Comments for submission the EPA-SAB concerning the draft document:

Toxicological Review of Acrylamide (CAS No. 79-06-1) In Support of Summary Information on the Integrated Risk Information System (IRIS)

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and the SAB Panel discussions during its meeting on March 10-11, 2008

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I have been retained by the North American Polyacrylamide Producers Association (NAPPA) to review the draft IRIS document with respect to its sections on mutagenicity, and make a presentation to the SAB at its March 10-12, 2008 meeting, regarding deficiencies or inconsistencies in the draft. In addition, I was asked to comment on the discussions and conclusions reached during the SAB proceedings, if warranted.

Acrylamide's and glycidamide's genotoxicity *in vitro* and *in vivo* is not in question. What is in question, and needs consideration in the IRIS document, is the interpretation of the data. My comments address the completeness and the accuracy of the interpretation of the data. This IRIS document is recognized by the EPA as being of high importance, as evidenced by the Agency's statements and the use of a special SAB Panel to evaluate the draft document. Therefore, every effort should be made to incorporate and address all the relevant publications. Some of these publications appeared after the draft document was prepared, but others have been available for a number of years; my reference list indicates which of my referenced articles have not been included in this draft document.

Brief summary of comments

The main points in of my comments are summarized here:

- The draft document does not given equal consideration to all relevant modes of action, as noted in the EPA draft *Framework for Determining a Mutagenic Mode of Action for Carcinogenicity: Using EPA's 2005 Cancer Guidelines and Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens*. For example, although the MOA related to glycidamide alkylation of DNA is discussed, there is no discussion of the data supporting induction of oxidative stress, and the interference with mitosis and meiosis, leading to aneuploidy as possible MOAs. There also are studies available to support the interpretation that the genotoxic effects of acrylamide and glycidamide may not be a consequence of the alkylation of DNA by glycidamide.

- There are a number of relevant published articles, dating back to 1995, and including articles published after the writing of this document, that are directly relevant to the genotoxic, and possibly carcinogenic, mode of action of acrylamide, that have not been addressed in the draft document.

- Acrylamide is a weak genotoxin in mouse somatic cells, and mouse and rat germinal cells. It is not genotoxic in rat somatic cells although the available carcinogenicity data is all from rat studies.

- Although the metabolite, glycidamide, forms DNA adducts, the WOE of the genetic toxicity data support the conclusion that the genetic effects seen can be explained by effects other than a direct genotoxic mechanism, specifically through interference with the mitotic and meiotic apparatus, through induction of an oxidative stress response and/or through protamine alkylation and disruption.

- The criticism of the dose-response analyses of the in vivo genetic toxicity of acrylamide by Allen et al. (2005) is inaccurate, and contradicted by the IRIS document itself and by comments made by the SAB Panel members. The Categorical Regression method used by Allen et al. was developed by the EPA to allow a synthesis of test data across related endpoints in situations where there are multiple studies on a chemical, and to bring a mathematical analysis to bear on

the dose-response issue. The doubling-dose analysis suggested by Dearfield (1995) may not be appropriate for analysis of chemical risk because it was originally developed for radiation risk assessment and presumes a linear relationship, whereas the Categorical Regression does not necessarily assume linearity. As was noted at the SAB meeting, the doubling dose analysis has not previously been used by EPA for chemical risk assessment.

- It is recognized that the various mutagenic modes of action discussed here are not mutually exclusive, and that acrylamide may act by different modes of action in different organs and cell types.

These points are expanded upon in the following pages.

1. The draft document does not given equal consideration to all relevant modes of action in addition to the genotoxic mode, as is recommended in the EPA's draft Framework for Determining a Mutagenic Mode of Action for Carcinogenicity (Sept. 2007).

The EPA draft *Framework for Determining a Mutagenic Mode of Action for Carcinogenicity: Using EPA's 2005 Cancer Guidelines and Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens* [EPA 120/R-07/002-A] states, (in §2.4.3.3) among other similar statements, "If there is evidence for more than one mode of action, each should receive a separate analysis."

The draft IRIS document analyzes only a possible genotoxic mode of action and does not address other possible modes other than a brief mention of aneuploidy, although the WOE supports the interpretation that the genetic damage seen in vitro and in vivo is a consequence of oxidative stress and/or interference with mitosis and meiosis. These alternate MOAs are supported by a number of publications that appeared before and after the draft IRIS document was prepared, and are critical to the interpretation of the genetic effects of acrylamide and glycidamide.

1a. Induction of oxidative stress

A number of recent in vitro studies have shown that the mutants isolated after treatment with acrylamide or glycidamide are primarily caused by loss of heterozygosity (LOH) and tend to be the effect of chromosome deletions (Koyama et al., 2006; Jiang et al., 2007; Mei et al., 2008). These deletions have been attributed by some of the authors to be the consequence of oxidative stress, i.e., increased levels of reactive oxygen species (ROS) and depletion of glutathione which is responsible for detoxification of active oxygen species.

An in vitro comet assay study (Thielen et al., 2006) using glycidamide in human lymphocytes was essentially negative unless the cells were treated with formamidopyrimidine-DNA-glycosylase (FPG) which recognizes 8-OHdG sites produced by oxidative damage and converts them to DNA strand breaks, detectable by the assay. The concentration and time-course of the induction of the oxidative damage sites paralleled the induction of *Hprt* mutants and shows that the oxidative damage sites may be more important than the guanine alkylation sites for the genetic effects. Similar weak comet assay responses were seen in some tissues of mice treated with acrylamide (Maniere et al., 2005).

Two recent studies on the induction of gene expression by AA and GA provides molecular-level support for the induction of ROS as the primary step leading to the genetic damage

(Clement et al., 2007; Hasegawa et al., 2008). In both human MCF7 cells and *C. elegans*, the principal increases in gene expression were genes associated with detoxification enzymes, including those in the glutathione and glutathione-*S*-transferase pathways, which are associated with oxidative damage. In the Clement study, *Mus81/Eme1*, which has been implicated in the repair of interstrand crosslinks by homologous recombination, was up-regulated (personal communication), but the *GADD45* gene, considered to be a first responder to DNA damage, was down-regulated. No DNA repair or cell cycle control genes were identified in the Hasegawa publication. The lack of response by the DNA damage-associated genes, and the dramatic increases in oxidative stress/damage-related genes suggests that alkylation of DNA by glycidamide may not be responsible for the subsequent genetic damage seen in the cells.

The studies in vitro and in vivo which have been interpreted as the basis of implicating DNA adducts in the genotoxic effects of acrylamide (Manjanatha et al., 2006; Besartinia and Pfeifer, 2003, 2004) are more consistent with oxidative stress than DNA adducts. The DNA adducts are not in the base pairing region, Oxidative stress produces the mutants observed in the afore mentioned studies.

1b. Interference with mitosis and meiosis

Both acrylamide and glycidamide interfere with microtubule motility (and therefore chromosome migration) and disassembly (required for motility) in vitro (Sickles et al., 1995, 1996, 2007). Acrylamide also induces increases in aneuploidy and polyploidy in mouse bone marrow cells (Gassner and Adler, 1996; Schriever-Schwemmer et al., 1997; Shirashi, 1978), and mitotic spindle damage in splenic cells (Backer et al., 1989). In male mouse germ cells it induces meiotic delay, and increases in aneuploidy and hypoploidy (Shirashi, 1978; Collins et al., 1992; Adler et al., 1993; Gassner and Adler, 1996).

2. A number of the responses being interpreted here as evidence for gene (point) mutations can be equally well interpreted as evidence for gross chromosome damage.

These include in vitro mammalian cell mutagenicity, in vivo mutations in the BigBlue transgenic mouse, and male germ cell specific locus mutations.

Mei et al. (2008) analyzed the mutations induced in mouse lymphoma L5178Y cells by acrylamide and glycidamide and found that 94% of the large colony mutants (which are considered to reflect gene mutations) and 100% of the small colony mutants (which are considered to reflect chromosome mutations) are deletions. Similarly, Koyama et al. (2006) showed that the majority of acrylamide-induced mutants in human lymphoblastoid TK6 cells were deletions, although the majority of glycidamide-induced mutants were not.

In the Manjanatha et al. (2006) BigBlue mouse study, the majority of the *cII* mutants induced by acrylamide were at a –GGGGGG– reversion hotspot in the gene and, without this hotspot, the results would not have been considered positive. *Hprt* mutations were induced in lymphocytes in the same experiment. Without further analysis, it is not possible to determine if these mutants were from point mutations or deletions, because, unlike the small *cII* locus which will does not allow recovery of most deletion mutants, the large *Hprt* locus allows the recovery of both deletion mutants and point mutations.

It should be noted that in this study, acrylamide was not mutagenic at the *cII* locus in vivo at the low dose (19 and 25 mg/kg/d for males and females, respectively) and the increase seen using

the hotspot mutations was only present at the high dose (98 and 107 mg/kg/d for males and females, respectively). This high dose was neurotoxic to the animals resulting in premature termination of the study. Similar neurotoxicity was not seen in animals exposed to glycidamide (88 and 111 mg/kg/d for males and females, respectively). The low GA dose of 25 mg/kg/d was not mutagenic at the *cII* locus in the males, but the low dose of 35 mg/kg/d induced a small, but nonsignificant increase in the females.

3. The relevance of glycidamide-guanine DNA adducts for acrylamide's genetic toxicity (and carcinogenesis)

During the discussion the afternoon of the second day of the SAB meeting, in response to a question from the SAB Panel as to the relevance of the GA-guanine DNA adducts for mutagenicity/carcinogenicity, Dr. Doerge responded that the NCTR's BigBlue mouse study (Manjanatha et al., 2005) showed a shift to GC→TA transition mutations in the *cII* gene, and that this shift was evidence of the GA-guanine adduct's relevance. However, in the analysis of the spectrum of responses in the BigBlue mouse study it is evident that if the frameshift mutations at the hotspot are removed, there was a shift from GC→AT transitions to GC→TA transversions, but there was no increase in total mutations induced by AA. It is not clear in the GA-treated mice if the mutagenic would have been judged significant when the hotspot mutations were removed from the computations. A similar shift from GC→AT transitions to GC→TA transversions was obtained with GA. Given the evidence for induction of oxidative stress by these chemicals, it is also possible that guanine-centered genetic effects could be mediated by 8-OH-dG, a well-characterized pre-mutagenic adduct.

In vitro studies with BigBlue mouse embryonic fibroblasts treated with acrylamide produced a doubling of the percent G>C transversions (from a control value of approx. 6%), considered by the authors to be a weak response, but a 16% decrease in G>A transitions (from a control value of approx. 37%) (Besaratinia and Pfeifer, 2003). None of the individual transitions or transversions was statistically significant. In a parallel study with glycidamide that used the same control value as the acrylamide study (Besaratinia and Pfeifer, 2004), there was less than a doubling of G>C transversions, but a statistically significant 2.9-fold increase in G>T transversions (from a control value of approx. 12%), and a non-significant 30% decrease in G>A transitions (from a control value of approx. 37%). [It should be noted that the values from the two Besaratinia and Pfeifer publications are approximations from bar graphs because the actual values were not provided in the text.]

It is recognized that some of the data from the above systems also support a gene mutation response, and that some have not been adequately analyzed to determine the nature of the mutation, although it is not known without further analyses, which of the mutants, if any, resulted from glycidamide adducts and which resulted from oxidative-damage adducts. Recent studies of gene expression changes induced by glycidamide in human cells in vitro (Clement et al., 2007), and gene expression changes induced by acrylamide in *C. elegans* (Hasegawa et al., 2008). The induced gene families were primarily associated with oxidative stress responses. These results suggest that alkylation of DNA by glycidamide may not be responsible for the subsequent genetic damage seen in the cells.

Other evidence also suggests that the GA-guanine adducts may be 'disconnected' from the genetic damage seen in vivo. For example, Maniere et al. (2005) noted that "the in vivo organ-specific damaging effect of acrylamide, as determined by the comet assay, was not concordant with the extent of glycidamide-DNA binding in the corresponding tissues."

In Sega's studies of alkylation in male germ cells by acrylamide (Sega et al., 1989) the authors concluded that "essentially all" of the sperm alkylation was protamine, and not DNA, alkylation. Dearfield et al. (1995) noted that "the levels of [] radioactivity bound to DNA did not change with time, while alkylation of protamines peaked (>10-fold increase) during the second week after treatment with acrylamide, which coincides with exposure of late spermatids and early spermatozoa." Dearfield further noted that the spermiogenic pattern of protamine alkylation corresponds with the sensitive sperm stages for the induction of dominant lethal effects, heritable translocations, specific locus mutations, and DNA strand breakage.

4. The increased heritable translocation frequency in male mouse germ cells and its implications for heritable risk assessment

There was concern expressed by the Panel that a heritable translocation test (Shelby et al, 1987), which reported an induced frequency of 24% at the low dose of 40 mg/kg/d for 5 days suggested a linear extrapolation to very low doses. The report by Adler et al. (1994) should also have been considered in this same context. The Shelby and Adler data sets (Shelby et al., 1987; Adler et al., 1994) are summarized in the following Table.

Summation of heritable translocation studies

Study	Daily dose	No. days	Cumulative dose	Translocation frequency
Shelby et al., 1987	0	–	0	0.2%* (1/8,095)
	40 mg/kg	5	200 mg/kg	24%
	50 mg/kg	5	250 mg/kg	39%
Adler et al., 1994	0	–	0	0.04%* (3/8,700)
	50 mg/kg	1	50 mg/kg	0.6%
	100 mg/kg	1	100 mg/kg	2.7%
	50 mg/kg	5	250 mg/kg	21.9%

* historical control value

When the Adler et al. fitted their data to a Weibull distribution, a non-linear distribution was obtained. Adler then used this distribution to estimate a doubling dose of approx. 25 mg/kg. This value can be compared to the 0.39 mg/kg value calculated from the 5x50 mg/kg dose by Dearfield in the Adler experiments in **§ 5.5.1** of the draft IRIS document. The difference between 0.39 and 25 mg/kg reflects the difference between assuming a linear (Dearfield) and non-linear extrapolation (Adler). In the IRIS document's description of the Adler result (p. 219, last sentence of the 1st paragraph), there should have been some mention that Adler's Weibull calculation showed that the dose response between 0 and 250 mg/kg was non-linear. Additional support for non-linearity of the heritable translocation response is the need for two independent chromosome breaks to produce the two chromosome ends that will eventually (and stochastically) rejoin to form the translocation, i.e., it is a 2-hit phenomenon. This aspect of the assay was addressed by Adler et al., 1994.

In addition to this additional value, the EPA should also attempt to calculate a doubling dose or risk factor based on the BMD_{10} values of 29 and 30 mg/kg for mice and rats, respectively, obtained by Allen et al. (2005) using the categorical regression integration of all the germ cell data.

5. The Table of Risk Extrapolation Factor (REF) scores is unsupported and not relevant

The REF score Table (**§ 5.5.1, p. 217**) was introduced in a series of publications in 1992 (ICPEMC, 1992). It was originally presented by Dearfield et al. (1995) who described them as "risk extrapolation factors … between rodent … experimental models and humans …." Its presence in the IRIS document is described as being for the same purpose. The Table's values are based on a not-well-supported, and somewhat arcane, characterization of different genetic toxicity tests and endpoints that is not adequately justified in the publication, and has not been used or examined since. There is no indication, either in the Dearfield et al., 1995 publication, nor in the IRIS document, as to how the REF values were derived or how they are to be used. However, the IRIS document notes the "considerable uncertainty" associated with the REFs and does not appear to use them for subsequent calculations.

The REF Table, and its associated discussion, should be removed from the document for the above reasons.

6. The dismissal of the analyses presented in Allen et al. (2005) is misplaced and inaccurate.

Many of the "serious (if not fatal) flaws and assumptions" [**§4.8.3.1**. *Hypothesized Mode of Action—Mutagenicity* (at pg. 151)] attributed to the Allen et al. (2005) categorical regression analyses of in vivo genetic toxicity data, are also used by the EPA in their analyses.

Briefly, Allen et al. used the EPA's Categorical Regression procedures to synthesize the results of all the in vivo chromosome damage and mutation data, rather than rely on only a single test or endpoint. The developed values were then used to calculate BMD10 values for the somatic cell effects reported in mice (all the somatic cell rat studies were negative) and separately for the germ cell effects in rats and in mice.

Some of EPAs criticisms of this study are addressed briefly, below.

- Allen et al. "… [assumed that] it is acceptable to apply a benchmark response of 10% [i.e., a BMD_{10}] to mutagenic events assumed to lead to tumor formation when the generally accepted "minimal" risk level for carcinogenicity is 0.0001% …."

Response:

It should be noted that **§5.4.3** [*Cancer Assessment*] of the draft IRIS document calculates and uses BMD_{10} and BMD_{20} values as starting values for estimating cancer risks. The Allen et al. study calculated the BMD_{10} values for somatic and germ cell effects but did not attempt to do risk assessment using these values. They represented only a synthesis of the in vivo data using accepted EPA procedures.

The BMD_{10} values for male germ cell effects were calculated by species and represented only a synthesis of the responses seen in the male germ cells. These values can be used in the IRIS estimates of germ cell risk [in addition to the doubling dose approach]

- "comparing results in mice with results in rats"

Response:

It is unclear why this is considered a failing of the Allen et al. study. Germ cell studies were available for both species, although the rat studies were primarily chromosome aberration studies. When the two species were analyzed independently the resulting BMD_{10} values were very similar

even though there were no reciprocal translocation or specific locus studies performed in the rats and glycidamide is produced at a substantially greater rate in rats than in mice. Such comparative analyses are vital for assessing inter-species variability.

- "assuming low-dose response relationships based on extrapolations from very high doses, and limited sample sizes"

Response:

This is the traditional approach for using rodent cancer and genetic toxicity data. The EPA in its analyses using the Dearfield table, used the same studies and data as were used in the Allen et al. report. This approach to the use of genetic toxicity data (and much other non-cancer toxicology data) is typical because it is rare to find chronic genetox studies. Allen et al. did not make assumptions about the low-dose response relationships; they simply plotted the data and commented on the potential confounding aspects of using short term exposures to model or assess chronic risk and addresses this concern.

- "disregarding the one hit, one tumor hypothesis."

Response:

The Allen et al. report developed BMD_{10} values for mouse somatic cell genetic toxicity, and separately for mouse and rat germ cell genetic toxicity. The somatic cell value is compared to the doses producing rodent cancer, but does not address the hits-per-tumor issue. Although, as the IRIS document notes, one-hit-one-tumor is a hypothesis, Dr. Jerry Rice, one of the consultants to the SAB was very emphatic in his comments on the second day of the meeting when he explicitly rejected this hypothesis, with a rare exception specifically in the case of retinoblastoma. He noted that tumor induction requires a minimum of two hits.

Traditionally, experimental data and their analyses are used to test, and drive, the hypothesis; the hypothesis is not supposed to dictate the interpretation of the data. It appears as if the Allen et al. approach to the data analyses, and their conclusions, are being criticized because they are not based on the one-hit/one-tumor model, or any other specific model, but, instead, trying to see where the data lead them.

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(* marked articles are not included in the draft IRIS document)

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