

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

Prepared by:

Annette M. Shipp, Ph.D.

Principal

ENVIRON International Corporation

Monroe, Louisiana

**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 PPAR $\alpha$  activation or  $\alpha$ 2 $\mu$ -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 homeostasis and its 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 sulfhydryl-rich proteins. Inhibition of uptake in this 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<sub>2</sub> 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<sub>3</sub> receptors that are located in the frontal cortex (Bouthenet *et al.*, 1991). D<sub>3</sub> 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<sub>3</sub> 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<sub>2</sub> 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 The production of TVMs is linked to the production of Leydig Cell Tumors (LCTs)

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 adenohipophyseal 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- $\beta$  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 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 matrix 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<sub>2</sub> receptors on the

pituitary lactotrophs, the cells that secrete prolactin, and inhibits prolactin release. Conversely, dissociation from the lactotroph D<sub>2</sub> 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- $\beta$ , 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 carberglyline (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  $\gamma$ -irradiation resulted in the production of fibroadenomas, while administration of estradiol followed by  $\gamma$ -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 AA's Contribution to Mammary Fibroadenomas

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<sub>2</sub> 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<sub>1</sub> 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<sub>1</sub> 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<sub>1</sub> receptor agonists, SKF3, SKF8 and CY. The levels of cyclic AMP (cAMP) were also significantly increased, an effect that is associated with D<sub>1</sub> receptor activation, in cells incubated with dopamine. No effects on baseline progesterone secretion were reported following incubation with propranolol (beta-blocker), domperidone (D<sub>2</sub> receptor antagonist), bromocriptine (D<sub>2</sub> receptor agonist), or bulbocapnine (D<sub>1</sub> receptor antagonist). However, incubation with bulbocapnine, but not propranolol or domperidone, inhibited the dopamine-stimulated secretion of progesterone. Results of binding assays conducted with SCH23390, a D<sub>1</sub> receptor ligand, indicated that a D<sub>1</sub> 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<sub>1</sub> receptors on the ovarian cells and not through the beta-adrenergic receptors. Similar results reported by Arakawa *et al.* (1994) also indicated that D<sub>1</sub> receptor agonists increased progesterone secretion from PMSG-treated rats and that incubation with D<sub>1</sub> receptor antagonists inhibited the dopamine-stimulated increase in progesterone secretion.

AA acting at the level of the D<sub>1</sub> 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<sub>1</sub> 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<sub>2</sub> 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<sub>1</sub> 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<sub>1</sub> 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 propranolol 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<sub>1</sub>-subtype (D<sub>1</sub>-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<sub>1</sub> receptor agonist SKF-38393. Increases in cAMP levels and in cell contraction, effects that were not inhibited by propranolol, were reported. Direct evidence for the presence of D<sub>1</sub>-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<sub>1</sub> 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<sub>3</sub> and T<sub>4</sub> 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<sub>3</sub> and T<sub>4</sub> 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 has 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<sub>3</sub> or T<sub>4</sub> 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<sub>3</sub>, T<sub>4</sub>, or TSH levels). As discussed previously, exposures to AA were associated with mild changes in T<sub>3</sub>, T<sub>4</sub> 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<sub>3</sub> and T<sub>4</sub> 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<sub>3</sub>

and T<sub>4</sub> 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<sub>3</sub>, T<sub>4</sub> 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<sub>3</sub> and T<sub>4</sub> 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 T<sub>3</sub> and T<sub>4</sub>. In the Friedman *et al.* (1999) study, there was a suggestion that AA decreased T<sub>3</sub> and T<sub>4</sub> 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<sub>4</sub> 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<sub>4</sub> with enhanced biliary excretion of T<sub>4</sub> may occur. A mode of action involving enhanced biliary excretion of T<sub>4</sub> by way of induction of UDP-GT 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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<sub>4</sub> 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<sub>1</sub> 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. New Occupational Studies:

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. *The 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/m<sup>3</sup>-years, the equivalent of one-day exposure at the then current OSHA permissible exposure level (PEL) of 0.3 mg/m<sup>3</sup>. 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. The entirety of the dietary intake studies.

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  $\geq 1$  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).**