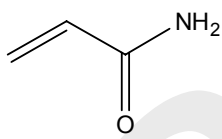


COMMITTEE ON MUTAGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

Fourth Draft Statement on the Genotoxicity of Acrylamide

Background

1. Acrylamide is a small, simple molecule ([Figure 1](#)). It is an α,β -unsaturated carbonyl with electrophilic reactivity. This means it can react with nucleophilic groups (amines, carboxylates, sulphhydryls etc) on biological molecules, such as proteins or DNA. *In vivo*, acrylamide may be metabolised to the reactive epoxide glycidamide, which is thought to have a role in acrylamide related toxicity.



[Figure 1: Acrylamide](#)

2. Acrylamide has been used as an industrial chemical since the mid 1950s. Polyacrylamides have a wide range of uses, including strengthening paper, as a flocculant for clarifying water and as grout to stop water leaks during tunnel construction. Occupational and accidental exposure of humans to acrylamide has resulted in neurotoxicity, which typically manifests as peripheral numbness (Shipp *et al.*, 2006).

3. Review of the available toxicology data on acrylamide was commenced during the 1990s in accordance with Council Regulation (EEC) 793/931 on the evaluation and control of the risks of “existing” substances. The resulting European Union (EU) Risk Assessment Report was prepared by the UK Health and Safety Executive (HSE), acting as rapporteur to the EU. The risk assessment report was finalised in 1999 and published in 2002, based on a literature review performed in 1995 with a few subsequent targeted searches (EU Risk Assessment Report, 2002).

4. In addition to its use as an industrial chemical, acrylamide is also known to be present in cigarette smoke, leading to estimated exposures in human smokers of approximately 3 $\mu\text{g}/\text{kg}$ bw/day based on level of haemoglobin-acrylamide adducts in blood samples, a well established biomarker of acrylamide exposure (Bergmark *et al.* 1997). The authors also noted that a background exposure of approximately 1 $\mu\text{g}/\text{kg}$ bw/day was observed in non-smoking controls. This unexpected result was explained when it was discovered that acrylamide can also form in certain types of food.

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5. This discovery came as the result of investigations following an industrial incident that occurred during construction of a railway tunnel in Sweden; seemingly unexposed 'control' individuals had unexpectedly high levels of haemoglobin-acrylamide adducts. Subsequent work has demonstrated that acrylamide forms when starchy foods, such as potatoes and cereals, are cooked at temperatures exceeding 100°C. Acrylamide forms as a reaction between the amino acid asparagine and reducing sugars that are naturally present in these foods (Törnqvist, 2005). Human dietary exposure cannot be avoided in a healthy balanced diet, although there are continuing efforts to understand factors affecting acrylamide formation in food, with a view to developing best practice to reduce acrylamide formation. A Food Standards Agency (FSA) exposure assessment in 2005 estimated that adult dietary exposure to acrylamide was 0.3 and 0.6 µg/kg bw/day for average and high level (97.5th percentile) consumers respectively. Toddler exposure was 1.0 and 1.8 µg/kg bw/day (Mason and Benford, 2007).

6. In response to discovery of acrylamide in food, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) reviewed the toxicity of acrylamide. JECFA considered acrylamide to be genotoxic in a range of studies and carcinogenic in two rat chronic feeding studies. Margins of exposure (MOE) were considered to be low and may indicate a human health concern (JECFA 2005). Based on UK exposure estimates, the MOEs range from 1000 for average adult consumers to 150 for high level toddler consumers (Mason and Benford, 2007). JECFA intends to re-evaluate the toxicity of acrylamide, if appropriate, following publication of a number of long term studies that are currently underway.

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Referral to COM

7. In January 2007, the HSE requested that the COM provide an opinion on the evidence regarding germ cell mutagenicity of acrylamide and the evidence regarding a threshold for germ cell mutagenicity with this chemical. The Committee was provided with a copy of the EU Risk Assessment Report and a submission from the Polyelectrolyte Producers Group (PPG), discussing the evidence regarding germ cell mutagenicity of acrylamide and the evidence for a threshold for germ cell mutagenicity with this chemical. A response was published in February 2007 (COM/07/S2). The COM was made aware of a response from the Polyelectrolyte Producers Group (PPG) to the chair (dated 8 May 2007) at the COM meeting of the 17 May 2007 (MUT/07/16 Annex 2) and agreed to a further evaluation of the genotoxicity data on acrylamide at the request of HSE. In view of the widespread dietary exposure to acrylamide, the Food Standards Agency requested that such a review should consider all available genotoxicity data on acrylamide.

Review Strategy

8. The COM agreed that the EU risk assessment review completed by HSE (EU Risk Assessment report 2002) could be used as a basis for the review, and for this to be extended with a systematic review of the scientific literature available subsequent to 1995.
9. The Secretariat drafted an overview of the EU Risk Assessment of acrylamide and outlined a strategy for the review of published literature in MUT/07/17. The search strategy was devised in order to identify all relevant studies that had not been cited in the EU Risk Assessment Report, and the last update to the search was performed on the 23rd September 2008. Details of this search strategy can be found in Annex A. Members reviewed the findings of the EU Risk Assessment Report and were content with the search strategy. Members were presented with a systematic review of data relating to the genotoxicity of acrylamide and glycidamide published after 1995, and other pre 1995 references that had not been included in the EU risk assessment report (MUT/08/02). This paper also provided an initial discussion of the acrylamide data, and this was extended to include the metabolite glycidamide in paper MUT/08/07.
10. The PPG were invited to give presentations at the October 2007 (MUT/07/16) and February 2008 (MUT/08/01) meetings and have submitted data and supporting references for Members to consider at several points during the review (as noted in relevant minutes and discussion papers). The PPG met with the Secretariat prior to each meeting in order to explain the Committee's procedures and to provide advice on the structure and content of the submissions, highlighting areas where more detail would be valuable to the Committee's deliberations. Members have been provided with minutes of these meetings as annexes to MUT/07/16, MUT/08/01 and MUT/08/06. The PPG submitted comments on the second draft Working Paper and a relevant abstract from a recent scientific meeting (McDaniel *et al.*, 2008); these were both tabled at the October 2008 meeting.

Discussion of the *in vitro* genotoxicity of acrylamide and glycidamide

In vitro Gene Mutation (Bacterial)

11. The EU risk assessment report concluded that negative results have been reported in standard bacterial mutagenicity tests both in [the](#) presence and absence of exogenous metabolic activation. Yang *et al.* (2005) reported a positive response in *Salmonella typhimurium* TA 98 in the presence and absence of exogenous metabolic activation and a positive result in TA 100 only in the presence of exogenous metabolic activation.
12. Emmert *et al.* (2006) did not record a positive response to acrylamide in *Salmonella typhimurium* YG7108pin3ERb5, a strain which has been

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engineered to express CYP2E1. A number of compounds which should have been mutagenic following activation by CYP2E1 also gave negative results which suggests that the test system may not be adequate. Previously, Hashimoto *et al.* (1985) investigated acrylamide and glycidamide in the presence and absence of Aroclor 1254 induced S9 liver homogenate. Acrylamide was negative in all strains \pm S9 but glycidamide was positive in strains TA100 and TA1535 \pm S9. TA1535 is the parent strain of YG7108pin3ERb5, the CYP 2E1 expressing strain used by Emmert *et al.* (2006) which was also not sensitive to acrylamide; so either the strain had lost sensitivity to glycidamide, or glycidamide was not formed in sufficient quantities to be detected in the Ames test.

13. It should be noted that Segerbäck *et al.* (1995) showed uninduced rat S9 produced a detectable level of glycidamide DNA adducts (see paragraph 43), although the addition of S9 was insufficient to give a positive result by Ames test by Hashimoto *et al.* (1985)

Conclusion: As reported in the EU Risk Assessment Report, *in vitro* gene mutation experiments in bacteria give results that are generally consistent with a lack of genotoxicity of acrylamide in this test system, in the presence and absence of S9 liver homogenate metabolic activation. The findings of Yang *et al.* are not sufficient to alter this conclusion. The available evidence from other studies casts doubt on the ability of S9 to catalyse the conversion of acrylamide to glycidamide in sufficient quantities for an effect to be detectable in the Ames assay. However, glycidamide, which forms as a result of oxidative metabolism *in vivo*, is clearly a DNA reactive mutagen in bacteria.

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In vitro Gene mutation (Mammalian)

14. Based on the reviewed data, the EU risk assessment report concluded that acrylamide is a direct acting mutagen in the available assays, probably causing clastogenic effects.

15. Besaratinia and Pfeifer (2003) examined mutation frequencies in the *cII* transgene in cultured Big Blue mouse embryonic fibroblasts following exposure to acrylamide (32 nM to 320 mM). A concentration related increase in mutation frequency was observed at 3.2, 32 and 320 μ M, peaking at 2-fold increase over control ($p \leq 0.001$); with mutation frequency reducing to background at higher doses. The authors suggested ed this may be due to cytotoxicity or a saturable process.

16. Besaratinia and Pfeifer (2004) subsequently compared the mutant frequencies and spectra in the *cII* transgene following acrylamide and glycidamide treatment. Treatment with either acrylamide or glycidamide led to statistically significant increases in mutation frequency ($p \leq 0.001$). A greater increase observed with glycidamide treatment and this was concentration related from 5 μ M to the maximum concentration of 5000 μ M. Mutation spectra (reported as the spectrum of individual mutated bases) were similar when acrylamide (320 μ M) was compared with glycidamide (500 μ M), but the

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relative proportion of G→T + C→A was increased compared to acrylamide and control samples, and T→G + A→C was decreased (the former being significant compared to control; $p \leq 0.001$). The authors suggested that the mutational spectra for acrylamide and glycidamide are consistent with N7-GA-Gua, N3-GA-Ade and N1-GA-dA adducts (these glycidamide adducts are discussed in depth in paragraphs 43 to 56).

17. Mei *et al.* (2007) investigated the effects of both chemicals on the Tk locus in mouse lymphoma cells. They reported significantly different ($p=0.018$) mutational spectra between acrylamide (16 mM) and glycidamide (4 mM), which were both significantly different from control ($p=0.0001$). However, in this paper, the mutation spectrum relates to extent of the deleted region on chromosome 11. Deletion of only the Tk locus accounted for approximately 60% of both acrylamide and glycidamide induced mutations; however, of the remaining mutations, acrylamide induced deletions were generally larger, extending further towards the kinetochore compared to glycidamide induced deletions. The possibility of recombination has not been investigated. This should be taken in the context of the DNA adduct data from the same paper, which showed N7-GA-Gua and N3-GA-Ade adducts were only detectable in glycidamide treated cells, consistent with a lack of metabolic competency in the mouse lymphoma cells. This suggests that the glycidamide mediated genotoxic effects of acrylamide would not be evident in this experimental system. This demonstrates the potential for direct mutagenicity of acrylamide that is not associated with the glycidamide DNA adduct mechanism (the authors propose an oxidative stress mechanism), but does not preclude a DNA reactive mechanism in metabolically competent cells.

18. Koyama *et al.* (2006) performed a similar study using human lymphoblastoid TK6 cells incubated with acrylamide (2.6 to 14 mM) or glycidamide (0.6 to 2.4 mM). These studies found a large difference between the response to acrylamide and glycidamide. Increases in mutant frequencies only reached significance at cytotoxic concentrations of acrylamide in one out of two studies; whereas there were significant increases in mutation frequency at sub-cytotoxic concentration of glycidamide, with a 20-fold increase above controls at 2.4 mM.

19. Baum *et al.* (2005) examined HPRT mutation frequencies in V79 cells exposed to acrylamide (0.1 to 10 mM) and glycidamide (0.4 to 2 mM). Acrylamide did not increase mutation frequencies in this experiment, whereas statistically significant increases were observed with glycidamide at 0.8 mM ($p < 0.01$), and 1.2 and 2 mM ($p < 0.001$)

20. Ao *et al.* (2008) examined HPRT mutation frequencies in HL-60 and NB4 leukaemia cell lines exposed to acrylamide (0.7 to 10 mM). A significant increase in mutation frequency was only observed at the highest concentration ($p < 0.05$). The mutation spectrum was different to the spontaneous mutation spectrum in control cells, where single exon and partial deletions were increased. There was no difference in spectra between the

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two cell lines. There was no evidence of metabolic competency of these cell lines. These results should be treated with caution as HL-60 cells have high levels of peroxidase activity, and thus high doses of acrylamide could induce oxidative DNA damage in a mechanism specific to this cell line. The relatively small deletions reported in this study could be consistent with a gene mutation response.

21. As with the Mei study, the Koyama, Baum and Ao studies were not performed with metabolic activation and the cells cannot be assumed to be metabolically competent; therefore, it is reasonable to assume the glycidamide mediated adduct mechanism would not operate in these *in vitro* model systems (although, unlike the study by Mei *et al.*, the Koyama, Baum and Ao studies did not analyse DNA adducts to confirm this hypothesis). This is a generic problem with assessment of chemicals requiring metabolic activation in mammalian cell mutation assays. For acrylamide, provision of DNA adduct data seems an appropriate biomarker for assessing intrinsic metabolic capacity of cells *in vitro*.

22. Glycidamide has been investigated alone by Johansson *et al.* (2005) with the data re-plotted by Silvari *et al.* (2005). This study compared HPRT mutation frequencies in base excision repair (BER) deficient and proficient Chinese hamster ovary (CHO) cells. Glycidamide was positive in BER proficient cells, but negative (and more cytotoxic) in BER deficient cells. The authors concluded that their study was not able to elucidate the type of lesion underlying the mutagenic effect.

23. In summary, these studies suggest that, in absence of metabolic activation, glycidamide DNA adduct mediated mutation does not occur. Glycidamide itself has been shown to be positive in all studies. Effective metabolic conversion of acrylamide to glycidamide has been demonstrated *in vivo* at low doses (see paragraph 50) by detection of hepatic glycidamide DNA adducts; therefore the *in vitro* data generated for glycidamide seem relevant to the risk assessment of acrylamide.

Conclusion: *In vitro* gene mutation experiments testing acrylamide in mammalian cells give results consistent with a non DNA adduct mediated effect in the apparent absence of metabolic conversion of acrylamide to glycidamide. However, glycidamide, which forms as a result of oxidative metabolism *in vivo*, is clearly a DNA reactive mutagen in mammalian cells.

The EU risk assessment report concluded that acrylamide is a direct acting mutagen in the available assays, probably causing clastogenic effects. The available data extend these conclusions. There is evidence to suggest a gene mutational effect of acrylamide in mammalian cells. However, in view of the uncertainties with regard to the mutagenic mode of action, it is not possible to say whether this is the result of a direct effect on DNA.

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In vitro cell transformation

24. The EU risk assessment report summarised a number of positive cell transformation assays of acrylamide both in presence and absence of exogenous metabolic activation. Park *et al.* (2002) reported a positive finding in Syrian Hamster Embryo (SHE) cells. It is noted that the effects were observed when the non specific CYP suicide inhibitor 1-aminobenzotriazole (ABT) was used. The authors suggest acrylamide induced a clastogenic effect responsible for the cell transformation and that glutathione depletion was partly responsible for this effect.

Conclusion: *In vitro* cell transformation assays are not recommended in the COM guidance (2000). The Committee considered that no definite conclusions can be reached based on these studies.

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In vitro chromosomal aberrations (mammalian cell)

25. The EU risk assessment report concluded that acrylamide is a direct acting clastogen in mammalian cells *in vitro*.

26. Yang *et al.* (2005) reported a dose related increase in chromosomal aberrations at 5, 10 and 50 mM acrylamide, in Chinese Hamster Lung (CHL) fibroblasts. This was reported in both the presence and absence of exogenous metabolic activation; however, a non-standard 22 h incubation with S9 liver homogenate was reported.

27. Martins *et al.* (2007) compared the frequency of chromosomal aberrations and sister chromatid exchanges (SCE) in V79 Mz cells treated with acrylamide (0.25 to 2 mM) and glycidamide (0.001 to 1 mM). Both chemicals resulted in an increase in aberrant cells (both including and excluding gaps), which was particularly evident at higher concentrations. The effect of glycidamide was approximately 2-fold greater than acrylamide at equimolar doses.

28. Glycidamide increased SCEs at concentrations at and above 0.01 mM, increasing to 10-fold above background at 1 mM; yet acrylamide only induced a significant increase in SCEs, a modest 1.6-fold, at the highest dose tested (2 mM). The SCE data were correlated to N7-GA-Gua and N3-GA-Ade DNA adduct levels in parallel cultures. There was a clear concentration related increase in N7-GA-Gua adducts at all glycidamide concentrations, with a similar increase in N3-GA-Ade becoming detectable at 0.25 mM. Neither adduct was detected in untreated control cells and only the N7-GA-Gua adduct was detected in cells exposed to acrylamide at 2 mM, and adduct levels were equivalent to cells treated with glycidamide at 0.001 mM.

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29. Glatt *et al.* (2005) reported a positive result for SCE formation in V79 cells containing transfected CYP2E1 (V-79 hCYP2E1-hSULT1A1 transfected)

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which was greater than the effect reported in V79 Mz cells. Thus overall there is evidence for both direct effects of acrylamide and effects mediated by glycidamide. The mechanism for the direct effects of acrylamide was not determined in these assays.

30. The DNA adduct data from Martins *et al.* suggest V79 Mz cells have negligible metabolic competency, which is consistent with the lack of CYP 2E1 activity for the conversion of acrylamide to glycidamide that was demonstrated by Glatt *et al.* Therefore, the effects of acrylamide seen in the Martins study are likely to be due to a mechanism independent of glycidamide adduct formation

Conclusion: The *in vitro* chromosomal aberrations study performed by Yang *et al.* found a similar rate of aberrations with acrylamide in the presence and absence of S9 liver homogenate; although uncertainty regarding the ability of S9 to metabolise acrylamide to glycidamide limits the usefulness of this study. The study by Martins *et al.* (2007) showed that glycidamide is modestly (approximately 2-fold) more clastogenic than acrylamide, yet there are substantial (2-3 orders of magnitude) differences in glycidamide adduct levels. This implies that acrylamide can cause clastogenicity by a mechanism independent of glycidamide DNA adduct formation in test systems lacking metabolic competency. It is reasonable to assume that this adduct independent mechanism of chromosomal aberration formation in cells that lack metabolic competency may operate alongside the glycidamide adduct mechanism in metabolically competent cells and *in vivo*.

The [biological significance of SCE is uncertain and this endpoint has been included in this review to help elucidate the mechanism of action of acrylamide](#). The mechanism of SCE formation appears to be related to adduct formation in the Martins *et al.* study, which is supported by the data from Glatt *et al.* using a cell line stably transfected with CYP2E1; although there is also evidence of a effect of acrylamide independent of glycidamide formation. However, [in view of the uncertain biological significance of SCE](#), few conclusions can be drawn from studies examining this endpoint.

The data indicate a clastogenic effect of acrylamide in the absence of metabolism to glycidamide but no definite conclusion regarding this glycidamide DNA adduct independent mechanism can be derived. Overall these data are consistent with the conclusion of the EU risk assessment report that acrylamide is a direct acting clastogen in mammalian cells *in vitro*.

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In vitro Micronucleus tests (mammalian cell)

31. No *in vitro* micronucleus assays were reported in the EU risk assessment report.

32. A study by Jie and Jai (2001) used Fluorescence *In Situ* Hybridisation (FISH) analysis of the chromosomal composition of micronuclei in mouse NIH3T3 fibroblasts. This indicated a clastogenic and aneugenic mode of

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action for acrylamide (~1.5 to 5.5 mM). No CYP2E1 activity or glycidamide adduct data were provided for NIH3T3 cells.

33. Baum *et al.* (2005) examined micronuclei in binucleated human blood lymphocytes exposed to acrylamide (0.5 to 5 mM) and glycidamide (0.05 to 1 mM). Although no significant effect was seen for either chemical, there was some limited evidence for an equivocal response of acrylamide in [lymphocytes from](#) some donors at the highest doses tested for both acrylamide and glycidamide.

34. Koyama *et al.* (2006) examined micronuclei in TK6 cells exposed to acrylamide (~2.6 to 14 mM) and glycidamide (~0.6 to 2.4 mM). A statistically significant increase was observed at the highest concentrations following both treatments. However, the authors noted [d](#) that the positive acrylamide concentrations exceeded [ed](#) the maximum concentration (10 mM) stipulated in the proposed guideline[†] (Aaron *et al.* 1994). This finding was not affected by co-incubation with phenobarbital and 5,6-benzoflavone induced rat liver S9 homogenate, indicating that either these cells have sufficient metabolic competence, or that S9 is unable to activate acrylamide.

35. Jiang *et al.* (2007) found significant concentration-related increases in micronuclei ($p \leq 0.05$) in Hep2G cells treated with acrylamide (0.625 to 2.5 mM). Statistically significant concentration-related increases in reactive oxygen species were also observed, measured by dichlorofluorescein formation assay; along with similar increases in immunoreactive 8-hydroxydeoxyguanosine (8-OHdG). The metabolic competence of this hepatoma derived cell line was not determined. This study provides some support for an oxidative damage mechanism; however, there is no evidence that this causes micronuclei, or indeed is the only mechanism by which acrylamide induces micronuclei.

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Conclusion: The *in vitro* micronucleus experiments give inconsistent results; although the three available studies that tested [ed](#) acrylamide were each performed in cells of various origins and the potential for differing metabolic competencies complicates the analysis. No studies were available that assessed [ed](#) metabolic competency of these cell lines. The study by Koyama *et al.* investigated both acrylamide and glycidamide and whilst acrylamide was equivocal in this assay, glycidamide was positive at the highest dose tested suggesting that, if formed, glycidamide is capable of inducing micronucleus formation in this test system.

No *in vitro* micronuclei studies in mammalian cells had been reported [in](#) the EU risk assessment report. While [le](#) studies have been performed subsequently, [n](#) inconsistent results mean that no conclusions may be drawn from these studies.

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[†] The revised draft test guidelines (OECD 487) recommend testing up to 50% cytotoxicity, which was ~7.5 mM in the Koyama study.

In vitro DNA damage (mammalian cells)

36. No comet assays had been reported in the EU risk assessment report. Baum *et al.* (2005) performed comet assays on human peripheral blood lymphocytes exposed to acrylamide (1 to 6 mM) and glycidamide (0.1 to 3 mM). Acrylamide exposure did not increase tail intensity, whereas there was a significant, concentration related increase in tail intensity following exposure to glycidamide (0.3 to 3 mM; $p < 0.001$). An extension to this study was reported by Thielen *et al.* (2006) who included formamido-pyrimidine-DNA-glycosidase (FPG) in the comet protocol in order to convert apurinic, apyrimidinic, ring-opened pyrimidines and oxidised purines, to single strand breaks. Human peripheral blood was exposed to glycidamide (0.003 to 0.3 mM) and, consistent with the Baum study, a significant increase in tail intensity was only seen at 0.3 mM. When the FPG incubation was included, a concentration and duration of glycidamide exposure related increase in DNA damage was observed at and above 0.01 mM.

37. Koyoma *et al.* (2006) performed comet assays in TK6 cells exposed to acrylamide (~6 and 14 mM) and glycidamide (0.6 to 2.4 mM). This yielded similar results to the Baum study, with a statistically significant ($p < 0.05$) concentration related increase in tail length at all glycidamide concentrations, which was not apparent in acrylamide exposed cells. If the DNA damage is the result of oxidative metabolism of acrylamide to glycidamide, these data would suggest that peripheral blood lymphocytes and TK6 cells lack metabolic competency.

38. Puppel *et al.* (2005) performed comet assays on hamster V79 cells, Caco-2 cells and primary rat hepatocytes that had been treated with acrylamide (0.1 to 6 mM) or glycidamide (0.001 to 0.6 mM). Following acrylamide treatment, significant increases in tail intensity were only observed in V79 ($p < 0.05$) and Caco-2 ($p < 0.01$) cells exposed to the highest concentration of acrylamide tested (6 mM for 24 hours). No effect was seen in the primary rat hepatocytes. All three cell types were sensitive to glycidamide with a concentration and generally duration of exposure related effect; following 24 h exposure, primary hepatocytes and V79 cells showed significant increases in tail intensity at 0.1 mM, with Caco-2 cells slightly less sensitive, showing significant increases at 0.3 mM.

39. The authors had hypothesised that differences in metabolic competency may be responsible for the differences in the level of DNA damage; therefore the presence of CYP2E1 was demonstrated in primary hepatocytes by Western blotting, but there was no immunoreactive protein detected in Caco-2 and V79 cells. A weakness of this study is that the CYP2E1 activity was not assessed and presence of protein does not necessarily indicate activity. It should be noted that, in this experiment, the cells that lack CYP2E1 protein are more sensitive to acrylamide, which is not consistent with the observed DNA damage being related to formation of glycidamide. The authors also hypothesised that the presence of glutathione (GSH) might sequester acrylamide which would have otherwise been metabolised to glycidamide in competent cells. Therefore DL-buthionine-[S,R]-sulfoximine (BSO) was used

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as an inhibitor of the GSH synthetic pathway to deplete the GSH pool in primary hepatocytes and V79 cells. These two cell lines were selected to investigate the effect of CYP2E1 expression. Co-exposure to BSO increased sensitivity of both cell lines to acrylamide related DNA damage, with a small but statistically significant increase at 0.5 mM in V79 cells and 1 mM in primary hepatocytes. Higher concentrations resulted in substantial cytotoxicity and precluded analyses at these concentrations. Caco-2 cells were not tested. Co-exposure to BSO did not cause a statistically significant change in sensitivity of V79 cells to glycidamide exposure, although this was not tested in either Caco-2 nor primary hepatocytes. Taken together, the data from Puppel *et al.* provide some evidence that oxidative stress may play a role in acrylamide, but not glycidamide, genotoxicity and cytotoxicity in this system. However, the uncertainty in the metabolic competency of the various cell types mean this study is hard to interpret.

40. Johansson *et al.* (2005) examined DNA damage in CHO cells exposed to glycidamide (0.5 to 8 mMh[‡]). Base excision repair (BER) and nucleotide excision repair (NER) deficient CHO cell lines were compared with wild-type cells. Glycidamide caused a concentration related increase in single strand breaks, as measured by detection of single stranded radiolabelled DNA in an alkaline DNA unwinding assay (Erixon *et al.*, 1979). At 8 mMh the amount of single strand breaks was equivalent to 30 Gy of γ radiation. Comparison of the various cell lines indicated that glycidamide adducts were likely repaired by short patch BER.

41. Ma *et al.* (2003) examined DNA damage by comet assay following exposure of HaCat cells to acrylamide (2 mM), in the presence and absence of the non specific CYP suicide inhibitor 1-aminobenzotriazole (ABT). The authors reported that comet rate and tail length in acrylamide treated cells were significantly reduced by ABT, suggesting these cells are capable of metabolising acrylamide to glycidamide. The full article was published in Chinese and additional details of the experiment are not available.

42. Blasiak *et al.* (2004) examined DNA damage in human peripheral blood lymphocytes that had been exposed to acrylamide (0.1 μ M to 0.05 mM) for three days, with a significant increase in DNA damage at and above 0.5 μ M. Chico Galdo *et al.* 2006 found elevated levels of DNA damage, assessed by alkaline comet assay, in primary thyroid cells from dog, sheep and humans, and in rat thyroid cell lines PC13 and FRTL5, which had been exposed to acrylamide (0.01 to 3 mM). The impact of metabolism in these cells and mechanisms of effects was not studied. Jiang *et al.* (2007) found significant concentration-related increases in DNA damage ($p \leq 0.01$) in Hep2G cells treated with acrylamide (2.5 to 20 mM). Statistically significant concentration-related increases in reactive oxygen species were also observed, measured by dichlorofluorescein formation assay; along with similar increases in immunoreactive 8-hydroxydeoxyguanosine (8-OHdG). The metabolic competence of this hepatoma derived cell line was not determined.

[‡] mMh = mM corrected for half life of glycidamide

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Conclusion: The evidence for *in vitro* DNA damage in acrylamide treated cells is inconsistent and interpretation of these data is complicated by the differing metabolic competencies of the cultured cells used in these assays. However, it is clear that glycidamide is able to damage DNA. These studies are consistent with a complex mode of action involving several possible mechanisms, including oxidative stress and DNA adduction.

DNA adduct formation *in vitro*, in cultured cells and *in vivo*

In vitro reactivity with DNA and free nucleotides

43. The formation of acrylamide related adducts has been investigated in *in vitro* experiments. As reported in the EU risk assessment report, Solomon *et al.* (1985) found direct adduction of acrylamide to calf thymus DNA when incubated at pH 7 for 40 days at 37°C. 2-Carboxyethyl adducts were detected at N1 and N6 of 2'-deoxyadenosine, N1 of 2'-deoxyguanosine and N3 of 2'-deoxycytidine. 2-formamidoethyl adducts at N7 of 2'-deoxyguanosine were also detected. These experiments only produced adducts after long incubation (40 days) with a high acrylamide concentration (68 mM).

44. Subsequent to the EU risk assessment literature search, Segerbäck *et al.* (1995) reported detection of the N7-(2-carbamoyl-2-hydroxyethyl)guanine (N7-GA-Gua) adduct following incubation of radiolabelled acrylamide in the presence of un-induced rat S9 liver homogenate for 2 hours at 37°C. Subsequent *in vitro* experiments with isolated DNA or free nucleosides treated with glycidamide have identified a range of adducts: N7-GA-Gua, N1-GA-Gua, N3-GA-Ade, N1-GA-dA, N6-GA-dA, N3-GA-dT and N3-GA-Cyd (Backman *et al.*, 2004 & 2007).

45. Gamboa da Costa *et al.* (2003) developed methods for detecting certain glycidamide DNA adducts. Adducts were detected in salmon testis DNA that had been exposed to glycidamide *in vitro*, in the ratio of 74:16:1 for N7-GA-Gua : N1-GA-dA : N3-GA-Ade.

DNA adducts in cultured cells

46. Mei *et al.* (2007) found a 60-fold difference between N7-GA-Gua and N3-GA-Ade adducts in glycidamide treated L51y8Y/Tk+/- cells, similar to the ratio reported by Gamboa da Costa *et al.* (2003), but could not detect either adduct in acrylamide treated cells. N7-GA-Gua and N3-GA-Ade adducts were found in cultured hamster fibroblasts (V79) treated with glycidamide (Martins *et al.*, 2006). N3-GA-Ade adducts were present at levels 100-fold lower than N7-GA-Gua. N7-GA-Gua adducts were detectable in cells treated with acrylamide but with reduced potency (2mM was equivalent to 1 µM glycidamide) and N3-GA-Ade were below the limit of detection (Martins *et al.*, 2006). Neither study used exogenous metabolic activation.

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47. Besaratinia *et al.* (2003 & 2004) reported the formation of DNA adducts in Big Blue mouse embryonic fibroblasts and normal human epithelial cells which had been exposed to acrylamide or glycidamide. The cell lines may have some CYP2E1 activity, although this was not investigated. Polymerase blocking lesions were mapped by PCR but the chemical identity of the adducts was not determined. The formation of DNA adducts was concentration-dependent, but there was no direct relationship between pattern of *cII* mutations and mapping of DNA adducts. DNA adducts formed following acrylamide treatment were reported to occur at similar locations in TP53 and *cII* to those formed from glycidamide.

DNA adducts in vivo

48. The EU risk assessment report cited evidence of DNA alkylation in the liver and, to a lesser extent, the testes. These studies were based on measuring DNA associated radioactivity. Segerbäck *et al.* (1995) found N7-GA-Gua adducts in the liver and other organs of rats and mice following an intra-peritoneal dose of radiolabelled acrylamide. Less DNA binding was detected in this assay than was detected in the studies referenced by the EU risk assessment report. The authors comment that their method only quantified N7-GA-Gua and that much of the DNA associated radioactivity was not associated with specific adducts since it appeared in the void volume of the column upon chromatographic purification).

49. Gamboa da Costa *et al.* (2003) used the methods that they had developed to quantify N7-GA-Gua and N3-GA-dA adducts in the livers of rodents exposed to acrylamide or glycidamide. The N1-GA-dA adduct could not be detected in cultured cells, or *in vivo*, even when converted to N6-GA-dA, due to limits in method sensitivity; so it is uncertain as to whether this adduct forms *in vivo* in the same ratio as detected in *in vitro* experiments (paragraph 45). As reported in later publications from these researchers, the limit of quantification (LOQ) for N7-GA-Gua and N3-GA-dA was in the region of 1 adduct in 10^8 nucleotides, with a limit of detection (LOD) approximately 0.5 adducts in 10^8 nucleotides. Intra-peritoneal administration of acrylamide to mice resulted in quantifiable levels of N7-GA-Gua and N3-GA-dA adducts (N7-GA-Gua being at a >70-fold higher level than N3-GA-dA, consistent with the *in vitro* data). Administration of glycidamide resulted in slightly increased levels of adducts but this was not consistently significant between studies and sex. Control mice were found to have low but detectable levels of N7-GA-Gua, which was probably the result of autoclave sterilisation of the rodent diet (Twaddle *et al.* 2004).

50. Subsequent studies compared intra-peritoneal dosing with oral gavage and dietary administration in mice and rats. These studies used low doses (0.1 mg/kg bw) which are closer to human mean dietary exposure, and ~500-fold lower than previous adduct studies (Doerge *et al.* 2005a, b & c). Notably, these studies found that dietary administration slightly attenuated acrylamide bioavailability but that oral (by gavage and also by dietary exposure) administration resulted in more extensive metabolism to glycidamide,

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probably the result of first pass metabolism. Furthermore, the levels of N7-GA-Gua adducts were proportional to the internal exposure to glycidamide (AUC) regardless of sex, species or chemical. This equated to ~2 adducts per AUC unit (μMh).

51. N7-GA-Gua and N3-GA-dA adduct levels were analysed in a range of tissues in rats and mice. Consistent with previous data, the N3-GA-dA adduct was present at ~70-fold lower levels than N7-GA-Gua. The authors commented that differences in adduct levels alone are not sufficient to account for the tissue specificity of the tumours in the carcinogenicity studies with acrylamide (Johnson *et al.*, 1986; Friedman *et al.*, 1995). However, the authors noted that limits of method sensitivity meant they could not examine the N1-GA-dA adduct, which has the potential to cause miscoding.

52. Doerge *et al.* (2005 a, b & c) also reported on the kinetics following gavage dosing of rats and mice. They found that acrylamide elimination had a similar rate constant to glycidamide formation, and glycidamide elimination had a similar rate constant to N7-GA-Gua adduct formation. Sub-chronic administration of acrylamide (~1 mg/kg bw/day for 28 days) to mice in drinking water showed an accumulation of N7-GA-Gua adducts in liver reaching a steady state of (3-400 adducts per 10^8 nucleotides) at 14 days. Similar data were obtained in rats although the levels slowly declined from the 14 day maximum in male rats.

53. Ghanayem *et al.* (2005a) compared adduct formation in CYP2E1 null mice following intra-peritoneal administration of acrylamide (50 mg/kg). N7-GA-Gua adducts were present in a 100-fold excess to N3-GA-dA adducts in treated wild-type mice. However, treated CYP2E1 null mice had detectable levels of the N7-GA-Gua adduct, albeit at lower (>50-fold) levels than wild-type mice. The authors suggested this shows that ~2% of acrylamide may be converted to glycidamide by a non CYP2E1 mediated mechanism in null mice.

54. Tareke *et al.* (2006) compared N7-GA-Gua adduct levels, haemoglobin adduct levels and internal glycidamide exposure, in rats and mice following sub-chronic administration in drinking water. They found the three parameters were each significantly associated with the others, suggesting that haemoglobin adducts may be a useful biomarker of liver adduct levels; although the short lifetime of erythrocytes (4 months in humans) means this is only an indication of recent exposure. Tareke *et al.* use the correlation between haemoglobin and liver DNA adducts to predict human liver DNA adduct level, based on the biomonitoring data available in the literature. This empirical relationship indicated that background dietary exposure to acrylamide may result in a steady state DNA adduct level of 0.2 to 0.3 adducts per 10^8 nucleotides. Allometric scaling based on body weight predicts 0.06 adducts per 10^8 nucleotides in a non smoking human.

55. Liver DNA adducts were also analysed as part of the *in vivo* micronucleus study (Davis and Recio, 2007) submitted by the PPG. The

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adducts data were presented to the Committee in MUT/08/01. In this study, mice were exposed to acrylamide (0.125 to 24 mg/kg bw/day) for four weeks by oral gavage. At doses below 4 mg/kg bw/day, the frequency of micronuclei in normochromatic erythrocytes and reticulocytes was generally indistinguishable from background, although there were some individual animal results below this dose that were significantly above background. However, there was a linear relationship between dose and liver N7-GA-Gua DNA adduct levels, with statistically significant ($p < 0.001$) increases above control levels seen at the lowest tested doses (see paragraph 67).

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In vivo germ cell DNA adducts

56. Holland *et al.* (1999) reported the formation of DNA adducts in sperm from mouse caudal epididymides using AMS analysis following intra peritoneal dosing of acrylamide (50 mg/kg bw). Xie *et al.* (2006) administered acrylamide (0.075-1000 µg/kg bw i.p.) to mice. Sperm DNA was isolated and AMS analysis used to investigate DNA adducts. A plot of log acrylamide adducts (ng/g) against log acrylamide dose showed a linear relationship for haemoglobin, serum albumin, protamine, sperm head and tail adducts, and sperm DNA adducts. It was noted that a dose level two orders of magnitude greater was needed to achieve DNA adducts at an equivalent level to protein adducts. For DNA adducts, a linear dose-response was noted above 7.5 µg/kg bw. This dose results in DNA adducts at ~0.1 ng acrylamide per g DNA, which represented the limit of detection in this experiment.

Conclusion: Whilst acrylamide has been shown to only weakly react with DNA *in vitro*, its reactive epoxide metabolite glycidamide can form a range of adducts. The studies cited in the EU risk assessment report used DNA associated radioactivity as a measure of adducts following treatment with radiolabelled acrylamide. Generally, the subsequent data specifically quantify levels of the N7-GA-Gua and N3-GA-Ade adducts, the former consistently occurring at levels ~60 to 100-fold greater than the latter. These adducts can be detected even at levels of exposure similar to the human mean dietary exposure; with evidence that acrylamide is more effectively metabolised to glycidamide at these low doses, and that interspecies and sex differences are reduced.

Whilst there is some debate as to the mutagenic potential of the N7-GA-Gua, this adduct cannot be completely discounted. Also the potential formation of O6-GA-Gua adducts warrants further investigation. The N1-GA-dA adduct, which has the potential to cause miscoding, was detected in reactions between glycidamide and purified DNA; however, problems with method sensitivity mean that it was not possible to quantify the N1-GA-dA adduct in cultured cells or *in vivo*.

N7-GA-Gua adducts appear to be related to the internal exposure (AUC) to glycidamide, either as a result of direct dosing with glycidamide, or forming as a result of acrylamide metabolism. Kinetic data suggest acrylamide elimination is related to glycidamide formation, and glycidamide elimination is

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related to N7-GA-Gua adduct formation. Experiments in mice have shown that there is a linear relationship between acrylamide dose and N7-GA-Gua adduct formation.

***In vivo* mutagenicity and DNA damage in somatic cells.**

In vivo Gene Mutation

57. The EU risk assessment report evaluated data from Myhr (1991) and Hoorn *et al.* (1993), who used the newly developed LacZ transgenic mutation assay (Muta™ Mouse) to investigate acrylamide (50 mg/kg bw/day for 5 days i.p.) mutagenicity *in vivo*. Bone marrow samples were examined for mutation frequency on days 3, 7 and 10 post dose. These studies were reported to be positive, although the EU risk assessment report concluded d that the full significance of the un-validated assay was unclear. Subsequently, the Muta™ Mouse model was used to assess the mutagenicity of acrylamide (50 or 100 mg/kg bw, i.p.) in liver, using lac-galE for mutant selection. Groups of 3-5 animals were dosed and killed at 3, 10 or 100 days post dose. A slight increase in mutation frequency at 50 mg/kg bw acrylamide was not interpretable given the negative findings at 100 mg/kg bw. There were no reports of toxicity in this study and ethylnitrosourea gave a positive result (Krebs and Favour 1997).

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58. The PPG identified additional unpublished Muta™ Mouse studies in liver, bone marrow and seminiferous tubules; which had been tabulated in Lambert *et al.* (2005). Mice were administered acrylamide i.p. (40 mg/kg bw/day for 17 days, or 125 mg/kg bw/day for 2 days), sampling 25 and 61 days post dosing. A significant increase in mutation frequency was only seen in the bone marrow of the 125 mg/kg bw/day treatment group, 25 days post dosing; all other tissues and sampling times gave no significant increase in mutation frequency. The mutations were not sequenced.

59. In a study by Manjanatha *et al.* (2006), groups of Big Blue male and female mice were given acrylamide (100 mg/l or 500 mg/l) or equimolar concentrations of glycidamide (120 mg/l or 600 mg/l) in drinking water for 4 weeks. This was equivalent to 19-35 and 88-111 mg/kg bw/day for low and high dose groups. Toxicity (hind leg paralysis) was observed in the high dose acrylamide group; therefore, treatment was stopped at 3 weeks. The effect resolved and the animals were included in the analysis. Spleen samples were taken for *hprt* mutation assay and genomic DNA was prepared from liver samples to assay *cII* mutation frequency. The *hprt* assay showed statistically significant increases in mutation frequency in both chemicals at all doses; the low dose group showed significant 3- and 8-fold increases in mutation frequency for acrylamide and glycidamide respectively ($p \leq 0.05$), with 16- and 25-fold increases at the higher dose ($p \leq 0.01$).

60. Mutation frequencies in the *cII* mutation assay were significantly increased ~2- and 2.5-fold in mice in the high dose acrylamide and

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glycidamide groups, respectively ($p \leq 0.05$); however, significant increases were not seen in the low dose groups. Mutant *cII* sequences from untreated animals were compared with those from high dose acrylamide and glycidamide treated animals. The mutation spectrum in acrylamide treated animals was significantly different to the control spectrum ($p = 0.0002$). The spectrum in glycidamide treated animals was also significantly different to the control spectrum ($p = 0.0001$); however there was no significant difference between the spectra of animals treated with acrylamide and glycidamide ($p \geq 0.57$).

61. These spectra show that acrylamide and glycidamide predominantly induce G→T mutations, which are consistent with N7-GA-Gua adducts (this adduct would be likely to undergo spontaneous depurination, and the resulting apurinic site would generally result in the incorporation of deoxyadenosine, causing a G→T transversion). However, A→G transitions, and A→T and A→C transversions were also increased in treated animals. These could be caused by N1- and N3-GA-Ade adducts. There was a significant increase in frameshift mutation frequency in both high dose treatment groups, from 19% to 35% ($p \leq 0.01$); these occurred mainly (80-90%) in the homopolymeric run of dG at bp 179-84. A similar effect was not observed in the study of Besaratinia *et al.* (2004). The authors suggested that this mutation is due to slippage of DNA polymerase; and highlighted that whilst increases in frameshift mutations were seen in the run of dG (bp 179-84), they were not seen in the run of dA (bp 241-6), which might indicate the presence of guanine but not adenine adducts. Overall the data were consistent with a gene mutation response of acrylamide mediated by metabolism to glycidamide.

62. A similar study using Big Blue rats has recently been reported as a poster at the Environmental Mutagen Society meeting (McDaniel *et al.* 2008). In this study male and female Big Blue rats were administered acrylamide (5 or 10 mg/kg bw/day) or equimolar concentrations of glycidamide in drinking water for 2 months. These doses were both lower than the lowest dose used in the Big Blue mouse study (equivalent to 19 to 35 mg/kg bw/day), which did not induce statistically significant increases in *cII* mutations. Neither chemical induced micronucleated reticulocytes in the rat study, although both induced small but significant increases in lymphocyte *hprt* mutant frequencies (2 to 3 fold, $p < 0.05$) with significant linear dose response trends. The mutation spectra were consistent with glycidamide DNA adducts. Preliminary results indicate that *cII* mutant frequencies in females are not increased in the mammary gland and liver; however, at the higher dose, glycidamide appears to increase mutation frequencies in the bone marrow and thyroid ($p < 0.01$), with increases also seen with acrylamide in the thyroid ($p < 0.05$).

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Conclusion: *In vivo* gene mutation experiments have given inconsistent results. Early studies by Myhr (1991) and Hoorn *et al.* (1993) in the Muta™ Mouse have shown an increase in mutation frequency following exposure to acrylamide, whilst Krebs and Favour (1997) report negative results in this model system. Lambert *et al.* (2005) summarise an additional Muta™ Mouse study examining the effect of acrylamide on several tissues and

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was generally negative; finding significant increases in mutation frequency only in the bone marrow at one sampling time point.

Big Blue Mouse studies by Manjanatha *et al.* (2006) have also shown a mutagenic effect in the *Hprt* gene at doses ≤ 35 mg/kg bw/day and in the *cII* transgene at doses ≤ 111 mg/kg bw/day for both acrylamide and glycidamide. There was no significant difference between the mutational spectra suggesting that acrylamide and glycidamide may cause mutations through a similar mechanism of action in this model system. These results are consistent with a mutational effect resulting from N7-GA-Gua adducts of glycidamide, although there is conflicting evidence for the possible contribution of N1 and N3 adducts of adenine. This is supported by similar results in a recent Big Blue rat study, although the COM reviews this preliminary, non-peer reviewed data with caution.

The study by Manjanatha *et al.* demonstrated increased mutation frequencies following dosing with either acrylamide or glycidamide. Furthermore, both compounds induced similar mutation spectra which is consistent with the expected DNA adduct profile, suggesting a glycidamide adduct based mutagenic mechanism. These data extend the conclusions reached in the EU risk assessment report.

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In vivo chromosomal aberrations

63. It was concluded in the EU risk assessment report that acrylamide produces chromosome aberrations in mice in bone marrow with limited evidence regarding splenocytes.

64. Kligerman *et al.* (1991) reported a significant increase in sister chromatid exchange (SCE) frequency (but not in the number of cells with a high frequency of SCEs) in spleen of C57BL/6 mice following i.p. dosing with acrylamide (100 mg/kg bw). Krishna and Theiss (1995) reported no evidence for chromosomal aberrations in bone marrow or spleen of rats dosed with 100 mg/kg bw acrylamide (route not given, only limited details available). Gassner and Adler (1996) reported an increase in the number of hypoploid 2nd metaphase cells in bone marrow of male mice following i.p. dosing at (80 and 120 mg/kg bw), which was statistically significant at the higher dose. Nesterova *et al.* (1999) reported a clastogenic effect in bone marrow from BALB/c and C57BL/6 mice following i.p. dosing with acrylamide (50 and 100 mg/kg bw for 5 days). No studies specifically testing glycidamide are available.

Conclusion: The *in vivo* chromosomal aberrations studies mainly show an increase in chromosomal aberrations following acrylamide administration. No studies specifically testing glycidamide are available, although it is likely that it would have been present in all of these studies as a result of metabolism *in vivo*. There is evidence of a clastogenic effect (Nesterova *et al.* 1999) and the evidence for hypoploidy reported by Gassner and Adler (1996) supports a potential aneugenic effect.

In vivo micronucleus assays

65. The EU risk assessment report concluded that acrylamide produced micronuclei in bone marrow and spleen in mice.

66. Paulsson *et al.* (2002) examined acrylamide (25, 50 and 100 mg/kg bw) administered i.p. to mice and rats. A linear dose related increase in micronuclei was detected by flow cytometry in mice, but not in rats. The authors noted that whilst acrylamide haemoglobin adducts levels were similar between rats and mice, glycidamide haemoglobin adducts were 3 to 6 times higher in mice than in rats. This indicates that rats have reduced metabolic conversion compared to mice and suggests that formation of glycidamide is required for micronuclei formation. In a follow up study, Paulsson *et al.* (2003) examined the dose-response relationship for glycidamide (15, 30 and 60 mg/kg bw) to investigate the species difference in sensitivity to glycidamide. In mice, a linear quadratic model fitted and, compared to their previous study, showed glycidamide had similar potency for inducing micronuclei, whether dosed as glycidamide or generated endogenously from acrylamide. The rat data were not clear, showing a non monotonic dose-response where the low glycidamide dose groups showed slight but significant ($p=0.001$) increases in micronuclei, with high dose group showing no significant increase above control. The authors suggested this may be due to bone marrow toxicity, as evidenced by the pronounced reduction in polychromatic erythrocytes. Glycidamide induced linear dose dependent increases in haemoglobin glycidamide adducts in both species.

67. Abramsson-Zetterberg (2003) also investigated the dose-response to acrylamide (2.5 to 100 mg/kg bw; 22 doses) in mice, using flow cytometry to detect increases in the frequency of micronuclei. A linear dose-response was observed and the DNA content of the micronuclei indicated a clastogenic, rather than an aneugenic response. This is in contrast to the microscopic evaluation by Schriever-Schemmer *et al.* (1997), where a clastogenic and aneugenic effect was seen in mice administered acrylamide i.p. (125 mg/kg bw). The PPG funded an additional micronucleus study, testing acrylamide at a range of doses (0.125 to 24 mg/kg bw/day, over 28 days), also using flow cytometry to detect micronuclei (Davis and Reico, 2007). Whilst the frequency of micronuclei was generally indistinguishable from control frequencies at doses below 4 mg/kg bw/day; the data fitted linear regression, quadratic regression and threshold models equally well. As noted in paragraph 55, there was a linear relationship between dose and liver N7-GA-Gua DNA adduct levels, with statistically significant ($p<0.001$) increases above control levels seen at the lowest tested doses.

68. Manjanatha *et al.* (2007) found significant increases ($p\leq 0.05$) in the frequency of micronuclei in Big Blue mice exposed to acrylamide (500 mg/l) and in mice exposed to an equimolar dose of glycidamide (600 mg/l) in drinking water for 4 weeks, equivalent to 88-111 mg/kg bw/day. Husøy *et al.* (2005) used conventional microscopic methods to evaluate the frequency of micronuclei in mice following subcutaneous administration of acrylamide or glycidamide (50 mg/kg bw), both resulting in significant increases in the

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frequency of micronuclei ($p \leq 0.001$). Yang *et al.* also used conventional microscopic methods following oral dosing with acrylamide (18 to 145 mg/kg bw), finding significant increases at and above 72.5 mg/kg bw ($p \leq 0.01$).

69. Ghanayem *et al.* (2005b) compared the frequency of micronuclei by flow cytometry, in CYP2E1 wild-type and null mice that had been administered acrylamide (25 or 50 mg/kg bw/day) i.p for 5 days. A significant dose-related increase was observed in wild type mice ($p \leq 0.001$); however, there was no effect in CYP2E1 null mice. This suggests that CYP2E1, and hence bioactivation of acrylamide to glycidamide, plays a role in the mechanism underlying the increase in frequency of micronuclei. This study also examined DNA adduct formation, finding that levels were 52- to 66- fold lower in CYP2E1 null mice, compared to wild-type mice (paragraph 53).

70. Kligerman *et al.* (1991) found a significant increase in micronuclei in binucleated splenocytes following i.p. administration of acrylamide to male C57BL/6 mice at 100 mg/kg bw, sampling at 24h. However, Krishna and Theiss (1995), testing acrylamide at 100 mg/kg bw to rats whilst developing a multi-tissue multi-endpoint assay, observed no micronuclei in the bone marrow or spleen. Dobrynzka and Gajewski (2000) found no evidence of micronuclei in PCEs in Pzh:Sfis mice administered acrylamide i.p. at 75 mg/kg bw i.p, sampling at 24,48,72h. No positive control data were reported. Abramsson-Zetterberg (2005) investigated the combined effect of viral infection with oral administration of acrylamide. Acrylamide did not significantly alter the micronucleus frequency in PCEs; however, dose was not reporting meaning this study is not interpretable.

Conclusions: The available *in vivo* micronucleus assays provide evidence of a clastogenic and possibly an aneugenic response to acrylamide. Evidence from CYP 2E1 null mice indicates that bioactivation of acrylamide to glycidamide forms part of the mechanism of action underlying the increase in frequency of micronuclei.

When evaluating the dose-response relationships derived by Abramsson-Zetterberg (2003), Paulsson *et al.* (2002), and Davis and Reico (2007); it is appropriate to apply the simplest modelling of the dose-response data (i.e. linear with no threshold) when other approaches give, at best, marginal improvements in dose-response analysis. It is possible that a no observed effect level for micronucleus formation could be determined from these studies but this may reflect the study design and sensitivity of the genotoxicity assay under consideration, rather than providing evidence of a biological threshold for genotoxic activity.

These studies expand the conclusions reached in the EU risk assessment report particularly with regard to dose-response assessment in mice.

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In vivo human biomonitoring

71. Kjuus *et al.* (2005) investigated chromosomal aberrations in workers exposed to acrylamide and N-methylolacrylamide whilst engaged in grout injection during railway tunnel construction. Blood samples were collected from 25 exposed and unexposed tunnel workers, matched for age, sex and smoking habits. No increases in chromosome aberrations or breaks were observed in exposed workers. There was a significant increase in chromosome gaps ($p=0.004$).

72. The authors assessed exposure by means of an “exposure-time index” which took into account the time spent in the tunnel and the time spent injecting grout over a 2 year period, this was time-weighted to account for differences in the level of grout usage. The exposed workers were stratified into two groups above and below the median exposure. There was no evidence of a dose-response relationship.

Conclusions: The single human biomonitoring study ([Kjuus *et al.* 2005](#)) identified in the systematic review provides no useful information on acrylamide genotoxicity due to the small sample size and the lack of robust exposure data.

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In vivo DNA damage assays

73. There were no DNA damage (comet) assays reported in the EU risk assessment report.

74. Maniere *et al.* (2005) used the comet assay to examine cells from blood, brain, liver, bone marrow, adrenals and testes from rats treated with acrylamide (18, 36 or 54 mg/kg bw). 24 hours post dosing, significant increases in DNA damage in blood leukocytes and brain were seen at 36 and 54 mg/kg bw, and testes at the higher dose ($p\leq 0.05$). No increase in DNA damage was observed in the other tissues. At earlier time-points (2 and 5 hours) significant increases were observed in the 54 mg/kg bw dose group, in all tissues except brain and testes. Klaunig and Kamendulis (2005) administered acrylamide (15 mg/kg bw/day) to rats for 7 days in drinking water and assessed DNA damage in liver, adrenals and thyroid. Significant increases in comet tail moment were observed for thyroid and adrenals. Zamorano-Ponce *et al.* (2006) examined bone marrow blast cells from mice treated with acrylamide (5, 20, 30 or 50 mg/kg bw). Statistically significant increases in comet tail moment were seen at all doses ($p\leq 0.01$).

75. Ghanayem *et al.* (2005a) examined DNA damage in blood leukocytes, liver and lung, in CYP2E1 wild-type and null mice that had been administered acrylamide (25 or 50 mg/kg bw/day) i.p for 5 days. Significant increases in comet olive tail moment were observed in the liver and blood leukocytes of wild-type mice, but not CYP 2E1 null mice. There was no effect in the lung

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cells. This suggests that CYP2E1, and hence bioactivation of acrylamide to glycidamide, plays a role in the mechanism underlying the increase in DNA damage in liver and blood leukocytes.

Conclusions: Comet assays performed on cells from animals dosed with acrylamide show an increase in DNA damage in various tissues, although there are some inconsistencies amongst some tissues. A study by Ghanayem *et al.* (2005a) using CYP 2E1 null mice indicates that bioactivation of acrylamide to glycidamide forms part of the mechanism of action underlying the increase in DNA damage in liver and blood leukocytes. These data extend the EU Risk Assessment Report.

In vivo DNA synthesis

76. Two studies were identified that examined induction of DNA synthesis (Lafferty *et al.* 2004; Klaunig and Kamendulis, 2005). These studies examined DNA synthesis in tissues generally identified as target organs in carcinogenicity studies by Johnson *et al.* (1986) and Friedman *et al.* (1995). Lafferty *et al.* (2004) investigated the effect of acrylamide in drinking water (2 or 15 mg/kg bw/day, for 28 days) on DNA synthesis in thyroid, adrenal medulla and testicular mesothelium, in male F344 and Sprague-Dawley rats. The cytochrome P450 inhibitor, 1-aminobenzotriazole (ABT), was used to assess the importance of CYP2E1 mediated bioactivation to glycidamide. Acrylamide induced DNA synthesis in all three tissues. The effect of ABT was less clear as, in some tissues, it induced DNA synthesis by itself (eg in thyroid follicular cells). However overall the authors suggested that oxidative metabolism or glycidamide did not appear to exclusively account for the induction of DNA synthesis.

77. Klaunig and Kamendulis (2005) measured DNA synthesis in F344 male rats dosed with acrylamide (15 mg/kg bw/day) in drinking water, for periods up to 28 days. An increase was seen in thyroid, testes, and adrenal medulla, but not in liver or adrenal cortex.

Conclusions: The *in vivo* DNA synthesis studies are likely to mainly detect DNA synthesis occurring during S-phase of the cell cycle during cell proliferation, rather than reparative unscheduled DNA synthesis. These studies indicate that acrylamide may induce DNA synthesis in several tissues. This may not be due exclusively to the effects of glycidamide or oxidative stress. No firm conclusions can be reached from these studies.

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***In vivo* mutagenicity and DNA damage in germ cells**

In vivo Germ cell Mutation assays

78. The EU risk assessment report summarised two specific-locus assays. In the first, a study by Russell *et al.* (1991), male mice received 5 daily doses

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of acrylamide i.p. (50 mg/kg bw/day). Specific locus mutations were observed when males were mated with untreated females 8-14 days and 15-21 days post treatment suggestive of an effect on late spermatid and early spermatozoal stages. The recovered mutations were associated with chromosomal aberration events. Acrylamide was not active in stem-cell spermatogonia. Ehling and Neuhäuser-Klaus (1992) dosed male mice i.p. with single doses of acrylamide (100 and 125 mg/kg bw). Increased mutation frequencies for late spermatid - spermatozoal stages were deletion type events and consistent with Russell *et al.*, although significantly increased mutation frequencies were also apparent in spermatogonia (≥ 43 days).

79. The EU risk assessment report also summarised a study that found no evidence for germ cell gene mutations in a preliminary validation of the *LacZ* assay, where testicular cell preparations were prepared from mice that had been administered acrylamide i.p. at 50 mg/kg bw/d for 5 days (Murti *et al.*, 1994). Subsequently, no effect was seen in the seminiferous tubules of Muta™ mice administered acrylamide i.p. (40 mg/kg bw/day for 17 days, or 125 mg/kg bw/day for 2 days), sampling 25 and 61 days post dosing (study details not reported but tabulated by Lambert *et al.* 2005).

Conclusions: Specific-locus assays indicate a positive response for acrylamide, specifically in late spermatid and early spermatozoal stages, and possibly in spermatogonia. Although transgenic mouse studies do not indicate a mutagenic effect on germ cells, the relevance of these findings and validity of these assays are unclear. None of these germ cell mutagenicity studies specifically investigated glycidamide or acrylamide metabolism.

In vivo Dominant lethal assays

80. Many dominant lethal assays were reported in the EU risk assessment report. Positive responses have been reported at dose levels of 3 mg/kg bw/day (oral) for 80 days, 25 mg/kg day (dermal) for 5 days and a single i.p dose of 125 mg/kg bw. The dominant lethal effects relate to effects on late spermatids and early spermatozoa.

81. A study by Working *et al.* (1987) examined the effect of acrylamide (30 mg/kg bw/day by oral gavage for 5 days) in F344 rats. Increased pre-implantation loss was observed over the first 4 weeks of mating and increased post implantation loss was seen over the first 3 weeks, consistent with an effect on spermatids and spermatozoa. Dobrynska *et al.* (1990) saw increased dominant lethality during the first week (*i.e.* an effect on spermatozoa) of mating following treatment at 125 mg/kg bw i.p. in Pzh:SF1SS mice, but not at 75 mg/kg bw. Holland *et al.* (1999) also observed increased pre-implantation loss during the first and second weeks post treatment in C57B1/6J mice administered acrylamide (40 and 50 mg/kg bw); although this novel study design and analysis is hard to interpret. Tyl and Friedman (2003) observed dominant lethality in the highest dose group in F344 rats administered 0.5, 2 and 5 mg/kg bw/day in drinking water for 10 weeks, as part of a multi-generation reproductive toxicity study. All germ cell

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stages would have been exposed in this study. Adler *et al.* (2004) found a dominant lethal effect during the first 12 days of mating post treatment with acrylamide (50 mg/kg bw; i.p.; for 5 days) in 102/E1xC3H/Ei)F1 mice. This is also consistent with an effect on spermatozoa and spermatids.

82. In a study by Sublet *et al.* (1989), male Long Evans rats were administered acrylamide (0, 5, 15, 30, 45, or 60 mg/kg) by oral gavage for 5 days and then mated serially to naive females. Treatment reduced fertility and increased pre- and post-implantation loss, primarily over the first 3 weeks post-treatment, consistent with the effects on spermatids and spermatozoa apparent in other studies. However, the authors provided evidence of reductions in sperm transport in the uterus during the first week of mating with modest but statistically significant alterations in sperm motility at week 3. This may indicate a non-genotoxic treatment related effect that may contribute to the observed dominant lethal effect; thus complicating interpretation of these dominant lethal assays.

83. Some studies have specifically investigated the role of glycidamide, or metabolism of acrylamide to glycidamide. Generoso *et al.* (1996) tested glycidamide (125 mg/kg bw) as a follow-up study to Shelby *et al.* (1986) which tested the same dose of acrylamide (summarised in the EU risk assessment report). Glycidamide induced a similar dominant lethal response to acrylamide, in that effects were restricted to late spermatids and early to mid spermatozoa; the authors considered this profile to be consistent with other alkylating agents that adduct to nitrogen positions on guanine and adenine and also to protamines.

84. Adler *et al.* (2000) investigated the effect of glycidamide formation on observed dominant lethality over four mating periods of 4 days. Acrylamide was administered to male mice (125 mg/kg bw; i.p.) with some groups of animals pre-treated with 1-aminobenzotriazole (ABT) to inhibit metabolism of acrylamide to glycidamide. ABT abolished the dominant lethal effect of acrylamide in the second mating. In the third mating there was a partial reduction in acrylamide induced dominant lethal effects. There was no effect of ABT in the fourth mating. The authors suggested that the dose of ABT may not have led to complete inhibition of acrylamide metabolism; however, it is also possible that a direct effect of acrylamide may occur in addition to the effect related to glycidamide. The authors noted reduced fertility in the study may have been associated with an effect of acrylamide on the mobility of sperm mediated by an effect on motor proteins. Interpretation of this study would have been aided by evidence of the degree of inhibition of glycidamide formation. Interpretation is also hampered by the evidence that treatment with ABT alone had an effect on sperm motility.

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85. Ghanayem *et al.* (2005c) undertook a dominant lethal study comparing male wild type and CYP2E1 null mice dosed with acrylamide (12.5 or 25 mg/kg bw/day; i.p for 5 days). The authors reported an effect of acrylamide on spermatids in wild type mice leading to a reduction in implants at both dose levels and confirmed the effect in a repeat experiment. There were no effects

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on percent pregnancy, mean number of implants per female, percent live fetuses/pregnant female or percent resorptions/pregnant female when CYP2E1 null males were treated with acrylamide. The authors concluded that glycidamide was the ultimate germ cell mutagen for dominant lethal effects and that this was likely to be due to binding to nucleophilic sites in chromatin in early spermatozoa. The precise mechanism was not elucidated.

Conclusions: A number of *in vivo* studies indicate that acrylamide can cause dominant lethality predominantly involving late spermatocytes and early spermatids. Glycidamide produces a similar dominant lethal effect. Chemical inhibition of cytochrome P450 mediated metabolism of acrylamide to glycidamide indicates that acrylamide may itself cause a dominant lethal effect in early spermatozoa, although this result may be due to incomplete inhibition of glycidamide formation. However, ablation of the CYP2E1 gene suggests that glycidamide is the ultimate germ cell mutagen. The possibility of a toxic effect resulting from carry-over of acrylamide in the semen cannot be excluded, although this is unlikely due to the rapid urinary excretion of free acrylamide.

In vivo Germ cell Heritable Translocation Assays

86. The EU risk assessment report summarised two positive heritable translocation assays. Shelby *et al* (1987) exposed male mice to acrylamide (40 and 50 mg/kg bw/day i.p. over 5 days), finding respectively 39% and 24% of males were sterile and heritable translocation carriers. The exposed germ cell stages were spermatids and spermatozoa. A follow-up study by Generoso *et al.* (1996) tested glycidamide at 100 mg/kg bw a dose which resulted in ~50% dominant lethality. Male mice were mated 3.5 to 7.5 days post exposure, so as to expose spermatids and spermatozoa. Cytogenetic analysis of clearly sterile males indicated a 20% translocation rate, compared to 0.06% historical control. The authors considered the findings of these studies were similar to the study involving acrylamide.

87. The second study summarised in the EU risk assessment report, by Adler *et al.* (1994) investigated the effects of acrylamide, either administered as single i.p. doses (50 and 100 mg/kg bw) or over 5 days (50 mg/kg bw/day). Animals were mated 7 to 16 days post exposure. There was an exposure related increase in heritable translocation carriers (0.6%, 2.7% and 22% respectively compared to 0.04% in controls). In a follow-up study, Adler *et al.* (2004) reported that five daily dermal doses (50 mg/kg bw/day) resulted in 9% heritable translocation carriers. Male mice were mated 1.5 to 8.5 days post exposure, thus exposing spermatid and spermatozoal germ cell stages.

Conclusions: These studies indicate that both acrylamide and glycidamide have the potential to induce heritable translocations in spermatids and spermatozoa.

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In vivo Embryo Abnormalities

88. Holland *et al.* (1999) examined the morphology of embryos (blastocyst/morula) from untreated female mice mated to males given i.p. injections of acrylamide (10-50 mg/kg bw/day; for 5 days). The animals were mated over a period of up to 5 weeks post dosing with acrylamide. Abnormal embryos (retarded cleavage, small number of blastomeres, embryos with lysis or abnormal cell structure and unfertilised eggs) predominated during weeks 1-3 mating at 40-50 mg/kg bw. In a separate experiment at 50 mg/kg bw/day, abnormal embryos were more frequent at week 2 compared to week 3 of mating. A dose-related effect was reported with an apparent NOEL of 10 mg/kg bw/day and 90% induction of abnormal embryos at 50 mg/kg bw/day. These data are largely consistent with the [results of](#) dominant lethal assays.

Conclusions: Investigations into the morphology of mouse embryos produced from matings where the males have been dosed with acrylamide are consistent with the available dominant lethal studies. No similar studies specifically investigate glycidamide or acrylamide metabolism, and this novel study design and analysis makes this study hard to interpret.

In vivo germ cell chromosomal aberrations

89. Studies summarised in the EU risk assessment report provide evidence for chromosomal aberrations (including complex rearrangements) and aneuploidy in mouse spermatogonia following either dietary administration (60 mg/kg bw/day; for 1-3 weeks) or i.p. dosing (100 mg/kg bw/day).

90. Marchetti *et al.* (1997) administered male mice (50 mg/kg bw/day; i.p.; for 5 days) and mated with untreated females at 2.5-48.5 days post final dose. Metaphase analysis of 1st cleavage division zygotes was undertaken. A post fertilisation reduction in the number of zygotes was reported on days 2.5-12.5 representing effects on late spermatids and early spermatozoa. Cell cycle delay was noted. Chromosomal aberrations were reported at the earlier time points, peaking at 6.5 days (spermatids/spermatozoa), and still statistically significantly increased at up to day 27.5 (pachytene spermatocytes). A wide range of chromosomal aberrations was reported. The highest level of acentric fragments and translocations occurred on day 6.5. The highest level of unbalanced translocations occurred on day 9.5. The response for balanced translocations was reported to be similar to that documented in other studies. The authors suggested that the time interval for inheritable/dominant lethal mutagenicity extended from late spermatids and spermatozoa to pachytene spermatocytes.

91. Schmid *et al.* (1999) found no evidence for an aneugenic effect when male mice were administered acrylamide (60 or 120 mg/kg bw; i.p.). Caudal epididymides were obtained 22 days post dose and examined by FISH

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analysis for aneugenic effects on sex chromosomes and chromosome 8. A positive result was obtained with colchicine.

Conclusions: Studies investigating germ cell chromosomal aberrations in mice exposed to acrylamide suggest that effects may occur in late spermatids, early spermatozoa and possibly pachytene spermatocytes. Further examination of the effect of acrylamide on this germ cell stage would be necessary to help interpret this novel finding. There was no evidence for an aneugenic effect following FISH analysis of sperm from mice dosed with acrylamide. None of these germ cell chromosomal aberrations studies specifically investigate glycidamide or acrylamide metabolism.

In vivo Embryo Micronucleus

92. The EU risk assessment report documented evidence for micronucleus formation in spermatogonia and spermatocytes of rats.

93. Titenko-Holland *et al.* (1998) administered acrylamide (50 mg/kg bw/day; for 5 days) to male mice, which were mated to untreated females for 5-17 days after end of treatment. Females were sacrificed 86-88h after mating to sample post meiotic embryonic cells for examination of morphology and micronucleus formation. FISH staining was performed to analyse centromere DNA content of micronuclei. The dose level used resulted in 10% loss of males at 24h post dose but no effect on mating. A large increase in the frequency of abnormal embryos (single cell, lysed blastomere and embryos having less than 10 cells). There was an increase in pyknotic and fragmented nuclei. There was a significant increase in micronuclei formation in treated normal and abnormal embryos. Both centromere positive and negative micronuclei were increased in treated embryos. The authors suggested both aneugenic and clastogenic mechanisms of action were involved in the observed response.

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Conclusions: Acrylamide can cause micronuclei to form in germ cells *in vivo*. The presence of both centromere positive and negative micronuclei indicates that acrylamide may have both aneugenic and clastogenic effects on germ cells.

In vivo Germ Cell Unscheduled DNA Synthesis (UDS)

94. The EU risk assessment report summarised two positive UDS studies in mouse and rat germ cells, with evidence for an effect on early spermatids. Generoso *et al.* (1996) administered male mice glycidamide (150 mg/kg bw) and observed a significant increase in UDS in early spermatids.

Conclusions: *In vivo* germ cell unscheduled DNA synthesis (UDS) assays indicate both acrylamide and glycidamide are able to induce DNA synthesis, particularly in early spermatids.

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Germ Cell DNA damage assays

95. The EU risk assessment report summarised a study where spermatozoa from mice treated with acrylamide (100 mg/kg bw) were analysed for single strand breaks by alkaline elution. Increased DNA damage occurred mainly in spermatids and pachytene spermatocytes. A subsequent alkaline elution study by Bjorge *et al.* (1996) used germ cells *ex vivo* from human and rat. No effect was seen in rat germ cells. A marginal increase was seen in human germ cells treated with acrylamide (1 mM).

Conclusions: Evidence of germ cell DNA damage *in vivo* indicates that spermatids and pachytene spermatocytes may be sensitive to damage by acrylamide. *Ex vivo* experiments show that human germ cells may also be susceptible. None of these germ cell DNA damage studies specifically investigated glycidamide or acrylamide metabolism.

Other in vivo germ cell assays

96. Gassner *et al.* (1995) administered acrylamide (80 or 120 mg/kg bw; i.p.) to male mice. Microscopic examination of male germ cells using immunofluorescent stains for spindle structure was undertaken. A significant increase in spindle disturbances was identified which predominantly comprised multipolar spindles. The authors considered that the effects of acrylamide cannot be assigned to interactions with specific elements of the spindle but possibly could represent binding to various spindle proteins.

Evidence for Acrylamide Mechanisms of Action

97. Interpretation of the genotoxicity studies testing acrylamide is made difficult by the multiple potential mechanisms that may cause genetic damage, represented in Figure 2.

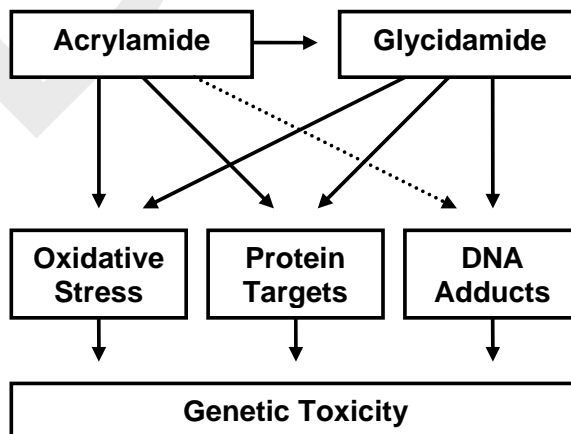


Figure 2: A diagram representing the complex interplay between the various potential mechanisms of acrylamide genotoxicity in somatic cells.

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98. It is possible that various genotoxic endpoints, such as micronucleus formation or chromosomal aberrations, may have differing sensitivity to the genotoxic mode of action mediated by each potential mechanism. This may explain the different apparent modes of action (point mutations, clastogenicity and aneugenicity) seen in the various assays.

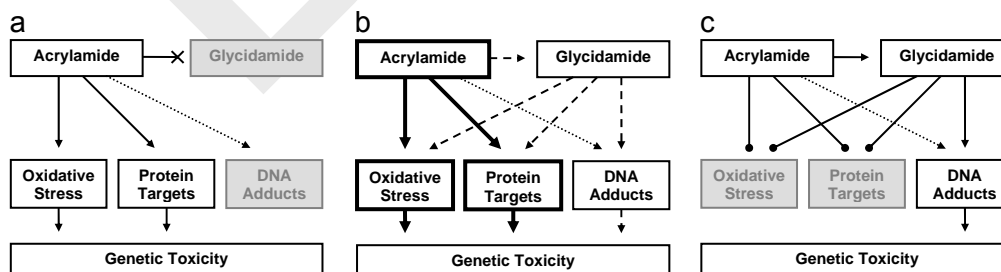
99. Differences in metabolic competency *in vitro* add an additional potential for inconsistencies. Effects related to glycidamide formation would be unlikely to be observed in a cell line which lacks metabolic competency, leaving only acrylamide mediated genotoxic effects to be observed (Figure 3a). This makes these models less relevant for assessing the genotoxic risk posed by acrylamide since the result cannot include the contribution of mutations resulting from glycidamide DNA adducts.

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100. The level of acrylamide exposure represents a potential complication in the assessment of *in vivo* studies. At high doses, where the hypothesised thresholds have been exceeded for protein target mediated genotoxicity and oxidative stress related DNA damage, one might expect the resulting genotoxic endpoints to be evident in a study. Conversely, in studies using low doses, or indeed at typical levels of human exposure, the threshold might not be exceeded and the related genotoxicity might not be evident. On the other hand, glycidamide formation is enzyme catalysed and therefore likely to be a saturable process. Bergmark *et al.* (1991) presented evidence that conversion of acrylamide to glycidamide is more efficient at low doses (51% at 5 mg/kg bw) than at high doses (13% at 100 mg/kg bw) on the basis of haemoglobin adduct measurements. This is supported by studies by Doerge *et al.* (2005a, b & c). This means that at high acrylamide doses where thresholds are exceeded and metabolism is likely to become saturated (Figure 3b), the relative contribution of glycidamide adducts to the overall genotoxic profile of acrylamide would be much less than at lower doses where the hypothesised thresholds have not been exceeded and metabolism to glycidamide is efficient (Figure 3c).

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Figure 3: A series of diagrams demonstrating the possible effects of (a) lack of metabolic activation, (b) high exposure level *in vivo* and (c) low exposure level *in vivo*. The cross represents lack of metabolism, the spots represent failure to reach the hypothesised threshold and the thick / dashed lines represent relative contributions of the various mechanisms of action.

Oxidative Stress

101. There is some evidence that oxidative stress may play a role in the genetic damage associated with acrylamide treatment. Glutathione depletion was shown to enhance DNA damage following acrylamide treatment (Puppel *et al.* 2005), and Jiang *et al.* (2007) found significant increases in immunoreactive 8-hydroxydeoxyguanosine (8-OHdG) together with increases in DNA damage in acrylamide treated cells. In support of this mechanism, Yousef and El-Demerdash (2006) have demonstrated a significant dose-related increase in glutathione S-transferase (GST) and superoxide dismutase (SOD) activity in the liver, lung, kidney and testes of rats treated with 0.5 to 500 µg/kg bw acrylamide; together with reductions in free sulphydryl groups and other biochemical changes indicative of oxidative stress which were also significant and dose-related. The doses used in this study were lower than those used in many of the *in vivo* genotoxicity studies assessed in this review, which suggests that oxidative stress may contribute to the genotoxicity evident in these studies.

102. It is plausible that some of the genotoxic effects seen following exposure *in vitro* and *in vivo* could be the result of oxidative stress. Since cells are normally able to tolerate and neutralise low levels of oxidative stress without adverse effect; it may be possible to identify a threshold that would protect against acrylamide related genetic damage occurring as a result of oxidative stress. However, the available evidence suggests that oxidative stress is not the only genotoxic mechanism resulting from acrylamide exposure. Therefore, it would be inappropriate to base a risk assessment solely on oxidative stress.

Effects on specific target proteins

103. It is thought that acrylamide and glycidamide are able to react with sulphydryl groups within cysteine residues of proteins and that this reaction is relatively non specific (Shipp *et al.*, 2006). Kinesins are sulphydryl rich proteins thought to regulate spindle dynamics during anaphase. Sickles *et al.* (2007) showed that acrylamide and glycidamide could significantly inhibit two bacterially expressed recombinant kinesins (KIFC5A, a motile kinesin; and KRP2, a depolymerising kinesin with an internal binding domain). Both chemicals were equipotent in reducing KIFC5A kinesin motility (0.1 to 1 mM), although glycidamide was more potent than acrylamide in reducing the depolymerising activity of KRP2 (1 to 10 mM). Kinesin inhibition occurred at similar concentrations to those used in many of the *in vitro* studies assessed in this review.

104. As with the oxidative stress mechanism, the available evidence suggests that inhibition of kinesin is not the only potential genotoxic mechanism that could arise from acrylamide exposure. Therefore, as with oxidative stress, it would be inappropriate for the risk assessment to be solely based on this mechanism. In addition, any consideration of a kinesin based mechanism would need to investigate the molecular mechanism of the kinesin

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inhibition, explore the redundancy of the binding site(s) on kinesin, the effects of partial inhibition of the total kinesin pool, and to determine a level of kinesin inhibition below which perturbations to the mitotic/meiotic spindle would not be expected.

105. It is probable that protein binding other than to kinesin may play a role in acrylamide related genotoxicity. Such protein binding is implicated in germ cell mutagenicity. Data from Sega *et al.* (1989) indicate that binding to sperm protamines may play a role in the observed dominant lethality, since protamine adduct but not DNA adduct levels correlate with dominant lethal mutation frequency. Protamines replace histones in spermatid DNA during mid to late spermatid stages. Some authors suggest that, in the early stages of histone replacement, adduction of acrylamide or glycidamide to “immature” protamines could prevent normal chromatin condensation, leading to stress within the DNA molecule and DNA breakage (Shipp *et al.*, 2006). Where studies testing acrylamide have examined the effect of acrylamide exposure on male germ cells at various stages of development, effects on late spermatids and early spermatozoa were often noted. This would be consistent with a protamine related mechanism, although further consideration of this mechanism would be necessary if genotoxic effects in germ cells were considered to be the critical target cell for acrylamide risk assessment.

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Metabolism of Acrylamide to Glycidamide

106. Studies by Doerge *et al.* (2005a & b) showed that acrylamide is metabolised to glycidamide in rats and mice. Oral exposure to acrylamide results in up to 7-fold higher relative systemic exposure to glycidamide compared to i.v. and i.p.; probably as a result of first-pass hepatic metabolism or other kinetic changes. There is also evidence that, as the dose level is reduced, the efficiency of conversion to glycidamide increases.

107. Oxidative metabolism of acrylamide to glycidamide is widely accepted to be mediated by CYP2E1; however, Ghanayem *et al.*, (2005a) found evidence of glycidamide DNA adducts in the livers of CYP2E1 null mice dosed with acrylamide, suggesting that glycidamide may also form *in vivo* by other mechanisms. It is unclear if this occurs in wild-type mice, or whether this is an adaptive change in the knock-out mouse; however formation of glycidamide by this pathway seems to be insufficient to cause dominant lethality (Ghanayem *et al.*, 2005c), micronuclei and DNA damage (Ghanayem *et al.*, 2005b) when tested in null mice. These experiments with CYP2E1 mice demonstrate that metabolism to glycidamide is required for these genotoxic endpoints.

108. Furthermore, these studies provide evidence that kinesin inhibition is unlikely to be a major factor in micronucleus formation, DNA damage and dominant lethality due to acrylamide. These genotoxic endpoints were not detected in CYP2E1 null mice; yet, since metabolism to glycidamide is a major route of acrylamide excretion, null animals would be expected to have

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much higher internal exposure to acrylamide. Plasma acrylamide concentrations in null mice were 137-fold higher than wildtype mice 6 hours after a single dose of acrylamide (50 mg/kg bw i.p.; Ghanayem *et al.* 2005a). Since Sickles *et al.* (2007) showed that both acrylamide and glycidamide inhibit kinesin, null animals might be expected to show equivalent or elevated levels of genetic damage resulting from kinesin inhibition rather than an absence of effect. It is possible that there may be other reasons why genotoxicity was not evident in null mice; for example, although urethane behaved as expected as a positive control in the micronucleus assay, elevated levels of acrylamide in the null mice might cause cell cycle inhibition which could have prevented cells from dividing during the interval between exposure and harvest.

Glycidamide DNA Adduct Formation

109. Once formed, glycidamide would be expected to inhibit kinesin activity (Sickles *et al.* 2007) and be also likely to cause oxidative stress in a similar manner to acrylamide. Whilst acrylamide only appears to adduct to DNA following prolonged exposure of DNA to high concentrations of acrylamide *in vitro* (Solomon *et al.*, 1985), the evidence for glycidamide DNA adducts is extensive. Doerge *et al.* (2005a, b & c) have reported N7-GA-Gua and N3-GA-Ade adducts in rats and mice, with the latter forming at ~70-fold lower levels than the former. Repeat dose studies in rats and mice exposed to acrylamide (~1 mg/kg bw day) showed that steady state was reached after 14 days in mice and female rats (males showed a slight decline from the 14 day maximum).

110. Koskinen and Plná, (2000) argued that, overall, the mutagenicity of alkyl adducts of N7-Gua and N3-Ade is 'rather small'. This is because although spontaneous depurination of the adducted base would result in incorporation of adenine against the apurinic site, the contribution of these apurinic sites would be small compared to the steady state of abasic sites and the fact that these lesions are constantly being repaired. The authors suggested these adducts may be a useful biomarker for more mutagenic adducts, such as the N1-Ade adduct, which would interfere with hydrogen bonding in the base pairing region. Gamboa da Costa *et al.* (2003) identified an N1-GA-dA adduct, forming at a ~5-fold lower level than N7-GA-Gua when DNA is treated *in vitro*; however, the authors could not detect this adducts in cultured cells or *in vivo* due to limits in method sensitivity. The formation of the N1-GA-dA adduct *in vivo* cannot be discounted.

111. Manjanatha *et al.* (2005) compared *cII* gene mutation spectra in Big Blue mice, and Besaratinia and Pfeifer (2003) performed similar experiments in fibroblasts derived from Big Blue mice exposed to either acrylamide or glycidamide. The mutation spectra induced by acrylamide and glycidamide *in vivo* closely resembled that of glycidamide *in vitro*. Increased G → T transversions predominated and were consistent with the miscoding potential of the N7-GA-Gua, but also increased A → G transitions, and A → C and A → T transversions were seen, suggestive of N3-GA-Ade and N1-GA-dA adducts.

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112. Davis and Recio (2007) have demonstrated a linear relationship between dose and liver N7-GA-Gua DNA adduct levels, with statistically significant ($p < 0.001$) increase above control levels seen at the lowest tested dose (0.125 mg/kg). Assuming the point mutations observed in the Manjanatha, and Besaratinia and Pfeifer studies occurred as a result of DNA adducts, the data from Davis and Recio would not be consistent with a threshold effect.

Germ Cell Genotoxicity

113. Overall there is evidence that the genotoxic effects of acrylamide are more potent in germ cells than somatic cells. This review has mainly focussed on the genotoxic effects of acrylamide on somatic cells, with a more limited consideration of germ cell effects. If there were to be sufficient evidence to support a threshold approach to acrylamide somatic cell mutagenicity, further consideration of the genotoxic effects on germ cells would need to be undertaken. Such a review would need to expand upon the evaluation of the mechanism of the effects on specific germ cell stages; acrylamide is reported to affect spermatids and spermatozoa in many studies, but there is also some limited evidence of effects in pachytene spermatocytes and spermatogonia which would require further investigation. Additional consideration would also need to be given to the effect of protamine binding on genotoxicity in germ cells.

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Overall Committee Conclusions

114. The EU risk assessment report concluded that acrylamide is an *in vitro* mutagen, and *in vivo* somatic cell and germ cell mutagen. The predominant effect was clastogenicity with some evidence for aneugenicity. The published evidence available since 1995 extends the effects of acrylamide to include identifiable glycidamide DNA adducts and gene mutations, detectable in cultured mammalian cells and somatic cells *in vivo*, and with mutation spectra which are consistent with those adducts. An element of the mutagenic effect of acrylamide, therefore, appears to be due to the formation of DNA adducts following metabolism to glycidamide.

115. Assessment of the genotoxic potential of acrylamide is complicated by multiple potential mechanisms, which include extensive protein binding / enzyme inhibition, oxidative stress and DNA adduct formation. It is plausible that each of these mechanisms may contribute to the genotoxicity of acrylamide. These mechanisms are not mutually exclusive.

116. Acrylamide is an *in vivo* mutagen. In order to move away from the default assumption that there is no level of exposure to this genotoxic carcinogen that is without some risk, it will be necessary to identify evidence of a threshold with supporting mechanistic data for all of the potential genotoxic mechanisms of acrylamide in somatic cells and germ cells. Based on the currently available evidence, it should be considered that there is no

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level of exposure to acrylamide that is without some risk, although we acknowledge that the genotoxic effects of exposure to very low levels of acrylamide are likely to be pragmatically indistinguishable from background.

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