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

REVIEW OF GENOTOXICITY OF ACRYLAMIDE

Discussion Paper comparing the genotoxicity of acrylamide and glycidamide

Referral to COM on acrylamide

1. The HSE requested a further evaluation from the COM regarding the information cited by the PPG in its letter to the chair of COM (dated 8 May 2007) (Annex 2 to this draft discussion paper). The Food Standards Agency have also requested that a consideration be given to all available genotoxicity data on acrylamide by COM. 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.

Background to COM review of acrylamide

2. HSE asked for an opinion on the evidence regarding germ cell mutagenicity of acrylamide and the evidence regarding a threshold for germ cell mutagenicity with this chemical in January 2007. A response to HSE was published in February 2007^a. 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 and agreed to a further evaluation of the genotoxicity data on acrylamide.

3. The COM considered a presentation from PPG on 'an analysis of the genotoxicity of acrylamide' at the October 2007 COM meeting. Additional data submitted by PPG following that meeting was presented in MUT/08/01.

4. The COM secretariat drafted an overview of the EU Risk Assessment of acrylamide and outlined a strategy for the COM review of published literature in MUT/07/17. The COM agreed that the EU risk assessment could form the basis of literature reviewed up to 1995, and that COM secretariat overview would focus on published literature from 1995 onwards. The COM agreed also agreed to review a number of specific research papers published prior to 1995 which had been identified by the secretariat but not included in the EU risk Assessment report. A reference list for the current review was subsequently distributed to COM members. A small number of newly published additional references were identified by COM members.

^a <http://www.advisorybodies.doh.gov.uk/com/acryla.htm>

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5. At the February 2008 meeting, the COM considered a second presentation from PPG on additional issues relating to acrylamide and glycidamide (MUT/08/01). The COM also received a systematic review of data relating to the genotoxicity of acrylamide and glycidamide published after 1995, and other references not included in the EU risk assessment report. This was complemented by an overview discussion paper relating to acrylamide genotoxicity (MUT/08/02).

6. The COM is now presented with a discussion paper comparing the genotoxicity of acrylamide and glycidamide (Annex A). Copies of certain key papers which have been identified as part of the review have been included in Annex B. Other papers identified as part of the systematic review are available from the secretariat, should members wish to view these in the course of evaluating this discussion paper. Members may also wish to consult the summaries of the reviewed papers, which they received in MUT/08/02. This is available on the COM website^{b,c}

Questions for COM

7. COM is asked to consider the following discussion paper (Annex A), which will form the basis for drafting the statement. Conclusions for each section have been drafted and members may wish to focus upon these paragraphs.

8. In addition, members may wish to consider the following question:

i) What further research may help clarify the role of glycidamide DNA adducts in acrylamide induced genotoxicity?

Secretariat May 2008

^b <http://www.advisorybodies.doh.gov.uk/pdfs/mut082a1t1.pdf>

^c <http://www.advisorybodies.doh.gov.uk/pdfs/mut082a1t2.pdf>

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

REVIEW OF GENOTOXICITY OF ACRYLAMIDE

Annex A: Discussion Paper comparing the genotoxicity of acrylamide and glycidamide

[This discussion document has been drafted to aid members in their consideration of acrylamide. It does not represent a formal view of COM]

Comparison of the genotoxicity of acrylamide and glycidamide

1. This is a discussion paper comparing the genotoxicity of acrylamide and glycidamide. It builds upon the previous paper discussing the genotoxicity of acrylamide (MUT/08/02)

In vitro Mutagenicity.

In vitro Gene Mutation (Bacterial)

2. During the February 2008 meeting, members considered that the paper by Yang *et al.* (2005) was not sufficient to alter the weight of evidence that acrylamide is not mutagenic in bacteria in the presence and absence of metabolic activation. 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 *E. coli* strain used by Emmert B *et al.* (2006) which was also not sensitive to acrylamide; so it seems that either the strain has lost sensitivity to glycidamide, or glycidamide was not formed in sufficient quantities to be detected in the Ames test (Sergerback *et al.* 1995 showed uninduced Rat S9 produces a detectable level of glycidamide DNA adducts, but Hashimoto *et al.* found that the addition of S9 was insufficient to give a positive result by Ames test).

Conclusion: *In vitro* gene mutation experiments in bacteria give results that are generally constant with a lack of genotoxicity in this test system, in the presence and absence of S9 liver homogenate metabolic activation. The available evidence from other genotoxicity studies casts doubt on the ability of S9 to catalyse the conversion of acrylamide to glycidamide. However, glycidamide, which forms as a result of oxidative metabolism *in vivo*, is clearly a DNA reactive mutagen in bacteria.

In vitro Gene mutation (Mammalian)

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3. Data reviewed in the EU risk assessment report concluded that acrylamide is a direct acting mutagen in the available assays, probably causing clastogenic effects. At the February 2008 meeting, Members concluded that the available data extended these conclusions and that 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, the COM could not support the definite conclusions outlined in the EU risk assessment report.

4. Besaratinia and Pfeifer (2003) examined mutation frequencies in the *cII* transgene in cultured Big Blue mouse embryonic fibroblasts. Members considered there to be a dose related increase in mutation frequency for both glycidamide and acrylamide treated cells. It was considered possible that, if these cells were metabolically competent, cytotoxicity may have reduced the metabolic competency, causing reductions in mutation frequency at higher doses.

5. Besaratinia and Pfeifer (2004) went on to compare the mutant frequencies and spectra in the *cII* transgene following acrylamide and glycidamide treatment. Both acrylamide and glycidamide had statistically significant increases in mutation frequency ($p \leq 0.001$), with a greater increase observed with glycidamide treatment. Mutation spectra (reported as the spectrum of individual mutated bases) were similar when acrylamide (320 μM) was compared with glycidamide (500 μM), but the relative proportion of G \rightarrow T + C \rightarrow A was increased compared to acrylamide and control samples, and T \rightarrow G + A \rightarrow C was decreased (the former being significant compared to control; $p \leq 0.001$). The authors suggest that the mutational spectra for acrylamide and glycidamide are consistent with N7-GA-Gua, N3-GA-Ade and N1-GA-dA adducts.

6. 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 mutations; however, of the remaining mutations, acrylamide induced deletions generally extended further towards the kinetochore compared to glycidamide induced deletions. 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 innate 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.

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7. 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, where increases in mutant frequencies only reached significance at cytotoxic doses in one out of two studies, and glycidamide where there were significant increases in mutation frequency at sub-cytotoxic doses, with 20-fold increase above controls at 2.4 mM.

8. 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 ($p < 0.001$)

9. Ao *et al.* (2008) examined hPRT mutation frequencies in HL-60 and NB4 leukaemia cell lines exposed to acrylamide (up to 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 that the spontaneous mutation spectrum in control cells, where single exon and partial deletions were increased. There was no difference in spectra between the two cell lines. There was no evidence of metabolic competency of these cell lines.

10. 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).

11. Glycidamide has been investigated alone by Johansson *et al.* (2005) with the data replotted 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.

12. In summary, these studies suggest that, in absence of metabolic activation, the glycidamide DNA adduct mechanism does not occur. Glycidamide itself has been shown to be positive in all studies. Effective metabolic conversion of acrylamide to glycidamide has been demonstrated at low doses (see paragraph 36) by detection of hepatic glycidamide DNA adducts; therefore the *in vitro* data generated for glycidamide seem relevant to the COM's risk assessment of acrylamide.

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

In vitro chromosomal aberrations (mammalian cell)

13. Data reviewed in the EU risk assessment report concluded that acrylamide is a direct acting clastogen in mammalian cells *in vitro*. At the February 2008 meeting, the Committee considered that the data indicated a clastogenic effect of acrylamide in the absence of exogenous metabolic activation but no definite conclusion on the mode of action could be derived. Overall these data did not extend the conclusions reached in the EU risk assessment report.

14. 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. SCE data show glycidamide increases SCEs at concentrations upwards of 0.01 mM, increasing levels 10-fold at 1 mM; yet acrylamide only induces a significant increase in SCEs, a modest 1.6-fold, at the highest dose tested (2 mM). This data was 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 the N7-GA-Gua was only detected in cells exposed to acrylamide at 2 mM, at levels equivalent to cells treated with glycidamide at 0.001 mM.

15. The COM generally places less weight on SCE data as the significance of this endpoint is uncertain. The DNA adduct data suggest V79 Mz cells have negligible metabolic competency (consistent with the lack of CYP 2E1 activity demonstrated by Glatt *et al.* 2005) for the conversion of acrylamide to glycidamide and that the effects of acrylamide seen in this test system are likely to be due to a mechanism independent of glycidamide adduct formation.

Conclusion: The *in vitro* chromosomal aberrations study performed 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 causes clastogenicity by a mechanism independent of glycidamide DNA adduct formation in this test system.

In vitro Micronucleus tests (mammalian cell)

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16. No *in vitro* micronucleus assays were reported in the EU risk assessment report. Members considered that the study by Jie and Jai (2001) using FISH analysis of chromosomal changes in NIH3T3 cells indicated a clastogenic and aneugenic mode of action for acrylamide (~1.5 to 5.5 mM). No CYP2E1 activity or glycidamide adduct data were provided NIH3T3 cells.

17. 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 some donors at the highest doses tested for both acrylamide and glycidamide.

18. 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). Statistical significance was observed at the highest concentrations following both treatments, although the authors note that the positive acrylamide concentrations exceed the maximum concentration (10 mM) stipulated in the 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.

19. 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 micronucleus formation, or indeed is the only mechanism by which acrylamide induces micronuclei.

Conclusion: The *in vitro* micronucleus experiments give inconsistent results; although the three available studies that test acrylamide were each performed in cells of various origins and the differing metabolic competencies confounds the analysis. It is apparent that the micronucleus end-point seems relatively insensitive to acrylamide compared to the clear effects seen in other measures of genotoxicity, such as chromosomal aberrations. No studies were available that test glycidamide, or assess metabolic competency of these cell lines.

In vitro DNA damage (mammalian cells)

20. No comet assays had been reported in the EU risk assessment report. At the February 2008 meeting, the Committee considered that the data were

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consistent with a complex mode of action involving a number of genotoxic effects of acrylamide and glycidamide.

21. 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.* 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.

22. Koyoma *et al.* (2006) performed comet assays in TK6 cells exposed to acrylamide (~6 and 14 mM) and glycidamide (0.5 to 2 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, this data would argue that peripheral blood lymphocytes and TK6 cells lack metabolic competency.

23. 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). 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 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.

24. 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 not 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-

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buthionine-[S,R]-sulfoximine (BSO) was used as an inhibitor of the GSH synthetic pathway to deplete the GSH pool in primary hepatocytes and V79 cells, so as 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. Neither Caco-2 nor primary hepatocytes were tested. 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.

25. Johansson *et al.* (2005) examined DNA damage in CHO cells exposed to glycidamide (0.5 to 8 mMh; i.e. mM corrected for half life of glycidamide). Base excision repair (BER) and nucleotide excision repair (NER) deficient CHO cell lines were compared with wildtype 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 *Mutat. Res.* 59, 257-71). 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.

26. 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 CYP inhibitor 1-aminobenzotriazole (1-ABT). The authors report that comet rate and tail length in acrylamide treated cells were significantly reduced by 1-ABT, suggesting these cells are capable of metabolising acrylamide to glycidamide. Unfortunately, the full article was published in chinese so additional details of the experiment are not available.

27. 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 0.5 μ M and above. 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).

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The metabolic competence of this hepatoma derived cell line was not determined.

Conclusion: The evidence for *In vitro* DNA damage in acrylamide treated cells is inconsistent and interpretation of this data is confounded 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 of action, including oxidative stress and DNA adduction.

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

28. Discussion paper MUT/08/02, presented at the February 2008 meeting, contained an overview of the DNA adduct data encompassing studies performed using acrylamide and glycidamide. This is briefly outlined in the following section:

In vitro reactivity with DNA and free nucleotides

29. 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 high acrylamide concentration (68 mM).

30. Subsequent to the EU risk assessment literature search, Sergerbäck *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. Several subsequent *in vitro* experiments with isolated DNA or free nucleotides 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.

31. Gamboa da Costa *et al.* (2003) developed methods for detecting the relevant 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

32. N7-GA-Gua and N3-GA-Ade adducts were found in cultured hamster fibroblasts (V79) treated with glycidamide. 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

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detection (Martins *et al.* 2006). A recent publication from the same group using L51y8Y/Tk^{+/-} cells found a similar 60-fold difference between N7-GA-Gua and N3-GA-Ade adducts, but could not detect either adduct in acrylamide treated cells (Mei *et al.* 2007).

33. 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 and glycidamide. The cell lines may have some CYP2E1 activity. Polymerase blocking lesions were mapped by PCR but the chemical identity of the adducts was not determined. The formation of DNA adducts was dose-dependent, but there was no direct relationship between pattern of *c//* mutations and mapping of DNA adducts. DNA adducts formed following acrylamide treatment were reported to occur at similar locations in TP53 and *c//* to those formed from glycidamide.

DNA adducts *in vivo*

34. 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. Sergerbäck *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 quoted in 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 (it appeared in the void volume upon purification).

35. 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 31). 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 10⁸ nucleotides, with a limit of detection (LOD) approximately 0.5 adducts in 10⁸ 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).

36. 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-

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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 administration resulted in more extensive metabolism to glycidamide, 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 ($\mu\text{M} \times \text{h}$).

37. 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 comment that differences in adduct levels alone are not sufficient to account for the tissue specificity of the tumours in the carcinogenicity studies. Although the authors note that limits of method sensitivity meant they could not examine the N1-GA-dA adduct, which has the potential to cause mis-coding.

38. 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.

39. Ghanayem *et al.* (2005) 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 suggest this shows that ~2% of acrylamide may be converted to glycidamide by a non CYP2E1 mediated mechanism in null mice.

40. 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 ~3 month lifetime of erythrocytes means this is only an indication of recent exposure. Attempts to model the data from rodent to human indicate liver adduct rates may be in the region of 0.06 to 0.3 adducts per 10^8 nucleotides.

41. Liver DNA adducts were also analysed as part of the *in vivo* micronucleus study (Davis and Recio, 2007) submitted by the Polyacrylamide Producers Group (PPG). The adducts data were presented to the Committee

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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 were indistinguishable from background (see paragraph 52). 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.

In vivo germ cell DNA adducts

42. Holland N *et al.* (1999) reported the formation of DNA adducts in sperm from mouse caudal epididymides using AMS analysis following dosing of 50 mg/kg bw. Xie *et al.* (2006) administered between 0.075-1000 $\mu\text{g}/\text{kg}$ bw i.p. 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 of two orders of magnitude was needed to achieve DNA adducts equivalent to protein adducts. For DNA adducts, a linear dose-response was noted above 7.5 $\mu\text{g}/\text{kg}$ bw. This dose results in DNA adducts at ~ 0.1 ng acrylamide per g DNA, which represents 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. The subsequent data generally specifically quantifies 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 exposures close to the human mean dietary exposure; with evidence that acrylamide is more effectively metabolised to glycidamide at these low doses, 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 warrant further investigation. The N1-GA-dA adduct, which has the potential to cause mis-coding, 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 experiments by Doerge *et al.* (2005c) suggest acrylamide elimination is related to glycidamide formation, and glycidamide elimination is related to N7-GA-Gua adduct formation.

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Experiments in mice have shown that there is a linear relationship between acrylamide dose and N7-GA-Gua adduct formation (Davis and Recio, 2007).

In vivo mutagenicity and DNA damage in somatic cells.

In vivo Gene Mutation

43. The EU risk assessment report evaluated data from Mhyr *et al.* (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 examined for mutation frequency on days 3, 7 and 10 post dose. These studies are reported to be positive, although the EU risk assessment report concludes 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 was 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).

44. At the February 2008 meeting, PPG provided a summary table of 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; with all other tissues and sampling times giving no significant increase in mutation frequency. The mutations were not sequenced.

45. In a study by Manjanatha *et al.*, 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 ($p \leq 0.01$).

46. Mutation frequencies in the *cII* mutation assay were significantly increased ~2- and 2.5-fold in mice in the high dose of 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 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 ($p \geq 0.57$).

47. 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 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 suggest that this mutation is due to slippage of DNA polymerase; and highlight 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.

Conclusion: *In vivo* gene mutation experiments have given inconsistent results. Early studies by Mhyr *et al.* (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 was generally negative; finding significant increases in mutation frequency only in the liver at one sampling time point.

Big Blue Mouse studies 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 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.

In vivo chromosomal aberrations

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

49. 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 on 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 ip dosing at (80 and 120 mg/kg bw), which was statistically 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 generally 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 hyperploidy reported by Gassner and Adler (1996) supports a potential aneugenic effect.

In vivo micronucleus assays

50. The EU risk assessment report concluded that acrylamide produced MN in bone marrow and spleen in mice. A number of studies were identified in the systematic review that was presented to the Committee in February 2008. These were generally positive. Several studies investigated the dose response relationship.

51. 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 rat and mouse, glycidamide haemoglobin adducts were 3 to 6 times higher in mice than in rats. This indicates that rats have reduced metabolic conversion, which 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 was not clear, showing a non monotonic dose response where the low dose groups showed slight but significant ($p=0.001$) increases in micronuclei, with

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high dose group showing no significant increase above control. The authors suggest 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.

52. 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). PPG funded an additional micronucleus study, testing acrylamide at a range of doses (0.125 to 24 mg/kg bw/day, over 28 days), using flow cytometry (Davis and Reico, 2007). Whilst the frequency of micronuclei was 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 41, 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.

53. Manjanatha *et al.* (2007) found significant increases ($p \leq 0.05$) in the frequency of micronuclei 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 sub cutaneous administration of acrylamide or glycidamide (50 mg/kg bw), both resulting in significant increases in the 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 ($p \leq 0.01$).

54. Ghanayem *et al.* (2005a) 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 adducts formation, finding that levels were 52- to 66- fold lower in CYP2E1 null mice, compared to wild-type mice (paragraph 39).

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

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of the mechanism of action underlying the increase in frequency of micronuclei.

When evaluating the dose response studies by Abramsson-Zetterberg (2003), Paulsson *et al.* (2002), and Davis and Reico (2007); it is appropriate to apply most simple modelling of the dose-response data (i.e. linear) when other approaches give, at best, marginal improvements in dose-response analysis. It is possible that a NOEL could be determined from these studies but this would reflect the study design and sensitivity of the genotoxicity assay under consideration, rather than providing evidence of a biological threshold for genotoxic activity. Furthermore, *in vitro* experiments may not be the most sensitive genotoxic endpoint compared to chromosomal aberrations.

In vivo DNA damage assays

55. There were no DNA damage (comet) assays reported in the EU risk assessment report. Maniere *et al.* (2005) examined 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 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 rats acrylamide (15 mg/kg bw/day) 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 of were seen at all doses ($p \leq 0.01$).

56. Ghanayem *et al.* (2005) 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 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.* (2005) 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.

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In vivo DNA synthesis

57. 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 meosthelium, 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 studies 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. The study was predominantly undertaken to investigate mechanisms of carcinogenesis in rat target organs.

58. 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 data 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.

In vivo mutagenicity and DNA damage in germ cells

In vivo Germ cell Mutation assays

59. In the EU risk assessment report summarised two specific-locus assays where single i.p. doses of acrylamide (100 and 125 mg/kg bw) and 5 daily doses (50 mg/kg bw/day) resulted in significant increases in late spermatids and spermatozoa. No evidence for a germ cell gene mutations was found in a preliminary validation *LacZ* assay at 50 mg/kg bw/d for 5 days (i.p). Similarly, no effect was seen in seminiferous in Muta™Mouse studies where the 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 (study details not reported but tabulated by Lambert *et al.* 2005).

Conclusions: Details of *in vivo* germ cell mutation assays are limited. Specific-locus assays indicate a positive response. Although transgenic mouse studies do not indicate a mutagenic effect on germ cells, the relevance and validity of these findings are unclear. No germ cell mutagenicity studies specifically investigate glycidamide or acrylamide metabolism.

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In vivo Dominant lethal assays

60. 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. There are a number of studies either not reviewed in the EU risk assessment report or post dating the report which provide essentially consistent results with those reported in the EU risk assessment document. Positive dominant lethal assays were reported by Working *et al.* 1987 (in F344 rats), Dobrynska (1990) in Pzh:SFISS mice, Tyl and Friedman (2003) in F344 rats and Adler 2004 in 102/ElxC3H/Ei)F1 mice.

61. Generoso *et al.* (1996) tested glycidamide (125 mg/kg bw) as a follow-up study to one testing 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.

62. 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 pretreated 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.

63. Ghanayem *et al.* (2005b) 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 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 binding to nucleophilic sites in chromatin in early spermatozoa. The precise mechanism was not elucidated.

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Conclusions: A number of *in vivo* studies indicate that acrylamide can cause dominant lethality predominantly involve late spermatids/early spermatocytes. Glycidamide produces a similar dominant lethal effect. Chemical inhibition of cytochrome P450 mediated metabolism of acrylamide to glycidamide indicates that there may be a dominant lethal effect in early spermatozoa, although this may be due to incomplete inhibition of glycidamide formation. However, ablation of the CYP2E1 gene suggests that glycidamide is the ultimate germ cell mutagen.

In vivo Germ cell Heritable Translocation Assays

64. The EU risk assessment report summarised two positive heritable translocation assays. Shelby *et al* (1987) tested acrylamide (40 and 50 mg/kg bw/day i.p. over 5 days) in male mice, finding respectively 39% and 24% of male mice were sterile and heritable translocation carriers. A follow-up study by Generoso *et al.* (1996) tested glycidamide at 100 mg/kg bw a dose which resulted in ~50% dominant lethality. Cytogenetic analysis of clearly sterile males indicating a 20% translocation rate, compared to 0.06% historical control. The authors considered these studies were similar.

65. 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). 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.

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

In vivo Germ Cell Embryo Abnormalities

66. 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 dominant lethal assays but suggest a lower NOEL for i.p dosing.

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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, although the i.p. NOEL of 10 mg/kg bw/day would appear to be lower than in conventional dominant lethal studies. No similar studies specifically investigate glycidamide or acrylamide metabolism.

In vivo germ cell chromosomal aberrations

67. Studies summarised in the EU risk assessment report documented 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).

68. 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 to be 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 reported 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.

69. 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 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 pachytene spermatocytes. There was no evidence for an aneugenic effects following FISH analysis of sperm from mice dosed with acrylamide. No germ cell chromosomal aberrations studies specifically investigate glycidamide or acrylamide metabolism.

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In vivo Embryo Micronucleus

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

71. 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 cells for examination of morphology and micronucleus formation. FISH staining was performed to analyse centromere DNA content of MN. 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 with <10 cells (cf 65% of embryos had 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 MN were increased in treated embryos. The authors suggested both clastogenic and aneugenic mechanisms of action were involved in the observed response.

Conclusions: Acrylamide can cause germ cell micronuclei to form *in vivo*. The presence of both centromere positive and negative micronuclei indicate that acrylamide has a both a clastogenic and aneugenic effect on germ cells. This is in contrast to the lack of an aneugenic effect in germ cell chromosomal aberrations assays. No germ cell mutagenicity studies specifically investigate glycidamide or acrylamide metabolism.

In vivo Germ Cell Unscheduled DNA Synthesis (UDS)

72. 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 UDS assays indicate both acrylamide and glycidamide are able to induce DNA synthesis, particularly in early spermatids.

Germ Cell DNA damage assays

73. 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

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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. No germ cell mutagenicity studies specifically investigate glycidamide or acrylamide metabolism.

Other in vivo germ cell assays

74. Gassner *et al.* (1995) administered acrylamide (80 or 120 mg/kg bw; i.p.) to male mice. Microscopic examination 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

75. Interpretation of the genotoxicity studies testing acrylamide is confounded by the multiple potential mechanisms that may cause genetic damage, represented in Figure 1. Interpretation of the genotoxicity profile is further confounded by the possibility that various genotoxic endpoints, such as micronucleus formation or chromosomal aberrations, may have differing sensitivity to the genotoxic effects mediated by each mechanism. Also, the difference between metabolic competencies of various test systems adds additional inconsistencies. For example, effects related to glycidamide formation would be unlikely to be observed in a cell line which lacks metabolic competency, leaving only acrylamide mediate genotoxic effects to be observed. Metabolism dependent pathways are coloured grey in Figure 1

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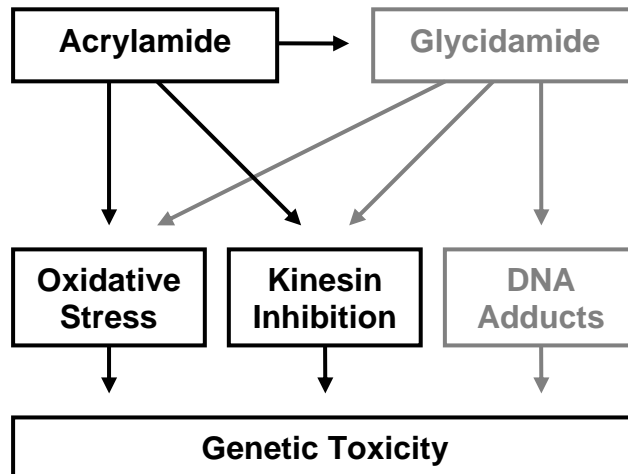


Figure 1: A diagram representing the complex interplay between the various potential mechanisms of acrylamide genotoxicity in somatic cells.

Oxidative Stress

76. 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 sulphhydryl groups and other biochemical changes indicative of oxidative stress which were also significant and dose-related. The doses used in this study were generally lower than those used in many of the *in vivo* genotoxicity studies assessed in this review.

77. 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.

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Kinesin Inhibition & Specific Protein Binding

78. Specific enzyme inhibition by acrylamide has also been proposed as a possible mechanism that could result in genetic damage. Kinesins are thought to regulate spindle dynamics during anaphase. Sickles *et al.* (2007) showed that acrylamide and glycidamide could significantly inhibit two bacterially expressed recombinant kinesins (KIFC4A, a motile kinesin; and KRP2, a depolymerising kinesin with an internal binding domain). Both chemicals were equipotent in reducing the KIFC4A 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.

79. As with the oxidative stress mechanism, the available evidence suggests that kinesin inhibition is not the only genotoxic mechanism resulting from acrylamide exposure. Therefore, as with oxidative stress, it would be inappropriate for the risk assessment to be solely based on kinesin inhibition. In addition, any consideration of a kinesin based mechanism would need to explore the redundancy of the binding site(s) on kinesin, the effects of partial inhibition of the total kinesin pool, and do determine a level of kinesin inhibition below which perturbations to the mitotic/meiotic spindle would not be expected.

80. Protein binding is also implicated in germ cell mutagenicity. Data from Segal *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 sperm DNA during mid to late spermatid stages. DNA damage studies reviewed in the EU Risk Assessment Report find increases in DNA damage in spermatids.

Metabolism of Acrylamide to Glycidamide

81. 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.

82. Oxidative metabolism of acrylamide to glycidamide is widely accepted to be mediated by CYP2E1; however, Ghanayem *et al.*, (2005) 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

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other mechanisms. It is unclear if this occurs in wild-type mice, or whether this is an adaptive change in the knock-out mouse.

Glycidamide DNA Adduct Formation

83. 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).

84. Evidence from methyl analogues (Koskinen *et al.*, 2000) has lead some authors to question the miscoding potential of the N7-GA-Gua and N3-GA-Ade. However, *in vitro* work by Gamboa da Costa *et al.* (2003) identified an N1-GA-dA adduct, which forms in the base pairing region of adenine and therefore has mis-coding potential. This adduct forms at a ~5-fold lower level than N7-GA-Gua when DNA is treated *in vitro* but could not be detected 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.

85. 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. The mutation spectra induced by acrylamide and glycidamide *in vivo* closely resembled that of glycidamide *in vitro*. Increased G → T and G → G transversions, and A → G transitions seen in these studies are consistent with the miscoding potential of the N7-GA-Gua, N3-GA-Ade and N1-GA-dA adducts.

86. Davis and Recio (2007) have demonstrated a linear relationship between dose and liver N7-GA-Gua DNA adduct levels, with statistically significant ($p < 0.001$) increases 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 occur as a result of DNA adducts, these data would not be consistent with a threshold effect.

Overall Conclusions: 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 DNA acrylamide related adducts and gene mutations, which are detectable in cultured mammalian cells and

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somatic cells *in vivo*. These effects appear to be mainly mediated by metabolism to glycidamide.

Assessment of the genotoxic potential of acrylamide is confounded by multiple potential mechanisms, which include 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; yet, these mechanisms are not mutually exclusive.

The default approach recommended by COM is to assume no threshold for *in vivo* gene mutational effects unless compound specific data can be provided to support such a mechanism. In order to move away from the default assumption that there is no safe dose for this genotoxic carcinogen, it will be necessary to identify robust thresholds for all of the genotoxic mechanisms of acrylamide.

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