, 1.06 (0 mg/kg bw) - 2.65 (100 mg/kg bw), not statistically different from negative control group; a significant dose-response relationship was observed (R^2-factor, 0.87). Experiment 2: range, 1.18±0.07 (0 mg/kg bw) - 1.90±0.26 (30 mg/kg bw), $p<0.001$ from 6 mg/kg bw onwards; a significant dose-response relationship (R^2-factor, 0.72). DNA content in micronuclei was low (FACS analyses).	Author concluded that "the dose response relationship did not disclose any sign of aneugenicity of AA, since dose response was found to be linear at the lowest doses".	Abramsson-Zetterberg 2003[1]
In vivo micronucleus test. Male CBA mice (n=4): single i.p. injection of AA at doses of 25, 50, and 100 mg/kg bw; male Sprague-Dawley rats (n=4/group): single i.p. injection of AA at a dose of 100 mg/kg bw. Animals were killed at various time points after treatment. Peripheral erythrocytes (mice) and bone marrow erythrocytes (rats only) were collected.	A significant linear increase in micronucleated peripheral erythrocytes was observed in mice (frequency ranged between 1 and 4 ‰). In rats no increases in micronuclei in bone marrow cells was observed compared to control.	Authors also observed an increase of hemoglobin adducts in treated mice and rats.	Paulsson *et al*. 2002[69]
In vivo micronucleus test in peripheral blood cells of male ICR mice. Animals were exposed to a range of AA (0 – 145 mg/kg bw) by oral gavage for a single time.	Frequency of micronucleated polychromatic erythrocytes was statistically significantly increased from 72.5 mg AA/kg bw and upwards ($p<0.01$).		Yang *et al*. 2005[84]

In vivo micronucleus test. Male CBA mice (n=4): single i.p. injection of glycidamide at doses of 0.18, 0.35, and 0.70 mmol/kg bw; male Sprague-Dawley rats (n=4/group): single i.p. injection at doses of 0.70 and 1.4 mmol/kg bw. Peripheral blood in mice was collected 48 hours after treatment. In rats, bone marrow erythrocytes were collected 24 hours after treatment. Negative controls were included.	A dose-related increase in micronucleated erythrocytes was observed in mice, but not in rats. In mice glycidamide has equality in potency compared to acrylamide.	In both mice and rats, glycidamide increased the amount of hemoglobin adducts in dose-dependent way. Authors suggest that lack of clear effect in rats may be due to toxic effects to the bone marrow at the examined exposure concentrations.	Paulsson *et al*. 2003[70]
In vivo spermatid micronucleus test using male Sprague Dawley rats. Animals were given a single i.p. injection of 50 and 100 mg AA/kg bw, or repeated injections of 50 mg AA/kg bw once daily for four days.	After single injection of AA, the frequency of micronuclei in spermatids was not significantly increased. After repeated exposure, AA significantly increased the frequency of micronuclei at 18 and 19 days after the last treatment. This indicates genotoxic activity in preleptotene spermatocytes and late spermatogonial stages.	Authors add that "DNA flow cytometry did not show cytotoxicity of AA to preleptotene sperma-tocytes, but a small increase in the number of stem cells. If spindle disturbance are caused by AA, as suggested, they were not detectable by induction of spermatid micronuclei in vivo 1 and 3 days after treatment of by treatment with AA of cultured segments of seminiferous tubules undergoing meiotic divisions *in vitro*".	Lähdetie *et al*. 1994[55]
In vivo spermatid micronucleus test using male Lewis rats. Animals were given a single i.p. injection of 50 and 100 mg AA/kg bw, or repeated injections of 50 mg AA/kg bw once daily for four days. Animals were killed at different time intervals.	After a single injection, AA statistically significantly increased the frequency of micronucleated perleptotene spermatids at 100 mg/kg bw (18 and 20 days after treatment). Also after *repeated exposure*, AA higher percentage of micronucleated cells was observed (day 19).		Xiao and Tates 1994[83]
In vitro SCEs in V79H3 cells. AA 1-2.5 mM for 24 hours.	A weak but statistically significant increase in SCE was observed.	Authors concluded that "AA thus seems to be a typical clastogenic rodent carcinogen without any gene mutation potential".	Tsuda *et al.* 1993[79]
In vitro chromosomal aberrations in Chinese hamster lung fibroblasts. Cells were exposed to AA from 1 to 400 mM for 24 hours.	The percentage of cells with chromosomal aberrations was significantly increased in a dose-dependent manner ($p<0.01$; only data presented up to 50 mM).		Yang *et al*. 2005[84]

In vivo dominant lethal and heritable translocations in C3H/E1 inbred mice. AA (50 mg/kg bw) was dermally applied (on the shaved backs) on 5 consecutive days to males. One to eight days after treatment they were mated to unexposed female 102/E1 mice. For the dominant lethal test, animals were also treated by i.p. injections of the same dose.	The percentage of dominant lethals after 1 to 3 matings were 81.7, 85.7, and 45.4% (i.p. injections), and 22.1, 30.6, and 16.5% (dermal exposure), respectively. Translocations. Of the 475 offspring that were screened 41 were carriers of translocations. The translocation frequency was 8.6%.		Adler *et al*. 2004[2]
In vivo heritable translocations in male C3H/E1 mice. Animals received a single i.p. dose of 50 and 100 mg AA/kg bw. Seven to 15 days after treatment they were mated with unexposed females 102/E1 mice.	The translocation frequencies were 0.6 and 2.7% after 50 and 100 mg/kg bw exposure, respectively (historical control, 0.04%).		Adler *et al*. 1994[3]
Detection of aneuploidy by multicolour FISH in mouse sperm after *in vivo* treatment with 60 and 120 mg AA/kg bw (i.p.injection).	AA did not induce increases of disomic or diploid sperm.		Citation in Adler *et al*. 2002[4] (original publ. Smid *et al*. 1999).
In vivo dominant lethal mutations in male mouse germ cells using CYP2E1-null and wild type mice. Male mice were given i.p. injections of 0.12.5, and 25 mg AA/kg bw for 5 consecutive days. Two days later they were mated with unexposed females. Endpoints examined included: number of implantations, no. of life and dead foetuses, no. of resorption moles, and no. of early and late dead embryos.	Wild type animals. Dose related increases in no. of resorption moles, and decreases in no. of pregnant females and living foetuses were observed in females mated with exposed males. CYP2E1-null mice. No changes in any of the parameters examined was found.	Authors concluded that "AA-induced germ cell mutations require CYP2E1 mediated epoxidation of AA".	Ghanayem *et al*. 2005[41]
1) *Bacillus sub*. spore-rec assay. AA 10-50 mg/disc. 2) Chromosomal structural change test. AA 2-5 mM for 24 hours. 3) Polyploidy test in V79H3 cells. AA 1-5 mM for 24 hours.	AA was strongly positive in inducing DNA damage.	Authors concluded that "AA thus seems to be a typical clastogenic rodent carcinogen without any gene mutation potential".	Tsuda *et al.* 1993[79]

DNA repair and synthesis

exposure conditions	results	remarks / conclusions	reference
In vitro primary hepatocyte DNA repair assay. Cells were obtained from male F344 rats, and treated with AA in medium for 24 hours. Concentrations used: AA and glycidamide: 0.01, 0.1, 1 and 10 mM. Experiment was repeated once. Cells with more than 5 nuclear grains were considered to be in repair.	The percentage of cells treated with AA and having more than 5 nuclear grains was not increased compared to negative control (3±3 (1 mM) versus 2±1). For glycidamide the percentage of cells with more than 5 nuclear grains increased significantly with increasing concentration compared to control (47±9 (10 mM) versus 2±1; $p<0.05$).	In human mammary epithelial cells AA produced a slight response under comparable exposure situation.	Butterworth *et al*. 1992[18]
In vivo DNA repair assay. Hepatocytes and spermatocytes were obtained from male F-344 rats, after they were exposed to AA or glycidamide by various exposure routes. Two to 12 hours after exposure animals were killed.	DNA repair expressed as percentage of cells with more than 5 nuclear grains. Single oral dose by gavage (AA 100 mg/kg bw). Hepatocytes: no DNA repair; spermatocytes: dose-dependent increase of DNA-repair. Repeated oral dose by gavage (AA 30 mg/kg bw). Hepatocytes: no DNA repair; spermatocytes: increased DNA-repair.	Increased DNA repair in spermatocytes.	Butterworth *et al*. 1992[18]
In vivo analysis of DNA synthesis, apoptosis and mitotic index. Male F344 and Sprague-Dawley rats (n=5/group/strain) were given AA in the drinking water for 7, 14, or 28 days. Concentrations tested were 0, 2, and 15 mg/kg bw. For analysis the thyroid, adrenals, testes and liver were harvested.	The thyroid and testicular mesothelium. DNA synthesis was significantly increased ($p<0.05$) in all animals given 15 mg/kg bw (7, 14, and 28 days), and in F344 rats given 2 mg/kg bw (7, 14, and 28 days). The adrenals. At all tested days, and in both animal strains a significant increase in DNA synthesis was observed at 2 and 15 mg/kg bw ($p<0.05$). In the liver no increased DNA synthesis was observed after F344 rats were given 2 mg AA/kg bw for 7 days (no other data on liver presented). No changes in apoptosis or mitotic index were observed in any of the tissues examined.	Authors conclude that "AA produced a selective increase in DNA synthesis that correlates with the previously reported tumor target tissues".	Lafferty *et al*. 2004[54]

Cell transformation

exposure conditions	results	remarks / conclusions	reference
In vitro SHE test (primary embryo cells). AA was applied at concentrations of 0.1 to 0.7 mM for 7 days. Positive and negative controls were included, as well as cotreatements with P450 and GSH inhibitors.	A dose-dependent increase in transformation was observed compared to negative control (medium) ($p<0.05$; at 0.5 and 0.7 mM AA). Cotreatement with ABT, a P450 inhibitor, did not result in different outcomes compared to those of AA alone. Cotreatment with BSO, an inhibitor of GSH synthesis, did significantly increase cell transformation compared to treatments with AA alone.	At 0.5 and 0.7 mM cytotoxicity did occur (lowered colony plating efficiency). The highest dose of BSO (5 µM) may have added to the cyto-toxicity of AA at 0.5 mM. Authors suggest that "AA itself, but not oxidative metabolites of AA appear to be involved in AA-induced cellular transformation".	Park *et al.* 2002[67]
In vitro BALB/c3T3 cells. AA 1-2 mM for 72 hours.	AA was strongly positive in the cell transformation test.		Tsuda *et al.* 1993[79]