NORTH AMERICAN POLYELECTROLYTE PRODUCERS ASSOCIATION

ATTACHMENTS TO

Comments on December 2007 US EPA External Review Draft
Toxicological Review of Acrylamide (CAS No. 79-06-1)
In Support of Summary Information on the Integrated Risk Information System

April 11, 2008

- April 1, 2008 statements from
    - Dr. Ernest Eugene McConnell
    - Dr. Annette Shipp
    - Dr. Errol Zeiger
- Costa, L. and Calleman, C. (1994). Determination of Hemoglobin Adducts Following Acrylamide Exposure. EPA/600/R-93/226. February 1994.
- Draft excerpts from a manuscript on kinesin activity
- Marsh, G. *et al.* (2007). Mortality Patterns Among Workers Exposed to Acrylamide: Updated Follow Up. *Journal of Occupational and Environment Medicine*, 49(1): 82-95.
- Wall, H. (2005). Acrylamide: A Two-Year Drinking Water Chronic Toxicity-Oncogenicity Study in Fischer 344 Rats – A Histopathology Peer Review and Pathology Working Group Review of Proliferative Lesions Involving the Mesothelial Lining Cells of the Tunica Vaginalis of the Testes in a Two-Year Drinking Water Chronic Toxicity-Oncogenicity Study in Fischer 344 Rats with Acrylamide – PWG Report. Unpublished Study by Experimental Pathology Laboratories, Inc. for SNF SAS and CIBA Specialty Chemicals. EPL Project No. 750-001. March 1, 2005.

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Mr. Robert Fensterheim
Executive Director
North American Polyelectrolyte Producers Association

I am writing in response to your request for me to comment on specific issues arising from the discussion by the EPA Science Advisory Board of the document titled "Toxicological Review of Acrylamide."

I am a board certified pathologist (ACVP) and toxicologist (ABT) with over 30 years experience in the design, conduct and interpretation of rodent bioassays. Over that period I have participated in more than 200 cancer bioassays and examined the histopathological slides from most of them. I was Chief of the Pathology Branch at the National Toxicology Program which was responsible for assuring the diagnoses from the NTP bioassays were accurate. Subsequently, I was the Director of the Toxicology Research and Testing Program of the NTP where I directed all aspects of their studies. Since leaving the NTP, I have continued to participate in the design and interpretation of numerous bioassays and act as a reviewer of the histopathologic slides from them. This experience has provided me with an insight into what these bioassays really mean and their limitations. It is in light of this experience that I offer my opinion on the following points:

1. Combining of tumor types.

2. The progression of adenomas to carcinomas.

3. What the pathology of acrylamide tells us about its mode of action.

1. Combining of tumor types.

The basis for the below comments and conclusions is derived from the National Toxicology Program's (NTP) procedures for combining tumors for determining a treatment-related effect in rodent bioassays and are described in detail in the paper by McConnell et al. (1986). This paper describes the rationale for why combining certain tumors together for statistical purposes is scientifically based and provides guidance for what should and should not be combined.

McConnell, E.E., Solleveld, H.A. and Boorman, G.A.: Guidelines for Combining Neoplasms as Evidence of Carcinogenicity in Long-term Toxicity and Carcinogenesis Studies. J. Natl. Cancer Institute 76(2):283-289, 1986.

For brevity, the following comments are restricted to the acrylamide (AA) studies of Johnson, *et al.*, 1986 and *Friedman et al.*, 1995.

1.1. Appropriateness of Combining Brain Tumors

The basic question in the IRIS document with regard to the brain tumors reported in the Friedman study is whether tumors referred to as "malignant reticulosis" should or should not be combined with glial cell tumors. The guidelines described by McConnell *et al*. (1986) and current practice by the NTP *clearly* suggest that they should not be combined to determine a treatment-related increase in brain tumors. The reason for this is that they arise from distinctly different stem cells. There are three types of gliomas that typically arise in the brains of rats, i.e. astrocytoma, oligodengrolioma and mixed gliomas. These glial cell tumors all arise from the same stem cell that is derived from the neuroectoderm and, therefore, it is appropriate to combine these tumors. In contrast, the stem cell for malignant reticulosis is entirely different, *i.e*. these tumors arise from the reticuloendothelial system, and are more in harmony with lymphomas and lymphosarcomas. In fact, the term reticulosis is no longer used for these lesions. They are more correctly termed "histiocytic sarcomas." Therefore, because of their fundamental difference in histomorphogenesis, they should not be combined with glial cell tumors.

Solleveld, H.A. and Boorman, G.A. 11. Brain. *In* Pathology of the Fischer Rat. Boorman, G.A. *et al*., Academic Press, 1990.

Solleveld, H.Y. and Zurcher, C. Neoplasms of the Central Nervous System. *In* Pathology of the Aging Rat, Vol 2. ILSI Press, 1994.

1.2. Appropriateness of Combining Mammary Tumors

The basic question in the IRIS document in regard to combining mammary tumors is whether it is appropriate to combine adenomas with fibroadenomas. At the time of the writing of the guidelines (McConnell *et al*., 1986), it was suggested that it was appropriate to combine adenomas with fibroadenomas because they were thought to arise from the same stem cell, *i.e.*, glandular epithelium. However, since that time it has been shown that these tumors are *clearly* different in their histomorphogenesis. The fibroadenomas arise from the ductal epithelium and are comprised primarily of hyperplastic fibrous connective tissue, have only small portions of a tubular (epithelial) component and rarely become malignant. In contrast, the adenomas arise from the acinar portion of the mammary gland, are composed primarily of epithelial cells and have the potential to progress to adenocarcinomas. This is why the current NTP policy is not to combine adenomas and fibroadenomas for determining a treatment-related effect.

2. Do Adenomas Progress to Carcinomas?

The basic premise behind the guidelines for combining tumors (McConnell *et. al*., 1986 and NTP convention) is that most adenomas have the ability to progress to adenocarcinomas. However, because they have the potential to progress does not mean that they will progress. Adenomas certainly have a different biological potential being classified as benign and why they are viewed much differently in the clinical setting with regard to surgical, etc. intervention. That is also why the NTP continues to differentiate benign from malignant tumors. The scientific basis for differentiating benign from malignant tumors is because it is generally recognized that carcinogenesis is a multistep process requiring a series of sequential mutational events. It is also well known that benign tumors can revert to normal tissue if the stimulus (chemical) for their induction is removed. As pointed out in the Guidelines for Combining Neoplasms (McConnell *et. al*., 1986) “Thus differentiating benign and malignant neoplasms may provide information relevant to the mechanistic action of the chemical.”

3. What the pathology of acrylamide tells us about its mode of action.

It is my opinion that the tumor data on acrylamide (AA) are more in line with a hormonal mode of action (MOA) for the induction of the tumors arising in the mammary gland, thyroid gland and tunica vaginalis testes rather than a direct genotoxic event as suggested in the IRIS document. My reasons for this opinion are as follows:

Although noted in the pathology tables reporting the tumors in the Johnson *et al.* (1986) and Friedman *et al*. (1995), the IRIS document does not give the proper weight or emphasis to the biological potential of the tumors of either the thyroid or mammary gland. Insight into the potential MOA can be learned by examining the pattern of the tumor response. As noted in both the studies, the tumor response was only statistically significant with regard to benign neoplasms of the thyroid (adenomas – both sexes) and mammary gland (fibroadenomas – females only). I find this very important data because, for me, it sheds light on the probable MOA. Although I consider all three types of tumors related in terms of a MOA, I will speak to mesothelial tumors of the tunica vaginalis testes (TVM) later in this document.

First, while I'm an advocate of combining benign and malignant tumors of the same tissue type for determining the carcinogenic activity of a material and have published the seminal paper on the subject (McConnell *et. al*., 1986), there is also a great deal more to this exercise than just combining the two morphological types. For example, in my experience most xenobiotics that appear to cause a neoplastic response in rodent models *via* a genotoxic MOA typically result in a malignant tumor response, often at the expense of the benign tumors. In fact, it is more common than not for the primary tumor response with genotoxic chemicals to be *primarily* malignant. In contrast, xenobiotics that cause neoplasia *via* an epigenetic mechanism, *e.g.*, perturbation of a hormonal pathway, irritation, etc., often cause a primary increase in benign tumors, although an increase in malignant tumors is possible depending on the potency of the agent.

Second, the organ distribution of the tumors can be important for understanding a potential MOA. Xenobiotic agents that cause neoplasia *via* a genotoxic MOA typically cause tumors at the site of highest concentration of the parent compound or the site of metabolism to the "active" metabolite, *e.g*., liver, or in an organ with a high affinity for the chemical or its metabolites, *e.g*., bone marrow, kidney, etc., or organs with active cell replication, *e.g*., liver, bone marrow,

intestine, etc. Chemicals that cause tumors *via* a hormonal MOA typically cause tumors in endocrine organs or organs directly influenced by hormones. Therefore, in the case of AA, the organ distribution of tumors in the thyroid and mammary gland would argue more for a hormonal rather than a genotoxic MOA. Also, if genotoxicity were the primary MOA one would expect tumors, especially of a malignant nature, in the liver because this is the site of primary metabolism and possibly other non-endocrine organs.

Finally, if the MOA was based on genotoxicity one would expect to see non-neoplastic "toxic" lesions, *e.g.*, liver necrosis, inflammation, etc. In addition, if AA caused tumors *via* a genotoxic MOA one would also expect to find an increase in tumors of the mammary gland of male rats, which was not the case. At a minimum, I think there would be dose-related pathology in the male mammary gland, which again was not the case.

At first glance, the mesothelial tumors of the tunica vaginalis testes (TVA) would appear not to fit into the hormonal MOA paradigm. However, there are two arguments that suggest that this is not the case. First, in my opinion there is nothing unique about the mesothelium that lines the testes or scrotum and that of the peritoneal cavity. The only unique feature is its location. The mesothelium is morphologically essentially the same as that which lines the rest of the abdominal and thoracic cavities. If the MOA for the TVMs were a genotoxic event, why would the TVMs only start in this location? If however, the MOA was hormonal, one could envision higher levels of the inciting hormone in the tunica vaginalis merely by its being in a microenvironment adjacent to the testes which often contain Leydig (interstitial) cell tumors in this strain (Fischer 344) of rat, *i.e.*, 90+%. It is known that these Leydig cell tumors are hormonally active, so local hormone levels could be higher than in the general circulation. In fact, the analysis by Iatropolis *et al*. (1998) that compared the amount of the testicle occupied by Leydig cell tumors to the development of TVMs shows a fairly strong correlation with the presence of the TVMs. A more in-depth discussion of this hypothesis can be found in the report of a Pathology Working Group (PWG) on the TVMs from the Friedman study, which I understand was earlier submitted to the Agency.

Iatropolis, M.J., *et al*. Microscopic evaluation of proliferative mesothelial lesions diagnosed previously as mesotheliomas of the tunica vaginalis testes. Unpublished report to Cytec Industries, October 14, 1998.

I would like to offer a second possible MOA for the TVMs that also deserves pointing out in the IRIS document. As pointed out by Iatropolis, there is a fairly strong correlation between the size of the Leydig cell tumors and the presence of TVMs. With this in mind I propose a further hypothesis for the restricted location of these mesotheliomas (TVMs). The scrotum of the rat "tightly" encloses the testes; is not pendulous like in higher developed species. Therefore, any enlargement of the testes results in an increase in testicular pressure on adjacent tissues. That being the case, the findings of Iatropolis suggest that it is possible that the testes bearing Leydig cell tumors are overall enlarged, and therefore cause increased pressure on the epididymus and its mesothelial covering. This pressure would in turn cause physical irritation of the adjacent mesothelium resulting in mesothelial hyperplasia and eventually TVMs. This is further supported by the fact that the Fischer 344 rat has both the highest incidence of both Leydig cell tumors and TVMs of any of the commonly used strains of rats. It is my understanding that this potential MOA was also pointed out in the same PWG report.

In summary, I believe the organ distribution and benign nature of the tumors found in the Johnson *et al.* (1986), and Friedman *et al.* (1995) oral acrylamide rat studies do not support a genotoxic MOA. I further propose that a hormonal MOA better fits the data, especially for the mammary gland and thyroid gland. This may also apply to the TVMs, but a further MOA such as physical irritation from an enlarged tumor-bearing testicle is also plausible.

Thank you for the opportunity to provide these opinions.

Sincerely,

Ernest (Gene) McConnell

Acrylamide: A Direct Acting Mutagen or Enhancer of Age-Related Tumors in Fischer 344 Rats?

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CHARGE QUESTION 20:

Are there other MOAs that should be considered? Is there significant biological support for alternative MOAs for tumor formation, or alternative MOAs to be considered to occur in conjunction with a mutagenic MOA? Please specifically comment on the support for hormone pathway disruption. Are data available on alternate MOAs sufficient to quantitate a dose-response relationship?

ANSWER:

While the USEPA attempted to follow the Human Relevance Framework (HRF), the application of that framework was incomplete and inadequate and, further, did not apply the same standard of evidence to the mutagenic mode of action (MOA) that was applied to the non-genotoxic MOAs. The following provides the basis for this answer.

I. Application of the First Step in the Human Relevance Framework (HRF): Is the weight of evidence sufficient to establish the MOA in animals?

The first step in the HRF may be the most important from a regulatory perspective. This initial step directs an investigator to identify the key events in the animal model, i.e., biological, cellular, or molecular, *by which a chemical* could cause the tumors noted. The

method by which this step is implemented is not explicitly defined but implicit in that step are the following approaches:

- Approach #1: When a tumor arises from a direct mutagenic event or arises because of excessive cytotoxicity or enhanced PPARα activation or α2μ-globulin accumulation, i.e., events that do not occur under normal physiological conditions, the initial step in the HRF works well and cause/effect may be discerned from only the chemical-specific data.
- Approach #2: When the increase in tumors occurs for those types of tumors that arise spontaneously, due to age-related or species/strain-related normal physiological changes, such as Leydig cell tumors in Fischer 344 (F344) male rats or liver tumors in B6F3C1 male and female mice, application of the HRF must employ a different starting point than just consideration of the chemical data. In order to understand the MOA for chemicals that act by enhancing or exacerbating either the progression (decreased latency) or incidence of spontaneously occurring age-related or strain/species-related tumors, the following must be considered:
    - The basic biology of that organ system along with physiological controls, such as feed back loops, that explains normal functioning;
    - The key steps in that biological/physiological flow of normal functioning that could be impacted by either changes due to aging or the application of an exogenous chemical resulting in changes in that cell or organ system's homeostasis; and,
    - The key step or biological "trigger(s)" (that is the obligatory precursor step) that provides the underlying stimulus, even in the absence of exogenous chemicals that "push" a normally functioning cell in an organ to become a neoplastic cell resulting in a tumor-containing organ. Stated differently, what are the biological/physiological changes that occur in the development of "spontaneously" occurring tumors in specific organs?

When an exogenous chemical acts by enhancing age- or species/strain-related tumors, the operative question is how does the chemical of interest add to the influence/effects of the naturally occurring biological "trigger"? When the underlying biology/physiology of the organ system in which the tumor is found is not considered, as is the case with these assessments, the conservative burden of proof lies with the exogenous chemical to

demonstrate that the MOA is understood. Invariably, there will be gaps that can then be used to claim that the MOA is undefined and uncertain. This is especially true for chemicals, such as acrylamide (AA), that may be opportunistic rodent carcinogens that are exacerbating age-related changes in a highly susceptible rat strain.

When these "pre-steps", that is, understanding the normal biology/physiology of a system and how that system changes with age, are included in the HRF, then one does not have to "connect all the dots" as the USEPA and others are expecting the acrylamide data to do, but rather one can use a wider array of data both for that physiological process and data from other chemicals that affect that process. In other words, while we may not have all of the data for a chemical for all of the "steps" in a biological cascade, we can integrate data for a chemical, in this case AA, and other chemicals to determine at what point in that biological cascade the chemical is likely acting and ask if that interaction influences what would be considered obligatory precursor events. ***It is less of a stretch to say that AA in aging male rats exacerbates the decline in circulating prolactin levels leading to and enhancing the biological cascade which results in an increase in spontaneous, age-related production of Leydig Cell tumors (LCT) (a known and well-accepted MOA for dopamine agonists in the production of LCTs) than to say that AA forms DNA adducts, which, by vaguely described and essentially unknown steps, results in tumors.***

Hence, application of this HRF framework ***requires an integration of all relevant data*** in a weight-of-evidence analysis of those data to: 1) discern patterns of effects that may be indicative of a potential MOA, in particular, is the potential MOA understood enough in general that approach #1 or #2 or a combination of both should be applied; 2) reconcile apparent conflicting data, in particular when studies in animals are not in agreement with epidemiological data; and, 3) determine the relevance of these findings to human health based upon the integration of data of pharmacokinetic and pharmacodynamic differences. While the USEPA in their recent evaluation of acrylamide applied the HRF, the Agency did not do so in a manner that integrated all of the relevant data or considered both approaches outlined above.

II. Discerning the Mode of Action for Acrylamide in the Production of Tumors in Fischer 344 Rats

Acrylamide produced significant increases in benign tumors in organ systems that are under, directly or indirectly, complex biological/physiological controls to ensure normal functioning, and, more importantly, are systems that undergo significant changes with age in the F344 rat. As discussed in more detail in Shipp *et al*. (2006), aging is associated with changes in the functional activity of many neuroendocrine systems in the F344 rat, among which are profound gender- and strain-specific changes in hypothalamic-pituitary pathway functioning that influences the balance of hormones that affect reproductive senescence (numerous references as cited in Shipp *et al*., 2006) and that affect thyroid function (Cizza *et al*., 1992; 1996). It is these age-related changes in these pathways, which are under hypothalamic-pituitary control, that form the underlying and unifying hypothesis that AA, by way of modulating neuroendocrine control, in particular and perhaps exclusively, dopaminergic tone, is exacerbating the production of age-related tumors in tissues under the control of this very complex biological/physiological system that are unique to the ageing F344 male and female rat. The following is a discussion of the potential MOAs for the observed tumors that were not adequately characterized in the USEPA draft submission.

1. Evidence that AA influences Dopaminergic Tone

If the underlying hypothesis is that AA acts by modifying dopaminergic tone that then influences the centrally-mediated processes for age-related functioning of selective systems, then two elements would need to be described: 1) that dopamine is an essential contributor to normal and age-related processes controlled by the hypothalamic-pituitary system, in particular in the maintenance of prolactin homeostatis and it's change with aging in the male and female F344 rat which are gender-specific; and 2) that AA can influence dopamine actions in these systems. The first element, dopaminergic influence on the pituitary, is well documented and not discussed further here but is summarized in the review by Shipp *et al*. (2006). It is well documented that AA modulates dopamine activity in rats (Agrawal *et al*., 1981; Ali *et al*., 1983; Bondy *et al*., 1981; Friedman *et al*., 1999; LoPachin *et al*., 2007; Srivastava *et al*., 1983; Uphouse and Russell, 1982; Uphouse *et al*., 1982; Yamada *et al*., 1995). The evidence for an influence on dopamine activity by AA is summarized as follows:

- LoPachin *et al*. (2007) demonstrated that AA inhibits uptake of dopamine into rat striatal synaptic vesicles likely by binding to sulfhydral-rich proteins. Inhibition of uptake in his region, and perhaps other regions, would allow dopamine to be active at the specific locus longer than under normal conditions, thereby, increasing the dopaminergic signal on those processes.
- While alterations in the levels of dopamine and its major metabolite, dihydroxyphenylacetic acid (DOPAC), were not found in some brain regions (e.g., the frontal cortex) (Ali, 1983; Ali *et al.,* 1983), significant increases in dopamine levels were found in the caudate nucleus (Ali, 1983).
- The change in dopamine levels in the caudate nucleus was dose-related and significantly increased after 20 days of daily injections of 10 or 20 mg/kg. The caudate nucleus in rats contains the same type of dopamine receptors as the pituitary (Cooper *et al.,* 1991), the D_2 receptor, which is thought to be the prime dopamine receptor in the pituitary. This difference could be explained by the relatively high levels of D_3 receptors that are located in the frontal cortex (Bouthenet *et al.,* 1991). D_3 receptors have been shown to function as autoreceptors in some areas of the brain and when activated, serve to decrease the production of dopamine (Levant, 1997). Thus, in areas rich in D_3 receptors, such as the frontal cortex, dopamine levels would be expected to be decreased following treatment of a compound such as AA that may modulate dopamine activity.
- Spiroperidol (a dopamine antagonist) binding significantly increased in rats following either single (25 mg/kg) or repeated doses (10 mg/kg for 10 days) of AA (Agrawal *et al.,* 1981; Bondy *et al.,* 1981; Uphouse and Russell 1981). Spiroperidol binding was significantly increased within 30 minutes following administration of AA, and remained elevated for 24 hours following a single injection of AA (Uphouse and Russell 1981). It has been suggested that the increase in spiroperidol binding is due to up-regulation of dopamine receptors from a previous nonaccessible pool (Agrawal *et al.*; 1981, Srivastava *et al.*; 1983, Uphouse and Russell 1981), and that the rapid response precludes de novo synthesis of receptors (Uphouse and Russell, 1981).

- Sensitivity to apomorphine, a D_2 dopamine agonist, is also altered in AA-treated rats. Additionally, administration of L-DOPA at high doses (500 or 1000 mg/kg/day for 7 to 14 days) resulted in increases in serum LH levels and decreases in prolactin levels (Yamada *et al.,* 1995). The authors suggested that the increase in serum LH appeared to be due to an increase in GnRH release by the hypothalamic-pituitary axis facilitated by the dopamine system (Yamada *et al.,* 1995).

2. *Understanding the Potential MOA for the Production of TVMs by Acrylamide*

2.1. TVMs are a tumor found mainly in male F344 rats.

One of the USEPA cautions in accepting an F344-specific MOA for AA in the production of TVMs was that acrylamide had not been tested in other strains or species. This is an example of the lack of integration of data by the USEPA that did not consider data in other strains or species with other chemicals to inform and address an apparent data gap. When individual pieces of information are considered together, there is a clear association between the production of LCTs in male F344 rats and the occurrence of TVMs. The incidence of spontaneous TVMs is higher in F344 rats, which have a high background rate of LCTs, than in the Sprague-Dawley and other rat strains, which have a lower spontaneous incidence of LCTs (Capen 1996; Maekawa and Hayashi, 1992; Takaki et al., 1989; Tanigawa et al., 1987). Further, a few of the chemicals, which produced increases in the incidence of TVMs in male F344 rats, such as ethylene oxide, were also tested in chronic bioassays in other strains of rats and did not produce TVMs in those strains (Berman and Rice 1979; Kari et al., 1989; Kurokawa et al., 1983; Snellings et al., 1984). Briefly:

- TVMs arise from the mesothelial cells that line the inner surface of the scrotum. No other mesothelial tissue in male rats was a target nor was this lesion seen in equivalent tissues in female F344 rats in either the Johnson *et al*. or Friedman *et al*. studies.
- The TVMs reported in these studies were histologically indistinguishable from background TVMs (Damjanov and Friedman, 1998; Iatropoulos *et al.,* 1998).

- Spontaneous occurrence of TVMs, while low, has been observed in a number of male rat strains (Gould, 1977; Tanigawa *et al.,* 1987).
- However, a survey of more than 400 bioassays performed by the National Toxicology Program indicated that TVMs were not found in male B6C3F1 mice evaluated at the same time as male F344 rats by the same route of exposure and similar protocols.
- These data indicate that the F344 rat is more prone to form TVMs, when compared with other strains of rats and that this may be an F344-specific response. The incidence of spontaneous TVMs is higher in F344 rats, which have a high background rate of LCTs, than in the Sprague-Dawley and other rat strains, which have a lower spontaneous incidence of LCTs (Capen, 1996; Maekawa and Hayashi, 1992; Takaki *et al.,* 1989; Tanigawa *et al.,* 1987).

2.2 <u>The production of TVMs is linked to the production of Leydig Cell Tumors (LCTs)</u>

Two mechanisms for the production of TVMs in male rats have been proposed (Tanigawa *et al.,* 1987):

- TVMs occur as a result of hormonal imbalances in the peritesticular area (O'Shea and Jabara, 1971).
    - There are age-related changes in the synthesis and/or secretion of gonadal and adenohypophyseal hormones (Turek and Desjardins, 1979).
    - Specifically, decreases in testosterone levels in the peritesticular area results in the changes/increases in localized growth factors, especially TGF-beta, which, unlike its action in epithelial cells, provides a proliferative signal to mesothelial cells.
- Enlargement of the LCTs produces a physical stimulus on the surrounding mesothelium in a manner related to a solid state/foreign body response, which has been associated with tumor formation (Tanigawa *et al.,* 1987; Ryan *et al.,* 1981).
    - Examples of solid state carcinogens are well known, notably, asbestos-induced mesotheliomas (Shabad *et al.,* 1974; Stanton and Wrench,

1972). While the mechanism of how asbestos-induced injury is translated into a transforming event leading to a tumor production is not known with certainty, recent data indicate that there are alterations in the expression of growth factors in transformed mesothelial cells (Gerwin *et al.,* 1987; Versnel *et al.,* 1988).

- In the reanalysis of TVMs reported in the Friedman *et al*. (1995) study by Iatropoulos *et al*. (1998), the degree of morphological progression of TVMs correlated with the size of LCTs. Further, according to Iatropoulos *et al*. (1998), TVMs were found only in animals with LCTs that occupied more than 75% of the testes. Of those TVMs rediagnosed as localized hyperplasia, the LCT occupied 25% or less of the testes.
- In the Friedman *et al*. (1995) study, there was a significant increase in the mean absolute testes weight in the two highest dose groups compared to controls, and the percentage of animals with testes reported upon gross examination as increased in size was also increased in the high-dose group.

These data suggest that the formation of TVMs in AA-treated rats was related to the occurrence and size of the LCTs. Therefore, the MOA for both spontaneous and chemically induced LCTs was considered. The integrating factor for these two proposed mechanisms of TVM formation –hormonal action or solid-state response ***– is that the development of LCTs and resultant alterations in the peritesticular microenvironment are obligatory precursor steps to TVM development.*** Whether that extracellular stimulus is due to altered hormonal balances in the peritesticular microenvironment that ensue in aged rats with advanced LCT development (the more likely mechanism) or through solid state pressure due to the increase size of the LCTs, production of these TVMs in either case is modulated by mesothelial autocrine growth factors, such as TGF-beta and the mesothelial cells response to these. Recent evidence indicates that growth regulatory pathways in mesothelial cells are absolutely different from those reported for other primary epithelial cell types (Gabrielson *et al.,* 1988). Unlike its inhibitory response in some epithelial cell lines, TGF-β stimulated DNA synthesis in cultured mesothelial cells (Gabrielson *et al.,* 1988). Further, these cells responded with increased DNA synthesis to other growth factors, namely platelet-derived growth factor (PDGF) and epithelial growth

factor (EGF). Mesothelioma cells lines have been shown to have increased mRNA levels of the PDGF gene when compared with normal mesothelial cells, which suggests that these may be autocrine growth factors for these neoplasms (Gerwin *et al.,* 1987).

2.3 Overview of LCT Production in the F344 rat.

General regulation of androgen balance and the production of LCTs can be summarized as follows:

- In the male rat reproductive system, in particular in F344 rats, maintenance of blood androgen hormone levels, primarily testosterone, and control of spermatogenesis are regulated by a complex feedback loop involving the hypothalamus, pituitary gland and the testes (Capen, 1996; Clegg *et al.,* 1997; Creasy and Foster, 1991; Herbert *et al.,* 1995; Prentice and Meikle, 1995).
- Decreases in testosterone levels in aging F344 rats result in a compensatory increase in the level of LH, the hormone that stimulates the Leydig cell to produce testosterone.
- In the aged F344 rat, age-related hormonal changes, in particular age-related decreases in prolactin in response to age-related increases in dopamine in TIDA neurons that directly affect the pituitary and changes in cellular receptor density in the testes have been reported in F344 rats (Amador *et al.,* 1985).
- Decreases serum prolactin levels result in a down-regulation of LH receptors resulting in an age-related decrease in testosterone levels (Cook *et al.,* 1999; Prentice *et al.,* 1992).
- A compensatory increase in LH from the brain caused by decreases in testosterone results in an up-regulation of LH receptors thereby resulting in Leydig cell hyperplasia (Amador *et al.,* 1985), and with continued stimulation by LH, eventually results in a progression from Leydig cell hyperplasia to neoplasia, a scenario unique to the rat, specifically F344 rats (Amador *et al.,* 1985; Cook *et al.,* 1999).

Dopamine agonists, such as mesulergine, norprolac and oxolinic acid, result in decreases in serum prolactin levels, which result in a down-regulation of Leydig cell LH receptors and subsequent decreases in serum testosterone levels, a mechanism unique to rodents (Alison *et al.,* 1994; Prentice *et al.,* 1992, Prentice and Meikle, 1995). To

compensate for the decrease in testosterone, LH levels increase, and if sustained, result in Leydig cell hyperplasia and LCTs.

2.4 AA's Contribution to Age-related Decreases in Prolactin and Testosterone

Several experiments indicate that AA could produce alterations in hormone levels that can impact several organs and organ systems. In particular, decreases in serum prolactin and testosterone levels, in combination with changes in testes size and weight, suggest a connection between AA's action at the neurotransmitter level and these effects. There is considerable evidence that AA produces a decrease in prolactin and testosterone levels in male F344 rats. This evidence can be summarized as follows:

- In the study by Friedman *et al*. (1999), dose-related decreases in testosterone to 53% of the control level occurred following a 14-day administration of 25 mg AA/kg to F344 rats beginning at age 8 weeks. After 28 days of treatment, testosterone levels were reduced in the 12, 19* and 25* mg/kg/day dose groups (* statistically different from control). After 14 days of treatment, dose-related changes in prolactin levels were noted in all groups except for the lowest dose group. The percentage decreases in prolactin levels from control values were 17%, 36%, 81%, and 87% in the 4.1, 12, 19*, and 25* mg/kg/day dose groups, respectively.
- Decreases in serum prolactin and testosterone levels following administration of AA have also been reported in other studies (Agrawal *et al.,* 1981; Ali *et al.,* 1983; Uphouse *et al.,* 1982). Dose-related decreases in testosterone and prolactin levels were observed following daily injections of 10 or 20 mg/kg for 20 days, and were significantly decreased in the 20 mg/kg/day group (Ali *et al.,* 1983).
- Administration of AA via a single intraperitoneal injection of 100 mg/kg to naïve (unhandled) F344 male rats resulted in a statistically significant decrease in prolactin levels, compared to controls (Uphouse *et al.,* 1982). Prolactin levels were not significantly decreased in animals that had been "gentled," that is, handled to reduce the stress response. This is consistent with other results that suggested that animals accustomed to handling had altered sensitivity to dopamine-acting compounds (Uphouse *et al.,* 1982).

In Summary. The biological/physiological cascade that is hypothesized to provide the connection between the LCT and the TVMs is as follows:

- The proposed MOA for AA is by enhancing the hormonal imbalance seen in aging male F344 rats by enhancing dopaminergic tone at the level of the pituitary gland resulting in a further decrease in prolactin levels. This results in a down-regulation of LH receptors on the Leydig cell, adds to age-related decreases in the synthesis of testosterone in the testes and secretion of testosterone into the peritesticular area, and the results in the formation of earlier and more metabolically active LCTs. The formation of LCTs results in further decompensation of hormone responsiveness and production resulting in a cascade of hormonal changes in the peritesticular microenvironment and subsequent formation of TVMs through a growth-factor receptor-mediated autocrine, growth factor response. Consequently, the formation of LCTs is considered an obligatory, precursor event for the formation of TVMs.
- Further decreases in the regional androgen levels as an indirect result of AA administration would accelerate and extend the spontaneous rate of TVM formation, even in the absence of exogenous genetic damage in these cells. In this case, it would be expected that the tumors would be morphologically the same as background, as indicated in the histopathological examinations (Friedman *et al.,* 1995; Iatropoulos *et al.,* 1998; Johnson *et al.,* 1986).

2.5 Relevance of TVMs to Human Health

The evidence suggests that the hormone/growth signal component involved in the promotion of these mesotheliomas is linked to the altered hormonal microenvironment in the male F344 rat peritesticular area. An analogous microenvironment in the human peritesticular area is not expected to occur for a number of reasons.

- The extent of the alterations in the hormonal levels in the peritesticular microenvironment of the male F344 rat by AA is strain-specific. According to Cook *et al*. (1999), "...compounds that induce LCTs in rats by disruption of the HPT axis pose a risk to human health, except for possibly two classes of compounds (GnRH and dopamine agonists). Because GnRH and prolactin receptors are either not expressed or are expressed at very low levels in the testes in humans, the induction of LCTs in rats by GnRH and dopamine agonists would appear not to be relevant to humans."

- There are significant differences between human and rat mesothelial cells, both in the production and the responsiveness to growth factors (Walker *et al.,* 1991). Depending on which growth factors and the role they play in mesothelioma development, the use of a rat mesothelioma model is not be predictive for humans (Walker *et al.,* 1991).

When considered in combination with the likely MOA for the production of TVMs in F344 male rats, the F344 rat is uniquely sensitive. The production of TVMs in the F344 rat is not relevant to humans. Therefore, the incidence of TVMs should not be considered further.

3. *Understanding the Potential Mode of Action of Acrylamide in the Production of Mammary Fibroadenomas in Female F344 rats.*

Fibroadenomas are common, spontaneously occurring tumors in aging female F344 rats that result from the consequence of aging in both mammary gland development and the normal physiology of the reproductive system in F344 rats. The changes in those systems in aging female F344 rats are reviewed in Shipp *et al*. (2006). The control of these processes is a complex series of events involving ovarian hormones, such as estrogen and progesterone, the pituitary gland hormone, prolactin, and in the case of the mammary gland, locally-produced growth factors and other cytokines released by the extracellular matrice surrounding and supporting mammary epithelial tissue that are likely involved in paracrine and autocrine control of cellular proliferation (see Shipp *et al.,* 2006). These events are briefly summarized in the next section.

3.1. Age-related Changes in Reproductive Function in Female F344 Rats

In the aging F344 rat, the complex system that controls hormone secretion begins to fail. In particular, unlike the male F344 rat, the dopamine inhibition of prolactin release is no longer effective in the aging female F344 rat. In the female F344 rat:

- Prolactin secretion is inhibited by dopamine, which is released from the TIDA neurons into the hypothalamic-hypophyseal portal vessels that supply the pituitary gland in response to high levels of blood or cerebrospinal fluid prolactin (Capen *et al,* 1991; MacKenzie and Boorman, 1990; MacLoed and Login, 1977; Meites, 1980; Stefaeanu and Kovacs, 1994). Through an undetermined mechanism, dopamine interacts with D_2 receptors on the

pituitary lactotrophs, the cells that secrete prolactin, and inhibits prolactin release. Conversely, dissociation from the lactotroph D_2 receptor results in a loss of inhibition and an increase in prolactin secretion.

- In the aging female F344 rat, there is a breakdown in the control of prolactin release possibly from the loss of or damage to TIDA neurons, loss of TIDA neuronal function or by some breakdown in the lactotroph, such that the cell is no longer responsive to dopamine (MohanKumar *et al*., 1997, 1998; Reymond 1990; Sarkar *et al.,* 1982, 1983). As a result of the deterioration of this system, prolactin levels are greatly increased and ovarian cyclicity is altered.
- Regardless of the mechanism, the end result is hyperprolactinemia in aged female rats, a condition that can then impact ovarian function and result in changes in the temporal patterns of ovarian hormone secretion.
- Prolactin stimulates corpus luteum function and stimulates the synthesis of progesterone in rats (Neumann, 1991). As a consequence, aging female rats with hyperprolactinaemia enter a state of pseudopregnancy in which elevated levels of prolactin and progesterone are sustained (Meites, 1980; Neumann, 1991).
- The ovaries of pseudopregnant rats contain numerous corpora lutea (Cooper *et al.,* 1986). As with pregnancy, pseudopregnancy is associated with high levels of progesterone and prolactin and low levels of estrogen (Demarest *et al.,* 1982; Huang *et al.,* 1976; Huang *et al.,* 1978; Lu *et al.,* 1979; Lu *et al.,* 1980; Peluso and Gordon, 1992). It is believed that in the pseudopregnant rat, high prolactin levels serve to maintain the corpora lutea. Thus, in pseudopregnancy, rather than regress, as occurs in a rat that is cycling normally, the corpora lutea persist and continue to secrete progesterone (Peluso and Gordon, 1992; Smith, 1980).
- Pseudopregnancy occurs at a much higher incidence in the aging female F344 rat (Estes and Simpkins, 1981; Estes, 1982; Peluso, 1992; Saiduddin, 1979) and as expected, the background rate of mammary fibroadenomas is higher in F344 rats than other strains.

3.2 Normal Mammary Gland Development in F344 rats

Growth, morphogenesis, and differentiation of the mammary gland in rats have been extensively studied and several comprehensive reviews have been published (Boorman *et al.,* 1990; Borellini and Oka, 1989; Cunha, 1994; Howlett and Bissell, 1993; Imagawa *et al.,* 1990; Russo *et al.,* 1982). Mammary gland growth and differentiation are under complex hormonal and cellular control and not all of these complex interactions are known with certainty, in particular the role of interactive growth factors in response to hormonal signals (Howlett and Bissell, 1993). A brief summary is presented here.

- During embryogenesis, epithelial morphogenesis is under mesenchymal control and apparently without other hormonal control (Howlett and Bissell, 1993; Sakakura, 1987). By birth, a basic ductal network, up to third- and fourth-order branching, has been established (Howlett and Bissell, 1993). Initially, the ducts are narrow and end in terminal ductules or small club-shaped terminal end buds (TEBs) (Boorman *et al.,* 1990; Russo *et al.,* 1982).
- During the first 3 to 4 weeks of postnatal life, ductal growth continues with elongation and branching of ducts within the newly formed mammary fat pad stroma and accelerates with TEBs forming at the tips of the ducts (Boorman *et al.,* 1990; Borellini and Oka, 1989; Howlett and Bissell, 1993). Elongation of the ducts occurs as a result of rapid growth in end buds, which, by turning and branching, give rise to the characteristic tree-shaped pattern of the mammary ductal system (Russo *et al.,* 1982).
- The number of TEBs quickly reaches a maximum and decreases thereafter as they differentiate into terminal ductules and alveolar buds (ABs) (Boorman *et al.,* 1990), with each TEB differentiating into three to five smaller ABs forming lobules, which begin to differentiate into alveoli (10 to 12 alveoli per AB) to form a lobule; and by approximately 6 to 8 weeks of age.
- During pregnancy, mammary gland development is completed with extensive lobuloalveolar growth to yield functional epithelial cells with the capacity to secrete milk (Howlett and Bissell, 1993).

3.3 Regulatory Control of Mammary Gland Development in the Female F344 Rat

Growth, morphogenesis, and functional differentiation of mammary epithelium depend on signaling from systemic hormones and on signals from the local tissue microenvironment to include, the extracellular matrix (ECM), the basement membrane and soluble cytokines (Cunha, 1994; Howlett and Bissell, 1993). These events have been reviewed by Shipp *et al*. (2006) and summarized briefly here.

- In the ECM of the mammary gland, regulatory signals are provided by two subcompartments of the mesenchyme/stroma (fibroblasts and adipocytes) and the subjacent basement membrane (Howlett and Bissell, 1993). Both mesenchymal cell types, adipocyte and fibroblasts, have distinct regulatory properties, and are separated from the epithelial cells by the basement membrane (Howlett and Bissell, 1993). Consequently, during prepuberty, postpuberty, and throughout adulthood, the ECM, in particular the stromal connective microenvironment, influences epithelial growth, ductal branching, epithelial differentiation, and lactation (Cunha, 1994; Howlett and Bissell, 1993).
- Lobuloalveolar growth and further differentiation of this ductal system during pregnancy and lactation require prolactin and progesterone and signals from the microenvironment from the basement membrane (Boorman *et al.*; 1990, Cunha, 1994; Howlett and Bissell, 1993). Progesterone has been shown to induce cell proliferation and to direct multilobular and lobuloalveolar branching (Darcy *et al.,* 1995; McGrath *et al.,* 1985).
- With each estrus cycle, additional TEBs form ABs with some lobuloalveolar growth and involution at the end of the cycle (Russo *et al.,* 1982). With each estrous cycle, in response to hormonal changes, the mammary gland in the female F344 rat undergoes further differentiation.

In summary, there is compelling evidence that ECM components work together with systemic hormones to direct the development of the mammary gland from embryogenesis through to adulthood and to regulate the function of mature mammary epithelial cells during pregnancy, lactation, and involution. These ECM cues arise from two separate subcompartments of the

mesenchyme/stroma (fibroblastic and adipocyte) and from the basement membrane, formed from mammary epithelial cells in response to cues from the fibroblast stroma. Mammary epithelium can be envisioned as being linked to other elements in the microenvironment, namely, the mesenchyme/stroma and basement membrane, to form a "dynamic and reciprocally interactive functional unit" (Bissell *et al.,* 1982).

3.4 MOA in the Production of Spontaneously Occurring Mammary Fibroadenomas in the Female F344 Rats

The connection between increased and sustained levels in prolactin and progesterone and the production of fibroadenomas can be understood when the role of progesterone in mammary differentiation is considered. Estrogen is required for ductal expansion in the prepubertal and postpubertal rat; however, progesterone is required for lobuloalveolar branching, differentiation, and growth. Increased differentiation of ABs into alveoli requires complex interactions between the epithelial cell, its surrounding basement membrane, and the stromal fibroblast support system in response to progesterone and other growth factors.

While stated as a sequence of events for purposes of discussion, this complex process can be viewed as interactive, reciprocal stimulation involving the readily differentiating alveoli. With progesterone stimulation acting most likely at the level of differentiating end bud, interaction between the stromal fibroblast, basement membrane, and epithelial cell progresses as each functions in the development of the lobuloalveolar structure (Howlett and Bissell, 1993). The fibroblast, in response to progesterone or to a mitogenic stimulus initiated by progesterone, is stimulated to produce the collagen matrix that provides support for the growing structure, as well as additional local signals to the basement membrane, which in turn mediates growth and differentiation of the alveoli into a functional unit capable of producing milk in response to other signals, such as prolactin. Complex growth factors may be involved in this fibroblastic response including paracrine stimulation by TGF-β, possibly released from epithelial cells or the basement membrane or by autocrine stimulation by fibroblast growth factor (FGF), which may aid in angiogenesis for the newly growing lobuloalveolar structure. While the exact interactive mechanisms are not known at this time with certainty, in pseudopregnancy the mammary gland is under sustained stimulation

from prolactin and progesterone, and as a consequence, produces a sustained proliferative growth signal to stromal fibroblasts eventually manifested as fibroadenomas. Hence, in this model for fibroadenoma development, pseudopregnancy is an obligatory precursor step, and the sustained, elevated levels of progesterone is the major hormonal trigger.

Other evidence includes:

- Elevated levels of prolactin and progesterone have been shown to increase the incidence of spontaneous mammary fibroadenomas in rats (Meites 1980), dogs (Neumann, 1991) and cats (Mol *et al.,* 1995).
- Agents that result in a decrease in prolactin levels, such as L-DOPA or ergot drugs (Meites, 1980) or carbergyline (Negishi and Koide, 1997), and/or, consequently, a decrease in progesterone levels, e.g., ovariectomy (Meites, 1980), resulted in either a decrease in the incidence or a regression of fibroadenomas in female rats.
- Chemicals known to inhibit dopamine, such as reserpine, decreased the latency period and increased the incidence of fibroadenomas (Meites, 1980)..
- Progesterone administration to mature female rats followed by γ-irradiation resulted in the production of fibroadenomas, while administration of estradiol followed by γ-irradiation produced primarily adenocarcinomas (Yamanouchi *et al.,* 1995). The authors concluded that the mechanism by which fibroadenomas were produced differed from the mechanism of adenocarcinoma formation in that adenocarcinoma formation was associated with high levels of estrogen.

3.5 <u>AA's Contribution to Mammary Fibroadenomas</u>

The predominant mammary tumor observed in the two-year bioassay for AA was the increased incidence in fibroadenomas in female F344 rats, which is the most common spontaneously occurring tumor in female F344 rats (Boorman *et al.,* 1990). Discerning AA's mode of action in the enhanced development of mammary fibroadenomas in female F344 rats requires an integration of all of the available data.

The pattern of the dose-response provides some insights into the potential contribution of AA to the increased incidence of mammary fibroadenomas. The incidence of mammary fibroadenomas was comparable to controls at doses below 0.5 mg/kg/day. A clear point of inflection in the dose-response curve was noted at 0.5 mg/kg/day, but rather than a linearly increasing trend above that dose, the responses plateaued, that is, the incidence in the 3.0 mg/kg/day dose group was the same as that seen in 0.5 mg/kg/day dose group. Moreover, the increases in incidence in fibroadenomas in the treated groups were not dramatic and were within the range of historical control values seen in other two-year bioassays (Boorman *et al.,* 1990; Goodman *et al.,* 1979; Maekawa *et al.,* 1983; Solleveld *et al.,* 1984). The shape of the dose-response curve suggests that:

- AA may be acting by a MOA that has a threshold or is at least nonlinear at low doses; or
- The MOA by which AA may be acting is through a saturable event(s) consistent with receptor interactions, e.g., once receptor occupancy has reached a level to trigger a response, further receptor interaction may not increase that response in a linear manner; or
- AA may be adding to that background biochemical stimulus operative in the production of spontaneously occurring fibroadenomas in the aging female rat.

Further, the fibroadenomas seen in the AA treated groups were histologically indistinguishable from those occurring spontaneously (Friedman *et al.,* 1995, Johnson *et al.,* 1986) and there was not a consistent statistically significant increase in the incidence of adenocarcinomas nor was there a significant effect on the latency of fibroadenomas. Taken together, these results suggest that AA is likely acting by contributing to changes in the levels of the key circulating hormones, namely progesterone. Consequently, a mode of action for AA would have to be complementary to the underlying mode of action for spontaneously occurring fibroadenomas, that is, resulting in alterations in circulating levels of prolactin and/or progesterone, and be consistent with the toxicity data for AA. Therefore, development of a MOA for AA must be based not only on the experimental data for AA, but also on the hormonal changes in the

aged female F344 rat, as they apply to changes in reproductive function and subsequent changes in mammary gland differentiation.

As noted above, the proposed mode of action for spontaneously occurring mammary fibroadenomas in the female F344 rat is one that is linked to the development of pseudopregnancy in the aged female. Age-related increases in prolactin levels, the release of which is refractory to dopamine inhibitory control in the aged female F344 rat, result in hyperprolactemia and, consequently, a sustained increase in progesterone release from the ovary. Hence, the ability of AA to contribute to the same biochemical cascade as is likely to be occurring in age-related fibroadenoma development, that is, modulation of prolactin levels or more likely progesterone levels, would be expected.

Administration of dopamine to male F344 rats has resulted in a decrease in prolactin levels, indicative of D_2 agonist activity (Agrawal *et al.,* 1981, Ali *et al.,* 1983, Friedman *et al.,* 1999, Uphouse *et al.,* 1982). However, no consistent changes in prolactin levels were seen in female F344 rats (Friedman *et al.,* 1999; Zenick *et al.,* 1986). Further, prolactin release becomes refractory to dopamine control in the aged female F344 rat, and it is likely that a weak agonist would not effectively influence prolactin secretion in the aged female F344 rat. Therefore, it is unlikely that AA is acting by increasing prolactin from the pituitary.

The more biologically plausible site for AA action is at the level of the D_1 receptor in the ovary. The control of steroidogenesis has historically been considered to be under the control of feedback loops that involve the gonadotropins LH and FSH. However, there are data that indicate that F344 rat ovarian cells contain dopamine receptors and, based on the results of studies conducted with dopamine agonists, there is evidence that dopamine is involved in the control of steroidogenesis in the female F344 rat (Arakawa *et al.,* 1994; Mori *et al.,* 1994; Negishi *et al.,* 1998). In the rat ovary, this control is thought to be mediated by the interaction between dopamine and D_1 receptors on ovarian cells (Mori *et al.,* 1994).

In a series of *in vitro* studies conducted using ovarian cells collected from pregnant mare serum gonadotropin (PMSG)-treated rats, Mori *et al*. (1994) reported dose-related increases in progesterone secretion with decreases in

estrogen secretion in cells incubated with dopamine or the dopamine D_1 receptor agonists, SKF3, SKF8 and CY. The levels of cyclic AMP (cAMP) were also significantly increased, an effect that is associated with D_1 receptor activation, in cells incubated with dopamine. No effects on baseline progesterone secretion were reported following incubation with propanolol (beta-blocker), domperidone (D_2 receptor antagonist), bromocriptine (D_2 receptor agonist), or bulbocapnine (D_1 receptor antagonist). However, incubation with bulbocapnine, but not propanolol or domperidone, inhibited the dopamine-stimulated secretion of progesterone. Results of binding assays conducted with SCH23390, a D_1 receptor ligand, indicated that a D_1 receptor was present in the PMSG-treated rat ovary cells. Binding of SCH23390 was inhibited by dopamine, SKF3, SKF8, CY and bulbocapnine. Based on these results, Mori *et al*. (1994) concluded that dopamine directly stimulated the rat ovarian cells to secrete progesterone, an effect that was mediated through D_1 receptors on the ovarian cells and not through the beta-adrenergic receptors. Similar results reported by Arakawa *et al*. (1994) also indicated that D_1 receptor agonists increased progesterone secretion from PMSG-treated rats and that incubation with D_1 receptor antagonists inhibited the dopamine-stimulated increase in progesterone secretion.

AA acting at the level of the D_1 receptor on the corpora lutea would result in an increase in progesterone that would add to the enhanced progesterone levels produced in response to naturally occurring hyperprolactemia in the aged pseudopregnant F344 female rat. While there is no direct evidence, i.e., receptor binding, that AA modulates D_1 receptor activity in the ovary, there is indirect evidence that such an interaction occurs with resulting increases in progesterone. In the reproductive study conducted by Zenick *et al*. (1986) in which female Long Evans rats were administered AA in the diet, only two observations were noted and only in the highest dose tested. These was a decrease in neonatal pup weight postpartum during lactation, which could be indicative of a decrease in lactation in response to a decrease in prolactin (the D_2 response mentioned in the above paragraph), and a slight delay in vaginal patency in female offspring. Delayed vaginal patency has been associated with decreases in estrogen levels. Such a decrease would be entirely consistent with action at the D_1 receptor on the corpora lutea and the resulting sustained release of progesterone. This effect was only seen

at the highest dose tested, 14 mg/kg/day. It can be surmised that if AA was affecting an increase in progesterone levels through D_1 interaction in the lower dose groups, the level of that increase was not sufficient to block feedback control of estrogen release. ***Lack of an effect on vaginal patency at the lower doses comparable with those administered in the bioassay is consistent with the suggestion that AA's contribution to progesterone levels was small and subtle, that is, the increases in fibroadenomas were within historical control ranges.***

The hypothesis for AA's mode of action in the development of mammary fibroadenomas in the female F344 rat is that AA, through dopaminergic modulation at the level of the ovary, enhanced age-related changes in progesterone levels leading to an increase in the background rate of mammary gland fibroadenomas. While the initiating stimulus for the induction of a sustained increase in progesterone levels may differ — hyperprolactinemia in aged female rats versus a dopaminergic contribution to steroidogenesis in the ovary — the consequence of both is the same, that is, increases in progesterone levels with the resulting cascade of biochemical events in the mammary microenvironment. AA's contribution to the increased incidence of mammary fibroadenomas is likely mediated through hormonal signals that would not only have a threshold and be under homeostatic control but would also be of questionable relevance to humans.

3.6 Relevance to Human Health

Mammary gland fibroadenomas in female F344 rats are spontaneously occurring, benign neoplasms that are formed in response to alterations in the complex balance between centrally and ovarian-mediated hormonal control in aging female rats. Age-related, centrally-mediated increases in prolactin levels result in increases in progesterone, and decreases in estrogen are ultimately manifested as sustained pseudopregnancy in the aging female F344 rat. The increases in prolactin and progesterone result in a sustained cell proliferative response in the stroma/fibroblast cells in the ECM in the mammary gland. This prolonged stimulation of these cells is the likely mode in the progression to fibroadenomas. The production of fibroadenomas is a species-specific response, normal to aged female F344 rats and rats of other strains, as well as mammals under luteotrophic control, such as cats and dogs. Both the mode of action of fibroadenoma formation in the aged female F344 rat, as well as the mode of

action by which AA may be enhancing the progression of this background response, are not relevant to humans for the following reasons.

Fibroadenomas in women result from an increase in estrogen or a decrease in progesterone, which is the opposite of the hormonal signals in rodents association with fibroadenomas production. Mammary tissue from different species has a very similar structural organization, although hormonal requirements for the growth and expression of the specific function of the gland vary among various strains or species (Borellini and Oka, 1989; Russo *et al.,* 1982). The evidence strongly indicates that production of spontaneously occurring fibroadenomas in the female F344 rat is mediated by an increase in prolactin and progesterone, with the subsequent biochemical cascade of proliferative responses in mammary stromal fibroblasts. Fibroadenomas in women are characterized by abnormal stromal connective tissue cells; however, epithelial cells of the ducts and lobules found in association with these stromal cells were identical to normal breast tissue (Smith and Oehme, 1991). Moreover, while not known with certainty, there is evidence that fibroadenomas in women are hormonally responsive tissues associated with any change that alters the balance between circulating estrogen and progesterone levels, such that an increase either in estrogen or a decrease in progesterone or both has been found to be associated with fibroadenomas. As reviewed in Smith (1991), patients with fibroadenomas and normal ovulatory cycles had significantly lower progesterone levels than controls even though estrogen levels were normal (Sitruk-Ware *et al.,* 1979). Plasma progesterone levels in women with fibroadenomas were also significantly reduced compared to normal controls matched as to menstrual cycles (Martin *et al.,* 1979). Further, in the group studied by Martin *et al*. (1979), plasma estradiol levels at the luteal phase were significantly higher in women with fibroadenomas and the level of estrogen receptors in fibroadenomas correlated with the degree of epithelial cell proliferation in the fibroadenoma. According to Smith (1991), human fibroadenomas are associated with a relative excess level of circulating estradiol over that of progesterone. In the rat mammary gland, the elevated level of progesterone, such as that produced during pseudopregnancy, is the obligatory step in the production of rat mammary fibroadenomas.

Pseudopregnancy in the rodent is an obligatory precursor step to fibroadenoma production. Age-related, centrally-mediated changes in prolactin levels resulting in pseudopregnancy in the aging female F344 rats result in the sustained release of progesterone in response to the luteotrophic effects of prolactin on the ovary. This is considered to be a precursor step in the production of fibroadenomas in rats. Increased levels of progesterone have also been shown to produce fibroadenomas in dogs and cats, species that are also under luteotrophic control (Neumann, 1991). Prolactin is not luteotrophic in primates, including humans (McDonald, 1980; Neumann, 1991); consequently, this sequence of events would not be expected to occur.

Dopamine receptors in the human ovary are unresponsive to dopamine agonists. There is evidence that the noradrenergic system, rather than the dopaminergic system, plays a role in the control of steroidogenesis in the human ovary. In a study by Bodis *et al*. (1993), human granulosa cells were incubated with norepinephrine or dopamine. Concentration-related decreases in progesterone secretion were reported in cells incubated with norepinephrine, and estradiol secretion was stimulated by dopamine in an inverse dose-related manner. The addition of the beta-adrenergic receptor blocker propanolol completely inhibited these effects, suggesting that these effects, including the effect of dopamine on estrogen secretion, were likely the result of the conversion of dopamine to norepinephrine and subsequent stimulation of noradrenergic receptors (Mayerhofer *et al.,* 1999).

The results of Mayerhoffer *et al*. (1999) confirmed that human ovarian cells contain D_1-subtype (D_1-R) receptors; however, these receptors were not associated with the control of steroidogenesis. In the Mayerhoffer *et al*. (1999) study, human granulosa cells were incubated with the selective D_1 receptor agonist SKF-38393. Increases in cAMP levels and in cell contraction, effects that were not inhibited by propanolol, were reported. Direct evidence for the presence of D_1-R receptors was demonstrated. However, neither basal and hCG-stimulated progesterone levels nor basal estradiol levels were significantly altered following incubation with SKF-38393. Based on these results, Mayerhoffer *et al*. (1999) concluded that in the human ovary, D_1 receptors did not play a role in the control of steroidogenesis. Consequently, administration of AA is not expected to alter

either progesterone or estradiol levels through dopaminergic modulation in the human ovary.

Mammary gland fibroadenomas in female F344 rats are spontaneously occurring, benign neoplasms formed in response to age-related alterations in the complex balance between centrally and ovarian-mediated hormonal control. Age-related, centrally-mediated increases in prolactin levels result in increases in progesterone and decreases in estrogen ultimately manifested as sustained pseudopregnancy in the aging female F344 rat. The increases in prolactin and progesterone result in a sustained cell proliferative response in the stromal/fibroblast cells in the ECM in the mammary gland such that the prolonged stimulation of these cells results in the progression to fibroadenomas. The hypothesis for AA's mode of action in the development of mammary fibroadenomas in the female F344 rat is that AA, through dopaminergic modulation at the level of the ovary, enhanced age-related changes in progesterone levels leading to an increase in the background rate of mammary gland fibroadenomas. While the initiating stimulus for the induction of a sustained increase in progesterone levels may differ - hyperprolactinemia in aged female rats versus a dopaminergic modulation in steroidogenesis in the ovary - the consequence of both is the same, that is, increases in progesterone levels with the resulting cascade of biochemical events in the mammary microenvironment. AA's contribution to the increased incidence of mammary fibroadenomas is likely mediated through hormonal signals that would not only have a threshold in the rodent but would not be relevant to humans. Consequently, the use of the incidence of mammary fibroadenomas quantitatively to determine a carcinogenic potency for AA is not warranted.

4. Understanding the Potential Mode of Action of Acrylamide in the Production of Thyroid Tumors

4.1 Normal Physiology of the Rat Thyroid and Modulation of the Physiology in the Production of Thyroid Tumors.

Of the three tumor types under consideration as to the MOA and relevance to human health, the production of Follicular cell thyroid tumors are among the most studied types of tumors produced in F344 rats. Discussions and descriptions of the

underlying mode of thyroid tumor development usually focus on the manner in which the production of thyroid hormones T_3 and T_4 could be altered, thereby resulting in the increase in TSH levels in response to decreased levels of thyroid hormones.

Several lines of evidence indicate that the level of TSH controls not only thyroid function and differentiation/growth but also follicular cell proliferation (Capen and Martin, 1989; Capen, 1992; Dumont *et al.,* 1992; USEPA - US Environmental Protection Agency, 1998). Less discussion, however, has been focused on the intracellular response to TSH levels, i.e., the mode by which TSH signals the cell to function or ultimately to proliferate. Complex biochemical cascades that influence cell function and differentiation versus cell proliferation differ among cell types and may vary across species (Dumont *et al.,* 1992).

There is experimental evidence that the effects of TSH on rat thyroid cells are primarily mediated by cAMP (Dere and Rapoport, 1986; Dumont *et al.,* 1992, Ealey *et al.,* 1987; Jin *et al.,* 1986; Lewinski, 1980; Medina *et al.,* 2000; Roger *et al.,* 1997; Saji and Kohn, 1990; Scheinman and Burrow, 1977; Tramontano *et al.,* 1988, Tramontano *et al.,* 1988, USEPA - US Environmental Protection Agency 1998; Wynford-Thomas *et al.,* 1987). However, TSH effects on function (i.e., thyroid hormone secretion and thyroid protein iodination) and effects on differentiation (i.e., iodine transport and thyroglobulin synthesis) occur at lower TSH concentrations than those required for thyroid cell proliferation (Roger and Dumont, 1984; Roger *et al.,* 1985). Moreover, in the rat follicular cell, only small changes in cAMP levels were required to induce cell proliferation (Dere and Rapoport, 1986). This is not surprising since in the rat, TSH levels and thyroid cell function are at near maximal levels (USEPA - US Environmental Protection Agency, 1998).

As reviewed by Shipp *et al*. (2006), thyroid tumors may result when the normal function of the thyroid to produce T_3 and T_4 is blocked. As indicated, this blockage may occur at one of several steps in this metabolic chain. While the site of action in this metabolic change differs, the consequences of each of these modes of action are the same-increased TSH levels due to the negative feedback at the level of the hypothalamus.

However, there is also evidence that increased cell proliferation, and with that the potential for tumor production, can occur by modes of action that do not result in an increase in TSH levels. Rather, these agents, as described above, act by enhancing the cAMP signal resulting in increased intracellular concentrations of cAMP.

Importantly for AA, little research as been conducted on the effects of aging on thyroid function and response to external stimuli. Cizza et al. (1992, 1996) investigated age-related alterations in hypothalamic-pituitary-thyroid function. Progressive follicular loss occurred as the F344 rat aged and was associated with significant and progressive decreases in free T4 and T3 levels without changes in TSH. This and other changes were described by Cizza et al. as an indication that in F344 rats, a progressive, centrally mediated decrease in thyroid function occurs with aging.

4.2 Effects of AA in the Thyroid and Potential MOA in Thyroid Tumor Induction.

When evaluated for thyroid function, AA produced morphometric changes (Khan *et al.,* 1999) and changes in indices indicative of proliferative responses, i.e., BrdU incorporation and PCNA expression (Klaunig, 2000), without significant changes in TSH levels (Friedman *et al.,* 1999; Khan *et al.,* 1999; Klaunig, 2000) or consistent changes in both sexes in T_3 or T_4 levels (Friedman *et al.,* 1999). The experimental data suggest that the effects of AA in the rat thyroid appear to be an increase in thyroid follicular cell proliferation without an appreciable alteration in function (e.g., changes in T_3, T_4, or TSH levels). As discussed previously, exposures to AA were associated with mild changes in T_3, T_4 or TSH levels, although there was evidence of proliferation in the thyroid gland (e.g., increased incorporation of BrdU, increased expression of PCNA and a microscopic appearance consistent with proliferation) without increases in TSH levels. Further, it is possible that TSH was increased but at the time points measured had returned to baseline levels.

Chemicals may alter thyroid hormone homeostasis by interfering with the synthesis and secretion of T_3 and T_4 or by increasing their metabolism. However, regardless of the mode of action, the biological response to these chemicals is a compensatory increase in TSH release, that if sustained may induce thyroid follicular cell hyperplasia that could progress to neoplasia. The data from the Friedman *et al*. (1999) study indicated that AA was associated with decreased T_3

and T_4 levels, but at doses that were much higher than the doses used in the bioassays (Friedman *et al.,* 1995; Johnson *et al.,* 1986). In the Friedman *et al*. (1999) study, there was some evidence that TSH was increased in the high-dose males after 28 days of treatment. Further, the morphometric changes in the thyroid gland reported by Khan *et al*. (1999) were suggestive of TSH stimulation of the gland. The absence of significant effects on T_3, T_4 and TSH levels after 28 days of treatment was in contrast with the findings reported for other chemicals, such as propylthiouracil, that interfere with thyroid hormone synthesis and metabolism, where marked decreases in T_3 and T_4 levels and increases in TSH levels were observed after a few days of treatment (Capen, 1996; McClain *et al.,* 1988; McClain *et al.,* 1989; O'Connor *et al.,* 1999). A number of potential MOAs were considered:

- *Disruption of thyroid peroxidase function*. AA, as with the sulfonamides that disrupt thyroid function by interfering with the thyroid peroxidase enzyme and coupling of iodine to tyrosine, has an amide functional group. It could be that the amide group is the functional group responsible for interference with the peroxidase enzyme and that AA is interfering with thyroid function at the peroxidase enzyme level. It could be that AA is a weak inhibitor of peroxidase and the coupling of iodine to the tyrosine residue, and rats treated with AA were capable of compensating for this inhibition.
- *Induction of microsomal enzymes.* Induction of the metabolism of thyroid hormones would reduce circulating levels of T3 and T4. In the Friedman *et al*. (1999) study, there was a suggestion that AA decreased T_3 and T_4 levels, which would be expected with microsomal enzyme induction. However, a number of factors indicate that this is unlikely:
    - The absolute and relative (to body weight) liver weights (increases in liver weights would be expected with induction of microsomal enzymes due to increased protein synthesis) were increased by approximately 6% and 12% in the high-dose males and 15% and 18% in the high-dose females, respectively, in the Johnson *et al*. (1986) bioassay but were not increased in the Friedman *et al*. (1995) study;
    - There is little evidence of induction of hepatic enzymes. In a study by Das *et al*. (1982), hepatic microsomal enzyme activity was statistically

significantly decreased in male Wistar rats. In contrast, El-Din *et al*. (1993) reported that hepatic S9 fractions obtained from rats that received a single intraperitoneal injection of 75 mg AA/kg were effective in the activation of known carcinogens when tested in the *Salmonella* reverse mutation assay.

  - Chemicals that induce hepatic enzymes, in addition to increasing liver weights, often result in microscopic changes in the liver. However, in the AA bioassays (Friedman *et al.,* 1995; Johnson *et al.,* 1986), there were no statistically significant dose-related microscopic liver changes reported.
  - Collectively, these differences, the absence of consistent increases in liver weights, the lack of microscopic changes in the livers of rats in the Johnson *et al*. (1986) and Friedman *et al*. (1995) bioassays and no firm evidence that AA induces microsomal enzymes, suggest that the induction of hepatic microsomal enzymes by AA and subsequent increased metabolism of thyroid hormone seems an unlikely mode of action for the production of thyroid tumors.

- *Glucuronidation.* Glucuronidation forming glucuronide conjugates of endogenous substrates, such as bilirubin and thyroid hormones, is catalyzed by UDP-GT (Parkinson, 2001). Increased glucuronidation and biliary elimination of T_4 results in disruption of the thyroid-pituitary axis and underlies the production of thyroid tumors (Kolaja *et al.,* 1999; Parkinson, 2001; Vansell and Klassen, 2001; Curran and DeGroot, 1991; McClain *et al.,* 1989). If AA acted by increasing UDP-GT activity, consequently and indirectly, increased glucuronidation of T_4 with enhanced biliary excretion of T_4 may occur. A mode of action involving enhanced biliary excretion of T_4 by way of induction of UDP-GL is operative in the rat and thought to be the basis of phenobarbital-induced thyroid tumors (Curran and DeGroot, 1991). No effect on hepatic UDP-GT activity measured in microsomal fraction was noted with administration of AA (Howland and Lowndes, 1979); however, AA was only administered for 14 days with activity measured on day 15.

- *Activation of cAMP.* There is no direct evidence that AA is capable of activating the pathway that leads to increased cAMP and subsequent thyroid follicular cell proliferation. However, there is indirect evidence that AA may activate this pathway. In a study by Abu-Jayyab *et al*. (1987), the selective dopamine D_2 receptor agonist bromocriptine (10 mg/kg via intraperitoneal injection) produced a slight decrease (3-6%) in cAMP levels in the thyroid gland of female rats *in vivo*. However, when the selective D_2 receptor antagonist sulpiride was administered (10 mg/kg via intraperitoneal injection), cAMP levels were significantly increased over control levels. These data suggest that the D_2 receptor may be involved in the control of cAMP production. This control may be a dopamine-mediated link to somatostatin production, which is involved in the regulation of TSH function, or perhaps as part of a linked pair of G protein receptors that, as indicated by Khan *et al*. (1992), would balance cAMP production. Therefore, it is possible that AA, by acting at a D-like receptor may activate adenylate cyclase. The activation of adenylate cyclase would result in an increase in the intracellular cAMP signal, which activates a cascade of biochemical events that results in increased DNA synthesis and cell proliferation. Further, activation of this pathway has been associated with increased PCNA (Dumont *et al.,* 1992; Roger *et al.,* 1997), and in rats exposed to AA, PCNA was increased (Klaunig, 2000).
- *Oxidative Stress.* Another potential mode of action is oxidative stress due to glutathione depletion in follicular cells (Chico Galdo *et al.,* 2004). AA induced morphological transformation in SHE cells after 7 days of treatment at concentrations of 0.5mM and higher and also resulted in reduction in glutathione levels (Park *et al.,* 2002). Cotreatment with AA and N-acetyl-L-cysteine (NAC), a sulfhydryl group donor, resulted in the reduction of AA-induced morphological transformation in SHE cells and the prevented reduction in glutathione levels. BSO treatment with AA enhanced the depletion of GSH. Co-treatment with ABT had no effect on transformation compared to administration of only AA. These results suggest that cellular thiol status (possibly glutathione) is involved in AA-induced morphological transformation.

Additional research is needed to determine the specific mode of action for the induction of benign follicular adenomas in the rat thyroid gland. ***While there is not clear evidence or mixed evidence of effects on thyroid hormones, it is important to note that all of these studies were short-term and not conducted in aged F344 rats. As demonstrated by Cizza et al. (1992, 1996) demonstrated that thyroid function in aged F344 rats is compromised. If AA is acting by exacerbating age-related changes in thyroid function, that action may only be manifested in older animals. Thyroid tumors were generally found in animals at terminal sacrifice and no decrease in latency was noted.***

The possible ways in which the thyroid homeostasis can be disturbed mentioned above are all likely to be processes that have thresholds, that is, a certain amount of disruption would be required before feedback loops result in sustained TSH stimulation of follicular cells resulting in the biological cascade of cell proliferation, hyperplasia, and neoplasia (USEPA - US Environmental Protection Agency, 1998). More recently, with respect to other chemicals, it has been suggested that thyroid tumors may be induced via a hormonal, non-genotoxic mechanism, even though the chemical is genotoxic (IARC - International Agency for Research on Cancer, 2001).

4.3 Relevance to Humans.

Several possible modes of action for AA's effects in rats have been considered that involve interference with thyroid hormone levels, either by interfering with production or increasing elimination. However, if AA was acting by interfering with thyroid peroxidase or by the induction of hepatic enzymes, both of which would induce an increase in TSH resulting in continued stimulation of the thyroid gland and potentially result in a carcinogenic response in the rat, there is no evidence that such a mode of action would result in a carcinogenic response in man (Alison *et al.,* 1994; Capen and Martin, 1989; Capen *et al.,* 1991; Capen, 1992, 1996; USEPA - US Environmental Protection Agency, 1998).

In an assessment of the relevance of thyroid follicular cell tumors observed in rodents and their relevance to human health, the USEPA (1998) noted that despite qualitative similarities in the control of thyroid hormone synthesis and secretion in

rodents and humans, humans were likely not as sensitive quantitatively to the development of thyroid tumors as a result of disruption of the pituitary-thyroid axis. The USEPA further noted that the presence of the high-affinity binding protein in humans was likely the reason for this quantitative difference. Although rats have binding proteins, these are low-affinity proteins, which allow the protein-bound thyroid hormone to be removed from the blood, metabolized and excreted more readily (USEPA - US Environmental Protection Agency, 1998). As a result, the half-life of T_4 is much shorter in the rat (1 day) than in the human (5 to 9 days). Consequently, the rat thyroid gland is chronically stimulated by TSH in order to compensate for the rapid turnover of thyroid hormone, and as a result, it is likely that increases in TSH would be more likely to induce growth and potentially neoplastic changes in the rat thyroid gland than in the human thyroid gland (USEPA - US Environmental Protection Agency, 1998). Moreover, thyroid follicular cell tumors induced by the chemical perturbation of the pituitary-thyroid axis, which would result in increases in TSH, were considered secondary to the effects on thyroid gland function, and represented nonlinear or threshold events (USEPA - US Environmental Protection Agency, 1998). Therefore, if in the rodent AA was acting by interfering with thyroid hormone synthesis via inhibition of thyroid peroxidase or by induction of hepatic microsomal enzymes, thereby increasing the metabolism of thyroid hormones, it is questionable whether these events would result in thyroid cancer in humans.

The third possible mode of action for AA involves the increase of cAMP levels. It is not known with certainty whether that increase would be through an adenylate cyclase-linked receptor independent of the TSH receptor (e.g., a D_1 receptor), by a heterotypic activation of the TSH receptor, or by direct stimulation of adenylate cyclase itself. The potential relevance of the activation of cAMP in humans is less clear. There are species differences in the control of the intracellular signal transduction pathways discussed above. It is important to note that any of these pathways may be directly dependent on another step in its own or other pathways (Dumont *et al.,* 1992). Consequently, stimulation of a pathway in one cell may induce factors that control these or other cells. Therefore, identical results seen in one system may have been attained though different pathways (Dumont *et al.,* 1992) and may differ among species (Raspe *et al.,* 1989). While activation of each

of these pathways may lead to a biochemical cascade through protein phosphorylations, increased protein and DNA synthesis, and ultimately cell proliferation, the relative activity of these differs among species (Dumont *et al.,* 1992). For example, cAMP is negatively controlled by norepinephrine, and the calcium-PI cascade is activated by acetylcholine through muscarinic receptors in the dog thyroid (Dumont *et al.,* 1992). Further, TSH activated the calcium-PI pathway in human thyroid cells, but not in dog or rat thyroid cells (Dumont *et al.,* 1992); however, this pathway only plays a minor role in proliferation in the rat and dog (Roger *et al.,* 1997). In human thyroid cells *in vitro*, the activation of cAMP resulted in a proliferative response, but only when accompanied by supraphysiological levels of insulin (Dumont *et al.,* 1992).

In the rat and dog thyroid cells, cAMP mediates both function/differentiation and proliferation. However, activation of function/differentiation occurs at lower TSH/cAMP concentrations than does proliferation. Essentially, concentrations of either TSH itself leading to increases in cAMP or increases in concentrations of cAMP by other means (e.g., activation of another adenylate cyclase-linked receptor) must exceed those required for normal function and growth and reach those that trigger proliferation. The rat has little buffering capacity with regard to the effects of changes in thyroid hormone levels or TSH levels. In order to maintain thyroid hormones within physiological levels, the rat thyroid gland is constantly stimulated with high levels of TSH (USEPA - US Environmental Protection Agency, 1998), i.e., the rat thyroid operates at near maximal capacity. Consequently, only small changes in cAMP levels may be required to exceed those required for normal function to those that stimulate cell proliferation. In contrast, rather than a proliferative response, human thyroid cells may respond with an increase in function or hypertrophy but, to date, no chemical has been identified in which alterations in thyroid function by a chemical agent have progressed to thyroid tumors. There are two possible explanations for potential species differences. First, in the humans, considerable buffering capacity is present, and the amount of change in TSH to move from normal function to proliferation may not be achievable given the requirements for insulin in this proliferative response in human cells *in vitro*. Secondly, it may be that *in vivo* either PKAI is not expressed in human thyroid cells or is far less

active in human cells compared to that in the rat thyroid cell. As a consequence, increased activation of PKAII would result in increased function/growth, but without activation of PKAI, a proliferative response leading to neoplasia would be absent.

The possible mode of action for AA's contribution to follicular cell tumors in the rat thyroid is not known with certainty. However, if AA were increasing circulating TSH levels or the intracellular levels of cAMP in the rat thyroid follicular cell via interaction with a receptor, this would represent a threshold or nonlinear mode of action. Further, if AA were acting through a receptor in the rat, it is possible that in the human thyroid, the receptor may not be present or could be linked to some other signal transduction pathway not associated with proliferation. In the absence of evidence that this pathway was unique to the rat and would not be operative in humans, the incidence of thyroid follicular cell tumors was considered in the quantitative assessment.

CHARGE QUESTION # 18.

Have the rationale and justification for the cancer designation for acrylamide been clearly described? Is the conclusion that acrylamide is a likely human carcinogen scientifically supportable?

ANSWER:

Emphatically no. The USEPA has undervalued the epidemiological data from several worker populations and from studies of literally tens of thousands of participants whose risk of developing one or more types of cancer was correlated with dietary intake of AA, and overvalued the animal data, in particular the value of the mouse studies. The decision to proceed with the HRF when either there is no epidemiological data or when that data are inadequate (which they are not) but positive animal data (mouse studies must be excluded in this case) are present should be conducted prior to the determination of the cancer designation. ***To determine that AA is a "likely human carcinogen" prior to examining thoroughly the MOA for the identified animal tumors (as is requested in Charter Questions 19 and 20) is inappropriate and sends the message to the reader that the classification was biased and preconceived.***

The answer to this question has three parts as discussed below.

I. Incomplete and inadequate use of the human data in the Weight of Evidence summary.

USEPA failed to consider the more recently published updates of the two occupational cohorts and one dietary study, the results of which strongly support the lack of carcinogenicity in workers exposed to acrylamide. These include:

1. <u>New Occupational Studies</u>**:**

Marsh *et al.* (2007) reported the most recent follow-up of workers in three US plants and one plant in the Netherlands, originally reported by Collins *et al*. (1989) and Marsh *et al*. (1999), adding an additional 8 years of follow-up of US workers and an additional 21 years of follow-up for the Dutch workers. No statistical increase in cancer mortality at any site, including the thyroid, testes, and CNS, was found for the period 1925 to 2002 nor was there evidence of an exposure-response relationship, even when evaluated by plant or when smoking history or time since first AA exposure were considered. T*he* authors concluded that *"acrylamide at the exposure*

levels experienced by these workers is not associated with elevated cancer mortality risks." One limitation of the study not improved by this additional follow-up is the high percentage of the cohort exposed for one year or less. The authors note that, *"on a relative exposure scale, our study included persons with the highest AMD exposures ever experienced by humans"* and further, *"short-term workers did not exhibit a differential mortality pattern often associated with increased mortality for both malignant and nonmalignant diseases."* The worker exposures were very well characterized in these cohorts. An acrylamide (AA)-exposed worker had to have been exposed to AA for greater than 0.001 mg/m3-years, the equivalent of one-day exposure at the then current OSHA permissible exposure level (PEL) of 0.3 mg/m3. Inhalation of AA at the OSHA PEL would result in estimates of AA intake in workers that is significantly greater than the estimated intake of AA from diet. **Perhaps the most notable in the update studies was conclusive the elimination of the pituitary cancer as a potential consequence of AA exposure. Any residual concern about this tumor type has been completely and totally eliminated.**

Swaen *et al.* (2007) provided an update of the worker cohort in a US plant originally examined by Sobel *et al*. (1986). The cohort size approximately doubled and had an additional 19 years of follow-up were included with 75% of the cohort exposed to AA for 10 years or more years. No statistically significant differences in cancer mortality were seen at any site that was attributed to AA even when the cohort was divided into cumulative dose or latency categories.

2. <u>The entirety of the dietary intake studies.</u>

USEPA failed to give adequate consideration to the epidemiological studies that evaluated the relationship between dietary intake and the incidence of several types of cancers and omitted other published data. As noted, Mucci *et al*., in a series of studies did not find an association between AA ingestion in foods and the incidence of a number of cancers, including mammary tumors. In their most recent study, which was not included in the USEPA draft, this group did not find an association between AA dietary exposure and the risk of colon and rectal cancers. This study used prospective data from the Swedish Mammography Cohort. The cohort was comprised of 61,467 women at baseline between 1987 and 1990. Through 2003, the cohort contributed 823,072 person-years, and 504 cases of colon and 237 of rectal cancer

occurred. After adjusting for potential confounders, there was no association between estimated acrylamide intake and colorectal cancer.

Taken together, these epidemiological studies, in particular the worker studies provide strong evidence that AA is not a human carcinogen at levels at which the general population would be exposed. AA exposure has been evaluated in US populations in workers at 4 separate work places exposed to AA at significantly higher exposures than would be expected to occur in US populations by way of dietary intake (Marsh *et al.,* 2007; Swaen *et al.,* 2007). Both studies concluded that AA was not associated with an increase in cancer mortality at any site investigated including the thyroid, testes, and CNS. Further, studies in a large population concluded that there was no increase in risk of several types of cancers including breast, ovary, or colo-rectal cancers from the ingestion of .AA in foods. ***The cancer designation should be changed to "Not expected to be a human carcinogen at low environmental or dietary exposures".***

II. The reliance on short-term mouse studies as evidence for AA's carcinogenicity in humans

The USEPA relied on data from mouse studies to conclude that AA is a carcinogen. However, these studies only provide evidence that AA may be a promoter of in extremely sensitive strains of mice and have severe limitations to generalize the potential for AA to be a human carcinogen at environmentally relevant exposure levels.

In one study, oral administration of acrylamide was associated an increased skin tumor yield in SENCAR and ICR-Swiss mice promoted with 12-*O*-tetradecanoylphorbol-13-acetate (TPA). The following should be considered:

- In both ICR-Swiss and SENCAR mice, there were no apparent dose-response relationships for skin cancer. Significant effects were noted only at high doses (total dose of 300 mg/kg), with only marginal, non-significant increases in tumor yield occurring at the lower doses
- The data in these studies were not expressed as incidence data (mouse with tumors/total number of mice), but where expressed as tumor yield data (total number of tumors/mouse). Tumor yield data are not used in the estimation of

carcinogenic potency. Further, in one of these mouse studies, no histopathological examination of the identified mass was conducted.

- In these assays, acrylamide did not elicit a response in the skin of SENCAR or Swiss mice in the absence of TPA or in the skin of BALB/c mice administered TPA, a strain of mouse relatively resistant to TPA promotion. SENCAR mice are a strain of mice bred specifically to be exceptionally sensitive to the induction of skin tumors.
- The induction of skin tumors in mice by TPA promotion occurs via a pathway that may not be operative in humans
- TPA-promoted skin tumors in mouse skin are not an appropriate model to assess the potential for skin tumors in humans and of questionable relevance in the assessment of the carcinogenic potential of acrylamide.

In the other study relied upon for their conclusion, Lung tumor yield was increased in AJ and SENCAR mice. The following should be considered:

- In A/J mice, there were dose-related increases in the incidence of lung tumors and lung tumor yield following repeated dosing with acrylamide
- Because A/J mice are highly susceptible to the formation of lung tumors, it was unclear if the finding in A/J mice were relevant to humans.
- No clear dose-response relationship was noted in the induction of lung tumors in ICR-Swiss mice
- Strain differences exists in the susceptibility to lung tumors
- The A/J mouse is more sensitive than other strains. BALB/c mice were completely resistant.

These data cannot be used to reliably predict the potential carcinogenesis of acrylamide in humans.

III. The reliance on benign tumors that do not progress to malignancy as evidence for carcinogenicity in humans.

Two chronic oncogenicity bioassays where male and female F344 rats were administered acrylamide (AA) in drinking water for up to two years have been conducted (Friedman *et al.,* 1995; Johnson *et al.,* 1986). The major findings in these

studies were significant increases in the incidence benign tumors: tunica vaginalis mesotheliomas (TVMs) in male rats, mammary gland fibroadenomas in female rats and thyroid follicular cell adenomas in male and female rats. The incidences were increased above the incidence in the respective control group and were considered related to AA treatment in rats; however, only the increased in the incidence of thyroid follicular tumors was outside of the historical control range as reported in the literature. Briefly, these results can be summarized as follows:

- ***Tunica vaginalis mesotheliomas (TVMs)*** are tumors of the mesothelial lining of the scrotum. Mesotheliomas and mesothelial hyperplasia are the most common spontaneously occurring tumors and nonneoplastic lesions, respectively, in the male F344 peritoneal cavity (Hall, 1990). Almost all of these are thought to arise from the tunica vaginalis mesothelium (Hall, 1990) and are considered by pathologists to be benign lesions that do not progress to malignancy**. These tumors should not be considered relevant to human health.**
- ***Mammary fibroadenomas*** are derived primarily from the stromal matrix (fibrous connective tissue) surrounding and supporting the epithelial ductal network in the mammary gland with, depending on the extent of involvement of adjacent epithelium, some ductular and alveolar epithelial involvement (Boorman *et al.,* 1990). Fibroadenomas are benign, endstage neoplasms; progression to or association with a malignant phenotype, such as adenocarcinoma, is rare, if it occurs at all (Boorman *et al.,* 1990). The incidence of fibroadenomas in all dose groups was within the historical control range reported for this response in female F344 rats [16% to 29% in two-year bioassays (Boorman *et al.,* 1990; Goodman *et al.,* 1979; Maekawa *et al.,* 1983; Solleveld *et al.,* 1984) and up to 57% in full life-span studies (Solleveld *et al.,* 1984)]. These tumors should not be considered relevant to human health.
- ***Thyroid follicular cell adenomas*** in male rats were significantly increased only in the high-dose (2 mg/kg/day) groups in both bioassays and the incidence in female rats was significantly increased in only the 1 and 3 mg/kg/day dose groups in the Friedman *et al*. bioassay, but was not significantly increased in female rats that received 2 mg/kg/day in any dose group in the Johnson study. The incidence of follicular cell adenocarcinoma was not increased in either bioassay in either sex in any dose group. Follicular cell tumors are not as common as other tumors in rats,

with spontaneous incidence rates ranging from 0% to 6% in control groups from other two-year or lifetime bioassays (Goodman *et al.,* 1979; Haseman *et al.,* 1998; Maekawa *et al.,* 1983; Solleveld *et al.,* 1984; Thomas and Williams 1994). The incidence of total follicular cell tumors in rats that received ≥1 mg/kg/day in one or both studies (with the exception of female rats in the Johnson study) was outside of the historical range commonly observed in this strain. The incidence of thyroid follicular cell adenomas observed in the high-dose groups of both bioassays was considered related to AA treatment.

The tumors that were increased in TVM, mammary fibroadenomas, and thyroid follicular cell adenomas are all benign tumors with only those in the thyroid having the possibility to progress to malignancy. USEPA guidance states that benign tumors should be considered on a case-by-case basis (USEPA, 2005). Those that do not progress to malignancy are not considered to be carcinogenic and should not be considered further in quantitative modeling. ***For that reason alone, if it human data were still considered inadequate, only the thyroid tumor data should have been considered to be of possible relevance to human health.***

CHARGE QUESTIONS #19:

Do you agree that weight of the available evidence supports a mode of carcinogenic action, primarily for the acrylamide epoxide, glycidamide (GA)? Has the rationale for this MOA the MOA been clearly and objectively presented, and is it reflective of the current science?

ANSWER:

No. The experimental mutagenicity data in a limited number of tests are sufficient to establish GA as a mutagen at high doses. These data, however, are not sufficient to establish a causal link between GA as the proximate carcinogenic agent acting by way of a mutagenic pathway leading to the production of the tumors that were consistently and significantly increased in F344 rats. . There are a number of issues and concerns with the USEPA analysis of the body of mutagenicity/genotoxicity data that have been discussed by Dr. Errol Zeiger and will not be discussed here.

REFERENCES

ALL REFERENCES CITED ARE FOUND IN SHIPP *ET AL.* (2006).

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)

December 2007

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.

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

Adler et al. Perturbation of cell division by acrylamide in vitro and in vivo. Mutat Res, 301, 249-254, 1993.

Adler et al. Dose response for heritable translocations induced by acrylamide in spermatids of mice. Mutat Res, 309, 285-291, 1994.

Allen et al. Dose-response modeling of in vivo genotoxicity data for use in risk assessment: some approaches illustrated by an analysis of acrylamide. Regul Toxicol Pharmacol, 41, 6-27, 2005.

Backer et al. The effects of acrylamide on mouse germ-line and somatic cell chromosomes. Environ Mol Mutagen, 13, 218-226, 1989.

Besaratinia and Pfeifer. Weak yet distinct mutagenicity of acrylamide in mammalian cells. J Natl Cancer Inst, 95, 889-896, 2003.

Besaratinia and Pfeifer. Genotoxicity of acrylamide and glycidamide. J Natl Cancer Inst, 96, 1023-1029, 2004

* Clement et al. Expression profile of human cells in culture exposed to glycidamide, a reactive metabolite of the heat-induced food carcinogen acrylamide. Toxicology, 240, 111-124, 2007.

Collins et al. Kinetochore-staining of spermatid micronuclei: Studies of mice treated with X-radiation or acrylamide. Mutat Res, 281, 287-294, 1992.

Dearfield et al. Acrylamide: A review of its genotoxicity and an assessment of heritable genetic risk. Mutat Res, 330, 71-79. 1995.

Gassner and Adler. Induction of hypoploidy and cell cycle delay by acrylamide in somatic and germinal cells of male mice. Mutat Res, 367, 195-202, 1996.

* Hasegawa et al. Acrylamide-responsive genes in the nematode *Caenorhabditis elegans*. Toxicol Sci, 101, 215-225, 2008.

ICPEMC (International Commission for Protection Against Environmental Mutagens and Carcinogens). A Method for Combining and Comparing Short-Term Genotoxicity Test Data. A Report from ICPEMC Committee 1. Mutat Res, 266(1), 1992. [entire volume]

* Jiang et al. Genotoxicity of acrylamide in human hepatoma G2 (HepG2) cells. Toxicol in Vitro, 21, 1486-1492. 2007.

* Koyama et al. Genotoxicity of acrylamide and glycidamide in human lymphoblastoid TK6 cells. Mutat Res, 603, 151-158, 2006.

* Maniere et al. DNA damage and DNA adduct formation in rat tissues following oral administration of acrylamide. Mutat Res, 580, 119-129, 2005.

Manjanatha et al. Genotoxicity of acrylamide and its metabolite glycidamide administered in drinking water to male and female Big Blue mice. Environ Mol Mutagen, 47, 6-17, 2006

* Mei et al. Genotoxic effects of acrylamide and glycidamide in mouse lymphoma cells. Food Chem Toxicol, 46, 628-636, 2008.

* Schriever-Schwemmer et al. Extruded micronuclei induced by colchicine or acrylamide contain mostly lagging chromosomes identified in paintbrush smears by minor and major mouse DNA probes. Mutagenesis, 12, 201-207, 1997.

Sega et al. Acrylamide binding to the DNA and protamine of spermiogenic stages in the mouse and its relationship to genetic damage. Mutat Res, 216, 221-230, 1989.

Shelby et al. Acrylamide: Induction of heritable translocations in male mice. Environ Mutagen, 9, 363-368, 1987.

Shirashi. Chromosome aberrations induced by monomeric acrylamide in bone marrow and germ cells of mice. Mutat Res, 57, 313-324, 1978.

* Sickles et al. Acrylamide arrests mitosis and prevents chromosome migration in the absence of changes in spindle microtubules. J Toxicol Environ Health, 44, 73-86, 1995.

Sickles et al. Direct effect of the neurotoxicant acrylamide on kinesin-based microtubule motility. J Neurosci Res, 46, 7-17, 1996.

* Sickles et al. Acrylamide effects on kinesin-related proteins of the mitotic/meiotic spindle. Toxicol Appl Pharmacol, 222, 111-121, 2007.

* Thielen et al. Genotoxicity of glycidamide in comparison to (±)-anti-benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide and α-acetoxy-*N*-nitroso-diethanolamine in human blood and mammalian V79-cells. Mol Nutr Food Res, 50, 430-436, 2006.

* U.S. EPA. *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; external peer review draft] September 2007.

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DETERMINATION OF HEMOGLOBIN ADDUCTS FOLLOWING ACRYLAMIDE EXPOSURE

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NOTICE

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ABSTRACT

The present project was undertaken to develop new methodologies for biological monitoring of exposure to the toxicant acrylamide in laboratory animals as well as humans. Methods were developed to measure the adducts of acrylamide and its epoxide metabolite glycidamide to cysteine in rat hemoglobin and to valine in human hemoglobin by means of gas chromatography/mass spectrometry.

Studies in rats indicated that both acrylamide and glycidamide adducts are formed following acute or chronic exposure to acrylamide, while only the glycidamide adducts are formed after exposure to glycidamide. Both adducts, in addition to acrylonitrile adducts, were measured in a group of Chinese workers exposed to acrylamide during its synthesis and polymerization. Significant signs of neurotoxicity were also found in this population.

Additional studies in rats indicated that acrylamide, but not glycidamide, is the proximate neurotoxicant while glycidamide may be responsible for the male reproductive and genotoxic/carcinogenic effects of acrylamide.

These studies suggest that these novel biomarkers to assess exposure to acrylamide are useful to assess potential health hazards (including possible cancer risks) due to exposure to acrylamide in occupationally exposed workers as well as in the general population.

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ABBREVIATIONS

AA	Acrylamide
AACys	S-(2-carbamoylethyl)cysteine
AAVal	N-(2-carbamoylethyl)valine
ANOVA	Analysis of Variance
ANVal	N-(2-cyanoethyl)valine
Bq	Bequerel
CEVal	N-(2-carboxyethyl)valine
CHEVal	N-(2-carboxy-2-hydroxyethyl)valine
EL_{50}	Effect Level, 50%
EI	Electron impact
EMG	Electromyography
ENMG	Electroneuromyography
eV	Electronvolt
FAB-MS	Fast Atom Bombardment Mass Spectrometry
GA	Glycidamide
GACys	S-(2-carbamoyl-2-hydroxyethyl)cysteine
GAVal	N-(2-carbamoyl-2-hydroxyethyl)valine
GC/MS	Gas Chromatography/Mass Spectrometry
Hb	Hemoglobin
HFB	Heptafluorobutyryl
HFBA	Heptafluorobutanoic Anhydride
HOEtVal	Hydroxylethyl Valine
HPLC	High Pressure Liquid Chromatography
LOEL	Lowest Observed Effect Level
MCV	Motor Nerve Conduction Velocity
MeOH	Methanol
NIn	Neurotoxicity Index
NMR	Nuclear Magnetic Resonance
NOEL	No Observed Effect Level
PCI	Positive Chemical Ionization
PFPITC	Pentafluorophenyl isothiocyanate
PFPTH	Pentafluorophenylthiohydantoin
SEM	Standard Error of the Mean
SCV	Sensory Nerve Conduction Velocity
TLC	Thin-Layer Chromatography
TLV	Threshold Limit Value
TWA	Time-Weighted Average
VU	Vibration Units

INTRODUCTION

Acrylamide

At room temperature acrylamide is a white crystalline solid with a high solubility in water. The high reactivity of its olefinic bond versus nucleophilic agents in Michael type additions is derived from its conjugation with an amide group. Industrially, acrylamide is produced by catalytic hydration of acrylonitrile and used mainly in the production of polymers such as polyacrylamide.

Humans are potentially exposed to acrylamide in industrial processes, grouting operations, synthesis of chromatography gels and leakage of the monomer from polyacrylamide used in the purification of drinking water. According to a survey undertaken by NIOSH, some 10,000 workers in 27 occupations are potentially exposed to acrylamide, including about one thousand persons involved in the synthesis of polyacrylamides and another thousand licensed to perform grouting operations. The number of laboratory workers exposed to acrylamide in the preparation of chromatography gels has been estimated to be as high as 100,000-200,000.

Since the early fifties the neurotoxic effects, involving both the central and the peripheral nervous system, have been the primary health concern of human exposure to acrylamide; close to 150 cases of human intoxication have been reported in the last 30 years (He et al. 1989). These reports have been paralleled by extensive studies in experimental animals, notably aiming at the elucidation of the mechanism of neurotoxic action of acrylamide (Miller and Spencer, 1985; Tilson, 1981). In more recent years, following the demonstration that acrylamide induces tumors in mice and rats, attention has been increasingly focused on the genotoxic and reproductive effects of the compound (Dearfield et al. 1988). Thus, acrylamide has been shown to be mutagenic *in vitro* in mouse lymphoma cells, and to give rise to heritable translocations and dominant lethal mutations in rodents. Unexpectedly for a compound producing these types of effects, acrylamide has consistently given a negative response in the Ames/*Salmonella* assay both in the absence and in the presence of an S-9 fraction.

Despite the fact that acrylamide has been shown to induce this wide range of adverse effects in mammalian species, few studies have investigated the role of metabolism in the toxicity of this compound and although reports have appeared indicating that acrylamide may undergo further metabolism, the structures of the intermediates generated have not been elucidated. From the point of view of quantitative risk assessment of human exposure this has represented a serious gap of knowledge, since the extrapolation of toxic effects in animals requires not only the identification of the agent(s) responsible for the induction of the effects, but, ideally, also information about the relationship between exposure dose and *in vivo* dose (defined as the time-integrated concentration of free electrophilic agent *in vivo*). In addition, since exposure to acrylamide may occur through several different routes, the total amount absorbed by a person may be very difficult to assess.

Determination of hemoglobin adducts for exposure assessment

The structural elucidation and quantitative determination of hemoglobin (Hb) adducts can be used to obtain this type of information. For example, in response to long-standing speculation about the possible involvement of a cytochrome P-450-generated agent in the toxicity of acrylamide, we were recently able to demonstrate the formation *in vitro* and *in vivo* in rats of glycidamide, an epoxide formed by oxidation of the olefinic bond of acrylamide, through the identification of S-(2-carboxy-2-hydroxyethyl)cysteine (**Fig. 1**) in hydrolyzed Hb samples from acrylamide exposed rats (Calleman et al. 1990).

Determination of Hb adducts as a way of assessing the relationship between environmental concentrations and *in vivo* dose, as well as for the dosimetry-based risk estimation of toxic effects, has been undertaken for several types of alkylating agents in humans. The advantages of such measurements are:

- Hb adducts are stable during the 4-month life span of the human erythrocyte, meaning that *in vivo* doses are integrated over a long and well-defined period of time.
- Large quantities of Hb are easily available from exposed animals and humans allowing for detection of low levels of adducts, e.g. by gas chromatography-mass spectrometry.
- Differences between species and individuals with regard to rates of uptake, metabolic activation and deactivation of electrophiles, as well as induction status of enzymes, are reflected in the levels of Hb adducts formed *in vivo*, thus providing a basis for extrapolation between species and dosimetry-based risk estimation.
- The relationship between dose in red blood cells and in target organs can be studied in experimental animals, and extrapolated to humans if due consideration is given to reaction kinetic and physicochemical parameters of the electrophile.

A method for the determination of Hb adducts of the parent compound acrylamide has been developed by Bailey et al. (1986) based on the liberation of S-(2-carboxyethyl)cysteine (**Fig. 1**) upon acid hydrolysis of Hb from acrylamide treated rats. Since, however, we are hypothesizing that glycidamide might be playing a crucial role in the induction of some of the toxic effects associated with acrylamide exposure, there was a need for the development of quantitative methods which would be applicable to both animals and humans and that could be used for the monitoring of both the adduct formed by acrylamide and by its epoxide metabolite.

AIMS

It was the **central aim** of this project to develop and apply methods for simultaneously determining the adducts formed by acrylamide and glycidamide in Hb as a means of assessing occupational exposure to acrylamide and to use these data in combination with dosimetric and metabolic studies in experimental animals undergoing toxicological tests to arrive at a risk estimation of human acrylamide exposure which may serve as a basis for regulatory action.

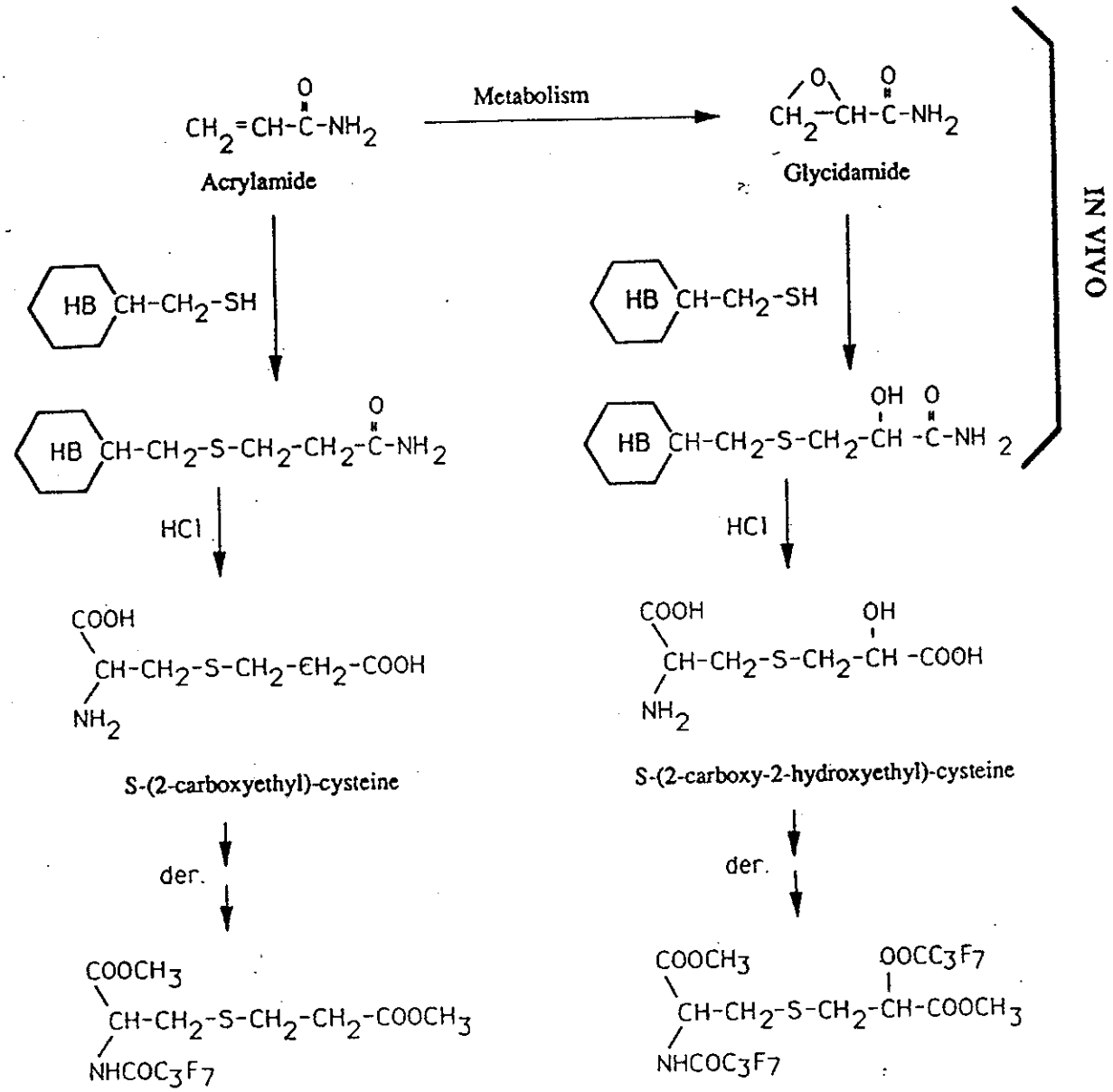

Figure 1. The reactions of AA and GA with cysteine residues in Hb. The products released during acid hydrolysis are *S*-(2-carboxyethyl)-cysteine and *S*-(2-carboxy-2-hydroxyethyl)-cysteine. The structures of the derivatized products are also shown.

In addition to the practical implications of the proposed methods for biomonitoring, acrylamide appears to be a suitable compound for the theoretical development of a risk model based on *in vivo* dosimetry because of the wide range of toxic effects it is known to induce. Since both acrylamide and glycidamide are reactive electrophilic compounds, it was not clear whether the various toxic effects associated with acrylamide exposure are induced by the parent compound, the epoxide, or the combined action of the two agents. Our finding that acrylamide was metabolized to glycidamide thus generated a few hypothetical solutions to theoretical problems particular to the toxicology of acrylamide.

The **specific aims** of this project were, therefore:

1. To develop a method for measuring the adducts of acrylamide and glycidamide to hemoglobin in rats and to determine both adducts following administration of acrylamide or glycidamide.

2. To develop a method for measuring the adducts of acrylamide and glycidamide in humans, measure adducts in workers occupationally exposed to acrylamide, and correlate adduct levels with neurotoxicity.

3. To evaluate and compare the neurotoxicity and reproductive toxicity of acrylamide and glycidamide in the rat.

METHODS

AIM 1

Synthesis of deuterated analogues

S-(2-Carbamoylethyl)-3,-3-d_2-cysteine was prepared in analogy with the non-deuterated compound (AACys), the synthesis of which was described in Calleman et al. (1990). Following derivatization with MeOH/HCl and HFBA (Calleman et al., 1978) the mass spectrum obtained upon electron impact ionization gave the fragments m/z = 419 and 205 interpreted as $[M]^+$ and $[M - C_3F_7CONHD]^+$, respectively, and the mass spectrum obtained when scanning for negative ions using methane as the reagent gas gave m/z = 418 $[M - H]^-$. Both spectra were in analogy with the nondeuterated compound. TLC, amino acid analysis, and GC/MS revealed an impurity consisting of ≈ 5% cystine.

S-(2-Carbamoyl-2-hydroxyethyl)-3,3-d_2-cysteine was prepared as described in Calleman et al. (1990) for the nondeuterated analogue (GACys). Following derivatization, the mass spectrum obtained upon electron impact ionization gave the fragments m/z = 572 $[M - CO_2CH_3]^+$, 417 $[M - C_3F_7CONHD]^+$, and 203 $[M - C_3F_7CO_2H - C_3F_7CONHD]^+$. Chemical ionization using methane as the reagent gas gave the positive ions m/z = 418 $[M - C_3F_7CONHD + H]^+$ and 632 $[M + H]^+$ and the negative ion 611 $[M - HF]^-$. The

spectra were in analogy with the nondeuterated compound. TLC, amino acid analysis, and GC/MS revealed cystine as an impurity of ≈ 5%. On amino acid analysis, three peaks were evident: two peaks, presumably the diastereoisomers (S)- and (R)-S-(2-carbamoyl-2-hydroxyethyl)-3,3-d_2-*dl*-cysteine (whose presence in approximately equal amounts was indicated by proton NMR spectroscopy), merging into one major peak corresponding to 90%; and a minor peak, possibly the regioisomer S-(1-carbamoyl-2-hydroxyethyl)-3,3-d_2-cysteine, corresponding to 10%.

The nondeuterated analogue gave the same pattern of peaks but did not contain any cystine. On GC/MS, the isomers were not resolved on the capillary column that was utilized.

Treatment of animals

Male Sprague-Dawley rats (130-260 g) were obtained from Tyler laboratories and kept on a 12-hr light/dark cycle with free access to food and water. AA (Eastman-Kodak, Rochester, NY) or GA (Polysciences, Inc., Warrington, PA) were dissolved in 0.9% NaCl and administered in a volume of 1 ml/kg body wt by ip injection. Control animals were given vehicle only. Blood was drawn into a heparinized syringe by cardiac puncture under ether anesthesia.

Groups of three rats were injected with AA (0, 0.5, 1.0, 5.0, 10.0, 50.0, and 100.0 mg/kg body wt) and blood was drawn 24 hr after the injection. In an additional experiment, a cumulative dose of 100 mg/kg body wt AA was given in different regimens to groups of four rats. The groups received a single injection of 100 mg/kg, 10 daily injections of 10 mg/kg, or 30 daily injections of 3.3 mg/kg. Blood was drawn 24 hours after the final injection.

Isolation of globin and analysis of hemoglobin adducts

Erythrocytes and plasma were separated by centrifugation of blood samples. The red blood cells were washed three times with 0.9% NaCl and lysed by addition of 1 vol of water. Cell membranes and debris were removed by centrifugation at 20,000g for 30 min. The supernatant was added to 10 vol of 1% HCl in acetone and the resulting precipitate was washed twice with acetone and once with ether and then air-dried.

Samples of 30 mg of globin were dissolved in 6 M HCl to a concentration of 10 mg globin/ml. The deuterated analogues, serving as internal standards for the quantitation, were added to the hydrochloric acid to give concentrations of 10 μmol/g Hb. The samples were hydrolyzed for 15 hr at 120°C in sealed Pyrex glass ampoules under vacuum ($<50 \times 10^{-3}$ mm Hg). The hydrolysates were evaporated to dryness, dissolved in 1.5 ml of H_2O), and incubated for 1 hr at 37°C. After adjustment of the pH to 9 with NaOH, the samples were applied to Dowex 1 x 8 (100-200 mesh, 1 x 15 cm, $HCOO^-$ -form) anion-exchange columns. The columns were washed with 80 ml of 5mM formic acid to remove most of the amino acids upon which the adducts were eluted with 60 ml of 100 mM formic acid.

The 100 mM eluates were evaporated and derivatized with MeOH/HCl and HFBA. Derivatized samples were dissolved in 0.5-1.0 ml of ethyl acetate before quantitation by GC/MS.

Gas chromatography/mass spectrometry analysis

The analyses were carried out using a Hewlett-Packard 5890A gas chromatograph linked to a Finnigan 4023 mass spectrometer. The operating conditions for the gas chromatograph were as follows: helium carrier gas at a constant pressure of 15 psi: temperature programmed from 1 min at 70°C, 15°C/min to 230°C, 5°C/min to 240°C, 20°C/min to 280°C, and holding for 1 min. A 30-m DB-5 (0.25 mm i.d., 0.25-μm phase thickness) fused silica capillary column was used. The mass spectrometer was operated in the electron impact mode at an ion source temperature of 250°C and an ionization energy of 70 eV. Chemical ionization mass spectra for the derivatized synthetical adducts were obtained with methane as the reagent gas at an ion source pressure of 0.30 Torr and a temperature of 220°C. Both positive and negative ion spectra were obtained at an ionization energy of 70 eV. For the analysis of the rat Hb samples, 1 μl of the ethyl acetate solutions was injected directly onto an on-column injector. AACys and its deuterated analogue were monitored at m/z = 202 and 203, $[M - C_3F_7CONH_2(HD)]^+$, respectively, and GACys and its deuterated analogue were monitored at m/z = 202 and 203, $[M - C_3F_7CO_2H - C_3F_7CONH_2(HD)]^+$, respectively. The retention time for AACys was 719 sec and that for GACys was 730 sec. Quantitation was based on the ratio between peak areas of the analyte and the internal standard. Correction for isotopic contributions and nonlinear response (at ratios <0.1) was made with a calibration curve prepared by mixing triplicates of seven rations between zero and one of known amounts of pure nondeuterated standard and the same amount of deuterated standards as added in the rat globin samples.

Alkylation of hemoglobin in vitro

The rates of reaction of AA or GA with Hb were determined in blood from nonexposed rats. Erythrocytes were separated from plasma by centrifugation, washed with saline, resuspended to the original blood volume using phosphate-buffered saline (PBS), pH 7.4, and incubated with either AA (1 mM) or GA (2 mM) at 37°C. Aliquots were taken from the incubation mixture at different times (5, 10, 15, 20, 40, and 60 min) and the reaction was terminated by adding 5 vol of ice-cold saline followed by centrifugation. The cells were washed twice with saline and lysed by the addition of water. Globin was isolated and the adduct levels were analyzed as described above.

AIM 2

Synthesis of alkylated valines

N-(-Carbamoylethyl)-L-valine (AAVal) was prepared by dissolving 1.76 g (15 mmole)

L-valine in 30 ml H_20 and 2.6 ml (18.6 mmole) triethylamine and adding 10 g (141 mmole) AA. The pH of the solution was ≈ 10. After 6 days at room temperature, the product was precipitated with acetone and recrystallized in H_2O/ethanol. The product (yield ≈ 32%) was judged to be pure based on TLC, amino acid analysis, ^{1}H-NMR and FAB-MS. The R_f-value on TLC silica plate, eluted with 2-propanol/H_2O 7:3, was 0.65. ^{1}H-NMR indicated δ 0.95 (6H, dd, valγ), 2.15 (1H, m, valβ), 2.65 (2H, 5, $CH_2CH_2CONH_2$), 3.2(2H, m, $NHCH_2CH_2$), 3.4 (1H, d, valα). FAB-MS gave the ion m/z = 189 $[M+H]^+$.

AAVal was derivatized with methanol/HCl and HFBA according to Calleman et al. (1978) and analyzed by GC/MS. The electron impact ionization spectrum gave fragments m/z = 381 $[M]^+$, m/z = 308 $[M-CH_2COOCH_3]^+$ and m/z = 212 $[M-C_3F_7]^+$ and chemical ionization with methane as the reagent gas gave the positive ions m/z = 382, 410 and 422 corresponding to $[M+1]^+$, $[M+29]^+$ and $[M+41]^+$. The spectrum was consistent with Structure A in **Fig. 2**.

AAVal was also derivatized with PFPITC according to Mowrer et al. (1986). On GC/MS, the pentafluorophenylthiohydantoin (PFPTH) gave m/z = 395 $[M]^+$ upon electron impact ionization. Chemical ionization yielded the positive ions m/z = 396, 424 and 436 ($[M+1]^+$, $[M+29]^+$ and $[M+41]^+$) and the negative ion m/z = 375 $[M-HF]^-$. See **Fig. 2**, structure B.

N-(2-Carbamoyl-2-hydroxyethyl)-L-valine (GAVal) was prepared in the same way as described for AAVal, but instead of AA, 3.74 g (42.9 mmole) GA was added. The yield was ≈ 44%. The product was judged to be pure by TLC, amino acid analysis, ^{1}H-NMR and FAB-MS. The R_f-value on TLC was 0.77 in the same system as for AAVal. ^{1}H-NMR indicated δ 0.95(6H, m, valγ), 2.2(1H, valβ), 3.25(2H, m, $NHCH_2CHOC$), 3.5 (1H, dd, valα), 4.4 (1H, m, $CH_2CHOHCONH_2$). FAB-MS gave the ion m/z = 205 $[M+H]^+$.

The product was derivatized with methanol/HCl and HFBA, and run on GC/MS. Five GC-peaks were evident in the approximate ratios 2:1:3:0.7:0.7. Upon electron impact ionization, peak number 2 gave the fragments m/z = 593 $[M]^+$, 242 $[M-C_3F_7]^+$, 308 $[M-CH(OOCC_3F_7)COOCH_3]^+$ and 299 $[CH_2CH(OOCC_3F7)COOCH_3]^+$.

Chemical ionization with methane as the reagent gas gave the positive ions m/z = 594, 622, 634 ($[M+1]^+$, $[M+29]^+$ and $[M+41]^+$). The structure of the derivative was consistent with C in **Fig. 2**. Peaks number 1, 3, 4 and 5 were interpreted as stereoisomers of structure E in **Fig. 2**.

Derivatization with PFPITC gave the PFPTH-derivative, structure D in **Fig. 2**, which on GC/MS gave the electron impact spectrum with a fragment at m/z = 411 $[M]^+$ while chemical ionization gave the positive ions m/z = 412, 440, 452 ($[M+1]^+$, $[M+29]^+$ and $[M+41]^+$) and the negative ion m/z = 391 $[M-HF]^-$.

N-(Carbamoylethyl)-d_8-L-valine (d_8-AAVal) and N-(2-carbamoyl-2-hydroxyethyl)-d_8-L-

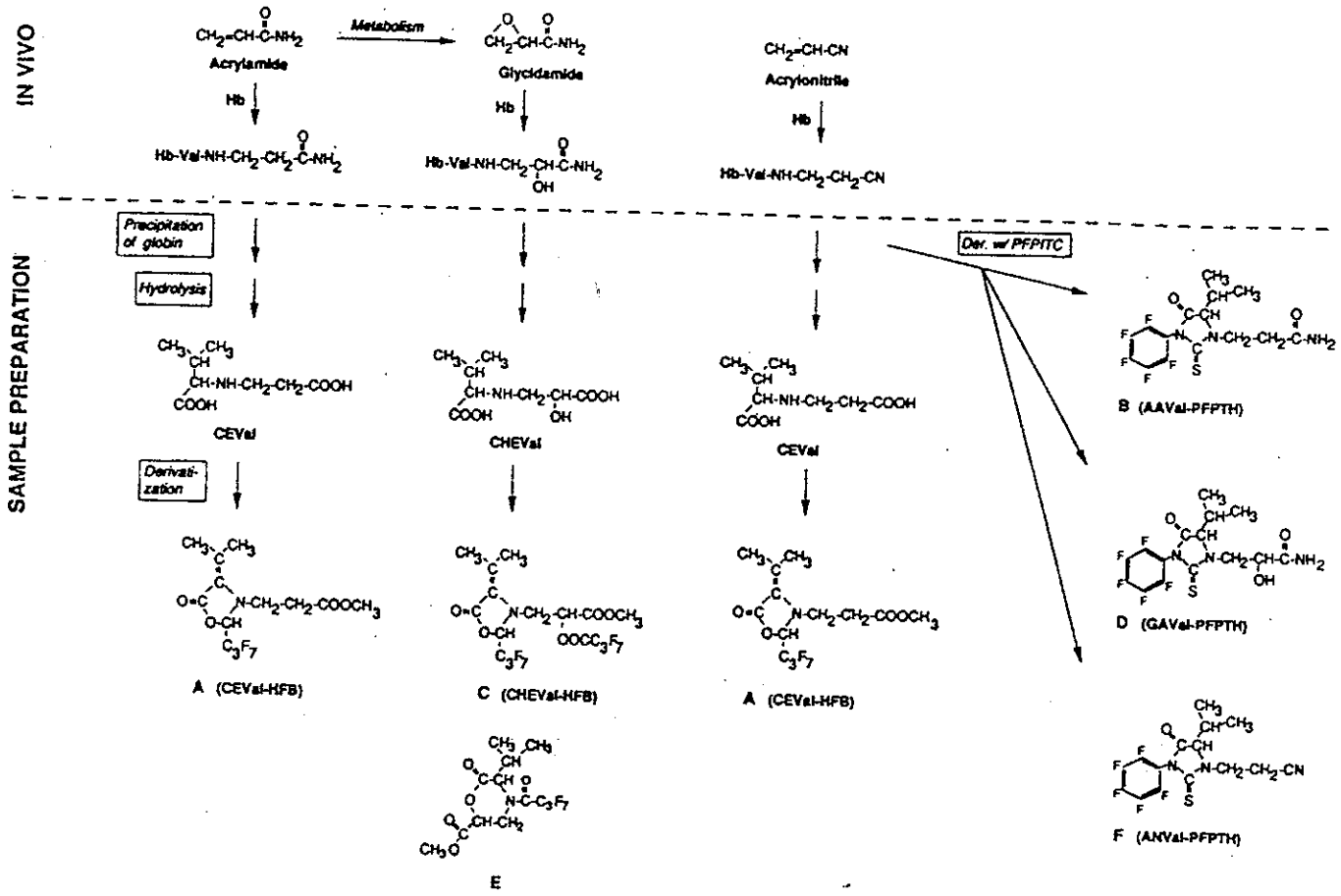

Figure 2. The reaction of acrylamide (AA), glycidamide (GA), and acrylonitrile (AN) with the N-terminal valine of hemoglobin *in vivo*. Following acid hydrolysis of globin, the reaction products of AA and AN with valine are released as CEVal, and the reaction product of GA with valine is released as CHEVal. Compounds A (CEVal-HFB), C(CHEVal-HFB), and E are formed after derivatization with methanol/HCl and HFBA. Following derivatization of globin with PFPITC, the compounds formed are B (AAVal-PFPTH), D (GAVal-PFPTH), and F(ANVal-PFPTH).

valine (d_8-GAVal) were prepared as the non-deuterated analogues (AAVal and GAVal), but with d_8-L-valine as starting material. The syntheses were made on a smaller scale, with a yield of 54% for d_8-AAVal and 38% for d_8-GAVal. The compounds were pure as judged by TLC. The mass spectra after derivatization with methanol/HCl and HFBA were in analogy with the non-deuterated compounds.

NMR spectroscopy and fast atom bombardment mass spectrometry (FAB-MS)

Proton NMR spectra were recorded as described in Calleman et al. (1990) and FAB mass spectra were acquired as described in Aim 1.

Analysis of Hb adducts in globin hydrolysates

Globin was isolated from whole blood as described previously. Samples of 100 mg of globin were dissolved in 6 M HCl to a concentration of 10 mg/ml. The deuterated reference compounds (d_8-AAVal, d_8-GAVal, d_2-AACys and d_2-GACys), serving as internal standards for the quantitation, were added. The concentrations of standards were 1 μmol/g Hb for the *in vitro*-treated Hb and 100 nmol/g Hb for human samples. The samples were hydrolyzed for 15 h at 120°C under vacuum. The hydrolysates were evaporated to dryness, dissolved in 2 ml H_20 and incubated for 1 hr at 37°C. The pH was adjusted to 9 with NaOH and the samples were applied to Dowex 1 x 8 anion exchange columns (1 x 30 cm, $HCOO^-$ -form, 100-200 mesh). The columns were eluted with 150 ml 12 mM formic acid followed by 60 ml 30 mM formic acid, 65 ml 60 mM formic acid and 100 ml 120 mM formic acid. The adduct-containing fractions (see Results) were evaporated and derivatized with methanol/HCl and HFBA. Derivatized samples were dissolved in 100 μl ethyl acetate from which 2 μl were injected for quantitation by GC-MS.

Analysis of hemoglobin adducts with the modified Edman degradation method

Samples of 50 mg of globin were derivatized according to Törnqvist et al. (1988). The amount of PFPITC reagent was increased from 7 to 10 μl. Globin alkylated with d_4-ethylene oxide was used as an internal standard, and was added to give a concentration of 10 nmol/g Hb of N-(d4-2-hydroxyethyl)valine (d_2-HOEtVal). The derivatized samples were dissolved in 50 μl toluene and 1 μl was injected for quantitative analysis by GC-MS.

Gas chromatography-mass spectrometry analysis

The analyses were carried out using a Varian 3400 gas chromatograph linked to a Finnigan 4500 mass spectrometer. The operating conditions for the gas chromatograph were as follows: helium carrier gas at constant pressure of 10 psi; temperature programming 100°C - 1 min - 20°/min - 240° - 10°/min - 320° - 2 min for PFPTH-derivatives. A 30 m DB-5 (0.32 mm i.d., 1 μm phase thickness) fused silica capillary column was used.

The mass spectrometer was operated in the electron impact mode at an ion source

temperature of 140°C and an ionization energy of 70 eV. In the chemical ionization mode, methane was used as the reagent gas at an ion source pressure of 0.40 Torr.

The HFB-derivatives of the valine adducts were analyzed in the positive ion chemical ionization mode. The derivative of CEVal and its deuterated analogue were monitored at m/z = 382 and 388 $[M+1]^+$, respectively, and the derivative of CHEVal and its deuterated analogue were monitored at m/z 622 and 628, $[M+29]^+$, respectively. The retention times for the derivatives of CEVal and CHEVal were 575 and 580 seconds, respectively. Quantitation was based on the ratio between peak areas of the analyte and the deuterated internal standard. The contribution of the deuterated internal standards of the valine adducts to the ions monitored for the non-deuterated compounds was 0.1%. The HFB-derivatives of cysteine adducts were analyzed in the electron impact ionization mode, as described before.

The PFPTH-derivatives were analyzed in the negative ion chemical ionization mode. AAVal-PFPTH was monitored at m/z = 375 $[M-HF]^-$ and d_4-HOEtVal-PFPTH at m/z = 352 $[M-HF]^-$. In addition to the valine adduct of AA, N-(2-cyanoethyl)valine-PFPTH (ANVal-PFPTH, **Fig. 2** structure F), the valine adduct of AN, was monitored at m/z = 274 $[M-103]^-$. The retention times for the PFPTH-derivatives of AAVal, ANVal and d_4-HOEtVal were 826, 701 and 692 seconds, respectively. The quantitation was based on the ratio between peak areas of the analytes and d_4-HOEtVal.

Alkylation of hemoglobin in vitro

The rates of reaction of AA or GA with N-terminal valine in human Hb were determined in blood from a non-exposed person, as described earlier. The incubation was made with AA (380 mM) or GA (100 mM) and aliquots were taken at different times (0-40 min.). The adduct levels were determined in hydrolyzed globin samples as described above.

Calibration

The analyses of PFPTH-derivatives were calibrated as follows: A calibration curve was prepared by addition of different amounts of *in vitro* alkylated (with AA or AN) globins, to 50 mg globin samples from a non-exposed person. The alkylation levels were 5 - 80 nmol/g Hb. d_4-Ethylene oxide alkylated globin was added to give 10 nmol d_4-HOEtVal/g Hb. The calibration curve for AA was slightly concave and for AN linear in the range studied. Calibration samples were run every day when unknown samples were analyzed in order to compensate for variations in sensitivity of the mass spectrometer.

The adduct levels of the *in vitro* alkylated globins were determined after acid hydrolysis and ion-exchange chromatography by the procedure described above. For the AA-globin, d_8-AAVal was added as internal standard. For the AN-globin, which was alkylated with ^{14}C-AN (168 Bq/nmol), the radioactivity in the peak corresponding to CEVal was counted following ion-exchange chromatography.

Field study in the People's Republic of China

Physical description of the factory

The acrylamide shop selected for this study was part of a large chemical factory employing 700 individuals in the city of Xinxiang, Hunan Province, China. The production of acrylamide, which had started in 1982, was operated in three shifts and amounted to 1,000 tons/year. The acrylamide production line operated as follows: acrylonitrile (bought from an external supplier) was converted to acrylamide (20% solution) by catalytic hydration with metal catalysts. The latter solution was then concentrated to give a 35% acrylamide solution, which was purified by cation and anion exchange. This solution is stored in large vessels and then transferred to smaller barrels: all these processes are performed in the same room, the "synthesis room." Barrels are then moved to an adjacent room, the "polymerization room," where acrylamide is mixed with acrylic acid, starch (35% of final product) and sodium allylsulfonate to generate a polyacrylamide co-polymer. After grinding and drying, the co-polymer is then bagged in another adjacent building, "the packaging unit." The use of the copolymer is primarily in the oil industry to fill the drilling tube and decrease friction and to harden soil and allow extraction of compact samples.

Air monitoring

The air concentrations of acrylamide were monitored twice during the summer months and twice in the month of September of 1991 when the study was undertaken. Each time several determinations were made. Acrylamide was sampled for 10 minutes (flow rate 3 l/min) by station sampling and determined by gas chromatography with a FFAP column and an electron capture detector following bromination. The detection limit of this method is 0.5 ng of acrylamide, equivalent to an air concentration of 0.03 mg/m^3 under the testing and sampling conditions used. The results of these analyses are shown in **Table 1a**. In the summer the air concentrations ranged from 0.3-8.8 mg/m^3 with an extreme value of 153.1 during the discharge of acrylamide (not included in **Table 1a**), whereas during the month of September when specimens for the determination of biomarkers were collected, a relatively narrow range of air concentrations of 0.11-1.64 was determined. Air levels of acrylonitrile were also monitored in June 1991 using a charcoal sampler and gas chromatography analysis (**Table 1b**).

Description of study populations

A total of 41 workers, 34 males and 7 females, occupationally exposed to acrylamide while working in the processes of synthetization, condensation, filling, polymerization, and maintenance, etc. were selected for the study. Depending on the primary location where

Table 1

1a. Air concentrations of acrylamide (mean ± SD, mg/m^3).

	Polymerization room	Synthesis
Summer	5.95 ± 2.55 (n = 6)	1.65 ± 0.89 (n = 8)
Autumn	0.58 ± 0.21 (n = 6)	0.61 ± 0.55 (n = 10)
Average	3.27 ± 3.29 (n = 12)	1.07 ± 0.88 (n = 18)

1b. Air concentrations of acrylonitrile (mean ± SD, mg/m^3)

	Polymerization room	Synthesis room
Summer	8.4 ± 3.7 (n = 6)	44.7 ± 37.9 (n = 6)

these workers performed their work, they were classified into synthesis workers, polymerization workers, packaging workers and ambulatory workers which included foremen, repairmen, etc. Two individuals who had only minimal exposure to acrylamide during the preceding 4- month period and two others who had been employed in the workshop for less than half a year, and thus were not expected to have had time to develop neurotoxic symptoms, were excluded from the regression analysis. The ages of the workers ranged between 18 and 42 years (mean 27 years). The exposure duration was 3 years on average with the shortest being one month and the longest 9 years. Ten workers had been exposed to acrylamide for more than 5 years. Their personal protection measures included gauze masks, cotton clothes, rubber gloves and boots.

The workers were brought into the hospital ward approximately 1 hr after they had started the morning shift. Blood was collected immediately upon arrival and the workers remained in the ward for medical examination and collection of urine for a 24-hr period.

For the Vibratron measurements the reference group consisted of 105 unexposed and healthy adults (51 males and 54 females ages 20-60 years) including mechanical workers and office personnel and for the ENMG a historical control group of 80 persons was used. Persons with diabetes and nervous diseases were excluded from both groups. For the biomarkers of exposure and signs and symptoms of neurotoxicity, ten unexposed individuals from the same city as the exposed group were studied.

Determination of mercapturic acids in urine

Urine samples were collected during 24 hrs and the volumes and specific gravities were determined. 1 ml of these samples was hydrolyzed with 1 ml of 6N HCl at 85°C for 4 hr. Under these conditions the N-acetyl group of the mercapturic acid as well as the amide group was hydrolyzed to give S-(2-carboxyethyl)cysteine. After centrifugation at 3,000g for 10 min, 0.1 ml of supernatant hydrolysate solution was evaporated to dryness under N_2 at 65°C. The residue was dissolved in 1 ml of water. 200-μl aliquots of the hydrolysate were mixed with 100 μl of the fluoraldehyde reagent. After 1 min, 10 μl of sample was subjected to HPLC. Urine samples were acidified with 1% HCl and frozen, and kept at -20°.

Solvent A was tetrahydrofuran-methanol-0.1 N sodium acetate (5:95:900, pH 7.2). Solvent B was methanol-water (9:1). The column was kept at ambient temperature, and 10 μl of standard or sample were injected into the column. Elution was achieved by running a gradient of 10% solution B over 8 min at a flow rate of 1.5 ml/min, then flashing the column with 90% solvent B at the end of each injection to eliminate the less polar compounds in urine.

Blood samples for hemoglobin adducts

The blood was drawn in vacutainer tubes containing EDTA as anticoagulant. Workers

were involved in the production of AA (synthesized by catalytic hydration of AN) and polyacrylamide, and had been exposed to AA and AN for 0.1 to 8 years. Blood samples were also collected from 10 control workers in the same city, who had not been exposed to AA or AN.

Measurements of vibration thresholds

A Vibratron II vibration sensitivity tester was used for the quantitative measurements of vibration threshold of bilateral index fingers and big toes of each subject in both groups at a room temperature of 22-25°C. The instrument consisted of two modules with vibrating posts and a controller. The testing procedure was a two-alternative forced choice procedure. While the position of vibration and intensity sequence were under control, the subjects were asked to press their fingers or toes against each rod and to determine which of the two rods was actually vibrating. The vibration settings of the five errors and the five lowest correct scores were then determined. The highest and lowest values of the ten scores were eliminated and the mean of the remaining eight scores determined the vibration threshold (1 vibration unit (VU) = 0.5 microns peak-to-peak amplitude).

Electroneuromyography (ENMG)

The ENMG was carried out with a Dantec 2000C electromyographic apparatus. The electric activities of the abductor pollicis brevis, abductor digiti minimi and anterior tibialis muscles, including insertion activity, spontaneous activity of motor units, and electrical activity during mild and maximal voluntary contraction, were recorded with concentric needle electrodes. The mean duration and mean voltage of 20 motor unit potentials and the percentage of polyphasic potentials in each muscle were determined. The duration of a motor unit was taken as the time for the oscilloscope tracing to return to the base line after its original deflection from the beginning of that potential. The voltage of a motor unit was taken as the peak-to-peak amplitude. The phase of a motor unit potential was defined by the number of deflections across the base line.

The minimum acceptable skin temperature of the hands and feet during the measurements of nerve conduction velocity and distal latency was 30°C. Bipolar skin stimulation electrodes were used in all the measurements of nerve conduction velocity. The voltage was always 30% supramaximal. For measuring maximal motor nerve conduction velocity (MCV), the median and ulnar nerves were stimulated at the elbow and the wrist, and the muscle action potential was recorded from the abductor pollicis brevis muscle and the abductor digiti minimi muscle with concentric needle electrodes. The peroneal nerve was stimulated at the knee and ankle, and the muscle action potential was recorded from the extensor digitorum brevis muscle by surface electrodes.

The sensory nerve conduction velocity (SCV) of the median and ulnar nerve was measured through the stimulation of the forefinger and the little finger, respectively, with ring electrodes. The sensory nerve conduction velocity of the sural nerve was recorded with a

surface electrode at midcalf, 14 cm from the stimulating electrode placed just below the lateral malleolus. The peak-to-peak amplitude of the evoked potentials was also measured.

The normal values of all related ENMG parameters were previously established in our laboratory using the same EMG apparatus and the same technique in 80 unexposed healthy adults.

Clinical and laboratory examinations

Physical and neurological examinations were conducted in both groups according to a protocol previously described (He et al. 1989). The laboratory studies included routine blood and urine tests, liver function (serum glutamate pyruvate transaminase and the thymol turbidity test) and serum hepatitis B surface antigen.

Questionnaire information

All subjects were interviewed with the aid of a structured questionnaire collecting information on demographic factors, smoking and drinking habits, height and weight, occupational history, past illnesses, reproductive history and family history.

Definition of Neurotoxicity Index

A neurotoxicity index (NIn) with a maximal score of 50 points was defined so as to provide a quantitative parameter expressing the peripheral neuropathy which could be correlated to the different biomarkers of exposure. The different observations scored in the Neurotoxicity Index are shown in **Table 2.** Clarifications for this Table include the following: (1) workers who had lost their pain or touch sensation got 1 to 3 points depending on the extent of this loss in fingers, hands, or forearms, (2) up to 2 points each for increased vibration thresholds in hands or feet as measured with the Vibratron instrument were given. Vibratron thresholds were compared to the corresponding control group with regard to age. If their ratios to the corresponding controls were 0-4 for toes or 0-2.5 for index fingers, a score of 1 point was given for each, whereas if the ratios were 4-8 or 2.5-5 for toes or fingers, respectively, 2 points were given for each, (3) for each observation of an ENMG abnormality 0.5 points were given, with a maximal possible total of 12 x 0.5 = 6 points.

Statistical methods

The chi-square test was used to analyze the symptoms and signs, and the Student's t-test was used in the analysis of ENMG parameters. The variance analysis and Q-test (Neuman-Keuls methods) were used in the comparison of vibration thresholds between the reference group and the acrylamide group, both with different age categories. Univariate and multivariate regression analysis was used to estimate correlation coefficients and levels of statistical significance for the relationship between the Neurotoxicity Index and the

TABLE 2

Definition of Neurotoxicity Index	
	Points
Numbness of extremities	1
Cramping pain	1
Loss of position sensation	2
Loss of pain sensation	0-3
Loss of touch sensation	0-3
Loss of vibration sensation:	
According to tuning fork	1
Vibration threshold in big toe	0-2
Vibration threshold in index finger	0-2
Clumsiness of hand	4
Difficulty grasping	4
Unsteady gait	4
Muscular atrophy	6
Decrease or loss of ankle reflexes	3 or 5
EMG	6
ENMG	0.5 per abnormality (max 6)
Maximum	Σ 50

biomarkers and ANOVA analysis was used for comparing the means of the different categories of workers.

AIM 3

Animals and treatments

Male Sprague-Dawley rats weighing 270-310g were divided into five groups and allowed free access to food and water. Rats were injected ip with AA or GA once a day for 8 days. The doses were 25 and 50 mg/kg for AA and 50 and 100 mg/kg for GA. Control rats were injected with the vehicle, distilled water (1 ml/kg). All animals were tested and sacrificed on day 9, 24 hr after the last injection. For the reproductive toxicity experiments, sexually mature Sprague-Dawley rats (350g) were utilized. Animals were administered either AA (50 mg/kg/day for 7 days) or GA (50 mg/kg/day for 14 days).

Neurotoxicity testing

Rotarod. The rotarod apparatus consisted of a horizontal plastic rod (6 cm in diameter) positioned 30 cm above a switch floor. The rod was divided by partitions so that 4 rats could be tested simultaneously. All rats were trained to stay on the rod (rotating at 20 rpm) twice daily for 5 consecutive days prior to the beginning of treatment. The cut off-time was 100 sec. The test was repeated 3 times at 20-min intervals and the average value of the measurements (in seconds) was utilized.

Hindlimb Splay. This method was similar to that described by Edwards and Parker (1977). Each rat was held in a horizontal position with the dorsal side up, 32 cm above a flat surface which was covered with a thin layer of sawdust. The rat was then dropped and the position of the fourth digit of each hindlimb on landing was marked and the distance between the toe(s) marks was measured. Values represent the average of 3 consecutive measurements.

Reproductive toxicity testing

Twenty-four hours after the last injection, animals were sacrificed by cervical dislocation under ether anesthesia and testis, epididymis and vas deferens were dissected and weighed. The head, body and tail of epididymis were minced and homogenized and the number of spermatozoa was determined. Sperm viability was measured as described by Bishop and Smiles (1957).

RESULTS AND DISCUSSION

AIM 1

Development of a GC/MS method for measuring Hb adducts of AA and GA

The reactions of AA and GA with cysteine residues of hemoglobin are shown in **Fig. 1.** Globin isolated from rat blood was subjected to acid hydrolysis and the adducts were released as S-(2-carboxyethyl)-cysteine and S-(2-carboxy-2-hydroxyethyl)-cysteine. The use of anion-exchange chromatography for purifying these particular adducts from the neutral and basic amino acids takes advantage of their extra carboxylic acid function. The hydrolysate was applied to the column and the most negatively charged amino acids were most strongly attached. With weak formic acid (5 mM) all amino acids with only one carboxylic acid group were eluted with the front at 5-20 ml, while 100 mM formic acid eluted the more strongly bound dicarboxylic acids. Since, however, both glutamic acid and aspartic acid are dicarboxylic acids, the 100 mM eluate contained, in addition to the two adducts, part of the glutamic acid content and all of the aspartic acid. The elution volumes were 70-90 ml for glutamic acid, 90 ml for AACys, 95-110 ml for aspartic acid, and 90-110 ml for GACys. After evaporation, the 100 mM eluates were derivatized for quantitation with GC/MS. Multiple ion monitoring in the electron impact ionization mode was chosen because with the instrument used it provided a higher sensitivity than the negative ion chemical ionization mode. The fragments monitored for the internal standard had one deuterium instead of the two deuteriums in the parent molecule due to loss of 1 deuterium atom in the fragmentation process (Calleman et al. 1990). The difference in m/z of only 1 between the analyte and the internal standard is not ideal since the contribution from the deuterated ion to the nondeuterated was 1% and the contribution from the nondeuterated ion to the deuterated was 10%. However, this was corrected for by use of the calibration curve.

The use of free deuterated amino acids as internal standards was based on the assumption that there is no preferential decomposition during acid hydrolysis of an amino acid depending on whether it is free in solution or exists as a peptide residue. Studies by Crestfield et al. (1963) on the close structural analogue S-carboxymethyl-cysteine in ribonuclease showed that 97, 103, 103, and 101% of this amino acid were recovered after 24, 48, 73, and 102 hrs of hydrolysis in 6 M HCl, respectively. The fact that the concentration of S-carboxymethyl-cysteine did not decrease after 24 hr when it existed free in solution indicates that this amino acid is equally stable as a free amino acid and as a peptide residue.

The synthetic standards of AACys and GACys for preparation of the calibration curve were quantified by amino acid analysis employing the response factor of leucine. The compounds contained no impurities. The detection limit for the GC/MS was 1.7 pmol of injected derivatized standard compound. The calibration curve on GC/MS was linear between the ratios of 0.1 and 1.0 with $R^2 = 0.999$ for both derivatized AACys and GACys.

Analyses in triplicate gave an average coefficient of variation (CV) of 6% for AACys and 8% for GACys. Despite the fact that deuterated internal standards were used, the GC/MS system exhibited a certain drift in the ratios determined between different occasions on which it was used. Thus, while one globin sample was processed and analyzed several times, the CVs for AACys (n=4) and GACys (n=5) were 2 and 4%, respectively, on a single occasion; the CV for repeated (n=5) injections of an identical sample on a different occasion was 2% for AACys and 13% for GACys. The CVs within the dose groups of rats were on average 13 and 15% for AACys and GACys, respectively. Background levels found in the globin from control rats were 0.15 (0.14-0.17) μmol/g Hb and 0.015 (0.010-0.019) μmol/g Hb for the AA and GA adducts, respectively. Administration of 1 mg/kg body wt acrylamide by ip injection to rats resulted in a statistically significant increase in adduct levels ($p<0.01$).

Acrylamide and glycidamide hemoglobin adducts following administration to rats

In a first experiment, rats were injected with different doses of AA and the adduct levels (AACys) in the blood were measured 24 hr after injection (**Fig. 3**). At this time all AA presumably had reacted since no parent compound was detectable by Miller et al. (1982) in any tissue after 1 day. The AA adduct level increased approximately linearly with the administered amount. The levels of the GA adduct (GACys), on the other hand, produced a concave curve (**Fig. 3b**). A typical mass fragmentogram from a rat treated with 10 mg/kg body wt of AA is shown in **Fig. 4**.

In a second experiment rats were administered increasing doses of GA. In this case the GACys adducts increased linearly with the administered amount (**Fig. 5**). On a mol/kg body wt basis, levels of GACys adducts were 3.2 times lower than the AACys levels measured following AA administration.

From these experiments, the percentage of the initial concentration of AA metabolized to GA was calculated (**Fig 6**). For this purpose, the concentration of GA (in mmol/kg) corresponding to the GACys adduct level in the AA-injected rats was calculated as if GA had been injected directly. This concentration was divided by the initial concentration of injected AA. At the lowest concentration of AA (5 mg/kg body wt) about 51% was converted to GA, while at the highest concentration (100 mg/kg body wt) only about 13% was metabolized to GA.

In an additional experiment, a cumulative dose of 100 mg/kg body wt AA was given as a single dose, distributed over 10 consecutive days as 10 mg/kg/day, or distributed over 30 days as 3.3 mg/kg/day. Twenty-four hours after the final injection, blood was drawn and the adduct levels of both AACys and GACys were measured. **Table 3** shows the measured adduct levels and, for comparison, the expected levels calculated according to Eq. (1):

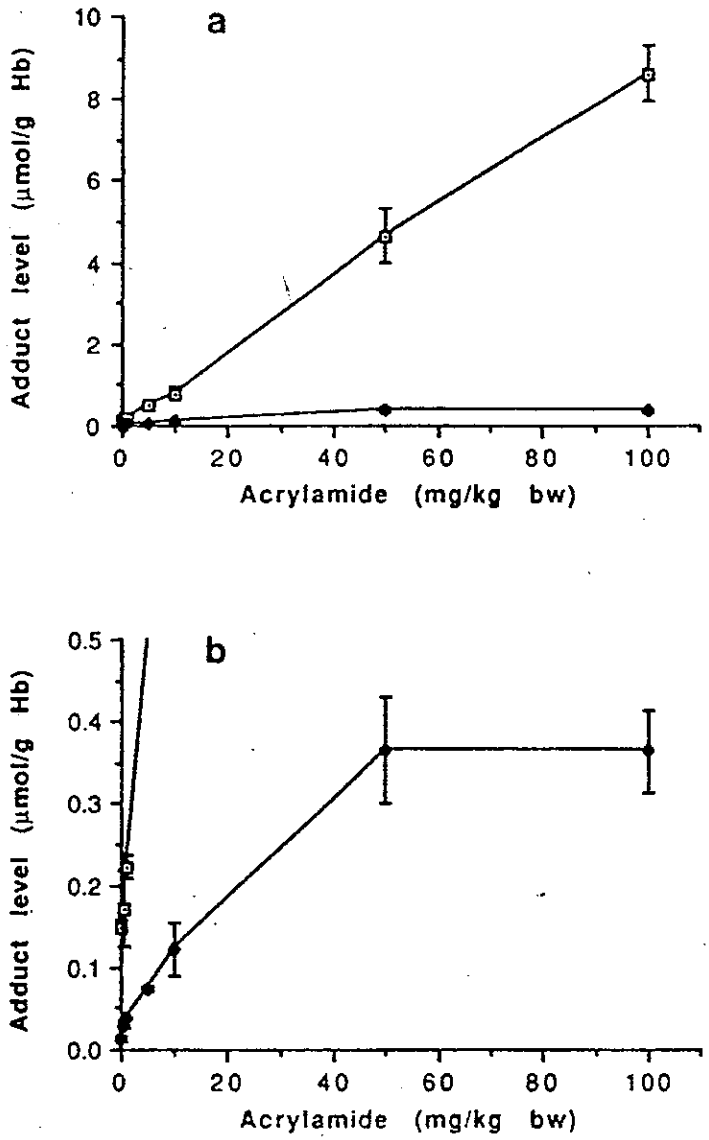

Figure 3. Hb cysteine adduct levels of AA and GA in rats injected with different single doses of AA.(□) Cysteine adduct of AA; (◆) cysteine adduct of GA. Symbols represent means ±SD of three to five animals. (a) Full scale. (b) Enhanced detail of (a).

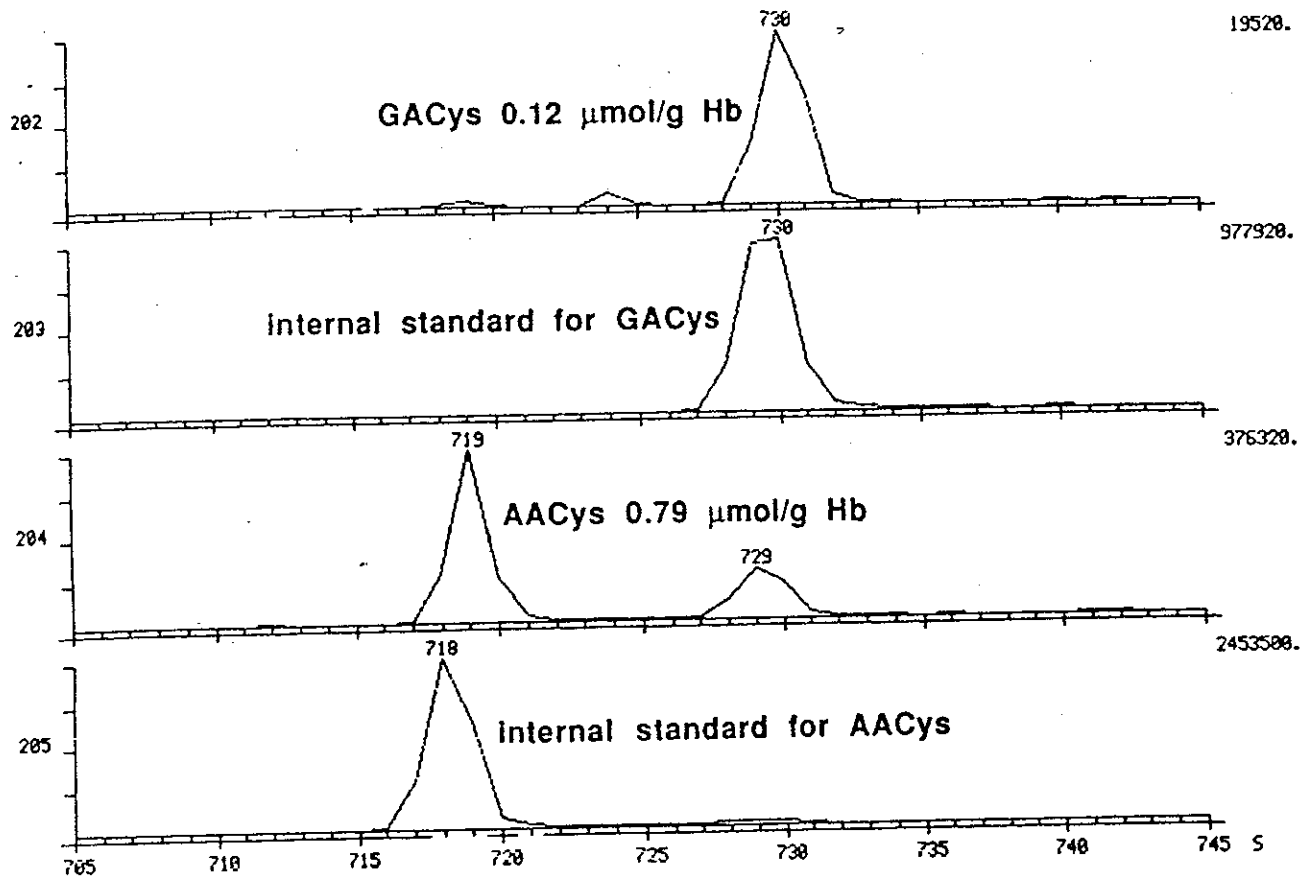

Figure 4 . A typical mass fragmentogram from a rat treated with 10 mg/kg body wt AA. The y-axis shows the intensities of the ions monitored, m/z = 202, 203, 204, 205, for the derivatized nondeuterated and deuterated forms of S-(2-carobxy-2-hydroxyethyl)-cysteine and S-(2-carboxyethyl)-cysteine. The x-axis shows retention time in seconds.

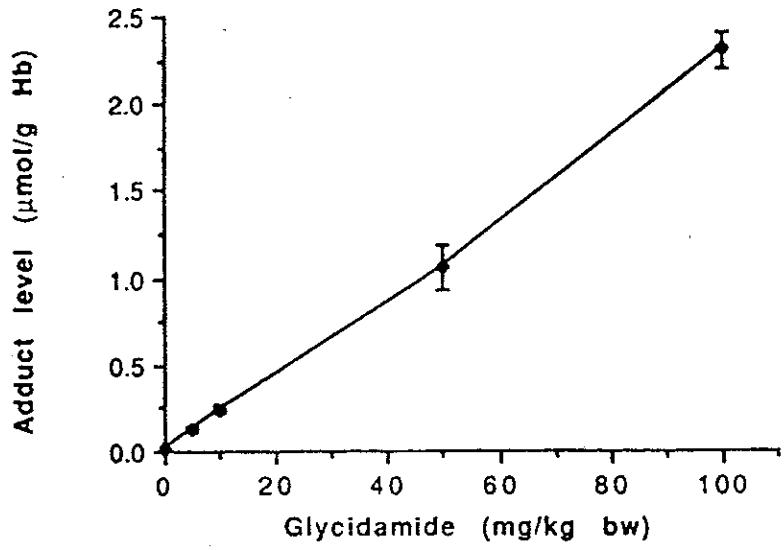

Figure 5. Hb cysteine adduct levels of GA in rats injected with different single doses of GA. Symbols represent means ± SD of three animals.

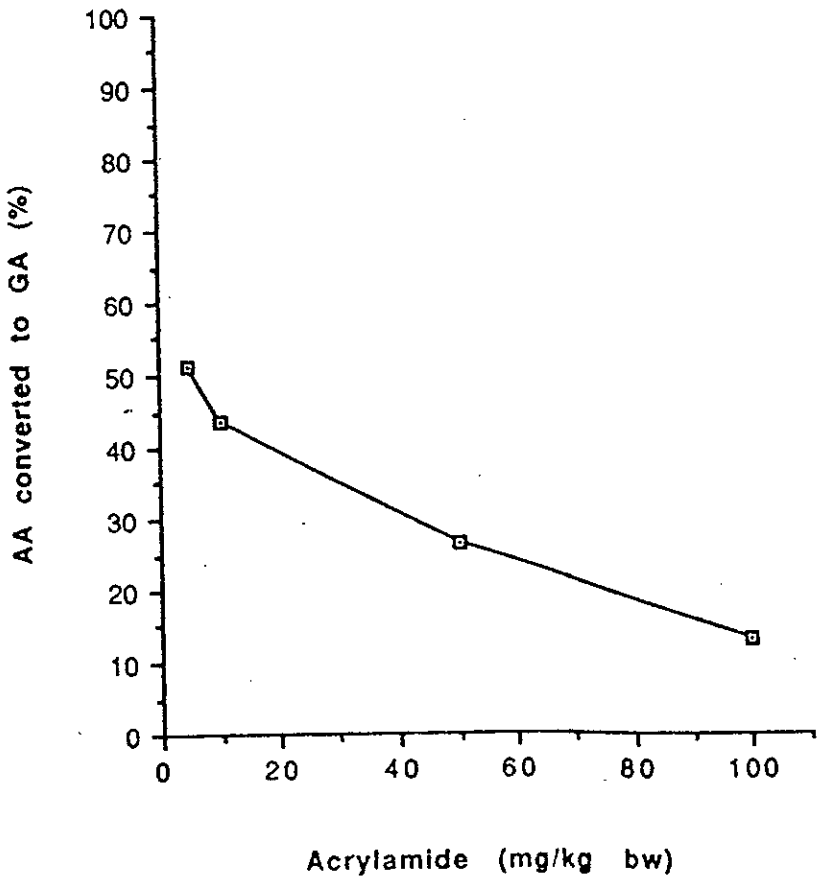

Figure 6. Conversion of AA to GA *in vivo* in rats injected with AA. The percentage is calculated in relation to the initial concentration of AA in the body. The fraction converted to GA is estimated from measured adduct levels of GA.

TABLE 3

Hemoglobin adducts in rats treated with different dose regimens of acrylamide

Dose rate (mg/kg body wt)	Measured adduct level (μmol/g Hb)		(SD, n)	Expected adduct level[a] (μmol/g Hb)
1 Day x 100	AACys[b]	8.62	(0.66, 5)	AACys 8.62
	GACys[c]	0.364	(0.049, 5)	GACys 0.364
10 Days x 10	AACys	5.72	(0.99, 4)	AACys 5.60
	GACys	1.00	(0.17, 4)	GACys 0.96
30 days x 3.3	AACys	3.58	(0.45, 4)	AACys 4.20
	GACys	0.749	(0.062, 4)	GACys 0.79

a The expected values were estimated from the data shown in Fig. 3 and were corrected for the replacement of old erythrocytes and the increase in blood volume due to the growth of the animal (Eq. (1)). The values of *a* in Eq (1) were 3.3 mg/kg/day, 0.23 and 0.044 μmol/g Hb for AA and GA, respectively, and, at 10 mg/kg/day, 0.645 and 0.1095 μmol/g Hb for AA and GA, respectively. The body weights increased from 132 to 380 g during the 30-day treatment and from 264 to 296 g during the 10-day treatment. The measured adduct levels were not significantly different ($p > 0.05$) from the expected adduct levels.

b AACys = The Hb cysteine adduct of acrylamide.
c GACys = The Hb cysteine adduct of glycidamide.

$$\{[RY]/[Y]\}_{acc} = \sum_{t=1}^{t_n} a(1 - \frac{t}{t_{er}}) \times b, \qquad (1)$$

where $\{[RY]/[Y]\}_{acc}$ is the accumulated level of adducts at the end of exposure, a is the daily increment of adducts based on the measured levels in the first experiment (dose-response of AA), t_n is the duration of the experiment in days, and t_{er} is the lifetime of the erythrocytes estimated to 61 days in the Sprague-Dawley rat (Derelanko, 1987). The factor b in the equation (body wt at time t_{n-t+1}/body wt at t_n) is used for correcting for the increase in total blood volume due to the growth of the animal which will cause a dilution of the adducts. This equation for the accumulation of Hb adducts during chronic exposure is based on the assumption that the Hb adducts are eliminated at the same rate as the erythrocytes. The measured adduct levels were not significantly different than the expected adduct levels.

Levels of AA adducts decreased as the dose rate decreased mainly because of the elimination of old erythrocytes and dilution of adducts due to body growth, but also because of a larger fraction of AA metabolized to GA. In fact, levels of GA adducts increased when the same cumulative dose of AA (100 mg/kg) was given as ten 10-mg/kg doses over a 10-day period, suggesting higher conversion of AA to GA. When the same amount of AA (100 mg/kg) was given over 30 days, however, although levels of GA adducts were higher than after the acute 100 mg/kg dosage, they were not as high as at the 10 x 10-mg/kg dosage regimen because of elimination of old erythrocytes and body growth.

The constants k_{cys} and k_{el} as well as $t_{1/2}$ are presented in **Table 4**. The second-order rate constants, k_{cys}, for the reaction between cysteine residues in hemoglobin and AA or GA were determined in vitro. These rate constants were calculated from $k_{cys} = [RY]/[Y]/D$, where [RY]/[Y] is the degree of alkylation measured in the experiment, D is the dose which may be calculated from D = [RX] x t, and [RX] is the concentration of AA or GA in the incubation mixture (Segerbäck, 1990).

The first-order rate constants for the elimination of AA and GA in the blood compartment, k_{el}, were estimated from adduct levels of AA or GA, when AA or GA was injected directly, using Eq. (2):

$$k_{el} = \frac{[RX]_0 k_{cys}}{[RY]/[Y]} \qquad (2)$$

where $[RX]_0$ is the initial concentration of the injected compound. Since AA distributes rapidly and evenly in the body to all tissues (Miller et al. 1982), its initial concentration may be estimated on the basis of 1 kg body wt = 1 liter; the same assumption is also made for GA since it is a highly water-soluble low-molecular-weight molecule. The k_{cys}

TABLE 4

Pharmacokinetic parameters of acrylamide and glycidamide in the rat

Compound	k_{Cys}[a] (liter/g Hb x hr)	k_{el}[b] (hr^{-1})	$t_{1/2}$[c] (hr)
Acrylamide	1.8×10^{-3}	0.37	1.9
Glycidamide	0.92×10^{-3}	0.48	1.5

a Second-order rate constant for the reaction with cysteine in rat Hb in vitro.

b Estimated first-order rate of elimination in the blood (Eq. (2)).

c Half-life *in vivo* in the blood. Calculated from $t_{1/2} = \frac{\ln 2}{k_{el}}$.

determined *in vitro* are used in Eq. (2) since the rates of reaction of AA or GA with Hb-cysteine *in vivo* are assumed to be the same as *in vitro*. The ratio [RY]/[Y] was taken from *in vivo* data assuming complete reaction of the parent compound within 24 hrs. The half-life is calculated from the relationship $t_{1/2} = \ln 2/k_{el}$.

General discussion of Aim 1

The spectrum of different amino acids in Hb which react with AA and GA is currently not known. There are several different nucleophilic amino acids such as histidine, cysteine, and N-terminal valine in hemoglobin (EPA, 1990) which may give rise to adducts useful for monitoring exposure to these electrophiles. The unsaturated bond of AA is, however, known to have high reactivity toward -SH groups in Michael type additions, which made cysteine adducts a natural choice for the development of our quantitative technique along the lines of the work of Bailey et al. (1986). Although the epoxide GA is expected to react significantly also with nitrogen- and oxygen-containing nucleophilic amino acids, the simultaneous determination of adducts formed by both agents provided an analytical procedure with the appeal of simplicity. Thus, a method was developed to determine the cysteine adducts formed by the two electrophilic agents.

The adduct levels of AA in our study are in good agreement with those determined by Bailey et al. (1986) in the concentration range 0-5mg/kg. The background level of AACys in that study, however, was 7 times lower than the background levels determined in our study (0.15 μmol/g Hb), which might be explained either by the use of different strains of rats (Wistar vs Sprague-Dawley) or by methodological differences, such as the use of different internal standards for quantitation.

The pronounced concavity of the GA adduct curve in rats injected with AA indicates that this metabolite is formed by a saturable process following Michaelis-Menten kinetics. It should be noted that because of our interest in providing data useful for relating hemoglobin adduct levels to neurotoxic effects, the dose range of injected AA and GA is rather high, compared to most previous studies of Hb adducts with other compounds. Thus, nonlinearities in the curve shapes, rarely observed at low doses, become evident.

As discussed previously (Calleman et al., 1990), the most likely candidate for the enzyme system responsible for the metabolic conversion of a simple alkene such as AA to GA is cytochrome P-450, although this hypothesis has not yet been tested. The saturable kinetics of this process may provide a possible explanation for the fact that AA has consistently given a negative response in the Ames/*Salmonella* test also in the presence of S-9 mix. Indeed, the case of AA could be similar to that of ethylene and propylene which are negative in the Ames test while their active metabolites, ethylene oxide and propylene oxide, respectively, are clearly mutagenic. It has been proposed that the negative responses of the two parent alkenes are due to the fact that their saturable metabolism may limit the maximal dose of epoxide generated in the test system to a level below what is required for a statistically significant response (Osterman-Golkar and Ehrenberg, 1982).

The experiments performed show that direct administration of GA results in the formation *in vivo* of the same adduct, S-(2-carboxy-2-hydroxyethyl)-cysteine, as that previously reported as evidence for the formation of this epoxide in AA-treated animals (Calleman et al. 1990). Adduct formation in GA-treated animals appears to be a linear function of the injected dose.

The origin of the background levels of adducts is not known. It is unclear whether they are produced as methodological artifacts or if they reflect true adduct levels. Since the adducts measured do not contain the amide group originally present, the adducts are not entirely specific to AA (or GA) exposure. For example, exposure to acrylonitrile has been shown to give S-(2-carboxyethyl)-cysteine in hydrolyzed Hb (Fennell et al., 1989). Intermediates in glycolysis, such as 3-phosphoenolpyruvate, 3-phosphoglycerate, and 1,3-diphosphoglycerate could theoretically give rise to S-(2-carboxy-2-hydroxyethyl)-cysteine following protein hydrolysis. S-(2-carboxy-2-hydroxyethyl)-cysteine (Ohmori et al. 1965) and S-(2-hydroxy-2-carboxyethyl)-cysteine (Yao et al. 1970) have been found in the urine of humans not known to be exposed to electrophilic substances. This indicates that the formation of the background levels of these specific adducts may be a result of endogenous processes. Background levels in humans and rodents of several other low-molecular-weight adducts have been found.

The hemoglobin binding index for AA to cysteine (6400 pmol (g Hb)$^{-1}$/μmol (kg body wt)$^{-1}$, is higher than for any other compound studied in the rat and is higher than that of GA (1820 pmol (g Hb)$^{-1}$/μmol (kg body wt)$^{-1}$), calculated at 50 mg/kg body wt of AA and GA, respectively. This difference is primarily explained by a lower reactivity of GA toward Hb-cysteine and a shorter half-life ($t_{1/2}$) in blood, compared to that of AA, thus giving a lower yield of adducts. The half-life for AA (1.9 hr) and GA (1.5 hr) reflects the sum of all reactions leading to the elimination of the compounds from the blood and is estimated on the assumption of a linear rate of elimination (Eq. (2)) in the concentration range studied. The half-life of AA is given as an average over the dose range 0.5-100 mg/kg body wt, although the elimination rate is expected to decrease with increasing administered doses of AA due to the saturable formation of GA. The half-life of AA in blood determined by Miller et al. (1982) and by Edwards (1975) was 1.7 and 1.9 hr, in good agreement with our data. The second-order rate constant for the reaction of AA with Hb was determined by Hashimoto and Aldridge (1970) to be 17 x 10^{-3} l x (g Hb x hr)$^{-1}$ [as calculated from a reported value of 18 l x (mol x min)$^{-1}$], as compared to 1.8 x 10^{-3} l x (g Hb x hr)$^{-1}$ in our experiments. The experimental conditions, however, were not identical in the two studies. In particular, the pH used by these authors was 8.0, higher than that used in our experiments (7.4); this is expected to increase the rate of reaction with cysteine residues because of the increased thiolate ion concentration at higher pH.

Possibly the most notable result of the present study is the high extent of metabolic conversion *in vivo* of AA to GA, reaching more than 50% at the lowest dose studied. As shown in **Fig. 6** there is a higher percentage of AA converted to GA at lower doses of AA. Consequently, if a toxic effect induced by AA is due to GA, the relative importance of the

risk for this toxic effect will increase at a lower AA exposure. For risk estimation, it will be, therefore, necessary to investigate which of the toxic effects induced by AA might be related to the parent compound, to the metabolite, or to a combination of the two. It is noteworthy that an injection of 50 or 100 mg/kg body wt AA will give the same tissue dose of GA as an injection of 10 mg/kg body wt GA. Thus, for a toxic effect which is exclusively caused by GA, we would expect the same effect if we inject 50-100 mg/kg body wt AA or 10 mg/kg body wt GA. This is based on the assumption that the tissue dose is directly related to the toxic effect in a dose-response relationship.

When AA was given in fractionated doses (i.e., in the chronic exposure regimen), a higher proportion of the cumulative tissue dose consisted of GA than after high-dose acute exposure (**Table 3**). Consistent with the results from the acute exposure experiments, this suggests that the risk for toxic effects caused by GA might be of greater importance at lower dose rates of AA in long-term exposure situations. In this experiment, the measured adduct levels were in good agreement with those calculated using the adduct levels from single injections of AA, suggesting that these particular adducts may be eliminated at the same rate as the erythrocytes. They appear, therefore, to be stable and useful for determination of tissue doses. In our calculation of expected values of Hb adduct levels, we have corrected not only for the elimination of old erythrocytes, but also for the dilution resulting from the increased body weight (Eq. (1)), and hence blood volume, during the course of the experiment. The apparently nonlinear decrease of Hb adduct levels often observed in rats following acute, single treatments of electrophilic agents (Neumann, 1981) may thus be explained by an increased blood volume during the course of the experiment rather than by a lack of stability of the adducts.

AIM 2

Development of methods for acrylamide and glycidamide Hb adducts in humans

Methods were developed for the determination of AA and GA adducts to N-terminal valine, both following total hydrolysis of the protein and by means of the modified Edman degradation procedure (**Fig. 2**).

In the first method, globin isolated from human blood was subjected to acid hydrolysis. In this procedure the amide groups originally present in AA and GA were converted to carboxylic acid groups during acid hydrolysis and thus valine adducts were released as N-(2-carboxyethyl) valine (CEVal) and N-(2-carboxy-2-hydroxyethyl) valine (CHEVal). The hydrolysates were applied to anion exchange columns and eluted stepwise with increasing concentrations of formic acid according to a modification of the method utilized in rats. Most of the normal amino acids, including glutamic acid, were eluted with 150 ml 12 mM formic acid. CECys eluted in the 60 ml 30 mM fraction, aspartic acid eluted in the 65 ml 60 nM fraction. CEVal eluted partly in the 30 mM fraction and partly in the 60 nM fraction, and these fractions were therefore pooled. The 100 ml 120 mM fraction contained

CHECys and CHEVal. The adduct-containing fractions (30 mM + 60 mM and 120 mM) were evaporated and derivatized with methanol/HCl and HFBA for GC-MS. The second-order rate constants for the reactions of AA or GA with N-terminal valine, k_{Val}, and k_{Cys}, in human Hb determined *in vitro* are presented in **Table 5**. In human Hb, AA is about 3 times more reactive towards cysteine than versus N-terminal valine, whereas GA is about equally reactive towards these two sites. The rate constant for the reaction of GA with N-terminal valine in Hb is comparable for human and rat. In contrast, the reactivity of cysteine in human Hb is 2 orders of magnitude lower than in rat Hb. This is explained by the presence of a reactive cysteine in position 125 of the b-chain in rat Hb, which is lacking in this position in human Hb (Hamboek et al., 1981).

Symptoms and signs in acrylamide exposed workers

The percentage of workers displaying different kinds of symptoms and signs are shown in **Table 6**. Fatigue (71%), numbness in hands and feet (71%), sweating (68%) of hands and feet and peeling of skin (59%) were the early and prominent symptoms in the acrylamide group and were much more frequently seen than in the reference group. The peeling of the skin in hands usually appeared within 2-30 days after topical contamination by aqueous acrylamide. Unsteadiness of grasping and walking were present in 6 and 10 acrylamide workers, respectively. Systemic symptoms such as dizziness, nausea, insomnia and headache were also found but their prevalences were not significantly different from controls.

On neurological examination, impairments of pain (54%), touch (46%) and vibration (41%) sensation were prominent in the distal part of lower limbs and hands of the exposed workers. Diminution or loss of ankle reflexes were found in 29% of the acrylamide group. There were 6 and 8 acrylamide workers having difficulties in standing on one leg and displaying a positive Rhomberg sign, respectively. None of these abnormalities were seen in the reference group. No cerebellar involvement was found in either group.

The results of a survey on the reproductive history of each subject showed no difference in fertility, abortion and birth defects in offspring between the acrylamide workers and referents.

Vibration thresholds

The vibration thresholds of index fingers and big toes in the reference group showed no difference between the two sides, nor between the two sexes (data not shown). However, the difference in vibration thresholds was significant among the 3 age-subgroups of referents, namely, < 31 years, 31-40 years, and > 41 years ($p<0.05$), showing an elevation of vibration thresholds along with the increase of age in unexposed healthy adults. A significant increase of vibration thresholds was found in acrylamide workers of all age groups (**Table 7**).

TABLE 5

Second-order rate constants for the reaction of acrylamide and glycidamide with amino acid residues in hemoglobin [liter (g Hb)$^{-1}$ hr^{-1}] x 10^6

Compound	Human k_{Val}	Human k_{Cys}	Rat k_{Val}	Rat k_{Cys}[c]
Acrylamide	4.4	14	N.D.[a]	1800
Glycidamide	11	9.5	12[b]	920

a Not determined

b GAVal was determined in globin from a rat treated with 100 mg/kg GA and k_{Val} was calculated from: k_{Val} = (GAVal x k_{Cys})/GACys.

c Data are taken from Table 4.

TABLE 6

Percentage of acrylamide-exposed workers and controls with symptoms of adverse health effects

	Acrylamide-exposed	Controls
Numbness of extremities	71	-
Fatigue	71	-
Sweating of hands and feet	68	-
Skin peeling	59	-
Menstruation disorders	4/7	-
Loss of pain sensation	54	-
Dizziness	44	-
Anorexia	41	-
Loss of vibration sensation	41	-
Nausea	39	-
Loss of ankle reflexes	29	-
Insomnia	29	10
Headache	27	-
Unsteady Gait	22	-
Loss of knee jerk	20	-
Unsteady Rhomberg sign	20	-
Loss of triceps reflexes	10	-
Loss of biceps reflexes	10	-

TABLE 7

Comparison of vibration thresholds (VU ± SD) between acrylamide workers and referents

Age (years)	Acrylamide Workers			Reference Group		
	n	fingers	toes	n	fingers	toes
<31	32	2.33±1.05*	5.13 ± 3.80*	42	1.20 ± 0.29	2.40 ± 0.85
31-40	9#	2.19 ± 0.90*	4.94 ± 2.74*	37	1.38 ± 0.37	2.85 ± 0.85
>41	0	-	-	26	1.49 ± 0.36	3.53 ± 0.97

* By variance analysis and Q test, $p < 0.01$.
# One worker aged 42 was included

Electroneuromyographic measurements

The mean duration and amplitude of motor units and the percentage of polyphasic potentials in muscles abductor pollicis brevis, abductor digiti minimi and anterior tibialis of the acrylamide group were within normal range. No spontaneous denervation potentials were found during mild contraction of sampled muscles. However, 20 out of 94 sampled muscles showed mixed patterns of recruitment during maximal contraction; 9 samples were of discrete pattern. Abnormal recruitment patterns were also seen in 75% of the 12 samples of anterior tibialis muscles of acrylamide workers.

In comparison with the reference values of ENMG parameters on a group basis, there was a prolongation of distal motor latencies in median, ulnar and peroneal nerves as well as a slowing of MCV of peroneal nerve in the acrylamide group. A prolongation of sensory distal latencies and a decrease of sensory potentials were also found in the median, ulnar and sural nerves, as well as a slowing of SCV of sural nerve in acrylamide workers (**Table 8**).

The neurotoxicity index as predictor of clinical diagnosis of peripheral neuropathy

The neurotoxicity index (**Table 2**) in the workers with a clinical diagnosis of peripheral neuropathy and those without are shown in **Figure 7.** As evident from the plots there is overlap only for two individuals between the neurotoxicity index and the clinical diagnosis, which seems to indicate that the neurotoxicity index as defined is a useful quantitative indicator of acrylamide-induced peripheral neuropathy. In the studied group no worker had a Neurotoxicity Index exceeding 30 points out of the maximal value of 50.

Hb adduct levels in humans exposed to acrylamide

The Hb adduct levels determined in the workers in the factory in Xinxiang are presented in **Table 9**. The AA and AN valine adducts were determined using the modified Edman degradation procedure. The GA valine adducts were determined after total hydrolysis of the globin. Since this method is very time-consuming, only 6 such samples were analyzed. Typical mass fragmentograms from the analyses are shown in **Fig. 8a and b**. All exposed workers had detectable Hb adduct levels of AA (0.3 - 34 nmol/g Hb) and AN (0.02 - 66 nmol/g Hb). GA adducts were detected in the samples from the 5 exposed persons (1.6 - 32 nmol/g Hb) but not in the control, and there was an increase in GA adducts with increasing AA adduct level with a linear regression correlation coefficient of $r = 0.96$ (**Fig. 9**). In controls, AA adducts were detected in 1 out of 10 samples (0.01 nmol/g Hb), and AN adducts were detected only in the smokers (in 4 out of 5 smokers, 0.03 - 0.14 nmol/g Hb). **Table 10** shows the average Hb adduct levels of AA in the workers according to their location in the factory together with the geometric means of the AA air concentrations. The Hb adducts of AN were also determined on coded samples in another laboratory (T. Fennell, C.I.I.T.). All results are shown in **Table 11.** In this parallel analysis ANVal adducts were detected also in controls, albeit at low levels.

TABLE 8

Comparison of Motor (MCV) and Sensory (SCV) Nerve Conduction between the Acrylamide and Reference groups (mean ± SD).

ENMG Parameters	Acrylamide group (N = 41)	Reference group (N = 80)
MCV (m/sec)		
Median nerve	56.8 + 7.2	59.6 + 5.0
Ulnar nerve	60.0 + 9.2	62.5 + 5.4
Peroneal nerve	43.8 + 8.5*	48.1 + 3.5
Motor Distal latency (ms)		
Median nerve	4.6 + 1.3*	3.5 + 0.4
Ulnar nerve	3.1 + 1.1*	2.3 + 0.3
Peroneal nerve	6.0 + 1.9*	3.9 + 0.5
SCV (m/sec)		
Median nerve	61.6 + 5.8	60.3 + 4.1
Ulnar nerve	61.9 + 6.4	66.9 + 4.3
Sural nerve	46.6 + 6.6*	56.8 + 4.0
Sensory Distal Latency (ms)		
Median nerve	2.8 + 0.4*	1.8 + 0.3
Ulnar nerve	2.5 + 0.8*	1.6 + 0.2
Sural nerve	3.0 + 0.8*	2.4 + 0.2

*$p < 0.02$ by Student's t-test

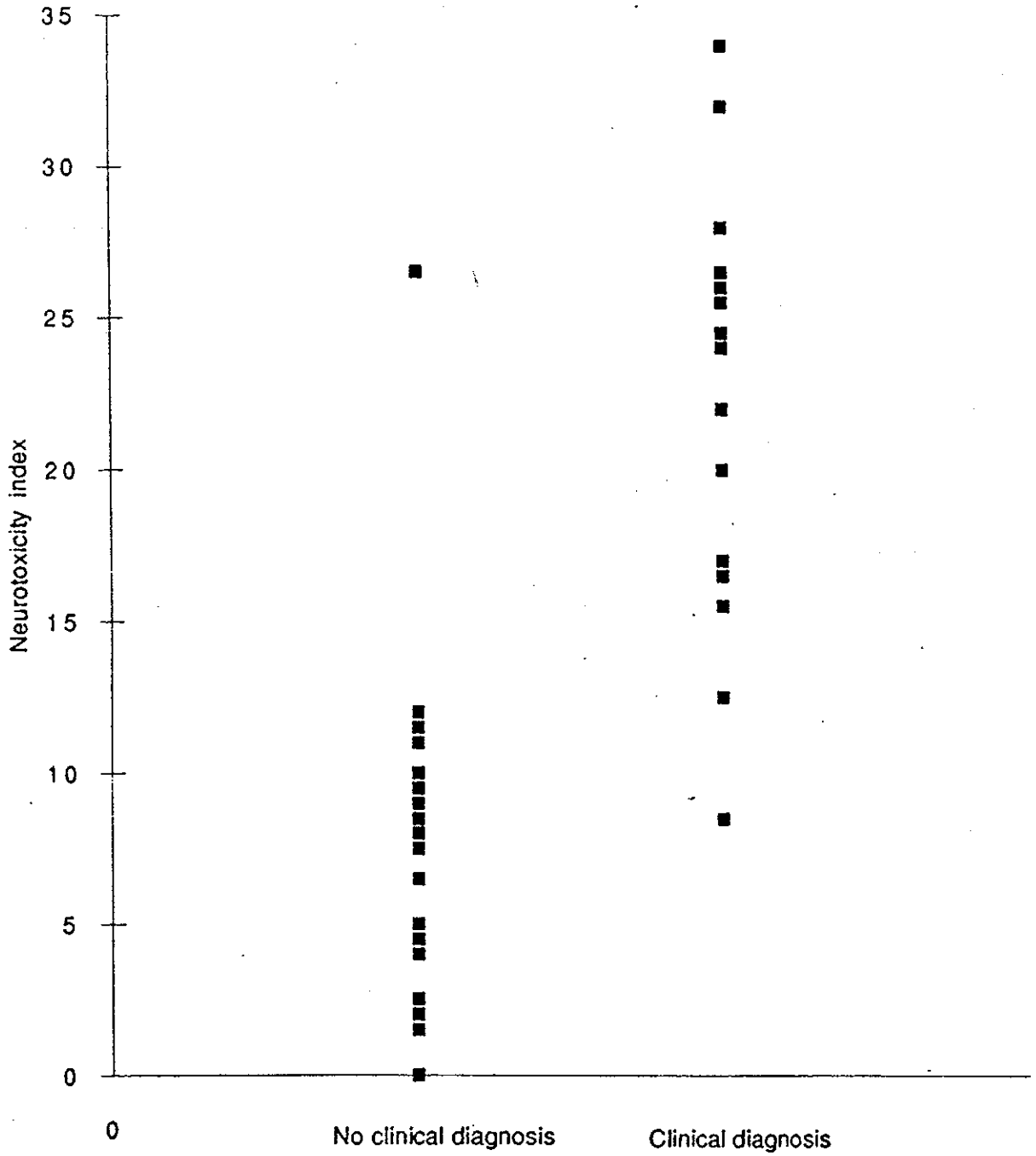

Figure 7. Neurotoxicity Index in workers with and without clinical diagnosis of peripheral neuropathy.

TABLE 9

Hemoglobin adducts of acrylamide (AAVal), glycidamide (GAVal) and acrylonitrile (ANVal) in workers

Subject No.	Exposure	Smoker	Hemoglobin adducts (nmol/g Hb)[c]			Job position
			AAVal	GAVal	ANVal	
C1	Control	no	nd[a]	nd	nd	
C2	"	no	nd	-[b]	nd	
C3	"	no	nd	-	nd	
C4	"	yes	0.01	-	0.14	
C5	"	no	nd	-	nd	
C6	"	yes	nd	-	nd	
C7	"	yes	nd	-	0.03	
C8	"	yes	nd	-	0.06	
C9	"	no	nd	-	nd	
C10	"	yes	nd	-	0.09	
E1	Exposed		6.9	-	17	Ambulatory
E2	"		6.7	-	22	Synthesis
E3	"		1.6	-	9.1	Ambulatory
E4	"		4.8	-	37	Polymerization
E5	"		2.6	-	4.3	Polymerization
E6	"		24	16	8.3	Ambulatory
E7	"		12	-	5.9	Polymerization
E8	"		11	9.3	0.12	Synthesis
E9	"		0.6	-	16	Packaging
E10	"		8.5	-	29	Synthesis
E11	"		34	-	66	Synthesis
E12	"		12	-	18	Synthesis
E13	"		5.6	-	6.4	Ambulatory
E14	"		6.7	-	5.9	Polymerization
E15	"		7.6	-	14	Synthesis
E16	"		5.8	-	32	Polymerization
E17	"		0.3	-	24	Synthesis
E18	"		11	-	2.7	Polymerization
E19	"		7.6	-	46	Polymerization
E20	"		4.2	-	26	Polymerization
E21	"		11	-	28	Ambulatory
E22	"		4.5	1.6	18	Packaging
E23	"		3.2	-	15	Packaging
E24	"		14	8.9	7.3	Polymerization
E25	"		3.7	-	7.2	Packaging
E26	"		10	-	58	Ambulatory
E27	"		8.5	-	31	Synthesis
E28	"		21	-	14	Synthesis
E29	"		5.4	-	12	Ambulatory
E30	"		1.3	-	24	No AA for 4 months
E31	"		4.9	-	2.5	Polymerization

Table 9 continued

E32	"	28	-	36	Synthesis
E33	"	7.1		0.02	Polymerization
E34	"	6.7	-	5.7	Polymerization
E35	"	3.8	-	7.9	Synthesis
E36	"	12	-	21	Synthesis
E37	"	7.5	-	15	Packaging
E38	"	21	-	20	Synthesis
E39	"	32	32	28	Synthesis
E40	"	0.6	-	1.4	Store house
E41	"	12	-	22	Ambulatory

[a] nd = Not detected
[b] Not analyzed
[c] The Hb adducts of AA and AN were determined with the modified Edman degradation method, and the adducts of GA were determined in hydrolyzed Hb samples.

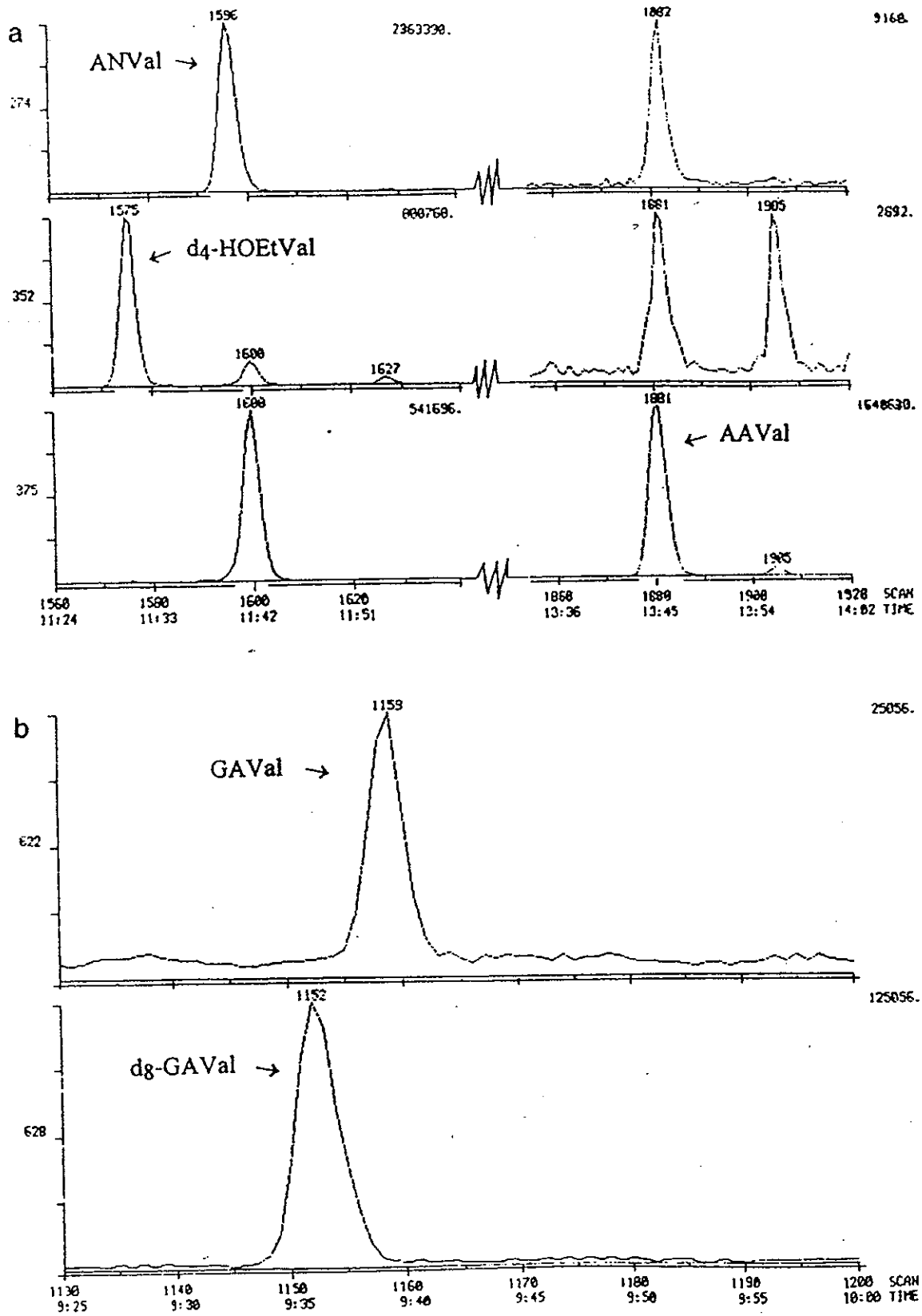

Figure 8. Mass fragmentogram of AA and GA valine adducts.

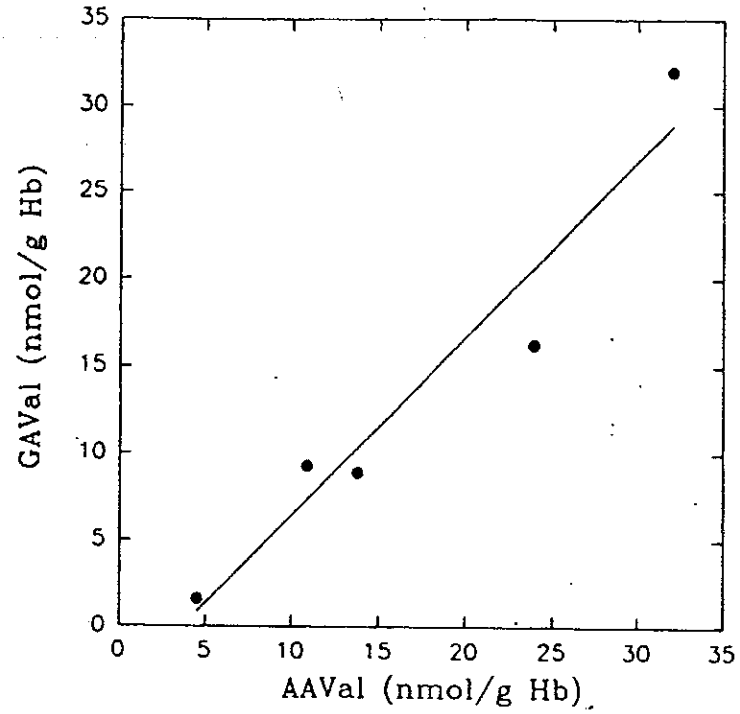

Figure 9. Correlation between AA and GA Hb adducts in workers exposed to AA.

TABLE 10

Air concentrations of AA and AAVal Hb adduct levels in workers in different locations

Location	AAVal[a] (nmol/g Hb)		AA air concentrations[b] (mg/m^3)	AAVal expected from air concentrations[c] (nmol/g Hb)
Controls	0.0 ± 0.0	(10)		
Packaging	3.9 ± 2.5	(5)		
Polymerization	7.3 ± 3.4	(12)	1.52 (0.19 - 8.8, 12)	0.93
Ambulatory	9.6 ± 6.	(8)		
Synthesis	14.7 ± 10.6	(14)	0.73 (0.11 - 3.01, 18)	0.44

[a] Mean ± SD (n)

[b] Geometric mean (range, n)

[c] Cumulative *in vivo* dose over 17 weeks calculated as:

1.52 mg $(m^3)^{-1}$ x (0.2 liter min^{-1} kg bw^{-1}) x (60 min hr^{-1}) x (8 hr day^{-1}) x (6 day $week^{-1}$) x 17 weeks =

1000 liter $(m^3)^{-1}$ x (71 mg $mmol^{-1}$) x 0.5 hr^{-1}

= 0.419 mMhr

Expected AAVal adduct level calculated as (cf. Equ. 1-2):

0.419 mMhr x 4.4 x 10^{-6} liter (g Hb)$^{-1}$ hr^{-1} x 1/2 = 0.93 nmol/g Hb

TABLE 11

Individual values for parameters predictive of adverse health outcome in workers exposed to acrylamide

nr	freeAA#	merc##	AAVal###	ANVal*	handvibr**	footvibr**	job	employment+	NIn
c1	nd	nd	0	0.09	1.2	3.3	control	0	0
c2	nd	2	0	0.15	1.6	2.8	control	0	0
c3	nd	nd	0	0.03	2.1	3	control	0	0
c4	65	1.6	0.01	0.33	2	1.5	control	0	0
c5	69	2.4	0	0.035	1.6	3.3	control	0	0
c6	62	2	0	0.17	1.6	2.3	control	0	0
c7	nd	3	0	0.25	1.3	2.1	control	0	0
c8	nd	2.4	0	0.3	1.8	4.3	control	0	0
c9	nd	3	0	0.05	1.1	4.2	control	0	0
c10	nd	2.4	0	0.41	1.3	3	control	0	0
9	nd	9	0.6	18.5	2.6	4.2	packaging	1.9	4
22	nd	33	4.5	22.2	1.3	3.1	packaging	1.75	8
23	nd	47	3.2	19.5	2.3	7.5	packaging	1.4	8.5
25	251	258	3.7	10.6	1.6	2.6	packaging	2.3	0
37	67	46	7.5	16.2	4.7	10.2	packaging	2.5	24
4	nd	27	4.8	33.2	3	5.7	polymerization	2.5	9.5
5	nd	19	2.6	8.6	1.6	2.9	polymerization	0.75	9
7	nd	15	12	8.7	2.3	5.2	polymerization	1.9	12
14	nd	19	6.7	21.6	1.7	4.5	polymerization	2.4	7.5
16	nd	15	5.8	27.5	2.6	4.3	polymerization	7	7.5
18	nd	17	11	7.8	1.1	2.4	polymerization	1.1	2
19	nd	35	7.6	39.6	3.1	6.2	polymerization	0.6	20
20	nd	16	4.2	31.2	1.4	3.3	polymerization	3.2	10
24	nd	78	14	10.1	1.2	1.4	polymerization	1.4	4
26	112	133	10	28.9	4.3	10.5	polymerization	8.2	22
33	nd	21	7.1	0.36	1.6	2.4	polymerization	6	8
34	78	27	6.7	9.6	1.8	2.5	polymerization	8.4	9
1	nd	9	6.9	18.2	2.8	7	ambulatory	8	8.5
3	nd	12	1.6	15.2	1.2	2.2	ambulatory	3.1	4.5
6	200	12	24	11.9	1.7	3.1	ambulatory	1.5	2
13	85	40	5.6	13	2.6	6	ambulatory	2.75	8.5
21	nd	52	11	21	2.3	5.9	ambulatory	3.2	12.5

TABLE 11 (Continued)

Individual values for parameters predictive of adverse health outcome in workers exposed to acrylamide

nr	freeAA#	merc##	AAVal###	ANVal*	handvibr**	footvibr**	job	employment+	NIn
29	nd	42	5.4	13.5	1.9	6.5	ambulatory	11.5	11
41	nd	129	12	20.1	4.3	4.4	ambulatory	4.5	32
2	nd	12	6.7	14.4	1.6	3.1	synthesis	3.5	16.5
8	141	42	11	0.22	1.4	27	synthesis	8	1.5
10	109	78	8.5	23.9	2.6	5.8	synthesis	3.75	26.5
11	130	39	34	26.9	5.6	15.2	synthesis	3.1	34
12	93	47	12	17.9	2.9	7.5	synthesis	3.1	26
15	nd	23	7.6	19.7	2.1	3.3	synthesis	1.6	15.5
17	65	9	0.3	22.2	2.5	5.7	synthesis	0.8	5
27	nd	94	8.5	25.9	3.6	7.5	synthesis	5.4	25.5
28	155	28	21	23.4	4.7	11.9	synthesis	7.9	24.5
32	45	109	28	26.8	4.2	18.9	synthesis	7.7	26.5
35	251	36	3.8	10.6	1.3	2.2	synthesis	2.5	2.5
36	140	163	12	22.4	2.3	5.7	synthesis	7.5	17
38	180	104	21	26	2.7	4.4	synthesis	3	28
31	nd	28	4.9	4.6	1.2	2.2	++	0.1	2
39	nd	318	32	32.7	1.5	1.9	++	0.25	6.5
30	43	nd	1.3	23.7	2	2.2	+++	1.2	12
40	nd	36	0.6	2.1	1.5	2.7	+++	3.5	11.5

ppm in plasma.
mercapturic acids, µmol/L urine.
nmol/g Hb (from Bergmark et al. 1993).

nmol/g Hb (from McNeela et al., in preparation).
Vibration units.

+ years.
++ Less than 6 months exposure to AA.
+++ not exposed to AA during the 4 last months.

nd = not detected.

Calculation of *in vivo* doses

Under the assumption that the adducts are stable during the lifetime of the erythrocytes, the daily increment of adducts in a chronic exposure situation, $([RY]/[Y])_d$, can be calculated as:

$$([RY]/[Y])_{ss} = ([RY]/[Y])d \times t_{cr}/2 \qquad (1)$$

where $([RY]/[Y])_{ss}$ is the steady-state level of adducts at the nucleophilic center, Y, and t_{cr} is the life-span of the erythrocytes, approximately 120 days in man (Osterman-Golkar et al., 1976). The daily *in vivo* dose, D, can then be calculated from:

$$D = ([RY]/[Y])_d / k_y \qquad (2)$$

where k_y is the second-order rate constant for the reaction of the electrophile with the nucleophilic center Y in Hb (in this case Y = $-NH_2$ of the N-terminal valine).

As an example, in worker No. E39, the daily *in vivo* dose of AA is given by:

$$D = \frac{32 \text{ nmol/g Hb} \times 2}{120 \text{ days} \times 4.4 \times 10^{-6} \text{ liter (g Hb} \times \text{hr)}^{-1}} = 0.12 \text{ mMhr/day} \qquad (3)$$

and in analogy, the daily *in vivo* dose of GA is calculated as 0.048 mMhr in the same person.

Other biomarkers of exposure

The individual data for the concentrations of free acrylamide in plasma (determined by K. Crofton, USEPA, HERL, RTP), S-(2-carboxyethyl) cysteine in urine and Hb adducts of acrylamide and acrylonitrile are shown in **Table 11** together with the job classifications of the different workers. For a majority of workers the levels of free acrylamide in the plasma could not be measured above the level of detectability. For the other biomarkers of exposure, statistically significant differences were observed between the exposed and control groups, which shows that they are in principle useful as biomarkers for workers heavily exposed to acrylamide and/or acrylonitrile. The group means for the different categories of workers are shown in **Table 12**.

Biomarkers of exposure as predictors of neurotoxicity

The relationships between free acrylamide in the plasma, mercapturic acids in the urine, hemoglobin adducts of acrylamide or acrylonitrile and the lifetime *in vivo* doses of acrylamide with the neurotoxicity index are shown in **Figures 10-14**. The correlation coefficients and statistical significances of these relationships are shown in **Table 13**, assuming linear relationships between the independent and dependent variables. The mean

Table 12

Biomarkers according to job classification

	Free AA*	merc ac.**	AAVal#	ANVal#	handVU##	footVU##	Life-time AA§	NIn
controls	65 ± 4	1.9 ± 1.1	0.0 ± 0.0	0.18 ± 0.13	1.6 ± 0.3	3.0 ± 0.9	0.0 ± 0.0	0.0 ± 0.0
packaging	159	78.6 ± 102	3.9 ± 2.5	17.4 ± 4.4	2.5 ± 1.3	5.5 ± 3.2	8.1 ± 6.6	8.9 ± 9.1
polymeriz	95	32.7 ± 36.4	7.7 ± 3.4	18.9 ± 12.8	2.1 ± 1.0	4.3 ± 2.5	27.0 ± 23.9	10.0 ± 5.8
ambulatory	143	36.6 ± 44.9	9.5 ± 7.3	16.1 ± 3.6	2.4 ± 1.0	5.0 ± 1.8	37.6 ± 21.9	11.3 ± 9.8
synthesis	131 ± 59	60.3 ± 45.8	13.4 ± 9.8	20.2 ± 7.7	2.9 ± 1.3	7.2 ± 5.1	68.3 ± 64.2	19.2 ± 10.6

* Means based only on the values above detectability, ppm
** Mercapturic acids, μmol S-(2 carboxyethyl)cysteine/L hydrolyzed urine
#nmol/ g Hb
Vibration units
§ Life-time *in vivo* doses of acrylamide; mMhr

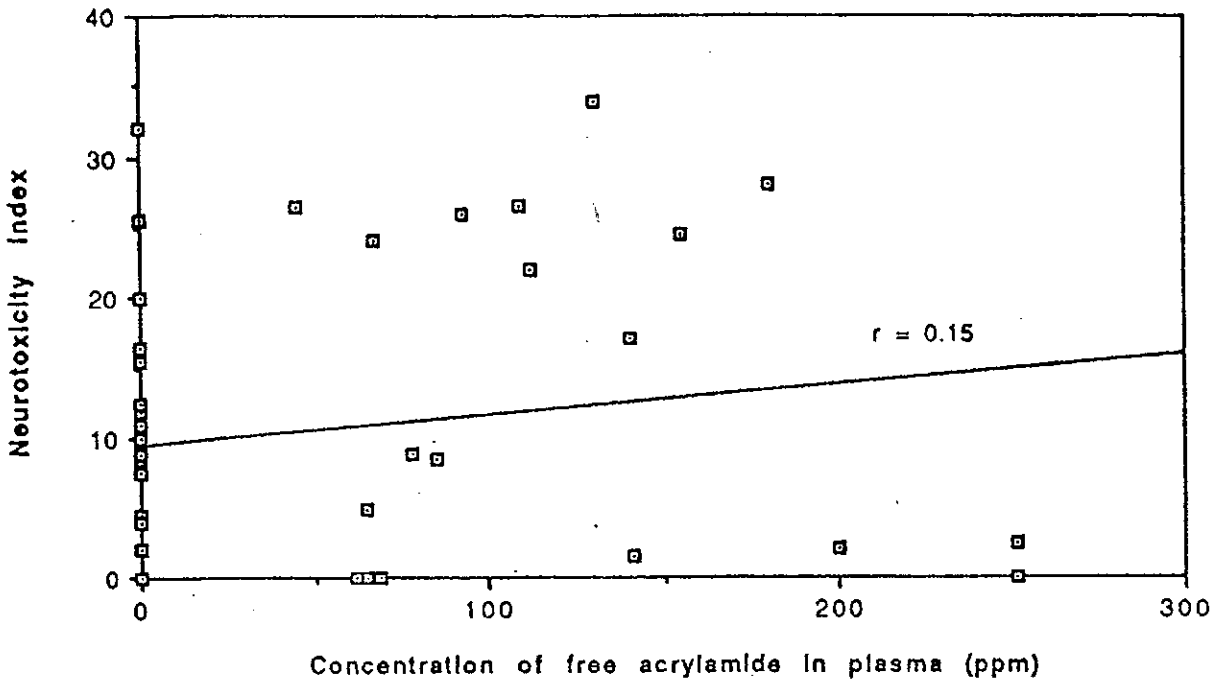

Figure 10. Correlation between neurotoxicity index and levels of free AA in plasma.

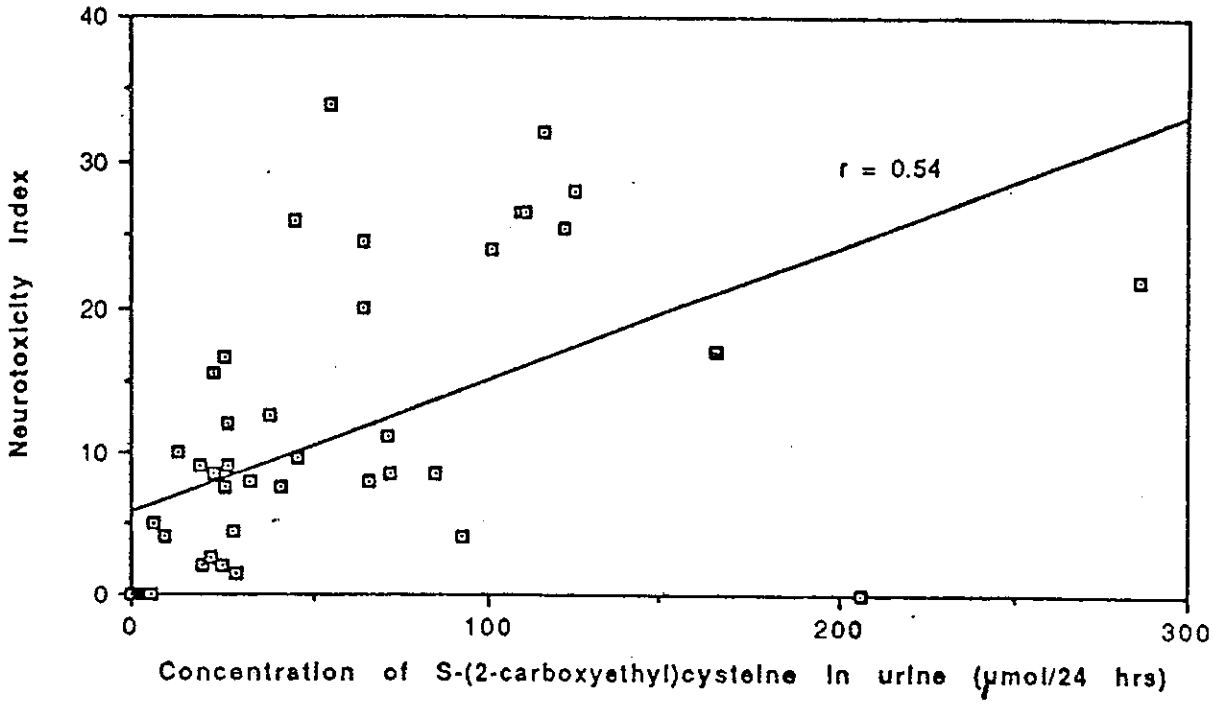

Figure 11. Correlation between Neurotoxicity Index and urinary mercapturic acid levels.

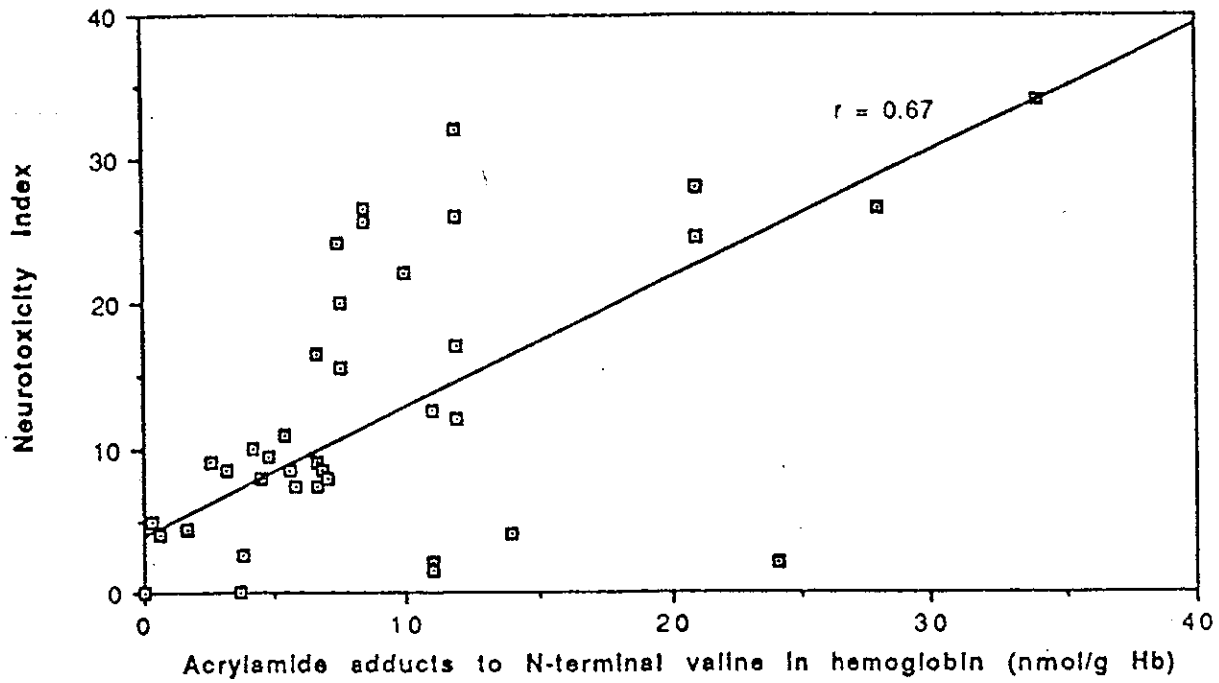

Figure 12. Correlation between neurotoxicity index and AA Val adducts.

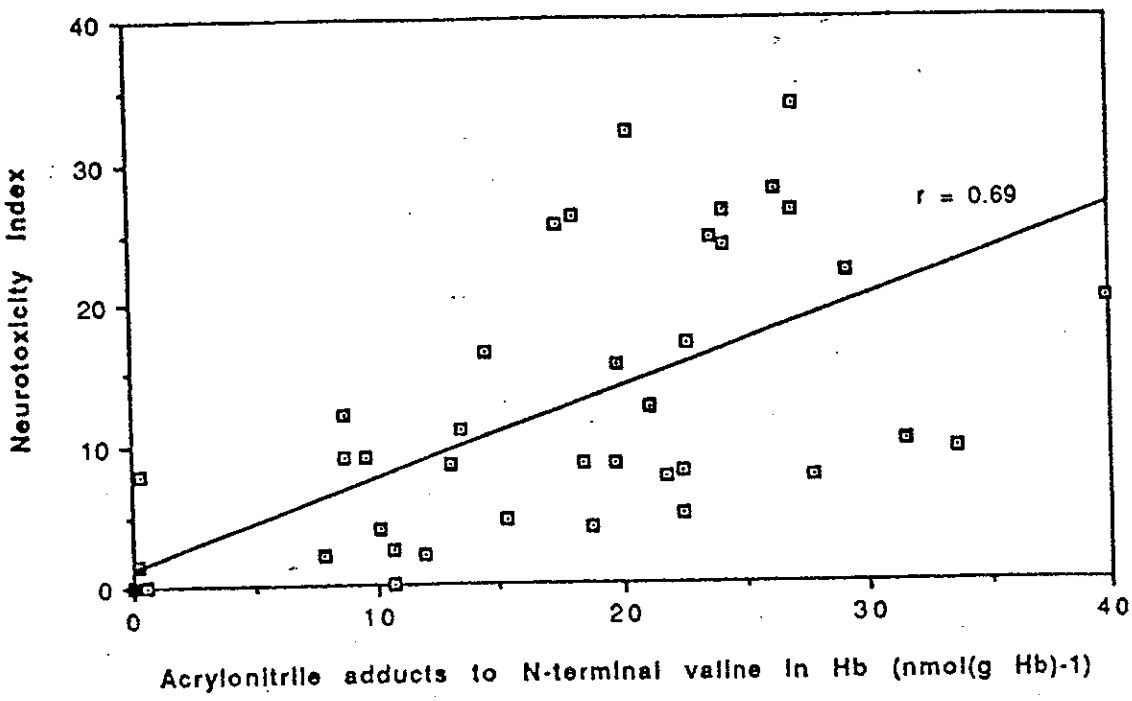

Figure 13. Correlation between neurotoxicity index and AN Val adducts.

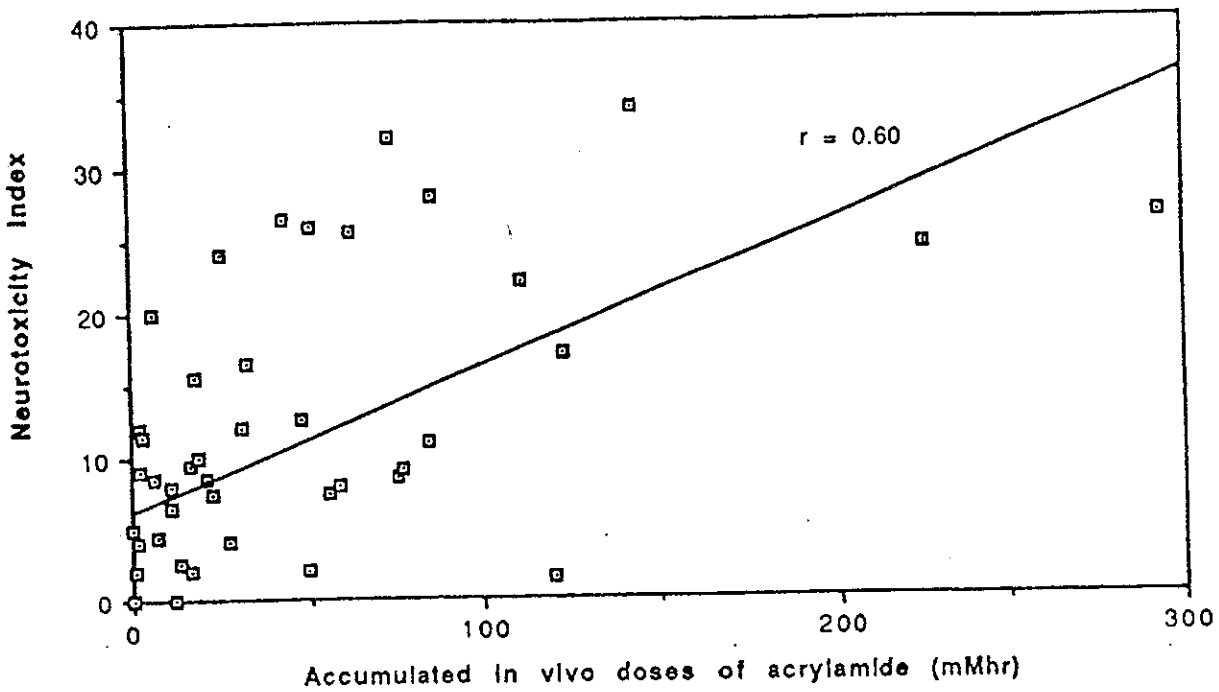

Figure 14. Correlation between neurotoxicity index and lifetime *in vivo* doses of AA.

TABLE 13

Correlation coefficients and levels of statistical significance for the relationships between different predictive parameters and the Neurotoxicity Index (NIn).

X variable	Y variable	Corr. Coeff.	P-value
free AA	NIn	0.15	0.31
merc	NIn	0.42	<0.01
AAVal	NIn	0.67	<0.001
AAVal x time	NIn	0.60	<0.001
ANVal	NIn	0.69	<0.001
ANVal x time	NIn	0.60	<0.001
time	NIn	0.44	<0.01
Foot Vibratron	NIn	0.69	<0.001
Hand Vibratron	NIn	0.81	<0.001
AAVal	ANVal	0.44	<0.01

values of the different biomarkers, as well as of different indicators of neurotoxicity in the different job categories, are shown in **Table 12**.

General discussion of Aim 2

Hemoglobin adducts

Electrophilic compounds react with nucleophilic amino acid residues in Hb, such as the SH of cysteine or the NH_2 of N-terminal valine. The activated double bonds of AA and AN react with nucleophilic sites in Michael type additions, whereas the epoxide GA reacts according to a classical nucleophilic substitution mechanism.

When analyzing samples from humans exposed to both AA and AN, the modified Edman degradation was our method of choice. The reasons for this were two-fold: first, this method is less time-consuming than the determination of adducts following total hydrolysis and ion-exchange chromatography; second, the total hydrolysis method does not distinguish between adducts originating from AA and AN, since both the 2-carbamoylethyl- and the 2-cyanoethyl- groups are converted to the 2-carboxyethyl- group in strong acid.

In our analyses, the amount of the reagent PFPITC was increased compared to the standard procedure of Törnqvist et al. (1988) because this has been shown to give a better yield of certain PFPTH-derivatives, and was therefore assumed to increase the yield of AAVal-PFPTH. d_4-HOEtVal was used as an internal standard because of the availability of a well-characterized standard globin alkylated with d_4-ethylene oxide. The overall response (a combination of yield in the derivatization and extraction procedure, and the response on GC/MS) was for AAVal-PFPTH somewhat lower (1.5-8 times) and for ANVal-PFPTH slightly higher (2-6 times) than for d_4-HOEtVal-PFPTH. The yield and extraction efficiency were not determined, but they were assumed to be constant for AAVal-PFPTH and ANVal-PFPTH versus the internal standard. The difference in response using GC/MS was compensated for by the use of calibration curve. Although the PFPTH derivative of GAVal was also formed and was detectable, the overall response was low (1-2 orders of magnitude lower than that of d_4-HOEtVal-PFPTH), and the quantitations were not reproducible. This might be explained by the fact that the GA adduct is very hydrophilic so that its PFPTH-derivative may be difficult to extract from the reaction mixture.

The AA and GA valine adducts were generally not detected in unexposed controls and a possible background could not be established. AN adducts were, however, detected in most smokers. Based on the lowest levels detected, detection limits for the method appear to be 0.01 nmol/g Hb (signal-to-noise ratio 6:1) and 0.02 nmol/g Hb (signal-to-noise ratio 12:1) for the AAVal-PFPTH and ANVal-PFPTH derivatives, respectively. These results show that the determination of AA and AN adducts using the modified Edman degradation method can be used as a tool to monitor total exposure to AA and/or AN.

As discussed above, adduct formation by GA to N-terminal valine was analyzed using the

total hydrolysis method, because of the low yields and hydrophilicity of its PFPTH-derivative. The detection limit of this method was about 1 nmol/g Hb. This acid hydrolysis method was used also to determine adduct levels in Hb treated *in vitro* with AA or GA for the determination of rate constants with cysteine-SH and valine NH_2. Background levels of the cysteine adducts were high in unexposed controls, probably due to methodological artifacts, and analysis of these adducts is suitable only for determination of high alkylation levels such as those in the *in vitro* experiment.

The valine adducts (CEVal and CHEVal) did not give the expected structures following derivatization with methanol/HCl and HFBA, apparently due to rearrangement reactions. The resulting derivatives are interpreted as being formed during the acylation, presumably by nucleophilic attack of the N-acyl-oxygen on the carbonyl of the "C-terminal ester" followed by elimination of CH_3OH. The derivatives of the d_8-analogues therefore contain only six of the original deuteriums in the d_8-valine as was shown by the fragmentation pattern on GC/MS. Comparison of mass spectra of the deuterated and the non-deuterated derivatives in the EI and the PCI modes proved to be essential in order to elucidate these structures. The CHEVal derivative gave four additional peaks on GC, whose structures were identified as stereoisomers of lactons, also formed during the derivatization. The use of deuterated standards compensated for variations in yield of the different derivatives. The quantitation of GA adducts was made on the same type of derivative as the one formed for CEVal.

While performing the analysis of GA valine adducts using total hydrolysis, CEVal was also determined and found to correspond well ($\pm$ 8 %) to the sum of the AA and AN valine adducts determined by the modified Edman procedure in the same individuals. As mentioned earlier, both AAVal and ANVal form CEVal upon acid hydrolysis of globin.

Based on their roles and positions in the production process we have subdivided the AA factory workers into four different categories: (1) workers involved in the synthesis of AA from AN and in the transfer of 35% AA solution into barrels, (2) ambulatory personnel such as foremen, maintenance workers, etc., with no stationary work place, (3) workers involved in the copolymerization of AA and acrylic acid, and (4) workers who packaged the copolymer after mixing it with starch. Among the workers with the lowest levels of AAVal adducts, one had been free of AA exposure for the past four months (No. 30) and one worked in the store house (No. 40). The air levels at different locations in the workshop were monitored on four different occasions prior to blood sampling. In September 1991 the AA air levels ranged between 0.1 and 1.6 mg/m^3, and in the summer months the levels ranged between 0.3 and 8.8 mg/m^3. Although the concentrations measured fall within a quite narrow range, an occasional level of 153 mg/m^3 was recorded during discharge of AA. The maximal allowable air concentration for AA in the workplace is 0.3 mg/m^3 both in the People's Republic of China and in the United States. A few conclusions may be drawn from the data presented. First, the average Hb adduct level of AA is related to the job description and to the location of the different workers in the factory. Second, it appears that only a small fraction of the total amount of AA absorbed by the workers

originated from inhalation of AA. Indeed, assuming a breathing rate of 0.2 l min^{-1} kg^{-1}, estimated for workers doing work involving moderate physical exercise (Calleman et al., 1978), and that the first-order rate of elimination of AA in humans is the same as in rats (0.5 hr^{-1}), it may be estimated that the observed Hb adduct levels in workers in the synthesis and polymerization rooms are 10-30 times higher than would be expected from the measured concentration of AA in the air. Our Hb adduct data are thus consistent with the notion that dermal exposure may be the predominant route of uptake, as suggested in several previous studies (EPA, 1988). In addition, the observation of skin peeling of the hands in several of the workers also suggests that dermal exposure is a significant route of uptake of AA.

The ratio of the *in vivo* doses of GA and AA was on average 3:10. In the rat, the percentage of AA converted to GA is about 60% following a low dose of AA, and the conversion of AA to GA decreases as the injected amount of AA increases (Bergmark et al. 1991). The metabolism of AA in the rat is therefore saturated at high administered amounts. It might be assumed that no saturation of metabolism of AA to GA occurs at the exposure levels found in this study in humans, because of the linear correlation between AA and GA adducts.

Recent preliminary experiments by D. Segerback (personal communication) showed that in mice and rats treated with AA, the major DNA adduct is N-7-(2-carbamoyl-2-hydroxyethyl)-guanine, which is formed by the reaction of GA with DNA. This is consistent with the hypothesis that GA may be the agent primarily responsible for cancer initiation following exposure to AA (Calleman et al. 1990). The findings that AA is metabolized to the genotoxic metabolite GA in humans, and that GA adducts are linearly related to AA adduct levels, suggest that exposure to AA might be associated with an increased cancer risk. However, a recent epidemiological study (Collins et al. 1989) has not found an association between AA exposure and an excess of cancer deaths, although the exposure levels determined in that study were much lower than in the present one. In this regard, it is of interest to note that the daily *in vivo* doses of AA and GA in the most highly exposed workers correspond to the *in vivo* doses in rats injected daily with 3 mg/kg AA. In cancer tests (Johnson et al., 1986), a daily dose of 2 mg/kg increased several types of tumors in Fisher 344 rats. In addition, exposure to AN is also expected to pose an increased risk of cancer. Therefore, follow-up studies using Hb adducts as biomarkers for monitoring reductions in the exposure to AA and AN as well as prospective studies of cancer incidences in workers heavily exposed to AA and AN are warranted.

Field study

As discussed before, the combination of uptake of acrylamide through different routes is a common characteristic of industrial exposure to acrylamide and speaks in favor of using biomarkers reflective of the total uptake of the substance through all routes as a basis for evaluating hazardous conditions in the workplace.

The biomarkers of exposure measured in this study reflect, however, different time-periods of exposure and are thus not expected to be equally useful for predicting an effect which is believed to be a function of a cumulative dose, or integrated concentration of the neurotoxic agent.

The concentration of free acrylamide in the plasma could only be detected in a few workers from each group except for the most extensively exposed group located in the synthesis room. It must be assumed that the levels of acrylamide detected in three of the controls reflect an artifact peak eluting at the retention time of the derivatized acrylamide, since no Hb adducts could be found from acrylamide in this group, and the levels detected (62-69 ppm) are only slightly above the detectability of this method (40 ppm). Given that this indicator reflects a momentary level and that blood sampling was performed at different times after the workers had left their work positions, the fact that free acrylamide could not be found in a high number of workers and that it correlated poorly with the Neurotoxicity Index, is not surprising. It is, however, noteworthy that the levels of free acrylamide in the plasma of the most highly exposed group are close to what would be expected from the Hb adduct values under the assumption of a rate of elimination of acrylamide from human plasma similar to that estimated in rats (0.5 hr^{-1}, Miller et al., 1982; Bergmark et al., 1991). Thus, the average level of acrylamide Hb adducts (14.7 nmol/g Hb) in this group corresponded to an average *in vivo* dose of 6.6 mMhr during the life-span of the erythrocytes (17 weeks in humans). This corresponds to a steady-state level of 580 ppm of free acrylamide in the plasma of workers working 8 hrs/day, 6 days/week. While quantitative inferences from these types of measurements should be made with great caution, the results seem to indicate that the rate of elimination of acrylamide is of the same order of magnitude in humans as in rats.

In contrast, the determination of mercapturic acids reflects exposure during approximately 1 to 2 days prior to urine sampling. In our study, the urine was hydrolyzed prior to analysis to yield the amino acid S-(2-carboxyethyl)-cysteine, the concentration of which reflects not only acrylamide, but also acrylonitrile conjugates, which are hydrolyzed to the same amino acid. In addition to this drawback, it should be kept in mind that mercapturic acids are detoxification products which are not necessarily proportional to the amount of reaction with target enzymes in nervous tissue. To the extent that interindividual variability in detoxification efficiency of acrylamide or acrylonitrile comes into play, the amount of mercapturic acids excreted in the urine may in fact be inversely related to the *in vivo* doses. Not surprisingly, the concentration of these mercapturic acids in 24-hr urine is a relatively poor predictor of the Neurotoxicity Index.

Hemoglobin adducts reflect the *in vivo* dose (integrated concentration) of an electrophilic substance during the 4-month period prior to blood sampling. As expected, this biomarker is better correlated with the neurotoxicity index than the other biomarkers of exposure determined in this study. Two peculiarities deserve attention in this regard: first, the fact that the Hb adducts formed by acrylonitrile correlate better with the Neurotoxicity Index than those formed by acrylamide, and second, that the lifetime *in vivo* doses of either agent

as estimated from the Hb adduct levels and time of employment do not correlate as well with neurotoxicity as do the Hb adducts themselves.

The good correlation between Hb adducts of acrylonitrile and the neurotoxicity index cannot be explained by a causative relationship since acrylonitrile exposure does not result in the different signs, symptoms and predictive parameters which were included in this index as defined here. Humans who have been exclusively exposed to acrylonitrile in occupational settings may present effects on the central nervous system but not the peripheral neuropathy characteristic of acrylamide exposure (F. He, personal communication to C. Calleman). However, although Hb adducts of acrylonitrile correlate better than those of acrylamide in univariate regression analysis, multivariate analysis gives the relationship: NIn = 0.59 (+0.14) x AAVal + 0.43 (+0.08) x ANVal, where both AAVal and ANVal are correlated with the NIn at the $p<0.01$.

The impairment of vibration sensation has been found to be an early sign of acrylamide neuropathy and proved to be related to the vulnerability of Pacinian corpuscles to acrylamide (LeQuesne, 1980). In this study, the prevalence of deficits of vibration sensation in acrylamide workers examined by a tuning fork (C 128) was 41%, whereas 58.5% of the same acrylamide group detected by a Vibratron II vibration sensitivity tester showed an increase of vibration thresholds which were higher than the upper limits of vibration thresholds of the reference group. This suggests that the Vibratron II vibration sensitivity tester is superior to tuning forks in terms of sensitivity, reliability and quantitation for the assessment of impairments of vibration sensation. It is therefore potentially useful in field screening of peripheral nerve dysfunction in acrylamide exposed workers. In fact, vibration thresholds in hands as determined by the Vibratron instrument show the best correlation with neurotoxicity of all the potentially predictive parameters. This good correlation is hardly unexpected given that loss of vibration sensitivity is a more sensitive indicator of peripheral neuropathy than either loss of pain or touch sensitivity and the Vibratron instrument may thus be a comparatively inexpensive and useful tool for identifying workshops with unacceptable exposure conditions.

The ENMG findings in acrylamide workers manifested as a prolongation of distal motor and sensory latencies and a decrease of distal sensory potentials in peripheral nerves indicate a damage to the distal part of the peripheral nervous system which coincides with the pattern of the central-peripheral distal axonopathy found in cats and rats dosed with acrylamide. The presence of abnormal recruitment patterns without any spontaneous denervation potentials of the sampled muscles suggests a mild damage to the motor fibers of the peripheral nervous system. On the other hand, there was a significant decrease of distal sensory potentials in 17 out of 41 acrylamide workers, the lowest being undetectable, indicating more involvement of sensory fibers than motor fibers in acrylamide neuropathy (Sumner and Asbury, 1974).

According to the diagnostic criteria for occupational acrylamide poisoning proposed in our previous studies (He et al., 1989) and based on the clinical and ENMG data available, 13

out of the acrylamide group under study were diagnosed with mild occupational acrylamide poisoning at a prevalence of 31.7%.

The results of this study provide evidence of neurotoxic effects of acrylamide in exposed workers, similar to those reported in previous studies (He et al., 1989, LeQuesne, 1980). The symptoms and signs presented in the acrylamide workers include numbness and weakness of hands and feet, excessive sweating of hands, impairments of vibration, pain and touch sensations, as well as diminution and loss of ankle reflexes. These clinical manifestations, usually preceded by peeling of skin from hands, are more prevalent than in the reference group and strongly indicate an involvement of the peripheral nervous system. The results of medical surveillance clearly indicate the necessity of prevention of acrylamide poisoning in the factory under study.

The neurotoxicity index was designed to be: (1) specific for acrylamide-induced peripheral neuropathy and to exclude such effects that might be due to acrylonitrile-induced effects on the central nervous system, (2) objective, i.e. independent of the subjective experience of the workers, and (3) reflective of the severity of the neurological disturbance. There was only minimal overlap in the Neurotoxicity Index between those individuals with a clinical diagnosis of peripheral neuropathy and those without.

Among the workers there was a better correlation between this neurotoxicity index and the levels of Hb adducts ($r = 0.67$) than with either the cumulative *in vivo* dose ($r = 0.60$) or the duration of employment ($r = 0.44$). However, in workers employed for more than 2 years, the correlation between neurotoxicity and the duration of exposure virtually disappeared ($r = -0.06$). The interpretation was thus made that the average dose-rate (as reflected by the Hb adduct levels for the 4-month period prior to blood sampling), rather than the cumulative *in vivo* dose, is the better predictive parameter for peripheral neuropathy in workers exposed for more than 2 years.

A risk analysis was hence performed which included only those workers who had been exposed to acrylamide for more than 2 years. If the percentages of these workers with different diagnostic indicators of neurotoxicity were plotted against their Hb adduct levels, sigmoid curves were evident reaching 100% in the group with the highest level of Hb adducts. From the plot, NOEL and LOEL and EL_{50} for clinical diagnosis of peripheral neuropathy were estimated to 2, 6 and 7.9 nmol AAVal (g Hb)$^{-1}$, respectively. Assuming a rate of elimination of acrylamide in man similar to that estimated in the rat, 0.5 hr^{-1}, these Hb adduct levels correspond to intakes of 0.3, 0.8 and 1.1 mg/kg/day, and further assuming a 100% uptake of airborne acrylamide and a rate of alveolar ventilation of 0.2 l min^{-1} (kg bw)$^{-1}$ in exposed workers, NOEL, LOEL and EL_{50} for peripheral neuropathy correspond to concentrations of 3, 8, and 10 mg/m^3, respectively. It may thus be concluded that in workers exposed for more than 2 years, the currently applied TWA of acrylamide, 0.3 mg/m^3, has a safety factor of 10 for adverse neurological effects. It should be also noted, however, that in the present study the primary route of exposure to AA appeared to be dermal.

AIM 3

Comparative neurotoxicity of acrylamide and glycidamide

Rats injected with the lowest dose of acrylamide gained weight, while those administered 50 mg/kg acrylamide or glycidamide did not (**Fig. 15**). Rats in the 100 mg/kg glycidamide group lost weight during the treatment. Animals in the two high-dose groups started to appear lethargic and ataxic after 5 injections (cumulative doses of 250 and 500 mg/kg for acrylamide and glycidamide, respectively). However, the appearance of the animals was quite different. While acrylamide-treated rats had their hindlimbs extended to both sides in the hindlimb splay test, glycidamide-treated rats routinely extended both legs to the same side.

Behavioral tests

The results of the rotarod testing are shown in **Fig. 16**. At the low doses, both acrylamide and glycidamide had no significant effect on the ability of rats to stay on the rod. Similar significant effects were, however, observed starting on day 6 in animals receiving either acrylamide (50 mg/kg) or glycidamide (100 mg/kg). On the other hand, in the hindlimb splay test a significant effect was seen only in animals treated with acrylamide (**Fig. 17**). At the 50 mg/kg dose, acrylamide increased the spread of the hindlimbs on landing after two doses, while at the 25 mg/kg dose a significant effect was seen after 8 injections. Neither dose level of glycidamide had any effect in this test. In the open field tests, no significant differences were found between control and treated rats at any time point, with regard to deambulation. However, animals in the 50 mg/kg acrylamide group showed a decrease in rearing on day 4. On day 8 the values for rearing (mean score $\pm$ SEM) were: control, 26.9 $\pm$ 9.4; glycidamide 50 mg/kg, 21.6 $\pm$ 9.6; glycidamide 100 mg/kg, 13.0 $\pm$ 2.6; acrylamide 25 mg/kg, 19.5 $\pm$ 5.1; acrylamide 50 mg/kg, 6.1 $\pm$ 2.9 (<0.05; n = 10/group).

Morphological observations

One of the most striking features at necropsy was urinary retention and distended bladders in rats dosed subacutely with acrylamide, as previously reported. The effect of acrylamide was clearly dose-dependent. Glycidamide appeared to cause some urinary retention but to a much lesser degree. In animals treated for 8 days with 50 mg/kg acrylamide or 100 mg/kg glycidamide, no apparent morphological alterations were seen at the light microscopic level in sections of the sciatic nerve. Therefore, additional groups of rats were injected with the same doses for 11 or 12 days to reach cumulative doses of 600 mg/kg (acrylamide) or 1200 mg/kg (glycidamide). Characteristic changes of acrylamide neurotoxicity were seen in the acrylamide group, with degeneration and axonal swelling, but not in the glycidamide-treated animals.

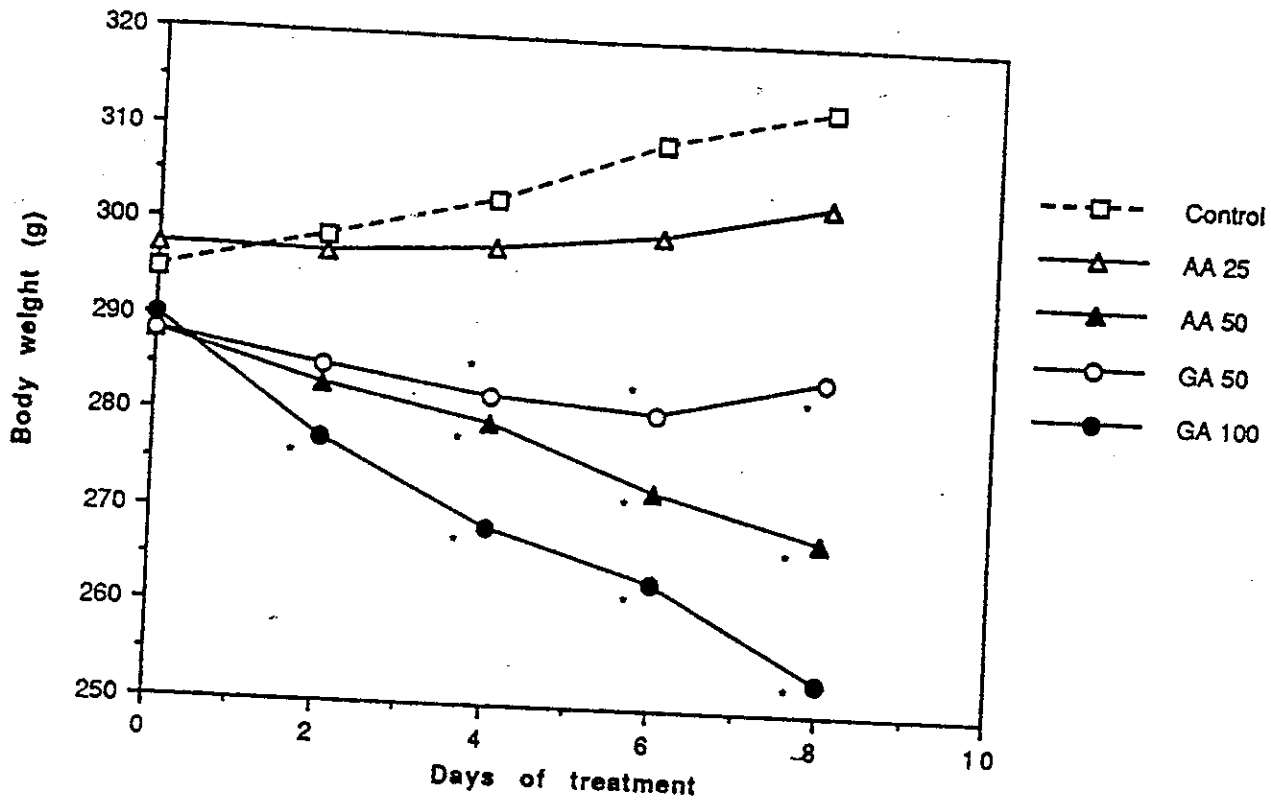

Figure 15. Body weights of rats treated with AA or GA.

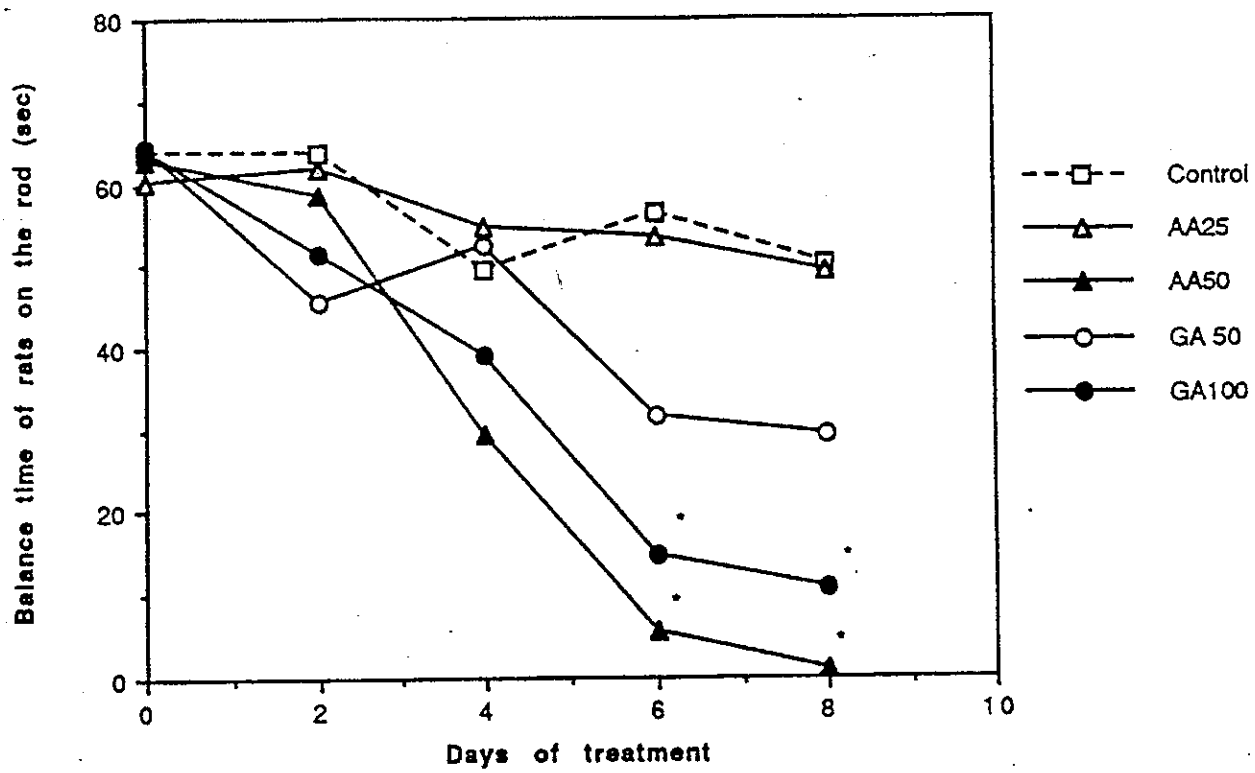

Figure 16. Rotarod test performance of rats exposed to AA or GA.

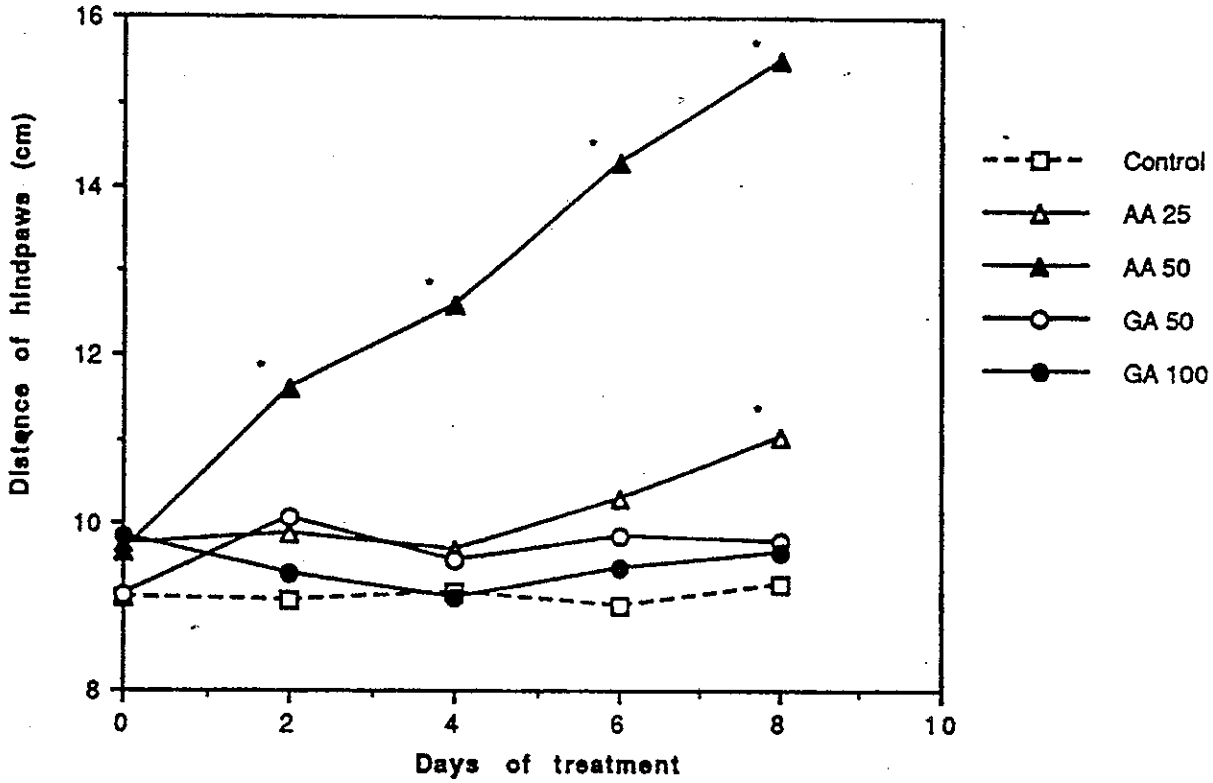

Figure 17. Hindlimb splay test in rats exposed to AA or GA.

Male reproductive toxicity of acrylamide and glycidamide

The effects of AA and its epoxide metabolite GA on the male reproductive system were determined after 7 and 14 days of treatment, respectively (50 mg/kg i.p./day). GA was administered for 14 days and no overt signs of toxicity were seen, except for a significant decrease in total body weight. Administration of AA was stopped after 7 days because at this time rats showed severe signs of neurotoxicity, i.e., hindlimb weakness and ataxia. Body weight following AA treatment was also significantly decreased.

GA significantly decreased epididymis weight, the ratio of epididymis weight/testis weight and the protein content of testicular tissue (**Table 14**), while AA exposure had no effect on these parameters. The vas deferens sperm count was significantly decreased by both AA and GA; however, only GA significantly affected viability (control, 33.1 $\pm$ 7.7% viable; GA, 3.6 $\pm$ 1.4% viable) (**Table 15**). DSP was affected in the epididymis only by GA, but no effect in the testis was seen for either treatment. Plasma testosterone levels were also unaffected by either treatment.

No differences in gross morphology and light microscopy of reproductive organs were seen with either compound. The seminiferous tubules were unaltered in their epithelium. Sertoli cells and interstitial cells appeared to be unaffected with either treatment.

General discussion of Aim 3

The main conclusion which can be drawn from the present results is that GA is involved in the reproductive toxicity of AA, but not in its neurotoxicity. Administration of AA, to a cumulative dose of 400 mg/kg, had a significant effect on the performance of rats in the rotarod test and in the hindlimb splay test, which is considered more specific for peripheral neuropathy (Edwards and Parker, 1977). Indeed, AA had a significant effect in this latter test even at the lower dosage regimen (25 mg/kg/da, for a cumulative dose of 200 mg/kg). GA, at the cumulative dose of 400 mg/kg, had no effect on either test, while at the cumulative dose of 800 mg/kg a significant effect was seen in the rotarod test. Animals in this group were very weak and lost weight compared to the other groups; the positive effect observed in the rotarod may, therefore, be due to general systemic toxicity rather than to a neurotoxic effect on peripheral nerves. The lack of effect in the hindlimb splay test seems to confirm this hypothesis. Furthermore, morphological observation at the light microscope level did not identify any damage to the tibial and sciatic nerve of GA 100 - treated rats.

Where male rats were given AA in a dosage regimen of 50 mg/kg/day for a cumulative dose of 350 mg/kg, mild signs of reproductive toxicity were present, as shown by a decrease of vas deferens sperm count. Longer treatments with AA were prevented by its severe neurotoxicity. On the other hand, when GA was given at the same dosage regimen for a cumulative dose of 700 mg/kg, signs of toxicity were minimal and no neurotoxicity was observed, although animals displayed significant toxic effects in the reproductive

TABLE 14

Effects of acrylamide and glycidamide on male reproductive organs in rats

	Control[a]	Acrylamide	Glycidamide
Body wt (g)	372 ± 3.7	330 ± 80[b]	344 ± 5.5[b]
Testis wt (g)	3.7 ± 0.1	3.4 ± 0.1	3.6 ± 0.1
Epididymis wt (g)	1.2 ± 0.04	1.1 ± 0.03	1.0 ± 0.04[b]
Testis wt/body wt (%)	1.0 ± 0.02	1.0 ± 0.03	1.1 ± 0.04
Epididy wt/testis wt (%)	33.5 ± 1.4	33.1 ± 1.1	29.1 ± 0.9[b]
Testis protein (mg/g testis)	96.6 ± 5.2	105.8 ± 4.8	77.4 ± 4.1[b]
Testosterone (ng/ml)	0.6 ± 0.2	0.8 ± 0.2	1.1 ± 0.4

[a] Results are mean ± S.E. of at least 4 animals. No significant difference between controls at 7 versus 14 days.
[b] Significantly different from control, $p \leq 0.05$.

TABLE 15

Effects of acrylamide and glycidamide on male reproductive parameters in rats

	Control[a]	Acrylamide	Glycidamide
Vas deferens sperm count (cell no. x 10^6)	14.3 ± 2.6	7.8 ± 0.6[b]	6.2 ± 1.2[b]
Sperm cell viability (% viability)	33.1 ± 7.7	32.4 ± 6.8	3.6 ± 1.4[b]
Testicular DSP (cell no. x 10^6)	67.6 ± 18.5	57.6 ± 10.2	66.2 ± 11.0
Epididymal DSP	365 ± 39.7	303 ± 25.8	68.8 ± 10.3[b]
Testosterone (ng/ml)	0.6 ± 0.2	0.8 ± 0.2	1.1 ± 0.4

[a] Results are mean ± S.E. of at least 4 animals. No significant difference between controls at 7 versus 14 days.
[b] Significantly different from control, $p \leq 0.05$.

system. The finding that GA causes reproductive toxicity agrees with the hypothesis of Sega et al. (1990) who postulated a role for this epoxide metabolite in the effects of AA on male gonads.

In summary, these preliminary observations suggest that peripheral neurotoxicity of AA may be ascribed to the parent compound, while reproductive toxicity may be due to the formation of the epoxide metabolite GA. Both compounds react readily with proteins and form adducts to hemoglobin (Calleman et al. 1990) and appear to have similar access to target tissues, while they may differ significantly in their reactivity with DNA. The cellular and molecular mechanisms involved in their toxicity in the nervous and reproductive systems need to be elucidated.

REFERENCES

Bailey E, Farmer PB, Bird I, Lamb JH, Peal JA. (1986). Monitoring exposure to acrylamide by the determination of S-(2-carboxyethyl)cysteine in hydrolyzed hemoglobin by gas chromatography-mass spectrometry. Anal. Biochem. 157, 241-248.

Bergmark E, Calleman CJ, Costa LG. (1991). Formation of hemoglobin adducts of acrylamide and its epoxide glycidamide in the rat. Toxicol. Appl. Pharmacol. 111, 352-363.

Bergmark E, Calleman CJ, He F, Costa LG. (1993). Determination of hemoglobin adducts in humans occupationally exposed to acrylamide. Toxicol. Appl. Pharmacol. 120, 45-54.

Bishop MWH, Smiles J. (1957). Induced fluorescence in mammalian gametes with acridine orange. Nature (London) 179, 307-308.

Calleman CJ, Bergmark E, Costa LG. (1990). Acrylamide is metabolized to glycidamide in the rat: Evidence from hemoglobin adduct formation. Chem. Res. Toxicol. 3, 406-412.

Calleman CJ, Ehrenberg L, Jansson B, Osterman-Golkar S, Segerback D, Svensson K, Wachtmeister CA. (1978). Monitoring and risk assessment by means of alkyl groups in hemoglobin in persons occupationally exposed to ethylene oxide. J. Environ. Pathol. Toxicol. 2, 427-442.

Collins JJ, Swaen GMH, Marsh GM, Utijian HM, Hashimoto K, Tanij H. (1989). Mutagenicity of acrylamide and its analogues in *Salmonella typhimurium*. Mutat. Res. 158, 129-133.

Crestfield AM, Moore S, Stein WH. (1963). The preparation and enzymatic hydrolysis of reduced and S-carboxymethylated proteins. J. Biol. Chem. 238, 622-627.

Dearfield KL, Abernathy CO, Ottley MS, Brantner JH, Hayes PF. (1988). Acrylamide: Its metabolism, developmental and reproductive effects, genotoxicity, and carcinogenicity. Mutat. Res. 195, 45-77.

Derelanko MJ. (1987). Determination of erythrocyte life span in F-344, Wistar, and Sprague-Dawley rats using a modification of the [^{3}H]diisopropylfluorophosphate ([^{3}H]DFP) method. Fundam. Appl. Toxicol. 9, 271-276.

Edwards PM. (1975). The distribution and metabolism of acrylamide and its neurotoxic analogues in rats. Biochem. Pharmacol. 24, 1277-1282.

Edwards PM, Parker VH. (1977). A simple, sensitive and objective method for early assessment of acrylamide neuropathy in rats. Toxicol. Appl. Pharmacol. 40, 589-591.

EPA (1988). Preliminary Assessment of Health Risks from Exposure to Acrylamide. Office of Toxic Substances, Washington D.C., 1988.

EPA (1990). Protein Adduct Forming Chemicals for Exposure Monitoring: Literature Summary and Recommendations. EPA/600/4-90/007.

Fennell TR, MacNeela MJ, Turner MJ, Swenberg JA. (1989). Hemoglobin adduct formation by acrylonitrile in rats and mice. Proc. AACR 30, 132.

Hamboek H, Fischer RW, Dilorio EE, Winterhalter KH. (1981). The binding of S-triazine metabolites to rodent hemoglobins appears irrelevant to other species. Mol. Pharmacol. 20, 579-584.

Hashimoto K, Aldridge WN. (1970). Biochemical studies on acrylamide, a neurotoxic agent. Biochem. Pharmacol. 19, 2591-2604.

He F, Zhang S, Wang H, Li G, Zhang Z, Li F, Dong X, Hu F. (1989). Neurological and electroneuromyographic assessment of the adverse effects of acrylamide on occupationally exposed workers. Scand. J. Work Environ. Health 15, 125-129.

Johnson KA, Gorzinski SJ, Bodner KM, Campbell RA, Wolf CH, Friedman MA, Mast RW. (1986). Chronic toxicity and oncogenicity study on acrylamide incorporated in the drinking water of Fisher 344 rats. Toxicol. Appl. Pharmacol. 85, 154-168.

LeQuesne PM. (1980). Acrylamide. In Spencer PS, Schaumburg HH, Eds. Experimental and Clinical Neurotoxicology. Williams and Wilkins Company, Baltimore, MD, 309-325.

Miller MJ, Carter DE, Sipes IG. (1982). Pharmacokinetics of acrylamide in Fisher-344 rats. Toxicol. Appl. Pharmacol. 63, 36-44.

Miller MS, Spencer PS. (1985). The mechanisms of acrylamide axonopathy. Annu. Rev. Pharmacol. Toxicol. 25, 643-666.

Mowrer J, Törnqvist M, Jensen S, Ehrenberg L. (1986). Modified Edman degradation applied to hemoglobin for monitoring occupational exposure to alkylating agents. Toxicol. Environ. Chem. 11, 215-231.

Neumann H-G. (1981). Significance of metabolic activation and binding to nucleic acids of aminostilbene derivatives *in vivo*. Natl. Cancer Inst. Monogr. 58, 165-171.

Ohmori S, Shimomura T, Azumi T, Mizuhara S. (1965). S-(b-carboxy-n-propyl)-L-cysteine and S-(b-carboxyethyl)-L-cysteine in urine. Biochem. Z. 343, 9-15.

Osterman-Golkar S, Ehrenberg L, Segerbäck D, Holestrom I. (1976). Evaluation of genetic risk of alkylating agents. II. Haemoglobin as a dose monotor. Mutat. Res. 34, 1-10.

Osterman-Golkar S, Ehrenberg L. (1982). Covalent binding of reactive intermediates to hemoglobin as an approach for determining the metabolic activation of chemicals: Ethylene. Drug Metab. Rev. 13, 647-660.

Sega GA, Generoso EE, Brimer PA. (1990). Acrylamide exposure induces a delayed unscheduled DNA synthesis in germ cells of male mice that is correlated with the temporal pattern of adduct formation in testis DNA. Environ. Mol. Mutag. 16, 137-142.

Segerbäck D. (1990). Reaction products in hemoglobin and DNA after in vitro treatment with ethylene oxide and N-(2-hydroxyethyl)-N-nitrosurea. Carcinogenesis 11, 307-312.

Sumner AJ, Asbury AK. (1974). Acrylamide neuropathy: Selective vulnerability of sensory fibers. Trans. Am. Neurol. Assoc. 99, 79-83.

Tilson HA. (1981). The neurotoxicity of acrylamide: An overview. Neurobehav. Toxicol. Teratol. 3, 445-461.

Törnqvist M, Kautiainen A, Gatz RN, Ehrenberg L. (1988). Hemoglobin adducts in animals exposed to gasoline and diesel exhaust. I. Alkenes. J. Appl. Toxicol. 8, 159-170.

Yao K, Suzuki M, Ohmori S. (1970). S-(2-hydroxy-2-carboxyethyl)cysteine in the normal human urine. Physiol. Chem. Phys. 2, 195-199.

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SUPPLEMENTARY NOTES
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ABSTRACT

The present project was undertaken to develop new methodologies for biological monitoring of exposure to the toxicant acrylamide in laboratory animals as well as humans. Methods were developed to measure the adducts of acrylamide and its epoxide metobolite glycidamide to cycsteine in rat hemoglobin and to valine in human hemoglobin by means of gas chromatography/Mass spectrometry.

Studies in rats indicated that both acrylamide and glycidamide adducts are formed following acute or chronic exposure to acrylamide, while only the glycidamide adducts are formed after exposure to glycidamide. Both adducts, in addition to acrylonitrile adducts, were measured in a group of Chinese workers exposed to acrylamide during its synthesis and polymerization. Significant signs of neurotoxicity were also found in this population.

Additional studies in rats indicated that acrylamide, but not glycidamide, is the proximate neurotoxicant while glycidamide may be responsible for the male reproductive and genotoxic/carcinogenic effects of acrylamide.

These studies suggest that these novel biomarkers to assess exposure to acrylamide are useful to assess potential health hazards (including possible cancer risks) due to exposure to acrylamide in occupationally exposed workers as well as in the general population.

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KEY WORDS AND DOCUMENT ANALYSIS

DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

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Draft excerpts from a manuscript on kinesin activity

Acrylamide was found to cause chromosomal aberrations and aneuploidy *in vitro* and *in vivo* (7). Acrylamide also induces genetically transmitted mutations as measured by heritable translocation test, dominant lethal test and specific locus test (8). However, acrylamide does not cause gene mutations as measured in enzyme mediated bacterial tests. Glycidamide is an extremely stable epoxide; so stable that it is found in mouse, rat and human urine (*9*). This epoxide reacts slowly with guanine to produce N-7-(2-carbamoyl-2-hydroxy-ethyl)guanine and, to a lesser extent with adenine to produce N-3-(2-carbamoyl-2-hydroxyethyl) adenine (10).

The mechanism by which acrylamide induces neurotoxicity is now well established (*13*). Acrylamide and its metabolite glycidamide are highly reactive with sulfhydryl groups in proteins (*14*). Alternatively, either the dependence of the organ or cell upon the function of the target protein or the sensitivity of specific proteins to sulfhydryl binding may determine the toxicological specificity of the acrylamide response. Therefore, concentration responses become essential to identifying critical site(s) of action of this toxicant.

We organized the discussion around four major themes: (1) data that suggests that DNA binding is not the basis for genetic anomalies, (2) the studies that demonstrated that kinesin binding is the mode of action in the peripheral nervous system, (3) the analogous kinesin studies conducted on genetic material and (4) the implications for these observations on risk assessment.

DNA alkylation and genotoxicity

Acrylamide binding to DNA is extremely slow; however, the epoxide metabolite glycidamide does bind DNA (16). In addition, the adducts formed by binding of glycidamide to DNA are not in the base-pairing region **(Figure 1** (*17*). The concept of low genotoxic potential of the N-7 adduct is supported by the data presented by Jenkins *et al*., (*18*) which indicates that N-7 alkyl adducts are effectively repaired and thus show threshold dose response relationships for mutation induction. Others have shown that alkylation of the N-7 and N-3 groups by methylmethane sulfonate are associated with thresholds in the formation of micronuclei and mutagenic potential (19). Therefore, the alkylation of DNA does not, a priori, dictate a mutagenic mechanism of action and genotoxic outcomes can vary dependent upon relative alkylation versus repair rate.

There appears to be an inconsistency between the time course of DNA adducts with the occurrence of DNA damage. The repair of DNA appears rapid as measured in the alkaline comet assay **(**20).**) When the DNA damage data at 5 and 24 hours are recalculated as percent of control (to account for differences in control (background), damage in the bone marrow, and liver are decreasing while the DNA adducts are increasing** (Table 1)**. In contrast, brain adducts were increasing over this time period while the adducts were decreasing**, (*20*). Therefore, DNA adducts and DNA damage as measured by the comet assay are not temporally correlated.

Human lymphocytes treated with acrylamide at concentrations ranging from 0.5-50 µM for 1 hour showed double strand breaks in DNA similar to hydrogen peroxide, the positive control (21). These were repaired within an hour showing double strand breaks in the DNA. This repair in DNA is in marked contrast to the pattern of DNA alkylation. The loss of bound of acrylamide follows a different time scale.

Treatment of 8-week old rats with 15 mg/kg of acrylamide in drinking water induced no increase in DNA strand breaks in liver (*22, 23*). In contrast, there was a significant increase in DNA breaks/labile sites in both the thyroid (50% over control) and adrenal (80% over control) glands as measured using the comet assay. The levels of DNA alkylation in all three organs are virtually the same (*20*). In contrast, acrylamide caused an increase in mutant frequency in the TK assay and MN test at a dose of 14mM while showing no DNA damage (*23*). In a loss of heterozygosity assay, this was accounted for by deletions. In V79 cells, acrylamide caused neither DNA damage, *hprt* mutations or micronuclei at concentrations of up to 6000 µM, 5000 µM or 10,000 µM, respectively (*24*). Collectively, these data suggest caution in extrapolation of DNA adduct level to the default conclusion that acrylamide or its glycidamide adduct produce genotoxicity. Additional data are required to determine if the DNA adducts are the cause of genotoxicity.

Prokaryotic cells are not mutated by acrylamide. Acrylamide has been extensively tested with and without metabolic activation in Salmonella. Acrylamide was not genotoxic at 5 mg/plate in one study (*26*), and was considered equivocal at 10 mg/plate (*25*). Glycidamide, on the other hand is a low potency genotoxicant and was found to be genotoxic at 50 µg/plate (*26*). One possible explanation is that acrylamide is not converted to glycidamide *in vitro*. Emmert et al.(*27*) transfected a methyl deficient strain of S. *typhimurium* with pin3ERb5. This plasmid contains cytochrome P450 reductase, cytochrome b5 and cytochrome P450 2E1. Allyl chloride, and 2-aminoanthracene, both substrates of cyp2e1, were genotoxic in this strain. Acrylamide at doses up to 10 mg/plate was not genotoxic in this strain showing that the prokaryote did not respond even when conversion to glycidamide took place. No satisfactory explanation for this

phenomenon has been proposed, although the lack of sensitivity of the prokaryotic cells could be a result of the lack of a cytoskeleton and therefore suggestive of a mitotic spindle mode of action.

Acrylamide was clastogenic to L5178Y and TK6 cells and did not produce point mutations when tested without S-9 (*23, 28)*. It was not tested with S9 in L5178Y cells. Measurement of colony size revealed that acrylamide induced only small colonies which are indicative of chromosome aberrations and not point mutations. This conclusion was confirmed with cytogenetic analysis. An unpublished NTP study of acrylamide in CHO cells resulted in induction of chromosome aberrations with S9; the response without S9 was judged "questionable" (NTP, 2007). Results showing chromosomal aberrations and mitotic spindle effects, and not point mutation, are a recurring theme in acrylamide genetic toxicology *(28)*). The compilation of the above results suggest that non-DNA adduct mechanisms have a more direct correlation with acrylamide induced genotoxicity than DNA adduct mechanisms. Deficiencies in the database are (1) the absence of concentration responses linking DNA adducts to mutations and (2) a void of data considering the impact of DNA repair mechanisms on DNA ACR adducts.

Literature Cited

7. Allen, B., Zeiger, E., Lawrence, G., Friedman, M., and Shipp, A., Dose-response modeling of in vivo genotoxicity data for use in risk assessment: some approaches illustrated by an analysis of acrylamide. *Regul Toxicol Pharmacol* **2005,** 41, (1), 6-27.

8. Dearfield, Kerry L., Douglas, George R., Ehling, Udo H., Moore, Martha M., Sega, Gary A. and Brusick, David J., Acrylamide: a Review of Its Genotoxicity and an Assessment of Heritable Genetic Risk. Mutat. Res. 330: 71-99.

9. Fennell, T. R., Sumner, S. C., Snyder, R. W., Burgess, J., Spicer, R., Bridson, W. E., and Friedman, M. A., Metabolism and hemoglobin adduct formation of acrylamide in humans. *Toxicol Sci* **2005,** 85, (1), 447-59.

10 . Doerge, D. R., da Costa, G. G., McDaniel, L. P., Churchwell, M. I., Twaddle, N. C., and Beland, F. A., DNA adducts derived from administration of acrylamide and glycidamide to mice and rats. *Mutat Res* **2005,** 580, (1-2), 131-41.

10. Sumner, S. C.; Fennell, T. R.; Moore, T. A.; Chanas, B.; Gonzalez, F.; Ghanayem, B. I., Role of cytochrome P450 2E1 in the metabolism of acrylamide and acrylonitrile in mice. *Chem Res Toxicol* **1999,** 12, (11), 1110-6.

11. Adler, I.-D., Baumgrtner, A., Gonda, H., and Friedman, M.A., 1-Aminobenzotriazole inhibits acrylamide- induced Dominant Lethal Effects in Spermatids of male mice. *Mutagenesis* **2000,** 15, (2), 133-136.

12. Ghanayem, B. I., Witt, K. L., Kissling, G. E., Tice, R. R., and Recio, L., Absence of acrylamide-induced genotoxicity in CYP2E1-null mice: Evidence consistent with a glycidamide-mediated effect. *Mutat Res* **2005**.

13. Sickles, D. W., Stone, J. D., and Friedman, M. A., Fast axonal transport: a site of acrylamide neurotoxicity? *Neurotoxicology* **2002,** 23, (2), 223-51.

14. Barber, D. S., Hunt, J. R., Ehrich, M. F., Lehning, E. J., and LoPachin, R. M., Metabolism, toxicokinetics and hemoglobin adduct formation in rats following subacute and subchronic acrylamide dosing. *Neurotoxicology* **2001,** 22, (3), 341-53.

15. Atay, N. Z., Calgan,Dilek , Ozakat1,Emine , and Varnali, Tereza Acrylamide and glycidamide adducts of Guanine. *Journ, Molec. Struct,:* **2005,** 738, 249-251.

16. Hemminki, K. and Lax, M., Stability of 7-alkyldeoxyguanosines of trichloropropylene oxide, epichlorohydrin and glycidol. Acta Pharmacol Toxicol (Copenh) 1986, 59:80-85.

17. Swenberg, J. A., Richardson, F. C., Boucheron, J. C., and Dyroff, M. C., Relationships Between DNA Adduct Formation and Carcinogenesis. *Env. Health Persp.***1985,** 62, 177-183.

18. Jenkins, G. J.; Doak, S. H.; Johnson, G. E.; Quick, E.; Waters, E. M.; Parry, J. M., Do dose response thresholds exist for genotoxic alkylating agents? *Mutagenesis* **2005,** 20, (6), 389-98

19. Doak, S. H., Jenkins, G. J., Johnson, G. E., Quick, E., Parry, E. M., and Parry, J. M., Mechanistic Influences for Mutation Induction Curves after Exposure to DNA-Reactive Carcinogens. Cancer Res. 67:3904-3911.

20. Maniere, I., Godard, T., Doerge, D. R., Churchwell, M. I., Guffroy, M., Laurentie, M., and Poul, J. M., DNA damage and DNA adduct formation in rat tissues following oral administration of acrylamide. Mutat Res 580:119-129.

21. Blasiak, J., Gloc, E., Wozniak, K., AND Czechowska, A., Genotoxicity of acrylamide in human lymphocytes. *Chem Biol Interact* **2004,** 149, (2-3), 137-49.

22. Lafferty, J. S., Kamendulis, L. M., Kaster, J., Jiang, J., and Klaunig, J. E., Subchronic acrylamide treatment induces a tissue-specific increase in DNA synthesis in the rat. *Toxicol Lett* **2004,** 154, (1-2), 95-103.

22. Klaunig, J. E.; Kamendulis, L. M., Mechanisms of acrylamide induced rodent carcinogenesis. *Adv Exp Med Biol* **2005,** 561, 49-62.

23. Koyama, N.; Sakamoto, H.; Sakuraba, M.; Koizumi, T.; Takashima, Y.; Hayashi, M.; Matsufuji, H.; Yamagata, K.; Masuda, S.; Kinae, N.; Honma, M., Genotoxicity of acrylamide and glycidamide in human lymphoblastoid TK6 cells. *Mutat Res* **2006,** 603, (2), 151-8.

24. Baum, M., Fauth, E., Fritzen, S., Herrmann, A., Mertes, P., Merz, K., Rudolphi, M., Zankl, H., and Eisenbrand, G., Acrylamide and glycidamide: genotoxic effects in V79-cells and human blood. *Mutat Res* **2005,** 580, (1-2), 61-9.

25. Zeiger, E., Haworth, S., Tests with preincubation modification of the Salmonella/microsome assay. In *Eval. short-term tests for carcin. Rep. of the inter. prog. on chem. Saf. coll. study on in vitro ass.. V*, 1985; pp 187-199.

26. Hashimoto, K., and Tanii, H., Mutagenicity of Acrylamide and its Analogues in Salmonella typhimurium. *Mut. Res.* **1985,** 158, (3), 129-133.

27. Emmert, B., Bünger, Jürgen, Keuch, Kristian, Müller, Michael, Emmert, Steffen, Hallier, Ernst, and Westphal, Götz A. , Mutagenicity of cytochrome P450 2E1 substrates in the Ames test with the metabolic competent S. typhimurium strain YG7108pin3ERb5. *Toxicology* **2006,** 228, (1), 66-76.

28. Moore, M. M., Amtower,A., Doerr,C., Brock,Karen H. and Dearfield,Kerry L., Mutagenicity and clastogenicity of acrylamide in L5178Y mouse lymphoma cells. *Environmental Mutagenesis* **1987,** 9, 261-267.

Figure 1: Double-stranded DNA showing the sites of DNA adduct formation including the important targets for alkylators (N7G, N3A, O6G, etc.) From Jenkins et al., 2005

Graphic representation of DNA base binding sites and their involvement in base pairing.

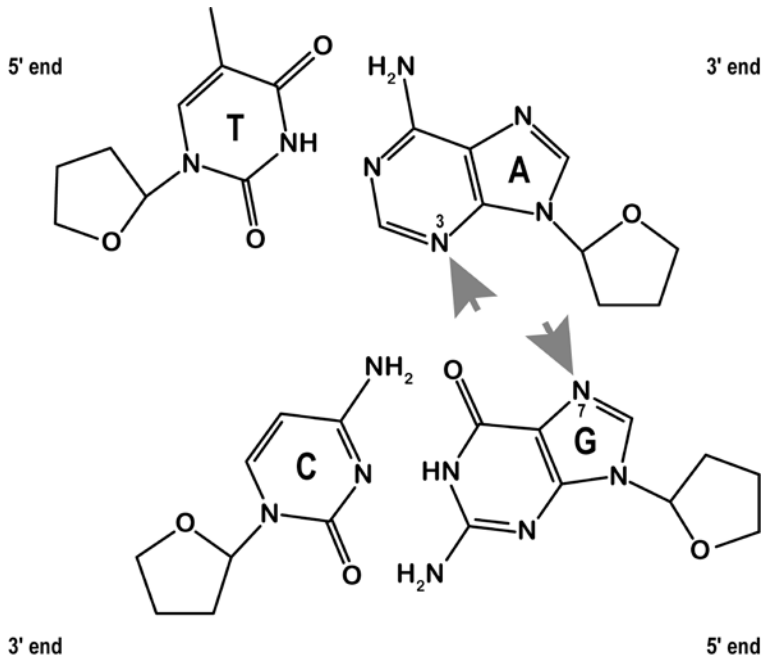

Table 1. Temporal Relationship Between DNA Adducts and DNA Damage[a]

Time After Treatment[1]	Bone Marrow Comet[2]	Liver Comet[2]	Brain Comet[2]	Liver 7-GA-GUA[3]	Brain 7-GA-GUA[3]
2 hours	113	143	86		
5 hours	229	206	186	5.1	3.2
24 hours	102	142	236	6.3	2.8
48 hours				2.9	2.6

[a.] Data from . Maniere, I., Godard, T., Doerge, D. R., Churchwell, M. I., Guffroy, M., Laurentie, M., and Poul, J. M., DNA damage and DNA adduct formation in rat tissues following oral administration of acrylamide. Mutat Res 580:119-129.

[1] Groups of rats were treated with 54 mg/kg acrylamide *po* and killed at the times indicated.

[2] Olive tail moment as % of concurrent control

[3] Expressed as molecules per 10^8 nucleotides. These data are estimated from a graph.

Mortality Patterns Among Workers Exposed to Acrylamide: Updated Follow Up

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Objective: *The objective of this study was to update the mortality experience of a cohort of workers with and without potential exposure to acrylamide (AMD) at three U.S. plants (n = 8508) and one plant in The Netherlands (n = 344).* ***Methods:*** *We computed standardized mortality ratios (SMRs) using national and local rates and modeled internal cohort rates to assess site-specific cancer risks by demographic and work history factors and several exposure indicators for AMD.* ***Results:*** *For the 1925–2002 study period, we observed both deficit and excess overall mortality risks among the U.S. cohort for cancer sites implicated in experimental animal studies: brain and other central nervous system (SMR = 0.67, confidence interval [CI] = 0.40–1.05), thyroid gland (SMR = 1.38, CI = 0.28–4.02), testis and other male genital organs (SMR = 0.64, CI = 0.08–2.30); and for sites selected in earlier exploratory analyses of this cohort: respiratory system cancer (RSC) (SMR = 1.17, CI = 1.06–1.27), esophagus (SMR = 1.20, CI = 0.86–1.63), rectum (SMR = 1.25, CI = 0.84–1.78), pancreas (SMR = 0.94, CI = 0.70–1.22), and kidney (SMR = 1.01, CI = 0.66–1.46). Except for RSC, attributed earlier to muriatic acid exposure, none of the mortality excesses was statistically significant. In the Dutch cohort, we observed deficits in deaths for all sites of a priori interest. An updated analysis of our previous exploratory findings for pancreatic cancer in the U.S. cohort revealed much less evidence of a possible exposure–response relationship with AMD.* ***Conclusion:*** *AMD exposure at the levels present in our study sites was not associated with elevated cancer mortality risks.* (J Occup Environ Med. *2007;49: 82–95*)

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Acrylamide (AMD) ($CH_2 = CHCONH_2$, CAS Registry Number 79-06-1) is a vinyl monomer that improves the aqueous solubility, adhesion, and crosslinking of polymers. Water-soluble polyacrylamides are the largest application of acrylamide and are used as additives by the wastewater, pulp and paper, textile, chemical, mining, and oil industries. Human exposure from these industrial applications is generally very limited.[1]

AMD is biotransformed in vivo to its epoxide, glycinamide, which has been reported to be genotoxic in a variety of in vitro and in vivo test systems.[2] AMD is carcinogenic to experimental mice and rats, causing tumors at multiple organ sites in both species when given in drinking water or by other means.[2–6] In 1994, the International Agency for Research on Cancer classified AMD as a probable human carcinogen (group 2A) based on sufficient animal evidence, inadequate human evidence, and evidence of a DNA reactive mechanism.[1] The U.S. Environmental Protection Agency has also classified AMD as a probable human carcinogen,[7] and the 11th U.S. National Toxicology Program Report on Carcinogens listed AMD as "reasonably expected to cause cancer in humans."[8]

Interest in the potential carcinogenicity of AMD was aroused in 2002 by reports that AMD is formed in significant quantities during high-temperature cooking.[9,10] This was followed by the 2003 reports that AMD is measurable at significant concentrations in many common human foods prepared at high temperatures by frying or baking[11] and that

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the mean daily intake of AMD is 38μg for males and 29μg for females.[12] AMD is formed during the cooking of foods primarily by the Maillard reaction between the amino acid asparagine and reducing sugars such as glucose.[13,14] Subsequent epidemiologic investigations and risk assessments of dietary AMD exposures have detected no evidence of adverse health effects.[15–19]

Only two epidemiology studies of AMD-exposed workers have been conducted to date. Recognizing the limitations of extrapolating findings of carcinogenicity bioassays across animal species or from animals to humans,[20] these studies were prompted by experimental animal studies that suggested AMD's carcinogenic potential based on an increased incidence of cancers, including those of the brain and other central nervous system (CNS), thyroid gland, other endocrine glands, reproductive organs, and mesotheliomas.[3–6]

The first epidemiology study, a small historical cohort study of 371 Dow Chemical Company employees assigned to AMD monomer and polymerization operations, found no statistically significant mortality excesses in the total cohort and no deaths for the implicated cancer sites.[21] This study did not include AMD exposure estimates for individual workers. The second study, a much larger historical cohort mortality study of 8854 male workers with potential exposure to AMD at three Cytec Industries Inc. (Cytec) chemical manufacturing locations in the United States and at one location in The Netherlands, was first reported in 1989 by Collins and coworkers.[22]

An 11-year update of the Cytec AMD cohort study (through 1994) that did not include The Netherlands plant was reported by Marsh and colleagues in 1999.[23] In the last update, no statistically significantly elevated mortality risks were observed for any of the implicated cancer sites, although mammary cancer mortality could not be evaluated in the all-male cohort. An exploratory analysis of four cancer sites (esophagus, rectum, kidney, and pancreas) selected because of their 20% or greater excesses in the AMD-exposed group and corresponding deficits in the unexposed group revealed a statistically significant 2.26-fold risk (95% confidence interval [CI] = 1.03–4.29) for pancreatic cancer mortality among workers with cumulative exposure to AMD greater than 0.30 mg/m^3-years but no evidence of a monotonic exposure–response relationship. A previously reported excess mortality risk of respiratory system cancer (RSC) at one U.S. plant remained elevated among workers with potential exposure to muriatic acid (relative risk [RR] = 1.50, 95% CI = 0.86–2.59).[23] The investigators of the Cytec cohort study have concluded that their results did not support the hypothesis that AMD is a human carcinogen.[22,23]

We extended and updated our investigation of the Cytec AMD cohort to continue surveillance of total and cause-specific mortality risks in relation to AMD exposure and to examine further our previous exploratory findings for pancreatic cancer. We report here the results of an 8-year extended update (1995–2002) of the U.S. AMD cohort and a 21-year extended update (1984–2004) of the AMD cohort from The Netherlands plant.

Materials and Methods

Study Population

Collins et al[22] and Marsh et al[23] have described details of cohort definition. In brief, the original AMD cohort included all male employees with full-time work experience at any of three U.S. plants (n = 8508) (Fortier, Louisiana; Kalamazoo, Michigan; Warners, New Jersey) or the Botlek, The Netherlands, plant (n = 346) between January 1, 1925, and January 31, 1973, with mortality follow up through 1983. In our first update of mortality through 1994,[23] we did not include the 346 workers from the Botlek plant because follow up was incomplete at the time of analysis. For the U.S. plants, company records were reviewed to update the work histories and the AMD and muriatic acid exposures of study members through 1994.

The current 8-year mortality update of the AMD cohort includes the 8508 workers from the original U.S. cohort and 344 workers from the Botlek plant.* Work histories and AMD exposures for Botlek plant workers were updated from 1984 through 2004 with the assistance of investigators from Maastricht University in The Netherlands and the personnel department of the Botlek plant. All U.S. workers had terminated before 1995.

Table 1 shows selected characteristics of the U.S. and Dutch study populations by plant. Among all four plants, the Warners facility is the oldest and largest contributing 81% of the total eligible study members and 82% of the total person-years. Most of the cohort members are white. Approximately one half of the U.S. cohort and one fourth of the Botlek cohort were short-term workers (defined as less than 1 year of employment). The Fortier, Kalamazoo, and Botlek plants included the larger percentages of long-term (20+ years) workers. Approximately 20% of person-years for U.S. workers were associated with AMD exposure compared with 79% of person-years for Botlek workers. Tobacco smoking history was unknown for 67% of the U.S. cohort and 28% of the Dutch cohort.

Cohort Tracing

The vital status of U.S. study members was determined as of December 31, 2002, using our modified, two-stage tracing protocol that is based on several conventional tracing sources, including the Social

*The original Botlek cohort reported by Collins et al (1989) included 346 workers. During the current update, two of the workers were determined to be female and ineligible for the study.

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TABLE 1
Characteristics of Study Plants and Populations Used in Mortality Update

Characteristics	US Plants				Botlek Plant
	Fortier	Kalamazoo	Warners	All Plants	
Plant startup date	1951	1930	1917	—	1965
AMD production dates	1966–date	1967–date	1954–1985	—	1966–date
Total subjects	1295	60	7153	8508	
White males	1172	57	6013	7242	344
Nonwhite males	123	3	1140	1266	0
Year of birth					
<1900	1	5	633	639	0
1900–1919	197	15	2602	2814	10
1920–1939	724	17	2908	3649	168
1940+	373	23	1010	1406	166
Year of hire (entry into study)					
1925–1939	0	5	1584	1589	0
1940–1949	0	12	2471	2483	0
1950–1959	730	17	1566	2313	0
1960–1969	396	10	869	1275	187
1970–1973	189	16	663	848	157
Year of termination from work					
1925–1949	0	1	3083	3084	0
1950–1969	561	12	2669	3242	63
1970–1989	551	39	1277	1867	178
1990–1994 (Botlek 2004)	183	8	124	315	103
Duration of employment*					
<1 yr	230	6	3932	4168	92
1–4.9	285	12	1537	1834	87
5–9.9	133	3	434	570	27
10–19.9	248	8	453	709	31
20+	399	31	797	1227	107
Person-yr†					
Total	47,791	2137	270,417	320,345	10,483
Unexposed to AMD	28,466	986	225,306	254,758	2134
Exposed to AMD‡	19,325	1151	45,111	65,587	8349
0.001–0.029 (mg/m^3-yr)	9030	441	7832	17,303	1653
0.03–0.29	9851	587	14,177	24,615	3394
0.30+	444	123	23,102	23,669	3302
Person-yr (1950–2002)					
Total	47,791	2031	239,335	289,157	
Unexposed to AMD	28,466	882	195,024	224,373	
Exposed to AMD†	19,325	1149	44,311	64,785	N/A
0.001–0.029 (mg/m^3-yr)	9030	439	7780	17,249	
0.03–0.29	9851	587	13,823	24,260	
0.30+	444	123	22,708	23,276	
Tobacco smoking status					
Never smoker	196	4	421	621	53
Ever smoker	550	25	1558	2133	194
Unknown	549	31	5174	5754	97

*Duration of employment computed as of last termination date (11/16/94) for U.S. plants and as of 12/31/04 for Botlek plant.
†1925–2002 for US plants; 1965–2004 for Botlek plant.
‡Person-yr refer to follow-up time not exposure time. Person-yr among unexposed includes follow-up time of workers before their first AMD exposure (ie, started employment in an unexposed job).
AMD indicates acrylamide; N/A, not applicable.

Security Administration (SSA), the National Death Index-Plus (NDI) system, and state vital statistics bureaus.[24,25] Subjects with "unknown" vital status were untraceable mainly due to missing Social Security numbers, a limitation of the original study due mostly to Warners employees hired before the establishment of the SSA. For deaths occurring before 1979 (beginning of NDI), death certificates were obtained from the corresponding state health departments and, to conform with the original study, were coded by a nosologist to underlying cause of death using the 8th revision rules of the International Classification of Diseases (ICD).

Table 2 shows the vital and working status of the U.S. cohort at the end of 2002 by plant. We identified a

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TABLE 2
Vital and Working Status of Study Populations by Plant

Vital Status	US Plants (1925–2002)				Botlek Plant (1965–2004)
	Fortier	Kalamazoo	Warners	All Plants	
Alive	879	27	2626	3532	233
Dead	458	33	4159	4650	71
Cause of death known (%)	448 (97.8)	33 (100)	3859 (92.8)	4340 (93.3)	71 (100)
Cause of death unknown	10	0	300	310	0
Unknown (%)	18 (1.4)	0 (0)	368 (5.1)	386 (4.5)	40 (11.6)*
Working status (as of 12/31/94 for US plants; 12/31/04 for Botlek plant)					
Working	0	0	0	0	16
Terminated	1255	57	6967	8279	322
Died while employed	40	3	186	229	6

*Includes 35 (10%) subjects who emigrated and 5 (2%) lost to follow up in The Netherlands.

total of 4650 deaths through 2002, an increase of 1368 deaths from the previous 1994 follow up of the U.S. plants. This includes 275 new deaths not identified in the previous 1994 update. Persons lost to follow up decreased from 513 persons (6.0%) in the original study to 386 (4.5%). Underlying cause of death was determined for 4340 or 93.3% of all deaths. Most of the 310 decedents with unknown cause of death were from the Warners plant and 124 of these died in New York City, where confidentiality rules largely restrict the release of cause of death information for research purposes.

Follow up of the Botlek population was conducted through municipal population registries and the causes of death were obtained from the Central Bureau of Statistics in The Netherlands. Of the 344 workers, 233 were alive at the end of 2004, 71 were dead, and 40 (12%) were lost to follow up, mostly because of emigration. The underlying cause of death was determined for all deaths and coded to the 9th revision rules of the ICD (Table 2).

Acrylamide Exposure Assessment

Exposures to AMD varied across plants, because each plant produced or used AMD differently. AMD is produced in two forms, wet AMD in aqueous solution and dry AMD that can be in either a powder or a solid pellet. The Warners plant had operations for producing both forms of AMD. The Fortier facility manufactured wet AMD monomer. Both plants used wet and dry AMD in the formulation of other products. Kalamazoo used only wet AMD. Potential exposure may have occurred during monomer and polymer production from inhalation of dry powder, AMD monomer, or aerosols of AMD solution and from dermal absorption of the AMD monomer and solutions. At the Botlek plant, acrylamide was made by bringing acrylonitrile in contact with water in the presence of a catalyst. Acrylamide was made in dry crystal form and in aqueous solutions. During 2004, the plant was converted to produce acrylamide in aqueous solutions only.

The historical reconstruction of quantitative AMD exposures detailed earlier[22] considered a worker as "exposed" to AMD if his cumulative exposure value was greater than 0.001 mg/m^3-years, the approximate equivalent of a 1-day average concentration exposure to the permissible exposure limit of 0.3 mg/m^3 in effect at that time. From the individual worker job histories and the job and time-specific exposure estimates, three time-dependent summary measures of AMD exposure were computed for each worker: duration of exposure (Dur) = the sum of the days spent in jobs with nonzero AMD exposure (in years); cumulative exposure (Cum) = the product of the number of days in each job and the estimated average daily exposure to AMD summed across all jobs (in mg/m^3-years), and average intensity of exposure (AIE) = the ratio of Cum to Dur (in mg/m^3). These summary measures are expressed as AMD_DurX, AMD_CumX, and AMD_AIEX, where X represents the number of exposure categories.

In the 1994 follow up, exposure estimates for the U.S. plants during the 1984–1994 update period were assigned using the 1983 AMD exposure values developed in the original study. Only few employees in the follow-up period would have potential exposure to AMD, because the Warners plant stopped AMD operations in 1985 and any extrapolation error would be small with minimal impact on our findings. This conservative operating assumption was also supported by a review of industrial hygiene monitoring data and by plant personnel knowledgeable of AMD processes during the follow-up period.

We did not update exposures for U.S. workers beyond 1994 for the current update, because all workers

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TABLE 3
Summary Statistics for AMD Exposure Measures by Plant, All Workers, 1925–1994

AMD Exposure Indicator*	US Plants				
	Fortier	Kalamazoo	Warners	All Plants	Botlek Plant
Acrylamide duration (AMD_Dur, yr)					
Minimum	0	0	0	0	0.00
25th percentile	0	0	0	0	0.122
Median	0	2.62	0	0	1.58
75th percentile	5.40	15.58	0	0	16.64
Maximum	34.62	41.67	40.99	41.67	37.75
Mean	4.16	7.62	1.20	1.69	8.01
Standard deviation	7.25	9.68	4.79	5.42	10.96
Coefficient of variation	174.3	127.1	400.5	319.9	136.8
Average Intensity (AMD_AIE, mg/m^3)					
Minimum	0	0	0	0	0.00
25th percentile	0	0	0	0	0.007
Median	0	0.003	0	0	0.032
75th percentile	0.010	0.008	0	0	0.284
Maximum	0.055	0.124	2.200	2.200	0.410
Mean	0.007	0.009	0.115	0.098	0.114
Standard deviation	0.011	0.019	0.364	0.336	0.139
Coefficient of variation	157.2	201.8	315.8	342.7	121.6
Cumulative (AMD_Cum, mg/m^3-yr)					
Minimum	0	0	0	0	0.00
25th percentile	0	0	0	0	0.006
Median	0	0.02	0	0	0.155
75th percentile	0.06	0.08	0	0	0.640
Maximum	0.81	0.72	32.31	32.31	6.82
Mean	0.04	0.08	0.29	0.25	0.569
Standard deviation	0.08	0.15	1.34	1.23	1.121
Coefficient of variation	190.7	180.8	458.3	487.6	197.0

*Computed from date of hire to earliest of date of termination, death or 12/31/1994 for US plants and 12/31/2004 for Botlek plant. AMD indicates acrylamide.

had terminated from employment by 1995 (Table 1). We also did not reevaluate mortality in relation to muriatic acid exposure at the Warners plant, because that was not the primary objective of the study. For the Botlek plant, work histories and corresponding AMD exposure estimates were updated for the 1984–2004 period by plant personnel familiar with past job classifications and AMD processes with scientific oversight provided by two of the authors (I.M., G.W.) and colleagues at Maastricht University. Table 3 shows for each plant selected summary statistics for the three AMD exposure measures. Because about one half of the U.S. and Botlek workers were short-term workers (<1 year), duration and cumulative AMD and muriatic acid exposures are generally small. The mean, average intensity of AMD exposure across all workers ranged from 0.007 mg/m^3 in Fortier to 0.115 mg/m^3 for Warners.

Statistical Analyses

Descriptive Analysis of Standardized Mortality Ratios

We examined the total and cause-specific mortality experience of the U.S. AMD cohort from January 1, 1925, to December 31, 2002; for Botlek, the observation period was January 1, 1965, to December 31, 2004. Cohort analyses were performed using a modified life table procedure from the Occupational Cohort Mortality Program (OCMAP-Plus).[26] Person-years at risk contributed by each study member were jointly classified by plant, race, age group, calendar time, duration of employment (Dur), and the time since first employment (TSFE). Person-year counts began at the date of hire and continued until date of death or the end of the 2002 or 2004 study periods. For workers lost to follow up, person-year counts stopped at the last date of known vital status, which was the date of employment termination or the date of emigration.

For the U.S. cohort, we computed expected numbers of deaths by multiplying U.S. male, average annual race, age, and time-specific standard population death rates (ICD 8 comparable) by the person-years at risk in the corresponding race–age–time intervals. U.S. male death rates covering the 1925–1989 observation period were obtained from an updated version of the cohort analysis software developed by Monson.[27] For the 1990–2002 period, corresponding U.S. rates were obtained for fully or nearly comparable categories from the Mortality and Population

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Data System (MPDS) maintained at the University of Pittsburgh.[28] For the Botlek plant, we used corresponding Dutch national mortality rates (ICD 9 comparable) provided by our Dutch coworkers. Comparability between the U.S. and Dutch cause of death categories was established to the extent possible.

For the U.S. plants, we also computed expected numbers of deaths based on MPDS rates for the male populations of the counties in which workers largely resided (for Warners—Middlesex and Union Counties, New Jersey; for Fortier—Jefferson Parish, Louisiana; for Kalamazoo—Kalamazoo County, Michigan). Due to MPDS data limitations, expected numbers of cancer deaths were limited to the period 1950–2001; noncancer deaths were limited to 1960–2001 (with 1962–1964 rates applied to 1960–1964 person-years and 2000–2001 rates applied to 2000–2002 person-years). Because local death rates usually provide the most valid external mortality comparisons (because they help to adjust for the social, cultural, and economic factors related to disease), all but the aggregate analyses were based exclusively on local rates. Moreover, because the counties involved represented large population areas, the local rates are measured with acceptable variability.

Mortality excesses and deficits were expressed as standardized mortality ratios (SMRs) along with their 95% CIs. SMRs were computed for subgroups of the cohort defined by plant, race, follow-up period, calendar period, year of hire, length of employment, and the time since first employment. SMRs were also computed for selected causes of death for categories of the AMD exposure measures with and without exposure lagging.[26] The statistical significance of SMRs was assessed using Poisson probabilities. All tests were done at the 0.05 significance level and no adjustment was made for multiple comparisons.

Relative Risk Regression Analysis

As detailed earlier by Marsh et al,[23] relative risk regression modeling was used to investigate the dependence of the internal cohort rates (modeled as time to death) for selected cancer sites on combinations of the categorical AMD exposure measures with adjustment for potential confounding factors. Data from the entire 1925–2002 study period were modeled. In brief, for each cancer site examined, risk sets were explicitly constructed from the cohort data file with age as the primary time dimension using the RISKSET program module in OCMAP-Plus.[26] To adjust for year of birth ("cohort") effects, risk sets were caliper-matched on year of birth. The time-dependent exposures were evaluated for each individual at each event time they were at risk. Conditional logistic regression models were fit using Stata v8.0[29] or LogXact v6.0.[30]

Demographic and exposure variables were first considered univariately as categorical variables to identify patterns of univariate associations with the outcome and to identify sparse data problems. Possible exposure–disease associations were then evaluated using a forward-stepwise approach to adjust for possible confounders. Effect modification was assessed to the extent possible. The statistical significance of each main effect (expressed as a global *P* value) and interaction was assessed with a likelihood ratio statistic. For the quantitative exposure variables that exhibited a monotonic pattern in the parameter estimates, a test for linear trend was conducted (expressed as a trend *P* value). All tests were done at the 0.05 significance level with no adjustment made for multiple comparisons. The small numbers of observed deaths for the cause of death categories of interest did not permit RR modeling for the Botlek cohort.

Results

General Mortality by Study Period

Table 4 presents for the combined U.S. cohort SMRs by cause of death and study period. During the 1995–2002 update period, we observed 1093 deaths, yielding a 5% overall deficit in total mortality compared with the general U.S. experience. For the combined 1925–2002 study period, we observed a statistically significant ($P < 0.01$) 7% deficit in total mortality based on 4650 observed deaths and 320,345 person-years. Results for the 1925–1994 period differed slightly from those reported by Marsh et al[23] due to the inclusion of 275 newly identified deaths.

For all malignant neoplasms combined and for many of the cancer site-specific categories examined, SMRs decreased in the 1995–2002 update period as compared with the SMRs in the earlier update. For pancreatic cancer, in particular, we observed a 38% deficit in deaths (nine deaths, SMR = 0.62, CI = 0.28–1.18) yielding a 6% overall deficit for the combined 1925–2002 period. SMRs also decreased in the update period for the three other cancer sites (esophagus, rectum, and kidney) identified as "exploratory" findings in our previous update,[23] resulting in small, not statistically significant excesses in the combined study period. Respiratory system cancer decreased from a statistically significant ($P < 0.01$) 19% excess in mortality for the 1925–1994 period to a not statistically significant 8% excess in the update period and remained as a smaller but still statistically significant ($P < 0.01$)17% excess in the combined 1925–2002 study period. Most RSC deaths were due to lung cancer.

For the other cancer sites of a priori interest in the combined study period (Table 4), we observed deficits in deaths for "cancer of the brain and other CNS" (SMR = 0.67, CI = 0.40–1.05) and "cancer of the testis and other male genital organs" (SMR = 0.64, CI = 0.08–2.30). No additional deaths from "thyroid gland" cancer were observed, yielding a not statistically significant excess based on three deaths (SMR = 1.38, CI = 0.28–4.02) for the com-

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TABLE 4
Observed Deaths and SMRs for Selected Causes by Follow-Up Period, All Workers, National Comparison, US Plants Combined

Cause of Death (ICD 8th Revision Codes)*	1925–1994			1995–2002†			1925– 2002†		
	Obs	SMR	95% CI	Obs	SMR	95% CI	Obs	SMR	95% CI
All causes (000–999)	3557	0.93‡	0.90–0.96	1093	0.95	0.89–1.00	4650	0.93‡	0.90–0.96
All malignant neoplasms (140–209)	913	1.06	0.99–1.13	291	0.97	0.86–1.08	1204	1.04	0.98–1.10
Buccal cavity and pharynx (140–149)	24	0.99	0.63–1.47	8	1.67	0.72–3.28	32	1.10	0.75–1.56
Digestive organs and peritoneum (150–159)	240	1.08	0.95–1.22	68	0.92	0.75–1.23	308	1.05	0.94–1.18
Esophagus (150)	32	1.27	0.87–1.79	9	1.01	0.46–1.91	41	1.20	0.86–1.63
Stomach (151)	48	1.24	0.91–1.64	8	0.99	0.43–1.95	56	1.19	0.90–1.55
Large Intestine (153)	72	0.97	0.76–1.22	33	1.29	0.89–1.81	105	1.05	0.86–1.27
Rectum (154)	26	1.31	0.86–1.93	4	0.94	0.26–2.40	30	1.25	0.84–1.78
Liver (155, 156)	13	0.72	0.38–1.24	4	0.45	0.12–1.16	17	0.63	0.37–1.02
Pancreas (157)	45	1.04	0.76–1.39	9	0.62	0.28–1.18	54	0.94	0.70–1.22
Respiratory system (160–163)	369	1.19‡	1.07–1.32	110	1.08	0.89–1.31	479	1.17‡	1.06–1.27
Larynx (161)	15	1.24	0.70–2.05	1	0.32	0.01–1.79	16	1.05	0.60–1.71
Lung (162, 163)	354	1.21‡	1.08–1.34	109	1.12	0.92–1.35	463	1.18‡	1.08–1.30
Bone (170)	2	0.69	0.08–2.50	1	2.30	0.06–12.83	3	0.90	0.19–2.63
Skin (172, 173)	10	0.67	0.32–1.23	4	0.74	0.20–1.89	14	0.69	0.38–1.15
Prostate (185)	73	0.98	0.77–1.24	38	0.93	0.66–10.28	111	0.97	0.79–1.16
Testis and other male genital organs (186, 187)	1	0.36	0.01–1.99	1	2.96	0.07–16.51	2	0.64	0.08–2.30
Bladder (188)	29	1.30	0.87–1.87	10	1.09	0.52–2.00	39	1.24	0.88–1.70
Kidney (189)	23	1.16	0.73–1.74	4	0.57	0.16–1.47	27	1.01	0.66–1.46
Brain and other central nervous system (191, 192)	15	0.69	0.39–1.15	3	0.55	0.11–1.62	18	0.67	0.40–1.05
Thyroid gland (193)	3	2.10	0.43–6.14	0	—	0.00–4.89	3	1.38	0.28–4.02
All lymphopoietic tissue (200–209)	62	0.80	0.61–1.03	22	0.74	0.47–1.12	84	0.78†	0.63–0.97
Lymphosarcoma and reticulosarcoma (200)	6	0.62	0.23–1.35	0	—	0.00–14.14	6	0.60	0.22–1.31
Hodgkin's disease (201)	9	1.33	0.61–2.52	1	2.13	0.05–11.85	10	1.38	0.66–2.53
Leukemia and aleukemia (204–207)	23	0.75	0.48–1.12	9	0.79	0.36–1.51	32	0.76	0.52–1.08
Other lymphatic tissue (202, 203, 208)	23	0.76	0.48–1.14	12	0.68	0.35–1.20	35	0.73	0.51–1.02
Benign neoplasms (210–239)	10	1.01	0.49–1.86	2	1.11	0.14–4.02	12	1.03	0.53–1.79
Diabetes mellitus (250)	47	0.76	0.56–1.02	37	1.18	0.83–1.63	84	0.91	0.72–1.12
Diseases of the circulatory system (390–458)	1569	0.91‡	0.86–0.95	383	0.78‡	0.70–0.86	1952	0.88‡	0.84–0.92
Nonmalignant respiratory disease (460–519)	196	0.76‡	0.66–0.87	99	0.77‡	0.62–0.93	295	0.76‡	0.68–0.85
Cirrhosis of liver (571)	83	0.96	0.76–1.19	9	0.79	0.36–1.50	92	0.94	0.76–1.15
All external causes of death (800–998)	251	0.72‡	0.63–0.81	20	0.77	0.47–1.19	271	0.72‡	0.64–0.81
Unknown causes (999.9)	202			108			310		
Persons	8508			4565			8508		
Person-yr	288,126			32,219			320,345		

*Monson life table program ICD 8th revision categories, labels, and codes for US plants for 1925–89; corresponding MPDS rates for 1990–2001.
†$P < 0.05$.
‡$P < 0.01$.
SMRs indicates standardized mortality ratios; ICD, International Classification of Diseases; Obs, observed; CI, confidence interval; MPDS, Mortality and Population Data System.

bined period. SMRs for some cancer site-specific categories increased in the update period; however, none of the resulting excesses in the update or combined study periods was statistically significant. Most of the nonmalignant cause of death categories shown in Table 4 reveal deficits in deaths for the update and combined study periods. Although not shown here, SMR analyses for the combined U.S. cohort by race, time period, year of hire, and duration of employment were essentially unremarkable for the a priori cancer sites and the other cause of death categories examined in Table 4.

General Mortality by Plant

Table 5 shows the 1950–2002 cancer mortality experience of work-

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TABLE 5
Observed Deaths and SMRs for Selected Cancer Sites by Plant, US Workers, 1950–2002, Local County Comparisons

Cause of Death (ICD 8th Revision Codes)*	Fortier			Kalamazoo			Warners		
	Obs	SMR	95% CI	Obs	SMR	95% CI	Obs	SMR	95% CI
All malignant neoplasms (140–209)	154	0.92	0.78–1.07	7	0.92	0.37–1.89	1031	0.98	0.92–1.04
Buccal cavity and pharynx (140–149)	5	1.13	0.37–2.64	0	—	0.00–21.31	27	1.03	0.68–1.50
Digestive organs and peritoneum (150–159)	39	1.08	0.77–1.48	0	—	0.00–1.96	264	0.88‡	0.77–0.99
Esophagus (150)	7	1.59	0.64–3.28	0	—	0.00–21.41	34	1.00	0.69–1.39
Stomach (151)	9	1.98	0.90–3.75	0	—	0.00–10.66	44	0.82	0.60–1.10
Large Intestine (153)	13	1.02	0.54–1.74	0	—	0.00–6.05	92	0.85	0.68–1.04
Rectum (154)	3	1.18	0.24–3.44	0	—	0.00–18.01	25	0.92	0.60–1.36
Pancreas (157)	7	0.98	0.40–2.02	0	—	0.00–9.86	47	0.90	0.66–1.19
Respiratory system (160–163)	62	0.89	0.68–1.14	5	1.92	0.62–4.49	409	1.20§	1.08–1.32
Bronchus, trachea, lung (162)†	58	0.87	0.66–1.12	5	1.99	0.64–4.63	392	1.21§	1.09–1.34
Prostate (185)	5	0.43	0.14–1.00	1	1.30	0.03–7.22	105	0.96	0.79–1.16
Testis and other male genital organs (172.5, 173.5, 186, 187)†	0	—	0.00–9.76	0	—	0.00–107.33	3	1.15	0.24–3.37
Bladder and other urinary organs (188, 189.9)†	3	0.85	0.18–2.49	0	—	0.00–15.28	36	1.07	0.75–1.48
Kidney (189.0, 189.1, 189.2)†	4	0.82	0.22–2.11	1	5.33	0.13–29.69	22	0.86	0.54–1.30
Brain and other central nervous system (191,192)	6	1.69	0.62–3.67	0	—	0.00–17.93	12	0.55‡	0.28–0.96
Thyroid gland and other endocrine glands (193, 194)†	1	2.59	0.07–14.44	0	—	0.00–197.80	5	1.80	0.59–4.20
All lymphatic and hematopoietic tissue (200–209)	14	1.02	0.56–1.71	0	—	0.00–4.35	67	0.74§	0.55–0.91
Leukemia and aleukemia (204–207)	3	0.58	0.12–1.70	0	—	0.00–12.86	29	0.82	0.55–1.18

*MPDS ICD 8th revision categories, labels, and codes for US plants.
†MPDS ICD codes differ from corresponding Monson codes.
‡$P < 0.05$.
§$P < 0.01$.
SMRs indicates standardized mortality ratios; ICD, International Classification of Diseases; Obs, observed; CI, confidence interval; MPDS, Mortality and Population Data System.

ers from each U.S. plant compared with the local male populations. Restricting the observation period excluded only 12 cancer deaths, all from the Warners plant. For certain categories, the observed number of deaths between Tables 4 and 5 may also differ due to the slight variations in the ICD codes used in the MPDS and Monson rates. Table 5 shows that 1031 or 86% of the observed cancer deaths occurred at Warners (SMR = 0.98, CI = 0.92–1.04); whereas, only seven were observed at Kalamazoo (SMR = 0.92, CI = 0.37–1.89). With the exception of a 20% excess for respiratory system cancer at Warners, previously attributed to muriatic acid exposures,[23] none of the plants reveals any statistically significant cancer-site specific mortality excess, including the cancer sites of a priori interest.

Table 6 shows the 1965–2004 mortality experience of the Botlek plant cohort based on the Dutch national population comparison. Shown are all cause of death categories of a priori interest and those with at least one observed death. For all causes combined and all neoplasms combined (includes benign neoplasms), we observed statistically significant ($P < 0.01$) 43% and 53% deficits in mortality, respectively. Deficits in deaths were also observed for most of the categories examined, including all the cancer sites of a priori interest. We observed not statistically significant excesses for bone and liver cancer based on only one or two observed deaths, respectively.

Cancer Mortality by Acrylamide Exposure Status

Table 7 shows the 1950–2002 cancer mortality experience of the U.S. cohort by AMD exposure status for the selected cancer sites examined in Table 5. Consistent with earlier updates,[22,23] we observed local county rate-based deficits in cancer mortality among both "unexposed" and "exposed" workers for all cancers combined and many cancer site categories, including "digestive organs and peritoneum" and central nervous system. In the previous update,[23] we selected for further exposure–response analysis any cancer site category with a 20% or greater mortality excess in AMD-exposed workers and corresponding deficit in

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TABLE 6
Observed and Expected Deaths and SMRs for Selected Causes, All Botlek Plant Workers, Dutch National Comparison

	1965–2004			
Cause of Death (ICD 9th Revision Codes)	**Observed**	**Expected**	**SMR**	**95% CI**
All causes (001–999)	71	124.6	0.57†	0.45–0.72
All neoplasms (including benign) (140–239)	21	44.3	0.47†	0.29–0.73
Esophagus (150)	1	1.4	0.69	0.02–3.87
Stomach (151, 152)	1	2.7	0.37	0.01–2.07
Large intestine (153)	3	3.1	0.96	0.20–2.80
Rectum (154)	1	1.1	0.89	0.02–4.94
Liver (155, 156)	2	0.8	2.42	0.29–8.73
Pancreas (157)	0	1.9	—	0–1.91
Lung (162, 163)	9	16.7	0.54	0.25–1.02
Kidney (189)	0	1.3	—	0–2.95
Bone (170)	1	0.1	13.21	0.33–73.59
Testis (186, 187)	0	0.1	—	0–24.73
Brain (191)	0	0.9	—	0–4.07
Thyroid (193)	0	0.1	—	0–40.19
Other lymphoma (202)	1	1.04	0.96	0.02–5.34
Diseases of the circulatory system (390–458)	34	46.5	0.73	0.51–1.02
Nonmalignant respiratory disease (460–519)	3	9.2	0.33*	0.07–0.95
All external causes of death (800–998)	5	5.3	0.95	0.31–2.22
All other causes of death (240–389, 580–799)	7	14.5	0.48*	0.19–0.99
Unknown causes (999.9)	0			
Persons		344		
Person-yr		10,483		

*$P < 0.05$.
†$P = 0.01$.
SMRs indicates standardized mortality ratios; ICD, International Classification of Diseases; CI, confidence interval.

unexposed workers. Three of the four cancer sites selected (rectum, pancreas, and kidney) remain here as candidates for further exposure–response analysis (esophagus not selected). No new cancer site categories were selected for additional exploratory analysis based on these screening criteria. Results of this same analysis for the Botlek cohort are shown in Table 8 for the cancer site categories with at least three observed deaths. Deficits in deaths were observed for most of the subgroups examined and none were selected for further exploratory analysis.

Table 9 shows for the U.S. cohort, observed deaths and local rate-based SMRs (1950–2002) for the three selected cancer sites by Dur, the time since first employment (TSFE), and the three exposure measures, AMD_Dur4, AMD_AIE4, and AMD_Cum4. The exposure categories for AMD_Cum4 are those used in the previous updates.[22,23] To help discern trends in SMRs, categories for the other variables were chosen to most evenly distribute the numbers of observed deaths simultaneously across the cancer sites examined. SMRs for each cancer site tend to increase with increasing values of both Dur and TSFE but remain as small, not statistically significant excesses in the highest categories. For the three AMD exposure metrics considered, category-specific SMRs are generally less than those observed in the previous update,[23] none was statistically significant and there is again no consistent evidence of an increased risk for cancer mortality in relation to AMD exposure. In particular, the pancreatic cancer SMR for workers in the highest AMD_Cum4 category (30+ mg/m^3-years) decreased from a statistically significant SMR = 2.26 ($P < 0.05$) to a not statistically significant SMR of 1.71.

We further examined pancreatic cancer mortality risks with relative risk regression models that related internal cohort rates to the potential confounding variables of interest and the categorical AMD exposure measures. Table 10 shows the observed number of deaths, the estimated RR and 95% CI, and the global and trend test (if warranted) P values for each category of the variables considered. All models are based on 54 cases and 12,225 noncases and adjusted for age and calendar time. The top section of Table 10 shows that none of the potential confounding variables considered is a statistically significant predictor of pancreatic cancer risk. Smoking history, a known, relatively weak risk factor for pancreatic cancer,[31] had a marginally statistically significant global test P value ($P = 0.087$) and RRs greater than 2.0 in the nonbaseline categories. The pattern of RRs for smoking history suggests that many workers with unknown smoking history were probably "ever smokers." Time since first AMD exposure had a relatively low global test P value ($P = 0.272$) and

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TABLE 7
Observed Deaths and SMRs for Selected Cancer Sites by AMD Exposure Status, All US Plant Workers, Local County Comparisons, 1950–2002

	Unexposed (<0.001 mg/m^3-yrs)			AMD-Exposed (0.001+ mg/m^3-yrs)		
Cause of Death (ICD 8th Revision Codes)*	Obs	SMR	95% CI	Obs	SMR	95% CI
All malignant neoplasms (140–209)	959	0.96	0.90–1.03	233	0.99	0.86–1.12
Buccal cavity and pharynx (140–149)	27	1.09	0.72–1.59	5	0.82	0.27–1.42
Digestive organs and peritoneum (150–159)	244	0.88†	0.77–0.99	59	0.97	0.74–1.25
Esophagus (150)	33	1.04	0.72–1.46	8	1.14	0.49–2.24
Stomach (151)	43	0.88	0.64–1.19	10	1.05	0.50–1.92
Large intestine (153)	91	0.91	0.73–1.12	14	0.64	0.35–1.07
Rectum (154)	21	0.84	0.52–1.29	7	1.40	0.56–2.89
Liver and biliary passages (155–156)	15	0.68	0.38–1.13	2	0.33	0.04–1.20
Pancreas (157)	38	0.78	0.55–1.07	16	1.41	0.81–2.29
Respiratory system (160–163)	383	1.16†	1.05–1.28	93	1.10	0.89–1.35
Bronchus, trachea, lung (162)	363	1.16†	1.04–1.29	92	1.14	0.92–1.40
Prostate (185)	100	0.99	0.80–1.20	11	0.55†	0.27–0.98
Testis and other male genital organs (172.5, 173.5, 186, 187)	3	1.22	0.25–3.58	0	—	0.00–6.53
Bladder and urinary organs (188, 189.9)	33	1.06	0.75–1.49	6	0.94	0.34–2.04
Kidney (189.0, 189.1, 189.2)	19	0.78	0.47–1.21	8	1.27	0.55–2.50
Brain and other central nervous system (191, 192)	15	0.73	0.41–1.21	3	0.58	0.12–1.69
Thyroid gland and other endocrine glands(193,194)	4	1.57	0.43–4.02	2	3.18	0.38–11.49
All lymphatic and hematopoietic tissue(200–209)	59	0.68†	0.51–0.87	22	1.04	0.65–1.58
Leukemia and aleukemia (204–207)	23	0.70	0.44–1.05	9	1.16	0.53–2.21
Persons		7565			2004	
Person-years		224,373			64,785	

*MPDS ICD 8th revision categories, labels, and (codes) for US plants.
‡$P < 0.01$.
SMRs indicates standardized mortality ratios; AMD, acrylamide; ICD, International Classification of Diseases; Obs, observed; CI, confidence interval; MPDS, Mortality and Population Data System.

TABLE 8
Observed and Expected Deaths and SMRs for Selected Cancer Sites, All Botlek Plant Workers, Dutch National Comparison, 1965–2004

	Unexposed (<.001 mg/m^3-yrs)			AMD-Exposed (.001+ mg/m^3-yrs)		
Cause of Death (ICD 9th Revision Codes)	Obs	SMR	95% CI	Obs	SMR	95% CI
All neoplasms (140–239) (includes benign)	7	0.74	0.30–1.52	14	0.40*	0.22–0.68
Large intestine (153)	2	3.03	0.37–10.96	1	0.40	0.01–2.25
Lung (162,163)	3	0.82	0.17–2.42	6	0.46	0.17–1.00
Persons		317			273	
Person-years		2134			8349	

*$P < 0.05$.
SMRs indicates standardized mortality ratios; ICD, International Classification of Diseases; Obs, observed; CI, confidence interval.

some evidence of an increasing trend (P for trend = 0.124).

For the three AMD exposure metrics considered, category-specific RRs are generally less than those observed in the previous update,[23] none of the models or individual RRs was statistically significant, and there is again no consistent evidence an increased risk for cancer mortality in relation to AMD exposure. For example, our previous univariate model for AMD_Cum4 (RRs = 1.00, 3.14, 0.77, and 2.63 [$P < 0.05$] for <0.001, 0.001, 0.03, and 0.30+ mg/m^3-years, respectively) now reveals much less evidence of a possible exposure–response relationship (updated RRs = 1.00, 2.03, 1.07, and 2.01, respectively). This finding was maintained in our bivariate model for AMD_Cum4 adjusted for the first time in this update for smoking history (RRs = 1.00, 2.08, 1.12, and 2.05). Even less evidence of an increasing risk with increasing AMD_Cum4 was found in our updated bivariate model adjusted for time since first AMD exposure (RRs = 1.00, 1.73, 0.88, and 1.59). Lagged

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TABLE 9
Observed Deaths and SMRs for Selected Cancer Sites by Duration of Employment, Time Since First Employment, and AMD Exposure Measures, All US Workers, 1950–2002, Local County Comparisons

	Rectum			Pancreas			Kidney		
	Obs	SMR	95% CI	Obs	SMR	95% CI	Obs	SMR	95% CI
Duration of employment (yrs)									
<1	8	0.63	0.27–1.24	22	0.82	0.51–1.24	10	0.73	0.35–1.34
1–	13	1.25	0.66–2.13	17	0.84	0.49–1.35	9	0.87	0.40–1.64
15+	7	1.05	0.42–2.12	15	1.17	0.66–1.93	8	1.20	0.52–2.37
Time since first employment (yrs)									
<20	3	0.71	0.15–2.06	4	0.66	0.18–1.68	2	0.58	0.07–2.09
20–	5	0.79	0.26–1.83	11	0.96	0.48–1.72	4	0.65	0.18–1.66
30+	20	1.04	0.64–1.61	39	0.92	0.65–1.26	21	1.00	0.62–1.52
AMD_Dur4 (years)									
Unexposed	21	0.85	0.52–1.29	38	0.78	0.55–1.08	19	0.78	0.47–1.22
0.001–	3	1.32	0.27–3.86	6	1.12	0.41–2.43	4	1.31	0.36–3.36
5–	2	1.15	0.14–4.14	6	1.55	0.57–3.38	3	1.37	0.28–4.00
20+	2	1.96	0.24–7.07	4	1.81	0.49–4.63	1	0.88	0.02–4.88
AMD_Cum4 (mg/m^3-yrs)									
<0.001	21	0.85	0.52–1.29	38	0.78	0.55–1.08	19	0.78	0.47–1.22
0.001–	1	1.43	0.04–7.98	3	1.65	0.34–4.83	1	0.88	0.02–4.92
0.03–	4	2.44	0.67–6.25	4	0.94	0.26–2.40	4	1.56	0.42–4.00
0.30+	2	0.75	0.09–2.71	9	1.71	0.78–3.25	3	1.15	0.24–3.36
AMD_AIE4 (mg/m^3)									
Unexposed	21	0.85	0.52–1.29	38	0.78	0.55–1.08	19	0.78	0.47–1.22
0.001–	4	2.96	0.81–7.58	5	1.34	0.44–3.14	2	0.86	0.10–3.10
0.02–	0	—	0.00–1.64	5	1.11	0.36–2.60	3	1.27	0.26–3.71
0.30+	3	2.08	0.43–6.09	6	1.85	0.68–4.03	3	1.77	0.37–5.18

$P < 0.05$.
SMRs indicates standardized mortality ratios; AMD, acrylamide; Obs, observed; CI, confidence interval.

forms (5, 10, and 20 years) of the three AMD exposure metrics produced a similar pattern of findings in SMRs and RRs as noted in Tables 9 and 10 (results not shown).

Discussion

Our initial AMD cohort study of U.S. and Dutch AMD production workers[22] and the previous 1994 update of the U.S. cohort[23] revealed little if any evidence that AMD exposure is related to increased cancer risks. This evidence for the absence of an association was supported by the many strengths of our study, including size, long observation period, excellent follow up and near complete death certificate ascertainment, quantitative AMD exposure estimates for individual workers, and excellent a priori statistical power (0.90+) to detect twofold or greater excesses for many causes of death categories examined.

The primary limitations of our study to date have been the relatively low a priori statistical power to detect twofold or greater excesses for the less frequently occurring cancer sites (ie, thyroid gland and male genital organs) implicated in experimental animal studies[3–6] and the inability to evaluate fully breast cancer mortality (another implicated site) in our exclusively male cohort. This limitation notwithstanding, we did observe two breast cancer deaths among male members of the U.S. cohort (Warners plant) who were unexposed to AMD (SMR = 1.21, 95% CI = 0.15–4.36). Another limitation of our study is that nearly one half of combined U.S. and Dutch cohorts were short-term workers (employed less than 1 year) and only 23% of the combined cohort worked 10 or more years. Consequently, most of the cohort had minimal potential for high cumulative AMD exposure. However, on a relative exposure scale, our study included persons with the highest AMD exposures ever experienced by humans. Contrary to many other occupational cohort studies, short-term workers did not exhibit a differential mortality pattern often associated with increased mortality for both malignant and nonmalignant diseases. The long length of follow up in this study may have mitigated the mortality influence of short-term workers.

The current study of the U.S. AMD cohort was strengthened further by 8 years of extended follow up that yielded in the total cohort 1093 observed deaths, including 291 from malignant neoplasms. We also included a 1984–2004 update of the original Dutch cohort that generated a total of 71 deaths (including 21 cancer deaths) for the overall 1965–2004 study period. The additional numbers of observed deaths also permitted more comprehensive statistical analyses such as adjustment for smoking history in our relative risk regression models for pancreatic cancer. The additional expected numbers of deaths due to "cancer of the brain and other central

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TABLE 10
Summary of Relative Risk Regression Analysis for Cancer of the Pancreas, US Plants, 1925–2002

			US Plants (54 cases, 12,225 noncases)			
Variable	**Category**	**Deaths**	**Risk Ratio**	**95% CI**	**Global Test (df)**	**Trend Test* (df)**
Univariate models†						
Race	White	47	1.00		0.001 (1)	
	Nonwhite	7	1.00	0.45–2.23	$P = 0.996$	
Year of hire	1925–1939	10	1.00			
	1940–1949	22	1.25	0.56–2.77		
	1950–1959	15	1.11	0.45–2.72	1.03 (3)	
	1960–1973	7	1.78	0.53–6.00	$P = 0.793$	
Duration of employment (yr)	<1	22	1.00			
	1−	17	1.01	0.54–1.94	1.23 (2)	0.93 (1)
	15+	15	1.43	0.73–2.79	$P = 0.541$	$P = 0.337$
Time since first employment (yr)	<20	4	1.00			
	20−	11	1.40	0.36–5.45	.25 (2)	
	30+	39	1.34	0.33–5.37	$P = 0.883$	
Smoking‡	Never	1	1.00			
	Ever	16	5.34	0.70–40.44	4.89 (2)	
	Unknown	37	3.38	0.46–24.83	$P = 0.087$	
Time since first AMD exposure (yr)	<20	42	1.00			
	20−	5	1.73	0.67–4.46	2.60 (2)	2.37 (1)
	30+	7	1.83	0.78–4.34	$P = 0.272$	$P = 0.124$
AMD_Dur4 (yr)	<.001	38	1.00			
	0.001−	6	1.35	0.55–3.32		
	5−	6	1.83	0.77–4.36	2.76 (3)	2.64 (1)
	20+	4	1.88	0.66–5.32	$P = 0.430$	$P = 0.104$
AMD_Cum4 (mg/m^3-yr)	<.001	38	1.00			
	0.001−	3	2.03	0.60–6.84		
	0.03−	4	1.07	0.38–3.04	3.81 (3)	
	0.30+	9	2.01	0.96–4.21	$P = 0.283$	
AMD_AIE4 (mg/m^3)	<.001	38	1.00			
	0.001−	5	1.58	0.60–4.00		
	0.02−	5	1.29	0.50–3.33	3.38 (3)	
	0.30+	6	2.32	0.95–5.64	$P = 0.337$	
Bivariable models‡						
AMD_Dur4 (yrs)	<.001	38	1.00			
	0.001−	6	1.36	0.53–3.53		
	5−	6	2.03	0.72–5.73	2.50 (3)	2.42 (1)
	20+	4	2.30	0.66–7.99	$P = 0.475$	$P = 0.120$
AMD_Cum4 (mg/m^3-years)	<.001	38	1.00			
	0.001−	3	2.08	0.60–7.22		
	0.03−	4	1.12	0.36–3.51	3.09 (3)	
	0.30+	9	2.05	0.84–5.02	$P = 0.378$	
AMD_AIE4 (mg/m^3)	<.001	38	1.00			
	0.001−	5	1.61	0.55–4.68		
	0.02−	5	1.32	0.45–3.88	2.49 (3)	
	0.30+	6	2.16	0.84–5.45	$P = 0.477$	
Bivariable models§						
AMD_Cum4 (mg/m^3-years)	<.001	38	1.00			
	0.001−	3	1.73	0.42–7.06		
	0.03−	4	0.88	0.23–3.36	1.44 (3)	
	0.30+	9	1.59	0.46–5.51	$P = 0.696$	
AMD_AIE4 (mg/m^3)	<.001	38	1.00			
	0.001−	5	1.23	0.35–4.30		
	0.02−	5	0.98	0.26–3.76	1.14 (3)	
	0.30+	6	1.78	0.50–6.37	$P = 0.768$	

*Trend tests performed only on exposure variables exhibiting a monotonic increase or decrease in parameter estimates.
†Risk sets matched on exact age and year of birth.
‡Risk sets matched on exact age and year of birth; models adjusted for smoking.
§Risk sets matched on exact age and year of birth; models adjusted for time since first AMD exposure.
CI indicates confidence interval; AMD, acrylamide.

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nervous system" increased to above 0.90 the statistical power to detect a twofold or greater excess from this a priori cancer site. The small, additional number of expected deaths for cancer of the "testis and other male genital organs" and thyroid cancer did not materially improve the statistical power associated with these two a priori sites (power = 0.40 and 0.21, respectively).

In the previous 1994 update, an exploratory exposure–response analysis of cancer sites with elevated risks among AMD-exposed workers (and a corresponding deficit among unexposed workers) revealed a statistically significant 2.26-fold risk for pancreatic cancer among workers with cumulative AMD exposure greater than 0.30 mg/m^3-years but little evidence of an association with increasing exposure. Our updated analysis of pancreatic cancer and two other cancer sites that again passed the screening criteria for further study (rectum and kidney) revealed much less evidence of a possible exposure–response relationship with AMD based both on SMRs and internal relative risk regression models adjusted for time since first AMD exposure or smoking history. In particular, the elevated pancreatic cancer excess in the highest cumulative AMD exposure category decreased to a not statistically significant 1.59-fold (95% CI = 0.46–5.51) excess in an RR model adjusted for time since first AMD exposure. In addition to the not statistically significant excesses in two of the three nonbaseline categories, we observed no evidence of an exposure–response relationship (RRs = 1.00, 1.73, 0.88, and 1.59 for <0.001, 0.001, 0.03, and 0.30+ mg/m^3-years, respectively). A similar conclusion was reached in the model adjusted for smoking history (corresponding RRs = 1.00, 2.08, 1.12, and 2.05).

Conclusion

The addition of 1093 observed deaths, more than 32,000 person-years, and a fourth study site to our historical cohort study of AMD production workers provided more definitive support for the conclusion that AMD exposure at levels experienced by these workers is not associated with elevated cancer mortality risks.

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References

1. International Agency for Research on Cancer (IARC). Acrylamide. In: *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Some Industrial Chemicals*. Lyon, France: IARC; 1994:60.
2. Rice J. The carcinogenicity of acrylamide. *Mutat Res*. 2005;580:3–20.
3. Bull R, Robinson M, Laurie R, et al. Carcinogenic effects of acrylamide in Sencar and A/J mice. *Cancer Res*. 1984; 44:107–111a.
4. Bull R, Robinson M, Laurie R, et al. Carcinogenic activity of acrylamide in the skin and lung of Swiss-ICR mice. *Cancer Lett*. 1984;24:202–212b.
5. Johnson K, Gorzinski S, Bodner K, et al. Chronic toxicity and oncogenicity study of acrylamide incorporated in the drinking water of Fischer 344 rats. *Toxicol Appl Pharmacol*. 1986;85:154–168.
6. Friedman M, Dulak L, Stedham M. A lifetime oncogenicity study in rats with acrylamide. *Fundam Appl Toxicol*. 1995; 27:95–105.
7. *Preliminary Assessment of Health Risks From Exposure to Acrylamide* [Draft]. Washington, DC: US Environmental Protection Agency, Office of Toxic Substances; 1988.
8. *National Toxicology Program, 11th Report on Carcinogens, Eleventh Edition, Carcinogen Profiles*. Research Triangle Park, NC: US Department of Health and Human Services; 2004.
9. Tareke E, Ryber P, Karlsson P, Ericsson S, Tornqvest M. Analysis of acrylamide, a carcinogen found in heated foodstuffs. *J Agric Food Chem*. 2002;50:4998–5006.
10. Mitka M. Fear of frying: is acrylamide in foods a cancer risk? *JAMA*. 2002;288: 2105–2106.
11. Svensson K, Abramsson L, Becker W, et al. Dietary intake of acrylamide in Sweden. *Food Chem Toxicol*. 2003;41:1581–1586.
12. Dybing E, Sanner T. Risk assessment of acrylamide in foods. *Toxicol Sci*. 2003; 75:7–15.
13. Mottram D, Wedzicha B, Dodson A. Acrylamide is formed in the Maillard reaction. *Nature*. 2002;419:448–449.
14. Stadler R, Blank I, Varga N, et al. Acrylamide from Maillard reaction products. *Nature*. 2002;419:449.
15. Bosetti C, La Vecchia C, Talmini R, et al. Food groups and laryngeal cancer risk: a case–control study from Italy and Switzerland. *Int J Cancer*. 2002;100:355–360.
16. Pelucchi C, Franceschi S, Levi F, et al. Fried potatoes and human cancer. *Int J Cancer*. 2003;105:558–560.
17. Mucci LA, Dickman PW, Steineck G, Adami HO, Augustsson K. Dietary acrylamide and cancer of the large bowel, kidney and bladder: absence of an association in a population-based study in Sweden. *Br J Cancer*. 2003;88:84–89.
18. Erdreich L, Friedman M. Epidemiologic evidence for assessing the carcinogenicity of acrylamide. *Reg Toxicol Pharm*. 2004;39:150–157.
19. Ruden C. Acrylamide and cancer risk—expert risk assessment and the public debate. *Food Chem Toxicol*. 2004;42: 335–349.
20. Haseman J, Lockhart A. Correlations between chemically related site-specific carcinogenic effects in long-term studies in rats and mice. *Environ Health Perspect*. 1993;101:50–54.
21. Sobel W, Bond G, Parson T, et al. Acrylamide cohort mortality study. *Br J Ind Med*. 1986;43:785–788.
22. Collins J, Swaen G, Marsh G, et al. Mortality patterns among workers exposed to acrylamide. *J Occup Med*. 1989; 31:614–617.
23. Marsh G, Lucas L, Youk A, Schall L. Mortality patterns among workers exposed to acrylamide: 1994 follow-up. *Occup Environ Med*. 1999;56:181–190.
24. Schall L, Marsh G, Henderson V. A two-stage protocol for verifying vital status in large historical cohort studies. *J Occup Environ Med*. 1997;39:1097–1102.
25. Buchanich J, Dolan D, Marsh G, Madrigano

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J. Under ascertainment of deaths using Social Security records: a recommended solution to a little-known problem. *Am J Epidemiol*. 2005;62:193–194.
26. Marsh G, Youk A, Sefcik S, Alcorn C. OCMAP-PLUS: a program for the comprehensive analysis of occupational cohort data. *J Occup Environ Med*. 1998; 40:351–362.
27. Monson R. Analysis of relative survival and proportional mortality. *Computers and Biomedical Research*. 1974;7:325.
28. Marsh G, Ehland J, Sefcik S. *Mortality and Population Data System*. University of Pittsburgh, Department of Biostatistics, Technical Report; 2004.
29. *Stata Statistical Software: Release 8*. College Station, TX: Stata Corp LP; 2003.
30. *LogXact Version 6.0—Software for Exact Conditional Logistic Regression*. Cambridge, MA: Cytel Software Corp; 2002.
31. Anderson K, Poter J, Mack T. Pancreatic cancer. In: Schottenfeld D, Fraumeni J Jr, eds. *Cancer Epidemiology and Prevention,* 2nd ed. New York: Oxford University Press; 1996.

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