TOXICOLOGICAL REVIEW
OF
ACRYLONITRILE
(CAS No. 107-13-1)

Prepared for
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Washington, DC

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This toxicological review document has been subject to an external peer review. A panel of independent peer reviewers was assembled by Toxicological Excellence for Risk Assessment (TERA), and a two-day meeting was held in Cincinnati, OH on September 22-23, 2003. Members of this panel include (in alphabetical order): Drs. John Christopher, Michael Dourson, Linda Erdreich, Susan Felter, Tim Fennell, Jeff Fisher, David W. Gaylor, Kannan Krishnan, Jeff Lewis, Alan Parrish, Jerry Rice, James E. Trosko, and Vernon Walker. The panel reviewed this document and reached consensus on the conclusions. The reader is referred to the notes of the meeting, which is available for download from TERA's website (www.tera.org), for a record of the issues that the panel discussed.

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ACRONYMS

AF	Adjustment factor
AMAP	Amplitude for motor nerve action potential
AN	Acrylonitrile
ASAP	Amplitude for sensory nerve action potential
BMD	Benchmark dose
BMDL	Lower confidence limit of the BMD
CEO	Cyanoethylene oxide
CHO	Chinese hamster ovary
CI	Confidence interval
DNA	Deoxyribonucleic acid
ED	Effective dose
EH	Epoxide hydrolase
FEL	Frank effect level
GD	Gestation day
GJIC	Gap junction intercellular communication
GSH	Glutathione
GST	Glutathione-S-transferase
GST-T1	Glutathione-S-transferase Theta-1
ip	Intraperitoneal
iv	Intravenous
LED	Lower confidence limit of the ED
LOAEL	Lowest observed adverse effect level
MCL	Mononuclear cell leukemia
MCV	Motor nerve conduction velocity
MF	Modifying Factor
MOE	Margin of exposure
mRR	meta-relative risk
NOAEL	No observed adverse effect level
O/E	Observed/expected
OR	Odds ratio
PC	Partition coefficient
P450	Cytochrome P450
PBPK	Physiologically based pharmacokinetic
PND	Post-natal day
RfD	Reference dose
RfC	Reference concentration
RR	Relative risk
SAB	Spontaneous abortion
sc	Subcutaneous

SCE	Sister chromatid exchange
SCV	Sensory nerve conduction velocity
SHE	Syrian hamster embryo
SIR	Standard incidence ratio
SMR	Standard mortality ratio
TWA	Time weighted average
UF	Uncertainty factor
UDS	Unscheduled DNA synthesis

EXECUTIVE SUMMARY

A toxicological review has been prepared for acrylonitrile (AN). Within this document, values for a reference dose (RfD), reference concentration (RfC), oral cancer slope factor, and inhalation unit risk are derived. These values reflect the best available science for AN and serve as up-to-date alternatives to the values derived by USEPA in their 1983 assessment of AN, as summarized on USEPA's Integrated Risk Information System (IRIS). Since 1983, USEPA has performed routine updates within the IRIS database up to 1991. However, the values for the RfC and oral and inhalation cancer potencies have changed little during this time-frame, and an oral RfD has not been derived. Over the past 20 years there have been numerous advances in our understanding of AN with respect to mechanism of toxic action, epidemiology, toxicity studies and pharmacokinetics that need to be incorporated into current toxicity assessments for AN. These advances address a number of the data needs identified by USEPA for AN in their 1983 assessment. This document evaluates and incorporates the current science into the derivation of an RfD, RfC, oral cancer potency and inhalation cancer potency for AN.

The document presented here is in a format similar to that found in Toxicological Reviews currently listed on IRIS. The sections include an introduction (**Section 1**), chemical properties (**Section 2**), toxicokinetics (**Section 3**), hazard characterization (**Section 4**) and dose-response evaluation (**Section 5**). The hazard characterization section provides an in-depth review of published studies on the non-cancer and cancer effects of AN exposures reported in humans and animals (**Sections 4.1** and **4.2**). The hazard characterization includes a synthesis and evaluation of the major toxicological effects of AN and likely modes of action (**Section 4.3**) as well as cancer weight-of-evidence (**Section 4.4**). The dose-response section (**Section 5.0**) provides the decisions made and calculations performed in deriving the RfD, RfC, oral cancer potency and inhalation cancer potency for AN. There are also appendices attached that provide more in-depth information for key aspects of the toxicological review.

Consistent with USEPA guidelines for dose-response assessment, emphasis is placed on the mode(s) of action (MOA) in this document for the purposes of guiding decision making at key steps of the assessment. The MOAs for AN indicate that many of the adverse effects of AN are attributed to the formation of one or more metabolites (*i.e.,* cyanoethylene oxide or CEO, cyanide). Therefore, the metabolism of AN is an important determinant of toxicity, which can vary across individuals and species. Although there may be small differences in the metabolism of AN in human populations due to genetic polymorphisms, the impact of these differences on susceptibility is likely to be small. More importantly, there do not appear to be important differences between males and females, and children are not expected to be at greater risk than adults based on kinetic factors determining the metabolic activation of AN. The metabolism of AN is complicated by a number of sources of nonlinear kinetics

and important species differences are identified with respect to the metabolism of AN. Furthermore, the metabolism of AN is influenced by the route of exposure, species to which it is administered, the magnitude of the exposure, and the vehicle in which it is administered. To help characterize many of the complexities associated with the metabolism of AN, physiologically based pharmacokinetic (PBPK) models have been developed for describing the kinetics of AN and CEO in rats and humans. These PBPK models were used to support the dose-response assessment for AN.

A comprehensive review of the epidemiology and toxicology literature for AN indicates that the principal noncancer endpoints of interest include neurological, irritation, hematological, reproductive/developmental and survival effects. Other effects (*e.g.*, kidney, adrenal toxicity) are generally associated with higher exposures to AN, or are not consistently identified as an effect for AN. The neurological and irritation effects of AN serve as the most appropriate endpoints for noncancer risk assessment. The mode of action for neurological effects may involve both parent chemical and the release of cyanide during metabolism. The mode of action for irritation effects is not known but may involve the binding of AN or CEO to cellular macromolecules or depletion of tissue GSH levels.

The carcinogenicity of AN has been evaluated in a number of epidemiology studies and animal bioassays. The weight-of-evidence from a large body of well conducted epidemiology studies do not support identifying AN as a human carcinogen. Follow-up studies conducted for a number of worker cohorts, including the one used to calculate the inhalation unit risk for AN in 1983, have not found any significant increases in cancer risk. The weight-of-evidence from animal bioassays identify AN as a multi-site animal carcinogen, producing tumors in the brain, forestomach, mammary gland, Zymbal's gland, and Harderian gland, in one or more species. Of the rodent tumor sites, the rat brain serves as the most important tumor site for cancer risk assessment. The mode of action for tumor induction in laboratory rodents is likely complex and could include multiple mechanisms, each of which could predominate at different doses. These mechanisms include likely indirect DNA damage (*e.g.*, caused by oxidative stress), possible direct DNA damage (*e.g.*, caused by the acrylonitrile metabolite CEO, or even by acrylonitrile itself), and epigenetic changes (*e.g.*, as indicated by inhibition of gap junction intercellular communication [GJIC] in the target tissues). The data are insufficient to rule out contribution from a direct DNA-reactive MOA for brain tumors, or to definitively identify a specific key event or MOA for brain tumors. Limited evidence for the genotoxicity of AN in humans and animals demonstrate important differences between the results of *in vitro* studies, particularly at cytotoxic concentrations and in the presence of a metabolic activation system, and the largely negative results of *in vivo* studies. The absence of measurable DNA adducts in rat brains argues strongly against a genotoxic mode of action for AN in producing rat brain tumors. Rather, a comparison of several modes of action employing the Hill criteria for causation indicates that rat brain tumors are likely the result of an epigenetic mode of action involving

oxidative stress, resulting from AN metabolism. The decision by IARC to downgrade the cancer classification for AN , reflects the negative epidemiology data sets, and supports the conclusion that AN at historical and present exposure levels is probably not carcinogenic to man.

Epidemiology data do not support an increased cancer risk from acrylonitrile exposure in exposed workers. In contrast, the experimental animal data clearly support the conclusion that acrylonitrile is carcinogenic in rodents. The proposed cancer modes of action in rodents involve general processes (e.g., oxidative stress, GJIC, DNA damage) that are known to occur in humans, and so the data are presumed to support the use of the rodent data in establishing a quantitative cancer risk value. Although the data are insufficient to rule out any contribution due to direct DNA reactivity, an overall weight of evidence evaluation does not support this as a predominant contributor to rodent carcinogenesis. These modes of action may not be mutually exclusive, and multiple modes of action may be operating at different dose levels. Furthermore, linear extrapolation from the animal data is not supported by the available epidemiology data. Based on this information, the overall weight of the evidence suggests that the cancer risk associated with the levels to which humans have been exposed in occupational settings is negligible, but that acrylonitrile may be carcinogenic to humans at higher doses based on extrapolation from rat studies.

Dose response assessments were conducted for AN for the purposes of deriving an oral RfD, an inhalation RfC, an oral cancer slope factor, and an inhalation unit risk. Within each dose-response assessment, a seven-step process was adopted for deriving AN toxicity values. Where possible, information regarding the toxicity, pharmacokinetics, and mode of action was used to guide decisions made at each decision point. The methods used in the dose-response assessments for AN are consistent with USEPA guidelines.

An **oral RfD of 0.05 mg/kg-day** was derived for AN based on neurological effects observed in rats. Benchmark dose methods were used to characterize the dose-response relationship expressed in terms of PBPK-derived internal dose measures. Confidence in the oral RfD for AN is considered to be medium-to-high. Confidence in the key study is considered to be medium primarily because the exposure regimen (gavage) does not reflect typical human exposures. However, the appearance of similar effects in rats from the same study following inhalation exposure to AN allays some of this concern. Confidence in the database is considered high since there are a number of well-conducted, chronic bioassays in both rats and mice, and a multigeneration reproductive toxicity study has been conducted. Confidence in the PBPK modeling is considered to be medium since the model has been validated in rats but not in humans, and because the model provides a good description of the kinetics of AN and CEO, but does not consider the kinetics of cyanide.

An **inhalation RfC of 0.7 mg/m^3** was derived for AN based on the irritation effects of AN in exposed workers. The categorical dose-response data for this endpoint were assessed in terms of external dose (atmospheric concentration). Confidence in the RfC value based on human data is considered medium to high. Confidence in the critical study is considered to be medium since it is based upon observations in exposed workers, but the exposure estimates may not accurately reflect historical exposures. Confidence in the database is considered high since there are a number of well-conducted, chronic bioassays in both rats and mice, and a multigeneration reproductive toxicity study has been conducted.

An assessment of the oral cancer potency of AN was conducted using the pooled data from twelve data sets (six from oral exposures and six from inhalation exposures) for brain tumors in rats. Benchmark dose methods were used to characterize the dose-response relationship expressed in terms of PBPK-derived internal dose measures, which allows for the combined use of oral and inhalation data set. For this data set, the LED05 was selected as the point of departure. Based upon the data on the mode of action for brain tumors in rats, and considering the absence of a significant positive response in a large epidemiology data set, a nonlinear method of extrapolation from the point of departure was used for the cancer dose-response assessment. Based upon a net uncertainty factor of 200 applied to LED05, **oral doses below 0.009 mg/kg-day are not expected to pose a significant risk to exposed human populations**. For the purposes of comparison and completeness, linear extrapolation from the point of departure yielded an oral cancer slope factor of 0.02 $(mg/kg\text{-}day)^{-1}$. Confidence in the oral cancer value is medium-to-high. Confidence in the key studies is considered to be high since they are well conducted, chronic bioassays, and because pooling the dose-response data from several studies provides a more complete description of the dose-response relationship. Confidence in the database for AN is considered to be high since there are several cancer bioassays for AN in the rat which provide consistent estimates of potency for brain tumors, and one bioassay conducted in mice which is inconsistent with rats with respect to brain tumors. Confidence in the PBPK modeling is considered to be medium since the model has been validated in rats but not in humans, and because the model provides a good description of the kinetics of AN and CEO, but does not consider the kinetics of cyanide.

An assessment of the inhalation cancer potency of AN was assessed using the same pooled data set for brain tumors in rats that was used to estimate oral cancer potency. Consistent with the oral cancer assessment, the LED05 value was used to support a nonlinear dose-response assessment. Based upon a net uncertainty factor of 220 applied to LED05, **inhalation exposures below 0.1 mg/m^3 are not expected to pose a significant risk to exposed human populations**. For the purposes of comparison and completeness, linear extrapolation from the point of departure yielded an unit risk of 1.6×10^{-6} $(ug/m^3)^{-1}$. Confidence in the inhalation cancer value for AN is medium-to-high. Confidence in the key studies is considered to be high since they are well conducted, chronic bioassays, and

because pooling the dose-response data from several studies provides a more complete description of the dose-response relationship. Confidence in the database is high because there are a number of large epidemiology studies and well-conducted cancer bioassays which lend support to this assessment.

The database to support a cancer risk assessment for acrylonitrile is unique in that it includes robust epidemiological data, bioassay data from two rodent species by two routes of exposure, a PBPK model, and extensive mechanistic data. It is important that all of these sources of data be integrated into the risk assessment for acrylonitrile. A strength of the epidemiology data includes the availability of several studies that contain reliable estimates of exposure. Robust rodent tumor data were integrated with a very large database of human epidemiology data demonstrating a lack of cancer in occupationally exposed workers. This approach allowed for a direct comparison of the exposure-response information in rodents and people in terms of internal dose, and supported departure from the default practice of linear extrapolation.

The conclusions of this assessment for AN are provided in **Table ES-1**.

Table ES-1. Summary of the Dose-Response Assessments for AN

Criteria	Value	Basis	Confidence
Oral RfD	0.05 mg/kg-day	Derived from a benchmark dose for neurological effects in rats, using a PBPK-derived internal dose measure, and a net uncertainty factor of 180	Medium-to-High
Inhalation RfC	0.7 mg/m^3	Derived from a NOAEL for irritation effects in human using a net uncertainty factor of 10. Value is supported by an RfC of 0.2 mg/m^3 based upon nasal irritation in rats	Medium-to-High
Oral Cancer Value	0.009 mg/kg-day	Based upon a nonlinear assessment of the pooled data for brain tumors in rats, using PBPK-derived internal dose measures, and a net uncertainty factor of 200 applied to the LED05 value. For the purposes of comparison and completeness, linear extrapolation from the point of departure yields a cancer slope factor of 0.02 $(mg/kg-day)^{-1}$.	Medium-to-High
Inhalation Cancer Value	0.1 mg/m^3	Based upon a nonlinear assessment of the pooled data for brain tumors in rats, using PBPK-derived internal dose measures, and a net uncertainty factor of 220 applied to the LED05 value. For the purposes of comparison and completeness, linear extrapolation from the point of departure yields a unit risk value of 1.6×10^{-6} $(\mu g/m^3)^{-1}$.	Medium-to-High

1. INTRODUCTION

This document presents background and justification for the hazard and dose-response assessment summaries in accordance with EPA's Integrated Risk Information System (IRIS). IRIS summaries may include an oral reference dose (RfD), inhalation reference concentration (RfC), and a carcinogenicity assessment.

The RfD and RfC provide quantitative information for noncancer dose-response assessments. The RfD is based on the assumption that thresholds exist for certain toxic effects. It is expressed in units of mg/kg-day. In general, the RfD is an estimate (with uncertainty spanning perhaps an order of magnitude) of a daily exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious noncancer effects during a lifetime. The inhalation RfC is analogous to the oral RfD, but provides a continuous inhalation exposure estimate. The inhalation RfC considers toxic effects for both the respiratory system (portal-of-entry) and for effects peripheral to the respiratory system (extra respiratory or systemic effects). It is generally expressed in units of mg/m^3.

The carcinogenicity assessment provides information on the carcinogenic hazard potential of the substance in question and quantitative estimates of risk from oral exposure and inhalation exposure. The information includes a weight-of-evidence judgment of the likelihood that the agent is a human carcinogen and the conditions under which the carcinogenic effects may be expressed. Quantitative risk estimates are presented in three ways. The *slope factor* is the result of application of a low-dose extrapolation procedure using a biologically-based model, or, in the absence of a biologically-based model, a Margin of Exposure (MOE) approach (if there is strong evidence of a nonlinear mode of action) or a linear default with a benchmark dose. The slope factor is presented as the risk per mg/kg/day.

The *unit risk* is the quantitative estimate in terms of either risk per µg/L drinking water or risk per $\mu g/m^3$ air breathed. Another form in which risk is presented is a drinking water or air concentration providing cancer risks of 1 in 10,000; 1 in 100,000; or 1 in 1,000,000.

Development of these hazard identifications and dose-response assessments for acrylonitrile (AN) has followed the general guidelines for risk assessment as set forth by the National Research Council (1983). EPA guidelines that were considered in the development of this assessment may include the following: the *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 1986a, 1999, 2003), *Guidelines for the Health Risk Assessment of Chemical Mixtures* (U.S. EPA, 1986b), *Guidelines for Mutagenicity Risk Assessment* (U.S. EPA, 1986c), *Guidelines for Developmental Toxicity Risk Assessment* (U.S. EPA, 1991), *Guidelines for Neurotoxicity Risk Assessment* (U.S. EPA, 1998a), *Proposed Guidelines for Carcinogen Risk*

Assessment (1996a), and *Reproductive Toxicity Risk Assessment Guidelines* (U.S. EPA, 1996b); *Recommendations for and Documentation of Biological Values for Use in Risk Assessment* (U.S. EPA, 1988); (proposed) *Interim Policy for Particle Size and Limit Concentration Issues in Inhalation Toxicity* (U.S. EPA, 1994a); *Methods for Derivation of Inhalation Reference Concentrations and Application of Inhalation Dosimetry* (U.S. EPA, 1994b); *Peer Review and Peer Involvement at the U.S. Environmental Protection Agency* (U.S. EPA, 1994c); *Use of the Benchmark Dose Approach in Health Risk Assessment* (U.S. EPA, 1995); *Science Policy Council Handbook: Peer Review* (U.S. EPA, 1998b); and memorandum from EPA Administrator, Carol Browner, dated March 21, 1995, Subject: Guidance on Risk Characterization.

2. CHEMICAL AND PHYSICAL INFORMATION RELEVANT TO ASSESSMENTS

AN is a clear, colorless liquid with a slightly pungent odor. Some relevant physical and chemical properties of AN are listed below:

CAS Registry number:	107-13-1
Empirical formula:	CH_2=CH-CN
Molecular weight:	53.06
Vapor pressure (20 °C):	106.7 hPa (Baxter, 1979)
	115 hPa (Kirk-Othmer, 1991)
	116 hPa (BASF AG, 1994)
	120 hPa (EC erdolchemiw, 1994)
	124 hPa (BG-Chemie, 1990)
Henry's law constan:t	$8.8x10^{-5}$ atm-m^3/mol (Mabey *et al.* 1982)
Water solubility:	73 g/L at 20 °C (American Cyanamid, 1959)
Log KOW :	0.25 (Praesi *et al.* 1979)
	0.08 (BASF, 1988)
	0.09 (Tanii and Hashimoto 1984)
	0 (Masuhara, 1981)
	0.3 (Tonogai *et al.* 1982)
	-0.92 (Vershueren, 1983)
Conversion factor:	1 ppm = 2.2 mg/m^3
Odor threshold:	47 mg/m^3 (Vershueren, 1983)
Melting Point:	-83 °C (Vershueren, 1983)
Boiling Point:	77.3 °C (Kirk-Othmer, 1991)
Density:	0.806 at 20 °C (American Cyanamid, 1959)
Flash Point:	0 °C (American Cyanamid, 1959)
Flammable limits:	2.8-28 vol/vol (Nabert and Schon, 1980)

3. TOXICOKINETICS RELEVANT TO ASSESSMENTS

The following sections summarize the information available regarding the absorption, distribution, metabolism, excretion, and pharmacokinetic models for AN. Where possible, sources of nonlinear kinetics, which are important to the mode of action (**Section 4.3**) and dose-response assessment (**Section 5.0**), are identified.

3.1 Absorption

AN is a small, water soluble molecule. The absorption of AN occurs via passive diffusion through the gastrointestinal tract, respiratory tract, or skin. Absorption is rapid and extensive following ingestion or inhalation of AN. Data available regarding the absorption of AN are discussed below by route of exposure.

Ingestion

In rats exposed to a single oral dose of 46.5 mg/kg AN, peak tissue concentrations were generally achieved within three hours of exposure, indicating that the oral absorption of AN is fairly rapid (Ahmed *et al.*, 1982, 1983). Following oral exposure of rats to 0.09 to 28.8 mg/kg and of mice to 0.09 to 10 mg/kg, the absorption of AN in rodents appears to be complete (Kedderis *et al.*, 1993). Based upon the amount of radiolabel excreted in the urine and feces up to 72 hours after dosing, absorption ranged from 82-100% in the rat, and from 85-100% in the mouse. There was no evidence of any dose-dependency on the extent of AN absorption. Results from an unpublished study in rats also indicate that AN is well absorbed, ranging from 90-98% (Young *et al.*, 1977).

Inhalation

Based upon urinary excretion data collected for six human male volunteers exposed to 5 or 10 mg/m^3 AN for eight hours, Jakubowski *et al.* (1987) report that approximately 52% of inhaled AN was retained. A higher retention of AN (91.5%) was reported in rats exposed to initial air concentrations of 1800 ppm (3,900 mg/m^3) AN, which became depleted in a biphasic manner reaching approximately 10-20 ppm after five hours of exposure in a closed chamber system. Based upon an analysis of time-course data for AN in chamber air, the amount of AN exhaled by the rats was estimated to be 8.5% (Peter and Bolt, 1984). Absorption was also essentially complete in Rhesus monkeys exposed to approximately 30 ppm AN for six hours (Peter and Bolt, 1984).

Dermal Contact

AN is well absorbed through the skin. The uptake of AN through skin in human volunteers was reported to be 0.6 mg/cm^2/hr (Rogaczewska and Piotrowski, 1968). A higher penetration rate of 3.6 mg/cm^2/hr was reported for human skin *in vitro* (van Hooidonk, 1986).

3.2 Distribution

Following absorption, AN is readily distributed throughout the body. Available data regarding the distribution of AN is summarized below via route of exposure.

Ingestion

Following oral or intravenous exposure to radiolabeled AN, most of the radiolabel accumulated in the liver, kidney, stomach mucosa, lung, and adrenal cortex and blood of rats and monkeys (Sandberg and Slalina, 1980).

Gut *et al.* (1981) determined half-lives of 61 and 70 minutes for AN radiolabel in blood and liver, respectively, of exposed Wistar rats. The elimination rates for the radiolabel were similar for intravenous, intraperitoneal, and subcutaneous exposures, although the metabolite composition differed for oral exposures.

In adult male Sprague-Dawley rats receiving a single oral dose of 46.5 mg/kg radiolabeled AN, the highest initial concentrations of radiolabel were observed in the stomach and intestines (Ahmed *et al.*, 1982, 1983). The red blood cells retained significant amounts of radioactivity for more than ten days after treatment, whereas the radiolabel declined sharply in plasma. Initially, the highest levels of radioactivity were found in the stomach and stomach content followed by the intestine. In liver, kidney, brain, spleen, adrenal, lung, and heart tissues the radioactivity of the acid soluble fractions declined while covalent binding to macromolecules remained unchanged. In subcellular fractions of liver, kidney, spleen, brain, lung, and heart, 20-40% of the total radioactivity was bound to nuclear, mitochondrial, and microsomal fractions whereas in cytosol only 6-14% was bound over a period of six hours. The position of the radiolabel show some differences, as when compared to [1-^{14}C]-AN administered to animals, the percentage of covalent binding of [2,3-^{14}C]-AN was significantly higher even 72 hours after dosing.

The irreversible binding of [2,3-^{14}C]-AN to proteins, RNA, and DNA of various tissues of male Sprague-Dawley rats after a single oral dose of 46.5 mg/kg was studied (Farooqui and Ahmed, 1983b). Binding of AN to proteins was extensive and was time dependent. Radioactivity in nucleic acids was registered in the liver and the target organs, stomach, and brain. DNA alkylation, which increased by time, was significantly higher in the target

organs, brain, and stomach (119 and 81 pmol/mg, respectively, at 24 h) than that in the liver. The covalent binding indices for the liver, stomach, and brain at 24 hours after dosing were, 5.9, 51.9, and 65.3, respectively. More recent studies have failed to confirm the binding observed to DNA (Pilon *et al.*, 1988a,b; Whysner *et al.*, 1998). It has been hypothesized that the label binding observed in the early study may be attributable to contamination of DNA with protein-bound radiolabel (Geiger *et al.*, 1983; Guengerich *et al.*, 1986).

The irreversible binding to tissue macromolecules was assessed in control and glutathione-depleted F-344 rats treated with an oral dose of 4 mg/kg [2,3-^{14}C]AN (Pilon *et al.*, 1988a). Glutathione was depleted in rat tissues by the administration of a combined intraperitoneal phorone/buthionine sulfoximine treatment (300 mg/kg and 2 mmol/kg, respectively) given 30 min prior to AN administration. The amount of total radioactivity recovered from brain, stomach (target organs), liver, kidney, lung, and blood (nontarget organs) was similar between control and glutathione-depleted rats. However, stomach, lung, blood, and liver showed an increase in total radioactivity content after glutathione depletion by phorone/buthionine sulfoximine treatment. Glutathione depletion also caused an increase in AN-derived non-dialysable radioactivity in liver, lung, kidney, stomach, blood, and brain macromolecules between six and 24 hours after the dose. There was no organ-specific accumulation of radiolabel in RNA in control rats. However, an increase in the radiolabel associated with RNA in the target organs but not in the nontarget organs was measured in glutathione-depleted rats.

The tissue distribution of CEO was investigated in F-344 rats and B6C3F1 mice following a single oral dose of 3 mg/kg (Kedderis *et al.*, 1993). Radioactivity from [2,3-^{14}C]-CEO was widely distributed in the major organs of rodents by two hours and decreased by 71-90% within 24 hours, demonstrating that there was no preferential tissue uptake or retention of CEO. CEO was detected in rodent blood and brain 5-10 min after an oral dose of 10 mg/kg AN, demonstrating that this metabolite rapidly circulates to extrahepatic target organs following exposure.

AN and CEO were detected in the blood of male Fisher-344 rats after administration of 3,10, or 30 mg AN/kg by gavage in water. AN and CEO were also measured in the liver and brain for the 10 mg/kg dose (Kedderis *et al.*, 1996). Peak tissue concentrations for AN and CEO were achieved within 0.2 hours. Although AN concentrations were comparable for both tissues (1-10 mg/L), CEO concentrations were considerably lower (~0.01-0.02 mg/L) in the brain compared to liver (0.05-0.08 mg/L).

Inhalation

The effect of GSH depletion on AN distribution was investigated in male F344 rats (Pilon *et al.*, 1988b). In this study, rats were given a 4 mg/kg dose of [2,3-^{14}C]-AN by inhalation. A combined phorone/buthionine sulfoximine treatment (300 mg/kg and 2 mmol/kg, respectively) was given 30 minutes prior to AN exposure to deplete GSH. Tissue uptake of AN into brain, stomach, liver, kidney, and blood was enhanced by glutathione depletion in exposed male Fischer 344 rats. However, GSH depletion caused a decrease in total radioactivity recovered in brain, stomach, liver, kidney, and blood and a concomitant decrease in the AN-derived nondialyzable radioactivity in these organs. In control rats, accumulation of radiolabel was greatest in brain RNA, but no radioactivity was detected in DNA of any organ examined. In GSH-depleted rats, the radiolabel concentration was higher in brain RNA than in the liver or stomach RNA, but was also 50% lower than that observed in brain RNA of control rats.

Following a three hour exposure of male Fisher-344 rats to 186, 254, or 291 ppm AN, AN and CEO concentrations were detected in blood, brain, and liver (Kedderis *et al.*, 1996). Concentrations of AN and CEO were generally higher in brain than in liver, and decreased rapidly in both tissues following cessation of exposure.

Other Routes

Radiolabel tends to be irreversibly bound to tissue proteins (Peter and Bolt, 1981). Within cells, radioactivity levels were higher in nuclear, mitochondrial, and microsomal fractions compared to cytosolic fraction. Binding was not dependent on NADPH, and occurred in heat-inactivated microsomes, indicating that metabolism was not required. Binding of the radiolabel was inhibited by the addition of thiols (GSH, cysteine, mercaptoethanol).

The distribution and accumulation of AN after the single ip injection of [2,3-^{14}C]AN were examined by whole-body autoradiography and by the determination of ^{14}C radioactivities in several tissues and subcellular fractions after a whole-body perfusion (Sato *et al.*, 1982). The radiolabel was seen strongly in blood, particularly in red blood cells, and in several tissues including lung, liver, and kidney. Longer retention of radioactivity in brain and muscle was observed. At the subcellular level a relatively high specific radioactivity was seen in cytosolic fractions of the brain, liver, and kidney.

Following iv injection of [1-^{14}C]-AN in the rat, high concentrations of total radiolabel were found in blood, liver, duodenum, kidney, and the adrenal glands (Silver *et al.*, 1987). Except for blood, there was a time-dependent decrease in total radiolabel in these tissues. Compared with other major organ systems, the levels of covalently bound radiolabel were lower in the adrenal glands.

An *in vitro* study was conducted to determine partition coefficients for AN and CEO (Teo *et al.* 1994). Active uptake of AN was observed in rat blood due to reaction with blood sulfhydryl groups, while CEO reacted with all tissues examined (rat blood, muscle, fat, liver, and brain). The active uptake processes were first order as evidenced by a linear decrease in the log of the vial headspace concentrations over time. Linear extrapolation of the log of the apparent partition coefficient to zero time, where the contribution of the active uptake process is zero, yielded an estimated partition coefficient of 487 for AN in blood. Equilibrium was achieved with AN after treatment of blood with diethyl maleate to modify blood sulfhydryl groups, with a partition coefficient of 512. The directly measured AN blood:air PC was 437, which compared well with the estimated values. Treatment of tissues with diethyl maleate or 2,4-dinitrofluorobenzene did not abolish the active uptake of CEO. However, pretreatment of tissues with CEO itself abolished subsequent CEO uptake. The CEO blood:air PC estimates obtained from zero time extrapolation of four CEO concentrations (1672 +/- 139) and from CEO pretreatment (1658 +/- 137, n = 8) were in good agreement.

The time course of AN and CEO in the blood of male Fischer-344 rats was measured for iv doses of 3.4, 47, 55, and 84 mg/kg AN (Gargas *et al.*, 1995). Peak AN levels (exceeding 100 mg/L at the highest dose) were achieved within 0.1 hours of exposure, whereas peak CEO levels (up to 0.1 mg/L) were achieved more slowly (~0.4 hours). A dose-dependent formation of hemoglobin adducts was observed, corresponding to approximately 2-5% of the administered dose.

The dose dependence of AN covalent binding to tissue protein, following a single acute exposure over a broad range of doses (administered subcutaneously), was investigated (Benz *et al.* 1997b). Covalent binding was a linear function of AN dose in the lower dose range (0.02-0.95 mmol/kg). The slopes of the dose-response curves indicated that tissues varied by nearly 10-fold in their reactivity with AN. The relative order of covalent binding was as follows: blood > > kidney = liver > forestomach = brain > glandular stomach > > muscle. Similar dose-response behavior was observed for globin total covalent binding and for globin N-(2-cyanoethyl) valine (CEValine) adduct formation. The latter adduct was found to represent only a small percentage (0.2%) of the total AN adduction to globin. Regression of tissue protein binding versus globin total covalent binding or globin CEValine adduct indicated that both globin biomarkers could be used as surrogates to estimate the amount of AN bound to tissue protein. At higher AN doses, above approximately 1 mmol/kg, a sharp break in the covalent binding dose-response curve was observed, and is explained by the nearly complete depletion of liver glutathione and the resultant termination of AN detoxification.

Following subcutaneous injection of 115 mg/kg [2,3-(14)C]-AN in male Sprague-Dawley rats, protein binding in the liver was reported (Nerland *et al.*, 2001). One set of bound

proteins was identified as glutathione-S-transferase, predominantly at a reactive cysteine site on one particular subunit (rGSTM1) of the enzyme. Mass spectral analysis of tryptic digests of the GST subunits indicated that the site of labeling was cysteine 86. The reason for the high reactivity of cysteine 86 in rGSTM1 was hypothesized to be due to its potential interaction with histidine 84, which is unique in this subunit. *In vitro* studies have shown that AN can bind to reactive cysteine sites in other proteins as well, including the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Campian *et al.*, 2002). AN irreversibly inhibits GAPDH in a temperature-dependent manner with second-order rate constants of 3.7 and 9.2 $M^{-1}s^{-1}$ measured at 25 and 37 degrees C, respectively. AN was found to inactivate GAPDH by covalently binding to cysteine 149 in the active site of the enzyme.

3.3 Metabolism

The metabolism of AN is an important determinant of toxicity, and has direct implications on the mode of action (**Section 4.3**). As depicted in **Figure 3-1**, AN is initially metabolized by two pathways: (1) conjugation with glutathione, either through catalysis with a cytosolic glutathione-S-transferase (GST) or nonenzymatically; and (2) epoxidation by microsomal cytochrome P4502E1 forming 2-cyanoethylene oxide (CEO) (Dahl and Waruszewski, 1989; Fennell *et al.*, 1991; Kedderis *et al.*, 1993; Burka *et al.*, 1994; Gargas *et al.*, 1995, Sumner *et al.*, 1999). The primary metabolites from both pathways are subject to further metabolism. The AN-glutathione conjugate can be converted to a mercapturic acid and excreted in urine. CEO, on the other hand, is metabolized by two pathways: (1) conjugation with glutathione, either through catalysis with cytosolic GST or nonenzymatically, forming conjugates on the second or third carbon; and (2) hydrolysis by microsomal epoxide hydrolase (EH).

The secondary metabolites of CEO can undergo further metabolism/decomposition. Of toxicological importance, cyanide can be released from the CEO metabolite generated by the EH pathway and from the GSH conjugate formed on the third carbon. Cyanide is detoxified by the mitochondrial enzyme, rhodanese, which uses sulfane sulfur (*i.e.*, thiosulfate) to form thiocyanate. Thiocyanate was detected in the blood and urine of volunteers exposed to 45 - 110 mg/m^3 AN for 30 minutes (Wilson and McCormick, 1949), and has been measured in the blood and brain of Sprague-Dawley rats exposed to 20, 50, 80, or 115 mg/kg AN by gavage (Benz *et al.*, 1997a). Peak blood cyanide concentrations were attained one hour after dosing in mice and three hours after dosing in rats (Ahmed and Patel, 1981). HCN has also been detected in exhaled breath of rats exposed to AN by the oral route–0.5% of a 46.5 mg/kg dose to rats (Ahmed *et al.*, 1982).

The release of cyanide appears to require CYP2E1 activity (Wang *et al.*, 2002; Kedderis *et al*., 1993). While the primary site of AN metabolism is the liver, other tissues have the capacity to metabolize AN. In rats, AN metabolism has been demonstrated *in vitro* in microsomal fractions from a number of tissue sites, including testes (Abdel-Aziz *et al.*,

1997), kidney (Mostafa *et al.*, 1999), lung (Roberts *et al.*, 1989), nasal tissue (Dahl and Waruszewski, 1989), small intestines (Subramaniam and Ahmed, 1995), and brain (Ahmed and Abreu, 1981). Human lung lipoxygenase has demonstrated an appreciable activity for metabolizing AN to cyanide *in vitro* (Roy and Kulkarni, 1999), suggesting that there may be additional enzymatic pathways leading to the formation of CEO and the release of cyanide from AN. In addition, nonenzymatic oxidation of AN may occur in tissues during periods of oxidative stress. For example, cyanide release was reported for a structurally similar chemical, acetonitrile, in the presence of reactive oxygen species (Mohamadin, 2001). In this *in vitro* study, a four-fold increase in peroxide levels resulted in a 35-fold increase in cyanide release.

The metabolism of AN is influenced by the route of exposure, species to which it is administered, the magnitude of the exposure, and the vehicle in which it is administered. Each of these complicating factors is summarized below.

- *Impact of Route of Exposure on Metabolism* - Based upon urinary metabolite data, conjugation with glutathione represents the predominant metabolic pathway for AN in rats exposed by gavage or iv or ip injection (Fennell *et al.*, 1991; Kedderis *et al.*, 1993, Tardif *et al.*, 1987). The primary metabolite observed in rats and other test species is N-acetyl-S-(2-cyanoethyl)cysteine (Dahm, 1977; Ahmed and Patel, 1979; Van Bladeren *et al.*, 1981; Ghanayem and Ahmed, 1982). Other major metabolites include thiocyanate and 4-acetyl-5-cyanotetrahydro-1,4-2H-thiazine-3-carboxylic acid (Langvardt *et al.*, 1980). However, the metabolism of AN exhibits a strong first-pass effect, and as such, the relative importance of each pathway depends upon the route of exposure. In rats, oxidation of AN, as indicated by urinary excretion of thiocyanate (% administered dose indicated in parentheses), was greater following oral exposures (23%) when compared to intraperitoneal (4%), subcutaneous (4.6%), and intravenous (1.2%) exposures (Gut *et al.*, 1981). Similarly, a marked influence of the route of administration on the pattern of metabolic excretion was reported in rats exposed to AN via inhalation, ip, or iv injection (Tardif *et al.*, 1987). After ip and iv injection, 2-cyanoethylmercapturic acid was the most important metabolite, whereas after inhalation exposure thiocyanate was the primary metabolite observed.

- *Species Differences in Metabolism* - With respect to species differences, mice and rats appear to form CEO at a greater rate (4x and 1.5x, respectively) compared to humans (Roberts *et al.*, 1991). Despite having a higher rate of CEO formation than rats, mice exhibited circulating levels of CEO that were approximately one third of the levels detected in rats (Roberts *et al.*, 1991), suggesting that differences exist between rats and mice with respect to CEO metabolism. Conjugation of CEO with GSH occurs faster in humans (1.5x) than in either mice or rats (Kedderis *et al.*, 1995). Additionally, hydrolysis of CEO by EH is very high in humans, but is virtually absent

in mice and rats (Kedderis *et al.*, 1995). However, EH activity can be induced in both rats and mice (Kedderis and Batra, 1993).

- *Dose-Dependency of Metabolism* - Several metabolic factors contribute to nonlinear kinetics for AN, including the presence of a saturable metabolic pathway and the depletion of cofactors required for metabolism. The existence of nonlinear kinetics complicates high-to-low dose extrapolation efforts required for human health risk assessment. With respect to dose-dependencies, urinary excretion of N-acetyl-S-(2-cyanoethyl)cysteine and S-(2-cyanoethyl)thioacetic acid across a wide range of oral doses (0.09-28.8 mg/kg) was increased in a non-linear manner (Fennell *et al.*, 1991; Kedderis *et al.*, 1993). GSH depletion has been observed in a number of tissues (brain, lung, liver, kidney, stomach, adrenal gland, erythrocytes) in rats exposed to AN (Cote *et al.*, 1984; Gut *et al.*, 1985, Benz *et al.*, 1997a, Vodicka *et al.*, 1990, Silver and Szabo, 1982). GSH depletion has also been reported in blood of human subjects exposed to AN (Jerca *et al.*, 1992). Depletion of GSH results in an increase in the flux of parent chemical metabolized via the oxidative pathway. For example, metabolism of AN to CEO following oral or inhalation exposures, as indicated by urinary thiocyanate, was enhanced approximately two- to three-fold in GSH-depleted F344 rats (using a phorone/buthionine sulfoximine pretreatment) compared to rats with normal GSH status (Pilon *et al.*, 1988a,b).

- *Effects of Vehicle on Metabolism* - Blood and tissue cyanide levels were measured in Sprague-Dawley rats one, three, and six hours after administration of a gavage dose of AN in saline, corn oil, safflower oil, mineral oil, olive oil and Tween-20. The use of a vehicle other than saline increased cyanide and thiocyanate levels (Farooqui *et al.*, 1995).

Consideration of factors affecting metabolism is important to the dose-response assessments for AN (**Section 5.0**), to ensure that efforts taken to perform both interspecies extrapolation and high-to-low dose extrapolation are appropriate.

3.4 Excretion

The excretion of AN and its metabolites occurs predominantly via the urine, with smaller amounts excreted in either the feces or exhaled breath.

Ingestion

When a single dose of [1-^{14}C]-AN was given orally to rats, approximately 27% of the administered dose was excreted in bile in six hours (Ghanayem and Ahmed, 1982). This amount was increased in rats who were either fasted overnight or pretreated with cobaltous

chloride. Pretreatment of rats with phenobarbital produced no change, while diethyl maleate pretreatment significantly decreased the portion of the dose excreted in bile in six hours.

In rats, following exposure to [1,2-^{14}C]-AN via oral or ip injection, the majority of the radiolabel (82-93%) was excreted in the urine (Sapota, 1982). A smaller percentage of the dose (3-7%) was exhaled unchanged in 24 hours.

Ahmed *et al.* (1982, 1983) report that rats given an oral dose of 46.5 mg/kg radiolabeled AN excreted approximately 40% of the radiolabel in urine, 2% in feces, 9% in expired air as $^{14}CO_2$, 0.5% as H^{14}CN and 4.8% as unchanged AN in 24 hours. Bile flow increased three times after the administration of AN and over a period of six hours, 27% of the radiolabel was recovered in bile.

After gavage administration of equimolar doses (0.87 mmol/kg) of [2-^{14}C]-methacrylonitrile (MAN) or [2-^{14}C]-AN to male F344 rats, substantial differences were observed in the excretion of these two chemicals (Burka *et al.*, 1994). Approximately 39% of the administered MAN dose was eliminated as CO_2 in 24 h after dosing, while only 11% of an equimolar dose of AN was eliminated as such. In addition, 31% of the MAN dose was exhaled as organic volatiles in 24 hours compared to less than 2% of an equivalent AN dose. HPLC analysis showed that AN is the only organic volatile exhaled by AN-treated rats. Urinary excretion of MAN was 22% compared to 67% of an equivalent dose of AN. The major urinary metabolite from AN results from direct conjugation with GSH, whereas the major urinary metabolite from MAN results from conjugation of the epoxide with GSH.

Male F344 rats and B6C3F1 mice were coadministered [1,2,3-^{13}C]AN (16-17 mg/kg) and [1,2,3-^{13}C]-acrylamide (21-22 mg/kg) after 0 or four days of administration of unlabeled AN or acrylamide (Sumner *et al.*, 1997). Rats and mice excreted metabolites derived from glutathione (GSH) conjugation with AN or acrylamide or derived from GSH conjugation with the epoxides CEO or glycidamide. For mice, an increased urinary excretion of total AN- and total acrylamide-derived metabolites ($p < 0.05$) on repeated coadministration suggested a possible increase in metabolism via oxidation. In addition, mice had an increased ($p<0.05$) percentage of dose excreted as metabolites derived from GSH conjugation after five exposures as compared with one exposure that may be related to a significant increase in the synthesis of GSH or an increase in glutathione transferase activity. No differences between one and five exposures for the rat were reported for AN.

Inhalation

AN and its metabolites have been detected in the urine of exposed workers. Urinary elimination of AN occurred predominantly during exposure, and was found at levels ~10-20 mole % of the urinary metabolites of AN. AN was also found in pre- and post-shift urine

samples of occupationally exposed workers (n = 34, median exposure = 78 mg/m^3) at levels exceeding those of non-exposed controls (Perbellini *et al.*, 1998). Similar results were found by Houthuijs *et al.* (1982) in a smaller group of workers (n = 15). Sakurai *et al.* (1978) also report urinary excretion of AN by workers.

Quantitative analysis of the dose-dependent urinary excretion of AN and its metabolites was carried out in male Wistar rats following inhalation exposure of the animals to 1, 5, 10, 50, and 100 ppm AN for eight hours (Muller *et al.* 1987). The excretion pattern of the compound and its metabolites was dependent on the exposure level; it is concluded that urinary determination of the unmetabolized AN and two of its metabolites, cyanoethyl mercapturic acid and thioglycolic acid, may be useful for biological monitoring of industrial exposure.

Adult male Sprague-Dawley rats were exposed acutely to 0, 4, 20, or 100 ppm AN via inhalation for six hours (Tardif *et al.* 1987). Urinary metabolites measured 24 hours after administration were 2-cyanoethylmercapturic acid, 2-hydroxyethylmercapturic acid, and thiocyanate. The relationship between excretion of total urinary metabolites and the degree of exposure was reasonably linear.

Other

The excretion of AN and its metabolites was investigated in male Sprague-Dawley rats following acute exposure to 0, 0.6, 3.0, or 15 mg/kg AN via iv, or ip (Tardif *et al.*, 1987). Urinary metabolites measured 24 hours after administration were 2-cyanoethylmercapturic acid, 2-hydroxyethylmercapturic acid, and thiocyanate. The relationship between excretion of total urinary metabolites and the degree of exposure was reasonably linear.

No specific sources of nonlinear kinetics were identified regarding the excretion of AN. In theory, nephrotoxicity could have an impact upon the excretion of AN and its metabolites in the urine. However, the nephrotoxic effects of AN appear to be associated with very high exposures (Rouisse *et al.*, 1986), and, therefore, are not expected to contribute to nonlinear kinetics at low doses.

3.5 Pharmacokinetic Models

Physiologically Based Pharmacokinetic (PBPK) Models

PBPK models have been developed for AN. The models have been refined over the past seven years, as summarized in the following papers:

- *Gargas et al. (1995)* - A physiologically based description for dosimetry of AN and CEO in the male F-344 rat was developed from *in vitro* data and studies of the iv

pharmacokinetics of AN and CEO. The dosimetry description includes *in vitro* estimates of the rates of reaction of AN and CEO with hemoglobin and blood macromolecules and the reaction of CEO with tissue GSH. Metabolic parameters for AN and CEO were estimated from iv pharmacokinetic studies. AN elimination from blood was described by saturable P450 epoxidation and first-order GSH conjugation. CEO elimination was described by first-order GSH conjugation. Calculation of hepatic clearance values shows first-pass hepatic extractions of 61 and 90% for AN and CEO, respectively. The dosimetry description accurately simulated the dose-dependent urinary excretion of AN metabolites derived from epoxidation to CEO and from direct GSH conjugation of AN. The dose-dependent formation of hemoglobin adducts from AN was also well simulated.

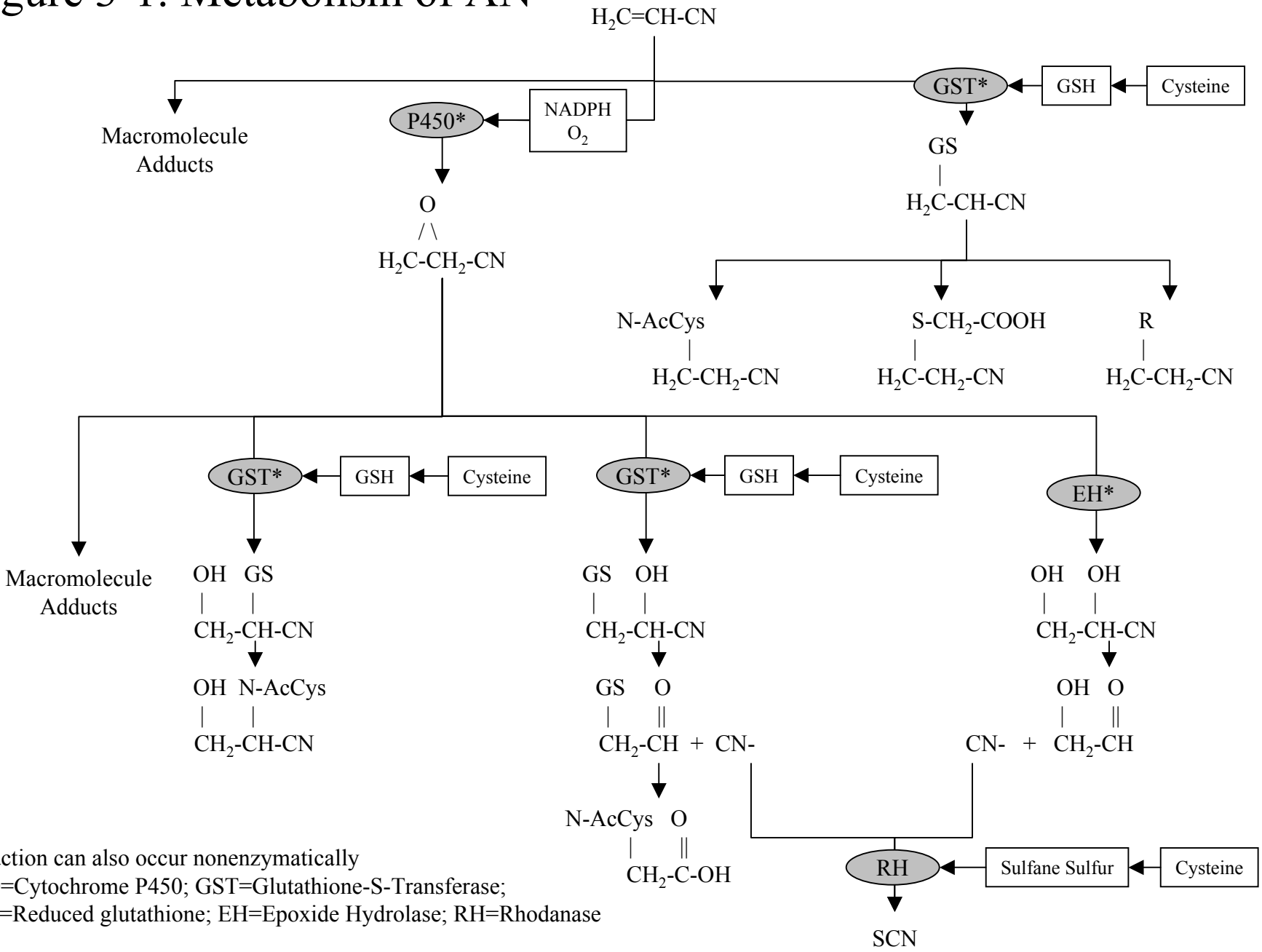
- *Kedderis et al. (1996)* - The physiologically based dosimetry description for AN and its mutagenic epoxide metabolite CEO in F-344 rats (Gargas *et al.*, 1995) was refined to include a physiological stomach compartment and the reactions of AN with tissue GSH. The second-order rate constant for reaction of AN and GSH at pH 7.3 was measured and included in the dosimetry description. Metabolic parameters for AN and CEO were estimated from oral bolus pharmacokinetic studies and previously obtained iv bolus data. AN elimination from blood was described by saturable P450 epoxidation and first-order GSH conjugation. CEO elimination was described by first-order GSH conjugation. The pharmacokinetic data were well simulated, although CEO blood concentrations after bolus oral dosing were somewhat overestimated. Sensitivity analysis of the dosimetry description indicated that the inhalation exposure route was much more sensitive to changes in metabolic and physiological parameters than either the iv or oral bolus routes. Therefore, inhalation pharmacokinetic data were obtained and compared to simulations of the dosimetry description. The dosimetry description accurately simulated the AN inhalation pharmacokinetic data, providing verification of the parameter estimates.

- *Sweeney et al. (2003)* - A physiologically based pharmacokinetic model of AN and CEO disposition in humans was developed and is based on human *in vitro* data and scaling from a rat model (Kedderis *et al.*, 1996). All of the major biotransformation and reactivity pathways, including metabolism of AN to glutathione conjugates and CEO, reaction rates of AN and CEO with glutathione and tissues, and the metabolism of CEO by hydrolysis and glutathione conjugation, were described in the human PBPK model. Model simulations indicated that predicted blood and brain AN and CEO concentrations were similar in rats and humans exposed to AN by inhalation. In contrast, rats consuming AN in drinking water had higher predicted blood concentrations of AN than humans exposed to the same concentration in water. Sensitivity and variability analyses were conducted on the model. While many parameters contributed to the estimated variability of the model predictions, the

reaction rate of CEO with glutathione, hydrolysis rate for CEO, and blood:brain partition coefficient of CEO were the parameters predicted to make the greatest contributions to variability of blood and brain CEO concentrations in humans. Expected variability in blood CEO concentrations (peak or average) in humans exposed by inhalation or drinking water was modest, with a 95th-percentile individual expected to have blood concentrations 1.8-times higher than an average individual.

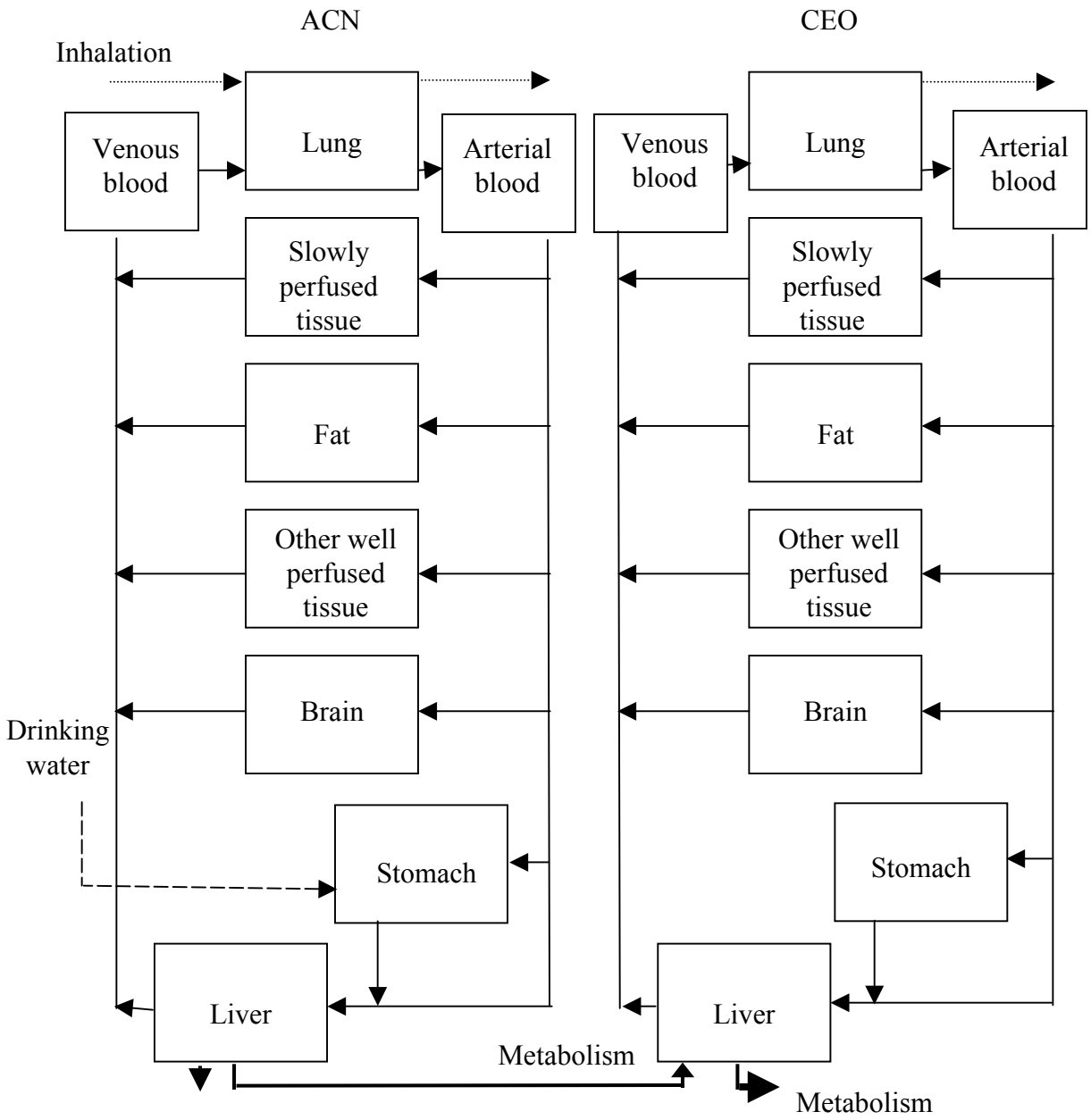
The PBPK models for AN in rats (Kedderis *et al.*, 1996) and humans (Sweeney *et al.*, 2003) are used in the dose-response analysis (**Section 5.0**) to allow for an evaluation of these relationships in terms of internal dose measures. The structure of the PBPK model is provided in **Figure 3-2**.

Figure 3-1. Metabolism of AN



*Reaction can also occur nonenzymatically
P450=Cytochrome P450; GST=Glutathione-S-Transferase;
GSH=Reduced glutathione; EH=Epoxide Hydrolase; RH=Rhodanase

Figure 3-2. PBPK Model Structure for AN and CEO



4. HAZARD CHARACTERIZATION

This section summarizes the information available regarding the toxicity and carcinogenicity of AN. This information is presented separately for studies in humans (**Section 4.1**) and in animals (**Section 4.2**). Summary and interpretation of both sources of information are provided in **Section 4.3** in an effort to identify key issues relating to the major effects and their modes of action for the dose-response assessment for AN (**Section 5.0**).

4.1. Studies in Humans

4.1.1 Non-Cancer Effects

A large number of epidemiology studies have been conducted on worker populations exposed to AN. While these studies focused primarily on mortality and morbidity rates for cancer (**Section 4.1.2**), some of these studies also reported on non-cancer endpoints. Human evidence from case reports and workplace surveys suggest that neuropathological and respiratory effects following exposure to AN is the critical non-cancer effect with the primary routes of exposure being inhalation and dermal contact with the chemical. It is evident that there is often co-exposure with other chemicals, which makes it very difficult to interpret the results of epidemiological studies in production and processing plants.

4.1.1.1 Reproductive Effects

Prior to 1990, no studies of reproductive or developmental effects in humans exposed occupationally or environmentally to AN by any route were located by ATSDR (1990); however, few specific studies have been carried out since that time.

Male-Mediated Reproductive Toxicity

According to BUA (1995), a decrease of the testosterone level in serum occurred in workers in Romanian factories who were exposed to AN and other unspecified chemicals. The extent of exposure or significance of the effect was not mentioned in the publication (Ivanescu *et al.*, 1990).

Female-Mediated Reproductive Toxicity

Dorodnova (1976) found no differences in gynecological health between 410 women exposed to AN in a polyacrylic fiber plant and that of 436 unexposed women. Levels of AN and duration of exposure were not stated.

Czeizel *et al.* (1999) evaluated the potential effects of AN exposure in people living in the surrounding region of a Hungarian AN factory. The rate and type of congenital abnormalities in 46,326 infants born between 1980 and 1996 to mothers living in the 30 settlements within a 25 km radius of the AN factory were examined. The ascertainment of cases with congenital abnormalities was based on the Hungarian Congenital Abnormality Registry complemented with the review of pediatric, pathology, and cytogenetic records. Three congenital abnormalities: *pectus excavatum* in Tata, 1990 to1992 (OR = 78.5; 95% CI = 8.4 to 729.6), undescended testis in Nyergesäujfalu between 1980 and 1983 (OR = 8.6; 95% CI = 1.4 to 54.3) and in Esztergom, 1981 to 1982 (OR = 4. 2; 95% CI = 1.3 to13.5), and clubfoot in Tata, 1980-1981 (OR = 5.5; 95% CI = 1.5 to20.3) showed significant time-space clusters in the study region. There was a decrease in risk of undescended testis with increasing distance from the AN factory. An unusual increase was found in the combination of oral cleft and cardiac septal defects in multi-malformed babies in Tatabäanya in 1990. However, the authors concluded that the results supported the null-hypothesis, *i.e.*, no effect of AN exposure for people living near the AN factory.

4.1.1.2 Neurotoxicity

Acute Neurotoxic Effects

Wilson (1944) reported that synthetic rubber workers exposed to "mild" concentrations of AN developed nausea, vomiting, and weakness. Headache and fatigue occurred in some cases.

Workers in a synthetic rubber manufacturing plant exposed to AN vapor at levels of between 16 (35 mg/m^3) and 100 ppm (219 mg/m^3) AN for 20 to 45 minutes experienced dull headaches, nausea, feelings of apprehension, and nervous irritability (Wilson *et al.*, 1948).

Wilson and McCormick (1949) identified nausea, vomiting, headache, and vertigo among other symptoms in workers at a synthetic rubber manufacturing plant following exposure to 'mild' concentrations of AN.

The death of a 10-year-old girl following dermal exposure to AN was reported by Lorz (1950). An AN preparation had been applied to the scalp of the child as a treatment for head lice. The child experienced nausea, headache, and dizziness. Death occurred four hours after application. The concentration was not specified in this case report.

Complaints of poor health, including headache, decreased work capacity, poor sleep, irritability, and poor appetite (during the first months of employment only) came from workers employed in the manufacture of AN (Zotova, 1975).

Zeller *et al.* (1969) reported that in 16 cases of acute inhalation of AN fumes by workers, nausea, vomiting, headache, and vertigo appeared within five to 15 minutes; none of the workers required hospitalization. Sartorelli (1966) also reported similar symptoms (*i.e.*, headache, vertigo, vomiting tremors, uncoordinated movements, and convulsions) in a worker who was exposed to AN vapors when a leakage occurred in a distillation apparatus. Symptoms disappeared after four days.

Signs of cyanide poisoning were exhibited by a man accidentally sprayed with AN. Dizziness, redness, nausea, vomiting, and hallucinations were reported to have occurred within 30 minutes of exposure (Vogel and Kirkendall 1984). The victim suffered subsequent hallucinations and convulsions. The primary route of exposure was dermal (or possibly gastrointestinal, given the nature of the incident). The patient was thoroughly bathed on three occasions, but required 15 antidotal treatments against cyanide poisoning over three days suggesting that AN or one of its metabolites was stored in tissues or skin and slowly absorbed. This episode gives some indication of the severity of the systemic poisoning via dermal exposure.

In a study with human volunteers exposed to AN at concentrations of 2.3 and 4.6 ppm for eight hours, no symptoms attributable to effects on the nervous system were observed (Jakubowski *et al.* 1987).

WHO (1983) and VROM (1984) summarized several cases of AN poisoning whereby workers exposed to AN concentrations > 5ppm (11 mg/m^3) suffered from headaches, vertigo, and limb weakness.

Chronic Neurotoxic Effects

Babanov *et al.* (1959, as reported in WHO, 1983) reported that workers exposed to AN concentrations at 0.6 to 6.0 mg/m^3 (0.3 to 3 ppm) for approximately three years suffered headaches, insomnia, general weakness, decreased working capacity, and increased irritability.

Ageeva (1970 cited in WHO 1983) reported depression, lability of autonomic functions (*i.e.*, lowered arterial pressure, increased sweating, changes in orthostatic reflex, etc.) in workers involved in AN production.

Sakurai and Kusumoto (1972) studied the health records of 576 workers from five AN fiber plants over a 10 year period. At exposure levels of 11 mg/m^3 (5 ppm) some subjective complaints such as headache, fatigue, nausea, insomnia were reported. The effects were positively associated with the length of exposure, but not with the exposure level or age of the workers. A total of 4439 examinations were made over the 10 years prior to 1970. The

576 workers were formed into two cohorts, one exposed to concentrations of AN of below 11 mg/m^3 (5 ppm), the other to below 45 mg/m^3 (20 ppm). However, in a later report by the same author (Sakurai *et al.*, 1978), it was stated that the study lacked adequate epidemiological design, the findings were based on routine health examinations, and the "exposure levels were not reliably reported" and may have been much higher. Exposure levels associated with these effects originate from before improved hygiene measures were introduced, and subsequent appraisal of this study indicates that many of the symptoms were associated with exposures well in excess of 5 ppm. Sakurai *et al.* (1978) stated that their findings were not contradictory to those of Wilson *et al.* reflecting the older and less controlled workplace environment where levels could be up to 20 ppm.

Stamova *et al.* (1976) studied workers' health in a polyacrylic fiber plant where AN exposures were around 10 mg/m^3, but could fluctuate to as high as 25 mg/m^3. An increased incidence of neurasthenic complaints (undefined) was reported. Other chemicals were present in the workplace and the length of exposure was not reported.

Ginceva *et al.* (1977) found no changes in the health status of a group of 23 men exposed to 4.2 to 7.2 mg/m^3 of AN for three to five years.

In the Sakurai *et al.* (1978) study, 102 workers whose exposure to AN exceeded five years and 62 matched controls, all randomly selected from six acrylic fiber factories in Japan were examined. The most highly exposed group had an average eight-hour exposure to AN of 9.1 mg/m^3. Medical examinations including multiple clinical chemistry measurements failed to detect any health effects attributable to AN.

In a study of 884 men occupationally exposed to AN, Kiesselbach *et al.* (1979) reported that mortality from nervous system diseases was not different than expected. There is some question as to the thoroughness of case ascertainment and follow-up in this study.

Buchter and Peter (1984) described a case of a 57 year old locksmith who was exposed to AN for 14 years. The man had also been exposed to prussic acid, ammonia, phosphoric acid, propylene, hydrochloric acid, and sulphuric acid. His complaints consisted of disturbance of memory, weakness, headache, dizziness, drowsiness, diminished vision and hearing, and low blood pressure. The diagnosis after examination was cerebrovascular insufficiency due to disturbance of the circulatory function, arterosclerosis, elevation of the erythrocyte sedimentation rate of unknown origin (no tumor), porphyrinuria, and noise induced hearing loss. In an effort tolerance test, the performance of the patient was only 10 to 20% of normal workers. However, the authors concluded that psychopathological development seemed to be more likely than chronic disease due to AN, as the patient was strongly convinced of his own inability to work, in spite of normal cardiopulmonary work capacity and only slight disturbance of the circulatory function.

A producer of AN reported 10 cases of skin complaints in employees (Bakker *et al.*, 1991). Paresthesia was reported in one patient.

In a more recent study (Kaneko and Omae, 1992), workers from seven factories exposed to AN at mean concentrations of 1.8 ppm (4 mg/m^3), 7.4 ppm (16 mg/m^3), and 14.1 ppm (31 mg/m^3), for a period of 5.6, 7, and 8.6 years, respectively, were questioned about their subjective symptoms by means of a questionnaire. A medical examination was not performed. Compared with non-AN workers, those questioned complained more often about headaches, fatigue, sweating, "heavy arms," and general weakness, but no clear differences were observed between the three exposure groups. No differences in neurotic status was found between those exposed and controls.

4.1.1.3 Genotoxicity

This section presents the information available for the genotoxicity of AN in human cells from *in vitro* and *in vivo* studies. Additional information regarding the genotoxicity of AN in animals and in nonmammalian cell systems is presented in **Section 4.2.6**. Interpretation of both sources of information is provided in **Sections 4.3** and **4.4**.

In Vitro Studies

Perocco *et al.* (1982) demonstrated that exposure of human lymphocytes to 0.5 mM AN (26.5 µg/ml) resulted in a significant increase in SCE. In contrast, Obe *et al.* (1985) was unable to demonstrate SCE-induction by AN after exposing fresh human lymphocytes to AN at concentrations of one and 10 µg/ml for 24 hours in the absence of S9 and for one hour in the presence of S9 from Arochlor-induced rat liver.

As part of an overall study to determine the possible mutagenic and genotoxic activity of AN, Rizzi *et al.* (1984) carried out a DNA repair assay using HeLa cells. In this study incorporation of [^{3}H]TdR into DNA was measured in four groups, namely control and AN-treated cells in the absence of hydroxyurea (-HU) and control and treated cells exposed to hydroxyurea (+HU). The results showed that the -HU/+HU relationship between treated and control cells and the value of +HU between treated and control cells were statistically significant at AN dose levels of 0.18 and 0.036 mM ($p < 0.01$ and $p < 0.09$ respectively). These results suggest that in both systems AN is a mutagenic and genotoxic agent at very low doses. In contrast, Martin and Campbell (1985) did not demonstrate unscheduled DNA repair in HeLa cells. Details of exposure levels and times were not provided in this study.

AN gave a positive response both in the presence and absence of metabolic activation in human lymphoblasts (TK6, *TK* locus). Using dose levels of five to 50 µg/ml, an exposure period of three hours in the presence of S9 (prepared from Arochlor-induced rat liver) and

20 hours in its absence, and an expression period of 72 hours, mutational frequency was increased 3.5 fold in the presence of S9 at both 40 and 50 µg/ml with relative survival at these exposure levels of 37% and 26%, respectively (Crespi *et al.*,1985). In the absence of S9, mutational frequency was increased two-fold compared with control at 15µg/ml, but only 1.3-fold at 20 µg/ml with marked cytotoxicity at this exposure level (18% survival). Mutagenic activity was also examined in the metabolically competent AHH-1 cell line (hypoxanthine guanine phosphoribosyl transferase locus) using dose levels of 5 to 25 µg/ml, an exposure period of 28 hours and an expression period of six days. AN also gave a positive response in this cell line, with an approximate 4.5-fold increase in mutational frequency over control at 25 µg/ml, at 16% relative survival, a response similar to that for the positive control benzo(a)pyrene (3.1 µg/ml) (Crespi *et al.*,1985).

The mutagenic potential of both AN and its metabolite CEO was also studied using the TK human lymphoblast cell line, the heterozygous thymidine kinase (*tk*) locus being the genetic marker, in the presence and absence of S9 from Arochlor-induced male Sprague Dawley rats (Recio and Skopek,1988). Cells were exposed for two hours with an expression period of six to eight days. AN was not mutagenic in the absence of S9, producing less than a two-fold increase in mutation frequency over a concentration range of 0.4 to 1.5 mM (21 to 80 µg/ml). In the presence of S9, a statistically significant mutagenic response (four-fold increase, $p < 0.05$) was seen at the highest exposure concentration assessed experimentally, 1.4 mM (74 µg/ml). Survival was reduced to approximately 10% at a concentration of 1.5 mM. 2-CEO induced a 17-fold increase in mutation frequency without metabolic activation at 100 µM. The results from these experiments confirm that AN is weakly mutagenic in mammalian cells, while the mutagenicity exhibited by CEO suggests that this metabolite may in fact be the ultimate mutagenic metabolite of AN.

In a follow-up study, CEO was used to treat human TK6 lymphoblasts (150 uM for 2h) (Recio et al., 1990). A collection of hprt mutants was isolated and characterized by dideoxy sequencing of cloned hprt cDNA. Base-pair substitution mutations in the hprt coding region were observed in 19/39 of hprt mutants: 11 occurred at AT base pairs and 8 at GC base pairs. Two -1 frameshift mutations involving GC bases were also observed. Approximately half (17/39) of the hprt mutants displayed the complete loss of single and multiple exons from hprt cDNA, as well as small deletions, some extending from exon/exon junctions. Southern blot analysis of 5 mutants with single exon losses revealed no visible alterations. Analysis of 1 mutant missing exons 3-6 in its hprt mRNA revealed a visible deletion in the corresponding region in its genomic DNA. The missing exon regions of 4 mutants (one each with exons 6, 7 and 8 loss and one mutant with a 17-base deletion of the 5' region of exon 9) were PCR amplified from genomic DNA and analyzed by Southern blot using exon-specific probes. The exons missing from the hprt mRNA were present in the genomic hprt sequence. DNA sequencing of the appropriate intron/exon regions of hprt genomic DNA from a mutant with exon 8 loss and a mutant exhibiting aberrant splicing in exon 9 revealed

point mutations in the splice acceptor site of exon 8 (T----A) and exon 9 (A----G), respectively.

AN's potential to induce sister chromatid exchange (SCE) and the induction of DNA single breaks was investigated using adult human bronchial epithelial cells obtained from autopsy specimens and used in the 3rd or 4th passage (Chang *et al.*, 1990). Cultures were exposed for 20 hours to levels of 150, 300, 500, or 600 µg/ml AN before being assessed for SCE and DNA strand breaks. Cytotoxicity (measured in terms of colony forming efficiency) was marked at the highest exposure level of 600 µg/ml, but the lower concentrations were not associated with toxicity. SCEs were significantly increased ($p < 0.01$) at dose levels of 150 and 300 µg/ml, the incidence of SCE per cell being 6.6 and 10.7 respectively, compared with 3.7 in unexposed control cultures, the incidence falling at 600 µg/ml due to the cytotoxicity. The extent of DNA single strand breaks appeared to be positively correlated with increasing levels of AN in the culture.

A human mammary epithelial cell (HMEC) DNA repair assay was performed in secondary cultures of HMEC by Eldridge *et al.* (1992). The secondary cultures of normal HMEC were derived from residual surgical material from mammoplasties of five healthy women. The cell line used has lost the ability to activate genotoxicants metabolically, but retained the capacity for DNA repair. Based on historical controls, any individual cell with greater than or equal to six nuclear grains (NG) was considered in repair. The population average NG for 25 to 80 cells was calculated for each slide, two slides per treatment group. The percentage of cells in repair was also calculated. The unpaired t-test for the equality of two means was used to compare NG between control and treated cultures. The Chi square test was used to test for significant differences in the percentage of cells in repair (IR) between control and treated cultures. A response was judged positive at $P < 0.05$ for NG and/or IR with an NG value greater than zero. Although CEO was cytotoxic to HMEC, it did produce a positive UDS response, confirming its genotoxic potential. In contrast no activity of AN was observed in the HMEC DNA repair assay (*i.e.*, a negative response), however, it was very cytotoxic in this assay.

In Vivo Studies

Thiess and Fleig (1978) examined chromosomal damage in peripheral lymphocytes of 18 workers exposed to AN for an average of 15.4 years. Workers were also exposed to styrene, ethylbenzene, butadiene, and butylacrylate. As in most human studies, the actual concentration of AN to which these workers were exposed was not reported. Average air concentrations of AN of 5 ppm (11 mg/m^3) were measured for the majority of the exposure period (approximately 10 years). These were considered representative of normal operating conditions, although higher peak exposures will have been present due to faults and manual operation. At the time the study was conducted, AN levels in the workplace had been

reduced to 1.5 ppm. The frequency of chromosomal aberrations in peripheral lymphocytes of AN workers was not increased compared to the unexposed controls.

Borba *et al.* (1996) measured chromosomal aberrations and SCEs in 14 workers employed in the continuous polymerization area and in 12 maintenance workers in an acrylic fiber plant. Twenty workers in administration in that plant served as a control group. The study provides no information on AN exposure level or duration of exposure, nor is information provided on exposure to other substances. There was no difference in SCEs between the two exposed groups and the controls. Maintenance workers were reported to show a higher incidence of chromosome aberrations than either the polymerization workers or the control group. However, the mixed exposures of the maintenance workers and the lack of increased aberrations in polymerization workers make it difficult to attribute the findings in the maintenance group to AN exposure.

4.1.1.4 Other Non-cancer Effects

General Mortality

No increase in overall or cause-specific mortality has been reported for AN workers in numerous epidemiological studies. No consistent relationship has been noted in terms of increasing dose or duration of exposure.

In a mortality study of 884 men occupationally exposed to AN, Kiesselbach *et al.* (1979) reported that the overall mortality for exposed workers was markedly lower than that of the comparison population (58 observed deaths versus 104 expected). Delzell and Monson (1982) reported a similar deficit in mortality among workers potentially exposed to AN (observed = 74, expected = 89.5; SMR = 0.8; 95% CI = 0.7 to 1.0). A number of these early studies suffer from small size and uncertain case ascertainment and follow-up, which may limit their usefulness, but a large number exist that basically come to the same conclusion.

O'Berg (1980) reported 89 deaths from all causes among workers exposed to AN at a DuPont plant which was higher than expected based on a company mortality registry (expected = 77.4), but lower than expected based on US (121.1) or state (156.0) rates. In a 1985 follow-up, O'Berg *et al.* continued to report a slightly increased total mortality in wage employees based on comparison with a company registry (observed = 139, expected = 117.8; SMR = 118), but no increase based on expected rates in the US population (observed = 139, expected = 171.4; SMR = 81.1). Chen *et al.* (1988b) reported on DuPont workers with exposure to AN and dimethylformamide and also observed a slight increase in mortality based on comparison with an internal company mortality registry (observed = 168, expected = 144.7). Chen *et al.* (1987) reported on another cohort of DuPont wage workers again reporting a deficit of deaths in the cohort (observed = 68, expected = 119.2 (US) or 87.8 (DuPont)). In

another follow-up study of DuPont workers, Collins *et al.* (1989) also reported a lower than expected mortality among AN exposed workers (145 observed, SMR = 0.67). The use of the company registry is limited by the fact that non-pensioners leaving DuPont employment were excluded and lost to follow-up so mortality may be under-reported. In a 1998 follow-up of DuPont workers using US rates for comparison (Wood *et al.*, 1998), a deficit in total mortality was reported (observed = 454; SMR = 69, 95% CI = 62 to 75).

A study of Dutch workers also reported a deficit in mortality. Swaen *et al.* (1992) observed 134 deaths in a cohort of 2,842 workers exposed to AN with 172.7 expected (SMR = 78; 95% CI = 65 to 92). Swaen *et al.* (1998) followed-up this study and observed 290 deaths versus 323.01 (SMR = 77.9; 95% CI = 79.7 to 100.7). In an additional follow-up of this cohort, 432 deaths were observed versus 466 deaths expected (Swaen *et al.*, 2004). This type of mortality deficit was again reported by Ives *et al.* (1993) for BP workers exposed to AN. Fifty-seven deaths were observed among this cohort given SMRs of 0.58 and 0.64 based on US and local county rates, respectively. In a recent UK mortality study of 2,763 male workers exposed to AN between 1950 and 1978 (Benn and Osborne, 1998), no significantly increased mortality was reported for all causes (409 observed, 485.5 expected; SMR = 84.2) was seen when compared to national rates. The largest mortality study yet conducted consisted of over 25,460 AN-exposed workers (Blair *et al.*, 1998). As with all previous studies using national rates, the workers had a lower than expected mortality (observed = 1217; SMR = 0.7, 95% CI = 0.6 to 0.7). Not surprisingly, the meta-analysis of AN mortality studies (n = 13) carried out by Collins and Acquavella (1998) found a mortality deficit (observed = 2,769, expected = 3,739.3; mRR = 0.8; 95% CI = 0.7 to 0.9). Such mortality deficits might have been due to healthy worker effect and insufficient follow-up time to observe effects in these cohorts in some cases, however, the larger, more recent studies appear to have had relatively complete case ascertainment and adequate follow-up.

Effects on the Respiratory Tract

The respiratory tract appears to be a target organ following inhalation of AN, both in man and in experimental animals. Based on the human experience (mainly as the result of accidental exposures to AN as a liquid or vapor), AN is an acute respiratory tract irritant causing effects such as irritation of the mucous membranes of the nose, eyes, and upper respiratory tract (Vogel and Kirkendall, 1984). In humans, the irritation of the throat and the respiratory tract appears to have a delayed action, with no sensation of irritation being felt in the initial period following exposure. Wilson (1944) reported that synthetic rubber workers exposed to "mild" concentrations of AN developed nasal irritation and an oppressive feeling in the upper respiratory tract. In a subsequent report, workers exposed to AN at concentrations of 16 to 100 ppm for periods of 20 to 45 minutes, developed irritation of the nose and throat and a feeling of fullness in the chest (Wilson *et al.,* 1948). In general

according to older publications (WHO, 1983), chronic AN exposure in workers caused, among other symptoms, dyspnea, coughing, irritation, and bronchitis.

More serious exposures have resulted in respiratory arrest and even death (Buchter and Peter, 1984). One reported fatal case involved a three-year old girl who slept overnight in a room recently sprayed with an AN-based fumigant. Respiratory malfunction and lip cyanosis were among the symptoms described prior to death (WHO, 1983).

Wilson and McCormick (1949) identified upper respiratory symptoms and nasal irritation, among other symptoms in workers at a synthetic rubber manufacturing plant following exposure to 'mild' concentrations of AN.

Human volunteers exposed acutely (eight hours) to AN at concentrations of 2.4 to 5.0 ppm (5.4 to 10.9 mg/m^3) exhibited no deleterious effects, indicating that AN is not very irritating to the respiratory tract at these concentration levels (Jakubowski *et al.*, 1987).

WHO (1983) and VROM (1984) summarized several cases of AN poisoning whereby workers exposed to AN concentrations > 5ppm (11mg/m^3) suffered from local effects such as irritation of the eyes, nose, throat, and respiratory tract.

Ginceva *et al.* (1977) found no changes in the health status of a group of 23 men exposed to 4.2 to 7.2 mg/m^3 of AN for three to five years.

Sakurai and Kusumoto (1972) studied the health records of 576 workers from five AN fiber plants over a 10 year period. At exposure levels of 11 mg/m^3 (5 ppm) irritation was reported. The effects were positively associated with the length of exposure, but not with the exposure level or age of the workers. A total of 4439 examinations were made over the 10 years prior to 1970. The 576 workers were formed into two cohorts, one exposed to concentrations of AN of below 11 mg/m^3 (5 ppm), the other to below 45 mg/m^3 (20 ppm). However, in a later report by the same author (Sakurai *et al.*, 1978), it was stated that the "exposure levels were not reliably reported." Subsequent appraisal of this study indicates that the symptoms of irritancy were associated with exposures well in excess of 5 ppm (EU, 2001). This reappraisal indicated that levels less than 10 ppm did not cause notable irritancy (EU, 2001). Sakurai *et al.* stated that their findings were not contradictory to those of Wilson *et al.* reflecting the older and less controlled workplace environment where levels could be up to 20 ppm.

Sakurai *et al.* (1978) investigated the health effects of exposure to AN in six acrylic fiber factories (including the five plants studied by Sakurai and Kusumoto, 1972). AN concentrations in air were measured in spot samples and personal air samples in these six acrylic factories on two consecutive days. In the best-controlled factories, air concentrations

of AN ranged from nondetect to 10 ppm. In factories with poorer controls on exposure levels, air samples as high as 100-200 ppm were reported. The median concentration for the highly exposed population of workers was reported to be 5 ppm (11 mg/m^3) based on spot samples, and an average of 4.2 ppm (9.5 mg/m^3) based upon personal air samples. Unexposed workers from the same factories were used as controls. Medical examinations were performed on 102 AN workers and 62 controls. Medical histories of these workers showed that many of the workers initially experienced irritation of the upper respiratory tract following exposure to AN in the years preceding this survey. A typical complaint when exposed to high concentrations of AN for a short duration was nasal discharge. These acute symptoms of irritation appeared to decrease gradually with time and became infrequent at the time of this study. Although some differences appeared to exist with respect to physical signs, none achieved statistical significance. The increased prevalence of irritative signs were associated with workers from a single factory with poorer controls on exposure levels. The gradual lessening of reported symptoms have been attributed to improved measures to reduce exposure. It should be noted however that in a later report by Sakurai (2000), it was stated that the "exposure levels were not reliably reported." These workers experienced irritation of the conjunctiva and upper respiratory tract following exposure to AN. However reappraisal of this study indicated that levels less than 10 ppm did not cause notable irritancy and the effects recorded were related to higher than 10 ppm exposure levels measured over years prior to this study being undertaken. A concentration of 3 ppm was considered to be a conservative, representative concentration of AN in the best-controlled factories where levels ranged from 1 to 10 ppm, and is considered a NOAEL value for the irritation effects of AN.

In a more recent study (Kaneko and Omae, 1992), workers exposed to AN at mean concentrations of 1.8 ppm (4 mg/m^3), 7.4 ppm (16 mg/m^3) and 14.1 ppm (31 mg/m^3), for a period of 5.6, 7 and 8.6 years respectively, were questioned about their subjective symptoms by means of a questionnaire. Exposures were estimated based upon two consecutive days of sampling, and, therefore, may not accurately depict actual exposures experienced by the workers. A medical examination was not performed. Compared with non-AN workers, those questioned complained more often about irritation of the mucosa and upper respiratory tract ("tongue trouble and choking lump in throat"), but no clear differences were observed between the three exposure groups. Because a dose-response relationship was not observed for these subjective symptoms, a NOAEL and LOAEL were not defined.

In a mortality study of 884 men occupationally exposed to AN, Kiesselbach *et al.* (1979) reported that mortality from respiratory diseases was not different than expected. Delzell and Monson (1982) also found no increased mortality among AN workers (observed = 5; SMR = 1, 95% CI = 0.3 to 2.4). Chen *et al.* (1987) reported no increase in mortality from respiratory diseases among DuPont workers exposed to AN (observed = 3; expected = 6 (US) and 3.3 (DuPont)). In a follow-up study, Collins *et al.* (1989) reported no increase in

respiratory mortality among DuPont workers exposed to AN compared to US expected rates (observed = 7, SMR= 0.69). Ives *et al.* (1993) reported a lower than expected death rate from respiratory diseases among AN workers (observed = 2; SMR = 0.42). Swaen *et al.* (1992) reported three deaths from respiratory disease in a cohort of 2,842 AN workers with 6.8 expected (SMR = 44; 95% CI = 11 to 129). The 1998 follow-up by Swaen *et al.* reported a slight increase in respiratory mortality over what was expected (observed = 17, expected = 14.04; SMR= 121.2, 95% CI= 70.6 to 194.1). However, this was not confirmed in a more recent follow-up in which 24 deaths for respiratory disease were observed and 25.3 deaths were expected (Swaen *et al.*, 2004). In a recent UK mortality study of 2763 male workers exposed to AN between 1950 and 1978 (Benn and Osborne, 1998), no significantly increased mortality was reported for all respiratory diseases (observed = 31, expected = 41.4; SMR = 74.8) or for bronchitis alone (observed = 13, expected = 12.2; SMR = 107) when compared to national rates. Blair *et al* (1998) reported lower than expected deaths from respiratory disease (observed = 40; SMR = 0.4, 95% CI = 0.3 to 0.6) and emphysema (observed = 6; SMR = 0.4, 95% CI = 0.2 to 1.0).

Effects on the Liver

AN is metabolized in the liver to potentially toxic metabolites (see **Section 3**); however, there is little data to indicate that the liver is a target organ for AN toxicity. In humans, mild jaundice accompanied by liver tenderness and low grade anemia lasting several days to four weeks has been observed after acute occupational exposure to AN vapors at high concentrations; however, the concentrations of AN to which workers were exposed were not reported (Wilson, 1944). The effects were fully reversible. Jaundice lasted for four weeks in one case with the individual still complaining of fatigue and lassitude after a year.

Workers in a synthetic rubber manufacturing plant exposed to AN vapor at levels of between 16 (35 mg/m^3) and 100 ppm (219 mg/m^3) AN for 20 to 45 minutes experienced mild jaundice among other symptoms; these effects subsided with exposure cessation (Wilson *et al.*, 1948).

Sartorelli (1966) reported an enlarged liver and pharyngeal congestion within one day of a worker's exposure to AN vapors from a leakage in a distillation apparatus. Symptoms disappeared after four days.

Sakurai and Kusumoto (1972) studied the health records of 576 workers from five AN fiber plants over a 10 year period. At exposure levels of 11 mg/m^3 (5 ppm) some changes in liver function tests were reported. The effects were positively associated with the length of exposure, but not with the exposure level or age of the workers. However, in a later report by the same author (Sakurai *et al.*, 1978), it was stated that the "exposure levels were not reliably reported."

Ginceva *et al.* (1977) found no changes in the health status of a group of 23 men exposed to 4.2 to 7.2 mg/m^3 of AN for three to five years.

In factory workers exposed to AN for 10 years or more, Sakurai *et al.* (1978) reported an increase in palpable livers of workers. However, the authors considered these results to be inconclusive because the increase was not statistically significant and subjective judgments were involved. Also, blood chemistry evaluations and liver function tests did not indicate liver damage.

In one study of a worker accidentally sprayed with AN indicated that transient injury to liver and muscle may have occurred, but the data are too limited to draw any firm conclusions (Vogel and Kirkendall, 1984).

WHO (1983) and VROM (1984) summarized several cases of AN poisoning whereby workers exposed to low AN concentrations (> 5 ppm or 11 mg/m^3) experienced slight liver enlargement and jaundice.

Effects on the Kidney

Workers in a synthetic rubber manufacturing plant exposed to AN vapor at levels of between 16 (35 mg/m^3) and 100 ppm (219 mg/m^3) AN for 20 to 45 minutes experienced kidney irritation among other symptoms; these effects subsided with exposure cessation (Wilson *et al.*, 1948).

Ginceva *et al.* (1977) found no changes in the health status of a group of 23 men exposed to 4.2 to 7.2 mg/m^3 of AN for three to five years.

Physical examination of workers exposed to AN vapors in the workplace for 10 or more years provided no indication of renal effects (Sakurai *et al.* 1978).

Chen *et al.* (1987) reported no increase in deaths due to genitourinary disease in DuPont wage workers exposed to AN based on comparison with US and company rates (observed = 1, expected = 1.2 (US) and 1 (DuPont)). Collins *et al.* (1989) reported no increased mortality from diseases of the genitourinary system in DuPont workers exposed to AN when compared to US expected rates (observed = 1; SMR = 0.44).

Effects on Gastrointestinal and Endocrine Systems

Ginceva *et al.* (1977) found no changes in the health status of a group of 23 men exposed to 4.2 to 7.2 mg/m^3 of AN for three to five years.

In a mortality study of 884 men occupationally exposed to AN, Kiesselbach *et al.* (1979) reported that mortality from gastrointestinal diseases was not different than expected. Questions about case ascertainment and follow-up make it difficult to interpret this study.

In a study of DuPont workers with exposure to both AN and dimethylformamide, Chen *et al.* (1985b) reported six deaths due to digestive diseases with an expected mortality of 4.8 based on a Dupont employee mortality registry. The difference was not significant. In a follow-up study, Chen *et al.* (1987) observed four deaths due to digestive diseases in a cohort of DuPont AN wage workers. The expected were 6.9 based on US rates and three based on DuPont company rates. Collins *et al.* (1989) reported a significant deficit in deaths due to digestive disease compared to US rates (observed = 4; SMR = 0.3).

Swaen *et al.* (1992) reported one death due to digestive diseases (expected = 5.93; SMR = 17, 95% CI = 0 to 94) among a cohort of 2,842 AN workers. In a follow-up study, Swaen *et al.* (1998) reported no increased mortality due to digestive diseases (observed = 6, expected = 10.96; SMR = 54.7, 95% CI = 20.0 to 119.1). This result was confirmed in a more recent follow-up of the same cohort in which 12 deaths from digestive disease were observed versus 15.8 deaths expected (Swaen *et al*., 2004). Ives *et al.* (1993) reported one death due to diabetes in a BP cohort exposed to AN (SMRs = 0.84 (US) and 0.67 (local counties). In a recent UK mortality study of 2,763 male workers exposed to AN between 1950 and 1978 (Benn and Osborne, 1998), no elevated mortality for endocrine, nutritional or metabolic diseases (observed = 5, expected = 5.8; SMR = 86.4), or for digestive diseases (observed = 8, expected = 13.8; SMR = 58.0) was seen when compared to national rates. Blair *et al.* (1998) also reported no increase in mortality due to diabetes in a cohort of 25,460 AN workers (observed = 9; SMR = 0.3, 95% CI = 0.2 to 0.6).

Effects on the Skin

Based on the human experience, AN is considered to be a skin irritant. A male laboratory worker spilled 'small quantities' of liquid AN on his hands, resulting in diffuse erythema on both hands and wrists after 24 hours, followed by blisters on the fingertips on the third day. His hands were slightly swollen, erythematous, itchy and painful and the finger remained dry and scaly on the 10^{th} day (Dudley and Neal, 1942).

Wilson *et al.* (1948) noted that direct skin contact with AN resulted in irritation and erythema and scab formation, with slow healing. Workers exposed to AN vapors at 16 to 100 ppm for 20 to 45 minutes complained of intolerable itching of the skin, but no dermatitis was observed.

Babanov *et al.* (1959, as reported in WHO, 1983) reported that workers exposed to AN concentrations at 0.6 to 6.0 mg/m^3 (0.3 to 3 ppm) for approximately three years suffered from

vocal cord inflamation and non-specific changes in the pale mucous membranes and skin. Zeller *et al.* (1969) reported on 50 cases of skin damage resulting from occupational contact with AN. A burning sensation developed within five minutes to 24 hours followed by reddening, which often blistered after one day. Babanov (1957) reported blistering at sites of contact six to eight hours after AN spilled on workers' legs. Serious skin burns developed on two workers who contacted a 5% AN solution while cleaning heated (50°C) apparatus.

Ageeva (1970 cited in WHO 1983) reported diffuse dermographia and increased sweating in workers involved in AN production.

Acute exposure to AN as a liquid or vapor causes a wide range of skin effects, including dermatitis in some of the non-fatal cases (Davis *et al.*, 1973). Skin contact with AN has also resulted in local irritation, erythema, swelling, blistering, and burns. These effects, however, generally reflect very high exposure levels following accidental exposure to a large quantity of AN.

Complaints of poor health, including skin irritation, (during the first months of employment only) came from workers employed in the manufacture of AN (Zotova, 1975).

Stamova *et al.* (1976) studied workers' health in a polyacrylic fiber plant where AN exposures were around 10 mg/m^3, but could fluctuate to as high as 25 mg/m^3. An increased incidence of skin diseases (undefined) was reported. Other chemicals were present in the workplace and the length of exposure was not reported.

Ginceva *et al.* (1977) found no changes in the health status of a group of 23 men exposed to 4.2 to 7.2 mg/m^3 of AN for three to five years.

In a study of Japanese workers exposed to AN, workers who may have been exposed to particularly high concentrations (inside polymerization tanks) experienced transient irritation of the scrotal skin. No concentrations of AN were specified (Sakurai *et al.* 1978).

Vogel and Kirkendall (1984) reported a case of a 24-year old man whose face, eyes, and body were sprayed by AN when a valve burst while he was unloading the chemical from a ship. In addition to other symptoms, he showed flushing and generalized erythema, but no skin rash was observed.

A producer of AN reported 10 cases of skin complaints in employees (Bakker *et al.*, 1991). Of these, five had irritant dermatitis while the other five proved to have an allergy to AN on patch testing. Paresthesia was reported in one patient.

Allergic Reactions

There is limited evidence of skin sensitization in humans following skin contact with AN in reports from industry; however, only a handful of such cases exist despite the many thousands of workers who have been exposed to AN. There is no data on respiratory sensitization for humans.

In a case reported by Hashimoto and Kobayashi (1961), skin lesions were first observed at the site of contact with liquid AN, which then spread rapidly to other neighboring regions. Several days after contact the lesions spread to other parts of the body that had not been in contact with the liquid. It was concluded that these later skin lesions were indicative of an allergic type response to the initial exposure to AN liquid.

A 27-year-old developed a rash on the same finger on which a AN-methyl methacrylate copolymer splint was used. Patch testing gave positive reactions to the copolymer and 0.1% AN (Balda, 1975).

Ginceva *et al.* (1977) found no changes in the health status of a group of 23 men exposed to 4.2 to 7.2 mg/m^3 of AN for three to five years.

A positive patch test for AN was determined in five employees of an AN processing and production plant who had contact dermatitis. The eight control individuals did not show any allergic reaction to AN (Bakker *et al.*, 1991).

Effects on the Eye

With regard to human experiences of acute exposure to AN as a liquid or vapor, a wide range of effects have been observed including irritation of the eyes. Based on the limited human experience (mainly as the result of accidental exposures), AN is considered to be a severe eye irritant.

Wilson (1944) reported that synthetic rubber workers exposed to "mild" concentrations of AN developed eye and nasal irritation among other symptoms. Workers in a synthetic rubber manufacturing plant exposed to AN vapor at levels of between 16 (35 mg/m^3) and 100 ppm (219 mg/m^3) AN for 20 to 45 minutes also experienced eye, nose, and throat irritation (Wilson *et al.*, 1948).

Five adults who spent the night in a room that had been fumigated with AN, and in which a child died of AN poisoning, complained only of eye irritation or showed no signs of AN poisoning (Grunske, 1949). The concentrations of AN in the air were not reported. Several

other instances of AN causing death in children, but only mild irritation in adults were reported by Grunske (1949), but not described in detail.

Lacrimation and visual disturbance have been described in some of the non-fatal cases (Davis *et al.*, 1973). These effects, however, generally reflect very high exposure levels following for example accidental release of a large quantity of AN.

WHO (1983) summarized various workplace studies (Zotova, 1975; Delivanova *et al.*, 1978; Enikeeva *et al.*, 1976; Ivanov, 1983) and found that blepharoconjunctivitis was reported at 5 ppm (11 mg/m^3) AN. Other non-ocular symptoms were also reported.

Ginceva *et al.* (1977) found no changes in the health status of a group of 23 men exposed to 4.2 to 7.2 mg/m^3 of AN for three to five years.

Sakurai *et al.* (1978) investigated the health effects of exposure to AN in six acrylic fiber factories. AN concentrations in air were measured in spot samples in these six acrylic factories on two consecutive days. On average, 102 samples were taken in each factory using subjects with at least five years exposure to AN. The median concentration for the highly exposed population of workers was reported to be 5 ppm (11 mg/m^3). Unexposed workers from the same factories were used as controls. Medical examinations were performed on 102 AN workers and 62 controls. Medical histories of these workers showed that many of the workers initially experienced irritation of the conjunctiva following exposure to AN in the years preceding this survey. These acute symptoms of irritation appeared to decrease gradually with time and became infrequent at the time of this study. Although some differences appeared to exist with respect to physical signs, none achieved statistical significance. The gradual lessening of reported symptoms have been attributed to improved measures to reduce exposure.

Vogel and Kirkendall (1984) reported a case of a 24-year old man whose face, eyes and body were sprayed by AN when a valve burst while he was unloading the chemical from a ship. In addition to other symptoms, there was mild conjunctivitis, but no corneal clouding. The fundascopic examination was normal.

Clinical Chemistry

Babanov *et al.* (1959, as reported in WHO, 1983) reported that, in workers exposed to concentrations at 0.6 to 6.0 mg/m^3 (0.3 to 3 ppm) for approximately three years, AN was immunosuppressive.

Ageeva (1970 cited in WHO 1983) reported a significant decrease in an 'epinephrine-like substance' and an increase in acetylcholine in AN-exposed workers.

Ginceva *et al.* (1977) found no changes in laboratory tests in a group of 23 men exposed to 4.2 to 7.2 mg/m^3 of AN for three to five years.

Sakurai *et al.* (1978) investigated the health effects of exposure to AN in six acrylic fiber factories. AN concentrations in air were measured in spot samples in these six acrylic factories on two consecutive days. On average, 102 samples were taken in each factory using subjects with at least five years of exposure to AN. The median concentration for the highly exposed population of workers was reported to be 5 ppm (11 mg/m^3). Unexposed workers from the same factories were used as controls. Medical examinations were performed on 102 AN workers and 62 controls. Clinical chemistry did not reveal any AN-related differences between AN workers and controls. An average urinary concentration of AN and thiocyanate ion of 0.36 mg/l and 11.4 µg/l, respectively, was measured.

Vogel and Kirkendall (1984) reported a case of a 24-year old man whose face, eyes, and body were sprayed by AN when a valve burst while he was unloading the chemical from a ship. In addition to other symptoms, there were increases in serum creatinine phosphokinase, transamines, and myoglobinuria, possibly as a consequence of tissue hypoxia.

Grigoreva (1990) reported a reduction in acid phosphatase, myeloperoxidase, and succinate dehydrogenase activity in peripheral blood leucocytes of workers exposed for more than 10 years to AN (concentrations unstated). Alkaline phosphatase activity was unchanged compared to controls, while the glycogen content was increased.

According to BUA (1995), a decrease of the testosterone level in serum occurred in workers in Romanian factories who were exposed to AN and other unspecified chemicals. The extent of exposure was not mentioned in the publication (Ivanescu *et al.*, 1990).

Hematologic Effects

Humans exposed to AN at concentrations where nausea, vomiting and weakness occurred (16 to 100 ppm for 20 to 45 minutes), were also reported to have low grade anemia and leucocytosis among other symptoms. However, complete recovery after cessation of exposure was reported (Wilson 1944; Wilson *et al.* 1948).

Ginceva *et al.* (1977) found no changes in laboratory tests in a group of 23 men exposed to 4.2 to 7.2 mg/m^3 of AN for three to five years.

No adverse hematological effects were detected in Japanese workers exposed to AN for 10 to 13 years at exposure levels averaging 2.1 to 14.1 ppm (Sakurai *et al.* 1978).

WHO (1983) summarized the workplace studies of Zotova (1975); Delivanova (1978); Enikeeva *et al.* (1976); and Ivanov (1983), which indicated that effects such as reduced hemoglobin levels, erythrocyte, counts and leucocyte counts occurred at 5 ppm (11 mg/m^3) in workers chronically exposed to AN.

Hematological effects of AN have reported changes in the blood count and reduced activity of T-lymphocytes (WHO, 1983). However, exposure to other chemicals can be assumed for at least some of these workers and generally the exposure conditions are inadequately characterized for direct causation to be determined (BUA, 1995).

Effects on the Cardiovascular System

In humans, tachycardia was among the symptoms described in a child (age three) who was exposed by sleeping in a room that had been fumigated with AN. The child died as a result of the exposure (Grunske 1949; WHO, 1983).

Babanov *et al.* (1959, as reported in WHO, 1983) reported that workers exposed to AN concentrations at 0.6 to 6.0 mg/m^3 (0.3 to 3 ppm) for approximately three years suffered pains in the heart region among other symptoms. Blood pressure was also reported to be reduced.

Ageeva (1970 cited in WHO 1983) reported lowered arterial pressure, labile pulse, and change in orthostatic reflex in workers involved in AN production.

Ginceva *et al.* (1977) found no changes in the health status of a group of 23 men exposed to 4.2 to 7.2 mg/m^3 of AN for three to five years.

Buchter and Peter (1984) described a case of a 57 year old locksmith exposed to AN for 14 years along with prussic acid, ammonia, phosphoric acid, propylene, hydrochloric acid and sulfuric acid. His complaints consisted of a disturbance of memory, weakness, headache, dizziness, drowsiness, diminished vision and hearing, and low blood pressure. The diagnosis after examination was cerebrovascular insufficiency due to disturbance of the circulatory function, arteriosclerosis, elevation of the erythrocyte sedimentation rate of unknown origin (no tumor), porphyrinuria, and noise induced hearing loss. In an effort tolerance test, the performance of the patient was only 10 to 20% of normal workers. However, these authors concluded that psychopathological development seemed to be more likely than chronic disease due to AN, as the patient was strongly convinced of his own inability to work, in spite of normal cardiopulmonary work capacity and only slight disturbance of the circulatory function.

In a mortality study of 884 men occupationally exposed to AN, Kiesselbach *et al.* (1979) reported that mortality from cardiovascular diseases was not different than expected. Delzell and Monson (1982) reported deficits in mortality from both circulatory disease (observed = 28, expected = 40.3; SMR = 0.7; 95% CI = 0.5 to 1.0) and cerebrovascular disease (observed = 5, expected= 5.4; SMR = 0.9; 95% CI = 0.3 to 2.2).

In studies of a DuPont worker cohort from various plants using or manufacturing AN, O'Berg (1980) first reported a slight, non-significant excess in mortality from heart disease (observed = 31, expected = 29.3), but a deficit in cerebrovascular disease mortality (observed = 3, expected = 5.1) based on comparison with a DuPont company mortality registry. A 1985 follow-up using US comparison rates (O'Berg *et al.*,1985) again reported a slight, non-significant excess in mortality from ischemic heart disease (observed = 54, expected = 52.9; SMR = 102.1), but a deficit in cerebrovascular disease mortality (observed = 4, expected = 6.6; SMR = 61). Chen *et al.* (1985b) studying DuPont workers with exposure to both AN and dimethylformamide reported a significant excess in mortality from ischemic heart disease (observed = 72, expected = 54.9), but a deficit in cerebrovascular disease mortality (observed = 4, expected = 6.6) based on comparison with a DuPont company mortality registry. However, in a subsequent study of AN workers, Chen *et al.* (1987) reported a deficit in circulatory disease mortality compared to either US or company rates [observed = 26, expected = 54.7 (US) and 44.6 (DuPont)]. Finally, Collins *et al.* (1989) reported a significant decrease in ischemic heart disease mortality among the most highly exposed workers (observed = 48; SMR = 0.71) and no excess cerebrovascular disease mortality among the same sub-group (observed = 4; SMR = 0.41).

In a 1992 Dutch study, Swaen *et al.* reported 59 deaths due to circulatory disease in a cohort of 2,842 AN workers versus an expected rate of 64.19 (SMR = 92; 95% CI = 70 to 119). The 1998 follow-up to this study reported 108 deaths due to circulatory disease in the worker cohort versus an expected rate of 119.03 (SMR = 90.7; 95% CI = 74.4 to 109.5) (Swaen *et al.*, 1998). This result was confirmed in a more recent follow-up of the same cohort in which 160 deaths from circulatory disease were observed versus 170.2 deaths expected (Swqaen *et al.*, 2004). Ives *et al.* (1993) reported a deficit in total cardiac mortality for BP workers exposed to AN [observed = 16, SMRs = 0.47 (US) and 0.5 (area counties)] and death due to ischemic heart disease [observed = 13, SMRs = 0.5 (US) and 0.5 (area counties)] and cerebrovascular disease [observed = 3, SMRs = 0.84 (US) and 0.89 (area counties)]. Mortality from hypertension was elevated (not significantly) based on one case [SMRs = 1.78 (US) and 2.93 (local counties)].

In a recent UK mortality study of 2,763 male workers exposed to AN between 1950 and 1978 (Benn and Osborne, 1998), no increased mortality was reported for all circulatory diseases (observed = 200, expected = 232.2; SM = 86.1) or for ischemic heart disease (observed = 151, expected = 167.9; SMR = 90.1) and cerebrovascular disease (observed = 27, expected

= 33.9; SMR = 79.1) when compared to UK national rates. The US study of 25,460 US AN workers conducted by Blair *et al.* (1998) reported deficits in mortality from both ischemic heart disease (observed = 374; SMR = 0.8; 95% CI = 0.7 to 0.9) and cerebrovascular disease (observed = 37; SMR = 0.5; 95% CI = 0.4 to 0.7). The meta-analysis of six AN mortality studies conducted by Collins and Acquavella (1989) also reported a deficit in mortality from ischemic heart disease (observed = 579, expected = 707.5; mRR = 0.8; 95% CI = 0.8 to 0.9).

Miscellaneous

Recently, a number of Chinese studies have examined the potential hazards of AN in exposed workers (Chen *et al.*, 2000; Xiao, 2000; Wu and Jin, 2000; Li *et al.*, 1999; Dong *et al.*, 2000a,b; Wang *et al.*, 2000). These studies are largely unpublished or published only in Chinese journals and untranslated, and the details of methodology, follow-up, and findings are limited. A variety of effects have been reported in these studies, including reproductive, neurological, genotoxicity, hematological, and systemic toxicity. However, due to limitations in these studies and a lack of sufficient detail presented, causality cannot be adequately assessed. The results of these studies appear contrary to the experiences in other countries with workers exposed to AN. A comprehensive review of these studies is beyond the scope of this document, and therefore they have not been included in the text below. Rather, the questions raised by these studies are subject to a separate, ongoing investigation being conducted by the international AN industry in cooperation with the National Institute of Child Health and Human Development.

4.1.2 Cancer Effects

A large number of epidemiology studies have been conducted regarding the potential carcinogenicity of AN in exposed worker populations. Some early epidemiology studies had limitations such as small size, poor exposure characterization, and short observation periods that make it difficult to draw conclusions about cancer risk. Potential confounding factors such as cigarette smoking or other occupational exposures were also not considered in most of these studies. The early studies also did not always or consistently report data or some potentially important cancers such as brain cancer (Collins and Acquavella, 1998). Additionally, only the largest of the early studies had sufficient statistical power to detect even a large elevation in the risk of rare cancers. Most seriously, exposure estimates were not attempted for most of the early studies, and in some studies it is likely that some of the subjects classified as exposed were, in fact, not exposed.

Notwithstanding the above-mentioned problems, the sheer number of worker studies, the availability of larger more recent studies including meta-analyses, and the internal consistency of the results overall, overcome the deficiencies of the individual studies (particularly the older ones). Two meta-analyses exist (Rothman, 1994; Collins and

Acquavella, 1998) that reach similar conclusions. These meta-analyses evaluated consistency, examined some potential confounding exposures, and produced risk estimates. Based on the weight-of-evidence, it can be concluded that there is little evidence that AN workers have increased cancer rates even though exposures in some groups of workers in the past were at levels that have caused tumors in rats (Ward and Starr, 1993; Collins and Strother, 1999). The extensive epidemiological information available does not support a causal relationship between AN and cancer in humans under conditions of occupational exposures.

These studies are discussed below according to date published, study population, and follow-up, and are summarized in **Table 4-1**.

4.1.2.1 Occupational Cohort Studies

Dupont Cohort

O'Berg (1980) followed 1,345 male workers employed in a DuPont synthetic textile (*i.e.*, Orlon) factory from 1950 to 1966. The study had a minimum follow-up period of ten years (1976). Morbidity and mortality rates were compared to those calculated for all DuPont employees. Exposure estimates to AN were divided into three qualitative groups (*i.e.*, low, medium, and high exposure). Although quantitative estimates of exposure levels were not assigned, exposures to AN were estimated to range from 5 to 20 ppm, based upon work records, interviews with work supervisors, and a survey of salaried workers. The analysis compared the incidence and death rate from total cancer and respiratory cancer of the AN workers with corresponding incidence and mortality data for all DuPont employees. Although the analysis examined duration of exposure to AN and job category, little detail on this relationship was provided in the analysis.

A total of 89 deaths were observed, compared with an expected number of 77.4 based on DuPont mortality rates. Twenty cancer deaths occurred compared to an expected number of 17.4 based on DuPont mortality rates. A total of 25 cancer cases were observed, including eight respiratory cancers and three prostate cancers. These values were greater than the expected number of cases (*i.e.*, 20.5, 4.4, and 0.9, respectively), based on DuPont incidence rates. It is unclear if smoking was considered as a confounder. Smoking information was available for all lung cancer cases, but one, and all of these were smokers (EU, 2001). All of the cancer cases, except for one non-respiratory cancer, occurred among 1,128 workers with six or more months exposure (standardized incidence ratio (SIR) = 126, standardized mortality ratio (SMR) = 113). A trend of increased cancer incidence was seen with increased duration of exposure and increased length of follow-up time, and the authors concluded that there may be an association between human lung cancer and AN inhalation exposure. Rothman (1994) points out that, although only 1% of the cohort was missed in the

Table 4-1. Summary of Risk Measures for Selected Cancer Types from Epidemiological Studies

Cohort Discription	Reference	Number	Study Period	Exposure Period	Quant. Exposure Estimates	Response Measure	Total Cancer	Lung	Brain/NS	Prostate	Other (Type)
Dupont Workers	O'Berg (1980)	1345	1956-1976	1950-1966	No	Mortality (O/E) Incidence (O/E)	20/17.4-24.5 (M) 25/20.5 (I)	8/6.1 (M) 8/4.4 (I) **8/3.4 (I -wage)**	NR NR	NR 3/0.9	NR 3/2.2 (colon)
	O'Berg *et al.* (1985)	1345	1957-1981(M) 1957-1983 (I)	1950-1966	No	Mortality (O/E) Incidence (O/E)	36/31.6 (M) 43/36.7 (I)	14/11.6 (M) 10/7.2 (I)	NR	1/1.0 (M) **6/1.5 - 1.8 (I)**	4/3.7 (lymph.M) 7/3.7 (lymph.I) 3/0.6 (bladder M) 4/1.7 (bladder I) 8/8.2 (digestive M) 5/7.4 (digestive I)
	Burke (1985a, unpublished)	472	1962-1983	NR	No	Mortality (O/E) Incidence (O/E)	4/2.8 (M) 13/9.1 (I) 10/7.9 (I-wage)	1/NR (M) 2/1.3(I)	NR	NR (M) 1/NR (I)	--
	Burke (1985b, unpublished)	700	1957-1983	NR	No	Mortality (O/E) Incidence (O/E)	5/4.7-6.2 (M) 11/10.1 -12.0 (I)	1/NR (M) 0/NR (I)	NR	NR	--
	Chen *et al* . (1987)	1083	1956-1981 (M) 1956-1983 (I)	1944-1970	No	Mortality (O/E) Incidence (O/E)	21/30-36.4 (M) 37/36.5 (I)	7/7.9-8.8 (M) 5/6.9 (I)	NR (M) 1/1.2 (I)	1/0.9 (M) 5/1.9 (I) **4/0.9 (I- wage)**	4/2.2-2.7 (lymph. M) 2/0.7 (lymph. I) 5/3.4 (colon I)
	Chen *et al* . (1988a)	1335	1950-1984	1950-1970	No	Incidence (O/E)	41/39.8	10/9.4	NR	6/2.7 **6/2.3 (wage)**	3/2.1 (baldder) 7/4.4 (lymph.)
	Chen *et al.* (1988b)	1335	1950-1982	1950-1970	No	Mortality (O/E)	37/36.3	14/13.2	2/1.6	NR	3/0.7 (bladder) 4/4.3 (lymph.) 8/9.1 (digestive)
	Wood *et al.* (1998)	2559	1944-1991	1944-1991	Yes	Mortality (SMR) Incidence (SIR)	78 (64-93) (M) 97 (79-118) (I)	74 (55-99) (M) 86 (54-130) (I)	113 (41-247) (M) 111 (30-285) (I)	129 (64-230) (M) 158 (82-276) (I)	115 (31-295) (bladder - M) 69 (19-177) (bladder - I) 57 (26-109) (lymph. - M) 0 (--) (lymph. - I) 69 (45 -100) (digestive - M) 89 (56 -134) (digestive - I)
Dutch Workers	Swaen *et al.* (1992)	2842	1956-1988	1956-1979	Yes	Mortality (O/E)	42/50.82	16/19.5	3/1.71	2/1.22	6/7.19 (digestive) 0/1.25 (bladder) 0/2.48 (lymph.)
	Swaen *et al* . (1998)	2842	1956-1995	1959-1979	Yes	Mortality (O/E)	97/110.78	47/42.82	6/3.45	4/4.8	3/3.07 (bladder) 11/15.1 (digestive)
	Swaen *et al* . (2004)	2842	1956-2001	1959-1979	Yes	Mortality (O/E)	146/164.5	67/62.5	6/4.8	8/8.7	5/4.6 (bladder)
UK Workers	Werner & Carter (1981)	1111	1950-1978	1950-1968	No	Mortality (O/E)	21/18.6	9/7.6 (total)□**3/0**	NR	NR	**5/1.9 (stomach)**
	Benn & Osbourne (1998)	2763	1950-1991	1950-1978	Yes	Mortality (O/E)	121/137.12	53/51.54	NR	NR	11/11.44 (stomach) 5/10.02 (lymph.) 11/8.75 (colon)
NCI/ NIOSH	Zack (1980, unpublished)	352	1952-1977	1952-1968	No	Mortality (O/E)	3/2.8	1/0.8	NR	1/0.03	--
	Gaffey & Strauss (1981, unpublished	325	1952-1977	1952-1953	No	Mortality (O/E)	4/11	2/3.7	NR	NR	2/0.3 (kidney)
	Collins *et al.* (1989)	1774	1951-1983	1951-1973	Yes	Mortality (O/E)	43/42.6	15/15.8	1/1.8	2/1.34	5/4.8 (lymph.) 8/10.25 (digestive)
	Blair *et al.* (1998)	15080 men 5190 women	1952-1989	1952-1983	Yes	Mortality (SMR)	0.8 (0.7-0.9)	0.9 (0.8-1.1)□**2.1**	0.7 (0.4-1.3)	0.9 (0.6-1.5)	0.8 (0.4-1.4) (stomach) 0.8 (0.4-1.8) (bladder)
	Marsh *et al* . (2001)	15080 men 5190 women	1952-1989	1952-1983	Yes	Mortality (SMR)	NR	0.74-0.9 (0.6-1.1)	0.69-0.74 (0.4-1.3)	0.92-0.95 (0.5-1.5)	0.79-0.85 (0.4-1.5) (stomach)
Individual Cohorts	Monson (1978, unpublished)	NR	NR	NR	No	Mortality (O/E)	NS increase	NR	NR	NR	--
	Kiesselbach *et al* .□ (1979)	884	1950-1977	1950-1965	No	Mortality (O/E)	20/20.4	6/6.9	NR	NR	4/3 (stomach)
	Theiss *et al.* (1980)	1469	1956-1978	1956-1978	No	Mortality (O/E)	27/20.5	11/5.6	NR	NR	4/1.7 (lymph.)
	Ott *et al.* (1980)	100	1940-1975	1940-1975	Yes	Mortality (OR)	NR	1/0.5	NR	NR	3/1.25 (leukemia)
	Herman (1981, unpublished)	1077	1951-1977	1951-1977	No	Mortality (O/E)	11/16.1 (total) 4/11.2 (wage)	1/3.7 (wage)	NR	NR	--
	Delzell and Monson (1982)	327	1940-1978	1940-1971	No	Mortality (O/E)	22/17.9	9/4.7 - 5.9	NR	NR	4/5.0 (digestive) 2/0.5 (bladder) 4/1.8 (lymph.)
	Stallard (1982, unpublished)	419	1960-1980	1960-1980	No	Mortality (O/E)	4/5.0	2/1.6	NR	NR	--
	Marsh (1983)	2490	1949-1976	1949-1966	No	Mortality (SMR)	NR	NR	NR	**153.6 (cohort)**	101.8 (digestive- cohort)

Table 4-1. Summary of Risk Measures for Selected Cancer Types from Epidemiological Studies

Cohort Discription	Reference	Number	Study Period	Exposure Period	Quant. Exposure Estimates	Response Measure	Total Cancer	Lung	Brain/NS	Prostate	Other (Type)
	Zhou and Wang (1991)	1811	1971-1988	1971-1988	Yes	Mortality (SMR)	1.25	NR	NR	NR	--
	Ives *et al.* 1993	894	1960-1988	1960-1985	Yes	Mortality (SMR)	0.91	1	NR	NR	1.15 (digestive)
	Mastrangelo *et al*. (1993)	671	1959-1990	1959-1988	No	Mortality (SMR)	NR	0.8 (0.1-2.9)	2.6 (.1-14.7)	NR	3.4 (0.4-12.3) (stomach)
Support Cohort & Case Control	Waxweiler *et al.* (1981)	4806	1942-1973	1942-1973	No	Mortality	NR	NR	NR	NR	--
	Siemiatycki *et al*. (1994)	484	1979-1986								
	Thomas *et al*. (1987)	300	1978-1981	NR	No	Mortality (OR)	300	--	0.9 (0.5-1.6)	--	--
	Ott *et al.* (1989)	129	1940-1978		No	Mortality (OR)	129	NR	NR	NR	**3.2 (non-Hodgkins OR)**
Meta-Analyses	Rothman (1994)	10835	1940-1988	1940-1979	No	Mortality (O/E)	224/218.2	85/79.4	NR	NR	--
	Collins & Acquavella (1998)	51844	1940-1996	1940-1991	No	Mortality (O/E) Incidence (O/E)	0.9 (0.8-0.9) (M) 1.0 (0.8-1.2) (I)	0.9 (0.8-1.1) (M) 0.8 (0.5-1.2) (I)	1.1 (0.8-1.5) (M) 1.1 (0.4-3.1) (I)	1.0 (0.7-1.5) (M) 1.4 (0.8-2.6) (I)	0.9 (0.6-1.2) (stomach - M) 0.3 (0.0-2.1) (stomach - I) 1.4 (0.9-2.0) (bladder - M) 0.8 (0.3-2.2) (bladder - I)
	EU, 2001	50476	1940-1996	1940-1991	No	Mortality (O/E) Incidence (O/E)	0.9 (0.8-0.9) (M) 1.0 (0.8-1.2) (I)	0.9 (0.8-1.1) (M) 0.8 (0.5-1.2) (I)	1.1 (0.8-1.5) (M) 1.1 (0.4-3.1) (I)	1.0 (0.7-1.5) (M) 1.4 (0.8-2.6) (I)	0.9 (0.6-1.2) (stomach - M) 0.3 (0.0-2.1) (stomach - I) 1.1 (0.7-1.7) (bladder - M) 0.8 (0.3-2.2) (bladder - I)

Bolded values indicate statistically significant results

ascertainment, this included two cases of lung cancer that were also omitted from the analysis. This raises questions about the validity of the ascertainment process and the analytical results. According to Doll (AN Epidemiology Conference, 1980), the 1980 O'Berg study raised some suspicion about the carcinogenicity of AN in humans, but did not amount to decisive evidence.

In a follow-up (1950 to 1981 for mortality and 1956 to 1983 for incidence) to the O'Berg (1980) study, O'Berg *et al.* (1985) studied cancer morbidity and mortality in 1,345 Orlon workers, who started between 1950 and 1966 at a DuPont textile fiber plant in South Carolina. The information in this study includes that of O'Berg (1980) and thus supercedes it. Total mortality was 155 observed deaths compared to 134.5 expected deaths based on company mortality rates. The same approximate excess (15%) extended to cancer mortality, where a total of 36 cancer deaths were reported (31.6 expected). Of these, 14 were from lung cancer (11.6 expected) for wage and salaried workers combined (12 lung cancers versus 10.2 expected among wage workers alone). Smoking habits were not considered. None of the cancer mortality rates were significantly increased compared to U.S. rates.

The cancer incidence findings were not very different from the cancer mortality findings. There were a total of 43 incident cases of cancer observed versus 36.7 expected on the basis of company-wide cancer incidence rates. For lung cancer, there were 10 cases observed versus 7.2 expected. This increase was less pronounced than reported previously and was not statistically significant (based on DuPont Company rate). There were fewer incident lung cancer cases than deaths because of the differing time periods for follow-up. For prostate cancer, six cases were observed compared to 1.8 expected for both wage and salaried workers. All six cases of prostate cancer occurred among wage workers, for whom the expected number was 1.5. Prostate cancer incidence was significantly increased, but no significant relationship between incidence and cumulative exposure was found. There were seven cases of lymphopoietic cancer versus 3.7 expected, and for the wage workers the respective numbers were six observed versus 2.9 expected.

The only analysis linking the amount of cumulative exposure or induction time to any effect was for lung cancer. This analysis showed seven cases of lung cancer versus 3.5 expected among workers with more than 20 years since first exposure (the category of longest duration), and six cases versus 2.8 expected for workers with the highest category of cumulative exposure. The highest ratio of observed to expected lung cancer was for those in the combined category of 20 or more years since hire and greatest cumulative exposure, in which there were four cases versus 1.8 expected. The primary difference between this study and the earlier O'Berg study (1980) is the finding on prostate cancer. Rothman (1994) concluded that the lung cancer excess, while modest overall, appeared to be related to the amount of exposure. He also points out that the relatively high SMR for all causes of death

combined may reflect incomplete death ascertainment from using the company registry which may influence the expected numbers.

Burke studied mortality in DuPont workers in Texas (1985a) and Tennessee (1985b). At the Texas facility (1985a), the cohort consisted of 472 male workers (including 423 wage workers). Vital status and incidence were determined in the same way as all DuPont studies (Social Security records and company registries). Among wage workers, there were 14 deaths with 13.7 and 22.5 expected based on company and US rates, respectively. Four deaths were due to cancer compared to 2.8 expected based on company rates. One death was from lung cancer and one death was due to prostate cancer, but no expected rates were provided. Thirteen incident cases of cancer were reported (9.1 expected from company rates); ten were among wage workers (7.9 expected). Two of these ten were lung cancer (1.3 expected) and one case of prostate cancer was reported (no expected number provided).

In the Tennessee plant (1985b), the cohort consisted of 499 wage and 201 salaried workers (N = 700 total). The methodology was identical to that used in the Texas study. There were 17 deaths observed with 21.9 and 34.2 expected based on company and US rates, respectively. There were five cancer deaths among wage workers (4.7 and 6.2 expected based on company and US rates, respectively) with one death from lung cancer (no expected number provided). There were 11 incident cases of cancer among wage workers (10.1 and 12.0 expected based on company and US rates, respectively). Both studies were among the smallest conducted and provide little information on exposure and methodology, limiting the amount of information that can be derived from them.

Cancer mortality and morbidity were evaluated in a cohort of 1,083 male synthetic textile (*i.e.*, Orlon) workers who started work between 1944 and 1970 at a different DuPont textile fiber plant than that studied by O'Berg *et al.* (Chen *et al.*, 1987). The observation period for latency covered the period from 1944 until 1981 for mortality based on US and DuPont rates and from 1944 until 1983 for morbidity based on DuPont rates. The follow-up period for the estimation of expected deaths covered only the period from 1957 until 1981. The mean observation time was 21.3 years.

There were 92 deaths observed in the cohort during the follow-up period, substantially fewer than the 124.0 expected on the basis of DuPont mortality rates and the 177.2 expected on the basis of rates for all white males in the United States. The deficit in deaths was more striking for salaried employees than for wage employees. No significant excesses in cancer mortality were found for specific cancer types, based upon either US and DuPont rates. Among the wage workers, there were 18 observed cancer deaths versus 20.4 expected from the DuPont rates and 24.1 expected from the United States rates. There were seven deaths from lung cancer among the wage workers, compared with 7.9 expected on the basis of the DuPont

rates. With respect to mortality, the total number of cancer deaths (wage and salary workers combined) was lower than expected (21 observed vs 30-36.4 expected).

There were 37 cases of cancer identified during the period 1956-1983 (36.5 expected). There was no excess seen for lung cancer (five cases observed versus 6.9 expected, p-value = 0.82). A significant increase in prostate cancer cases was reported (five observed vs. 1.9 expected, p-value = 0.04 - based on company rates) and concentrated in the most recent years of follow-up. Mortality from prostate cancer was not increased (one observed, 0.9 expected).

Both O'Berg *et al.* (1985) and Chen *et al.* (1987) reported an increased incidence of prostate cancer. O'Berg *et al.* found no relationship between cumulative exposure and the incidence of prostate cancer. Chen *et al.* stated that three of the prostate cancer cases had a latency period of more than 20 years. Both O'Berg and Chen used the internal DuPont Cancer Register for estimating the expected prostate cancer morbidity, which does not include retired workers or those who left the employ of DuPont. The DuPont studies also did not consider smoking habits.

The cancer incidence of DuPont workers exposed to AN or dimethylformamide (DMF) was investigated (Chen *et al.*, 1988a). A total of 2,530 workers were exposed to DMF only between 1950 and 1970, 16 workers to AN only between 1950 and 1966, 1,329 to both chemicals, and 1,130 to neither chemical (controls). Forty-one cancer cases were identified in the group of workers exposed to the chemicals alone or in combination, compared to an expected number of 39.8. The incidence of prostate cancer was significantly elevated in workers exposed to both chemicals. However, no dose-response relationship was observed for either chemical. With respect to cancer mortality in workers exposed to both chemicals, a total of 37 cancer deaths were observed compared to an expected number of 36.3 (Chen *et al.*, 1988b). No significant excesses were reported for any specific cancer type. Results from these two studies were not included in the meta-analysis of Collins and Acquavella (1998) because the earlier paper (Chen *et al.*, 1987) provided data on all DuPont AN workers.

A further DuPont study combined and updated the O'Berg (O'Berg, 1980; O'Berg *et al.*, 1985) and Chen *et al.* (1987) studies to study the 2,559 Orlon male workers from both plants over the time period from 1944 to 1991 (Wood *et al.*, 1998). This study assessed the risk of cancer mortality and incidence in the cohort with a vital status follow-up 99% complete through 1991.

Since the production processes at these two facilities were identical, a single exposure assessment procedure was developed with the objective of standardizing exposure classifications across work areas and job titles in the two plants. The following data were used for the exposure assessment: 1) a general history of each plant; 2) process descriptions of where AN was used and engineering and operating changes in the process that would

impact potential sources of exposure; 3) a matrix of work area names and job titles held in the relevant production areas during the years when AN was used; 4) documentation of personal protective equipment used; 5) air sampling data, both area and personal; 6) plant production records; and 7) details of work conditions and practices as described by long-term employees (including retirees).

After relevant job titles at each plant were standardized, a panel of long-term employees reviewed all job title/work area designations for their respective plants. The appropriateness of the assumptions made by the industrial hygienists were assessed and workplace conditions were further elaborated, such as whether or not the odor from AN was detectable during normal operations (approximately 20 ppm) or workers experienced symptoms of exposure such as headaches and nausea (greater than 20 ppm).

Area and personal air monitoring data were routinely collected beginning in 1975. These data, with consideration of requirements for use of personal protective equipment, were the principal factors used to estimate the ppm levels of job assignments at the two plants after 1975. Changes in processes, engineering, and ventilation were confirmed to be reflected in the monitoring data. In order to estimate the exposure levels prior to 1975, information related to the above named changes as well as the panel of knowledgeable employees (and prior employees) who could detail working conditions, were the primary sources of information. An estimate of exposure was made in ppm AN for a 40 hour work week for each potentially exposed job title/work area combination by time period.

The exposure estimates were ranked into four groups (*e.g.*, low, moderate, high, and very high) based upon the distribution of all jobs at both plants. The arithmetic mean mid-points for the four groups were 0.11 (0.0001 to 0.2) ppm, 1.10 (0.2 to 2.0) ppm, 11.0 (2.0 to 20.0) ppm and 30.0 (20.0 to <100.0) ppm, respectively. The average duration of exposure for workers in the cohort was 7.6 years. The mean cumulative exposure in ppm-years was 61.4 at Plant 1 and 52.1 at Plant 2, while the mean cumulative exposure was 57.6 ppm-years for the total cohort. More than 50% of Plant 1 cohort were exposed prior to 1956, while 23.2% of Plant 2 cohort were exposed prior to 1956 (*i.e.*, before similar processing to Plant 1 commenced at Plant 2 in 1957).

SMRs were used to assess cancer mortality using the US population and the registry for all DuPont employees as a basis for comparison. SIRs have been used to evaluate cancer incidence using the DuPont employee registry. Overall, vital status was unknown for only 1.2% (17) of the Plant 1 cohort and 0.5% (6) for Plant 2 cohort. Approximately 18% of the combined cohort was deceased at the end of follow-up, giving a total of 454 deaths. Death certificates were obtained for all but two of the 454 identified decedents. The cohort for both mortality and morbidity analyses is restricted to males who were potentially exposed to AN for at least six months (females are not included in the analysis because only 25 exposed

female employees were identified). Indicators of exposure used in the analysis of mortality and morbidity of specific cancer sites of interest are as follows: 1) latency (less than 20 years and 20 years or more of observation); 2) duration of exposure; 3) highest level of exposure ever experienced; and 4) cumulative exposure (ppm-years).

Overall mortality proved to be lower than expected compared to both the US population and the DuPont employee population (observed = 454, SMRs = 69 (95% CI = 62 to 75) and 91 (95% CI = 84 to 99) for the US and DuPont, respectively). All cancer death rates with the exception of prostate cancer were lower than the US and DuPont population referents (observed = 126, SMRs = 78 (95% CI = 65 to 93) and 86 (95% CI = 71 to 102) for the US and DuPont, respectively). The SMRs for prostate were slightly elevated, but were not significantly different from expected (observed = 11, SMRs = 129 (95% CI = 64 to 230) and 106 (95% CI = 53 to 189) for the US and DuPont, respectively). Similarly, the SMRs for brain and CNS cancer were slightly elevated, but not significantly so (observed = 6, SMR = 113 (95%CI=41-247) compared to U.S. referents). Other specific cancers were also not significantly elevated from referent populations and were in some cases significantly lower than expected (*i.e.*, respiratory cancer - observed = 47, SMRs = 74 (95% CI = 55 to 99) and 89 (95% CI = 65 to 181) for the US and DuPont, respectively). Other results showing the same trend include digestive cancer (observed = 27, SMRs = 69 [95% CI = 45 to 100] and 72 [95% CI = 48 to 105] for the US and DuPont, respectively), urinary cancer (observed = 7, SMRs = 91 [95% CI = 36 to 187] and 90 [95% CI = 36 to 185] for the US and DuPont, respectively), and lymphatic or hematopoietic cancers (observed = 9, SMRs = 57 [95% CI = 26 to 109] and 55 [95% CI = 25 to 105] for the US and DuPont, respectively). Analyses of all cancers, and prostate, respiratory, and digestive cancer mortality by indices of exposure also did not show any significantly associated increases nor a consistent pattern suggestive of a dose-response relationship. Based on DuPont rates, the SMR for prostate cancer mortality showed an inverse association with latency, duration of exposure, highest exposure, and cumulative exposure. The patterns in cancer incidence were also studied and were dissimilar to that seen in the mortality study in some cases. While total cancer incidence did not show a dose-response relationship with latency, duration, exposure, or cumulative exposure, prostate cancer did show a trend with latency and exposure (although not with duration or cumulative exposure). Lung cancer incidence and deaths were generally less than expected by exposure duration and across cumulative exposure categories. Despite initial findings of the earlier studies that were suggestive of a relationship between exposure to AN and certain cancer types (*i.e.*, lung, prostate), the most recent follow-up study of these workers suggest a slight deficit for most cancer types, and does not support the existence of such a cancer risk due to AN exposure.

W.F. ten Berge (1998) pointed out that the Wood *et al.* study had exposures that were on average 10 times higher than in the Blair *et al.* (1998) study. Based on his analysis of the Wood *et al.* study, he concluded that the occurrence of 126 cancer deaths versus 161.5

expected deaths makes it highly improbable that AN is a carcinogen in man. The probability for RR >1 was calculated to be 2.2×10^{-3}. For lung cancer, 47 deaths were observed versus 63.5 expected. The probability for the lung cancer RR>1 was calculated to be 1.87×10^{-2}. The observed-expected ratios were always smaller than one for all cancer mortalities except for prostate cancer. This increase (observed 11, expected 8.53) was not significant. A trend of an increased prostate cancer mortality or morbidity with increased cumulative exposure also could not be demonstrated. The relationship for much higher cumulative exposures (between 0.1 and more than 100 ppm years) was studied both for cancer morbidity and cancer mortality (*i.e*., prostate, lung, intestines) using both the general USA vital statistics (for cancer mortality) and the internal DuPont company rates (for both cancer mortality and morbidity). A consistent significant trend with cumulative exposure could not be detected for cancer morbidity or mortality (*e.g*., prostate, lung, intestines) despite the much higher exposures present compared to the Blair *et al.* (1998) study.

Dutch Cohort Studies

Swaen *et al.* (1992) studied a cohort of 2,842 workers from eight chemical plants situated within the Netherlands. The control group consisted of 3,961 unexposed workers from a nitrogen fixation plant. The chemical workers were exposed to AN for at least six months between January 1, 1956 and July 1, 1979, and followed until 1988. The exposure assessment was a hybrid methodology consisting of measurements from some companies and indirect assessments based on interviews with key personnel.

Jobs were classified according to exposure level ranges. Use of respiratory protection was documented for various jobs, but it was not taken into account when the exposure scores were assigned. Therefore, actual exposure was probably lower than assessed exposure in many cases. This is particularly so for workers with high levels of peak exposure, for which respiratory protection was mandatory. Both average and peak exposure levels were taken into account when the jobs were classified. Cumulative doses were calculated for each worker according to the job exposure assessments and the time spent in specific jobs. The mean observation time was about 17 years, and the observed cancer mortality in the exposed cohort was similar to the expected mortality. Specific analyses were carried out to investigate dose-response relationships and latency for total mortality and lung cancer mortality.

Overall, in the exposed cohort, there were 134 deaths observed, with 172.2 expected according to national mortality rates (SMR = 0.78). Approximately the same ratio of observed to expected deaths was found for the unexposed cohort as for the exposed cohort. There were 42 cancer deaths in the exposed cohort, with an expected number of 50.8 (SMR = 0.83). No significant increases in mortality were reported for any specific cancer type. For lung cancer, there were 16 deaths in the exposed cohort compared with 19.5 expected (SMR

= 0.82). Workers were stratified according to exposure level (<1, 1-10, >10 ppm-years) and latency (<10, 10-20, >20 years). When lung cancer risk was examined according to three categories of dose, there was a marginal increase (eight deaths versus 7.2 expected) in the highest category and deficits for the lower dose categories. The authors attributed the rising SMR by dose category to a waning of the healthy worker effect for longer-term employees. This interpretation was supported by the trend in the ratio of observed deaths for all causes combined to expected deaths by dose category; for low dose (<1 ppm-years), the ratio was 0.67; for moderate dose (1-10 ppm-years) it was 0.78; and for high dose (≥10 ppm-years) it was 0.83. There were two prostate cancer deaths in the exposed cohort, compared with 1.22 expected. These two deaths occurred in the group with the highest exposure and the longest 'latency.' No significant trends with either exposure level or latency were observed. Overall, no indications were found of a carcinogenic effect in this cohort of workers exposed to AN. The findings from this study support a lack of an effect of AN exposure on lung cancer risk. However, the excess for prostate cancer, although not significant, is reminiscent of the findings of several other reports (Berg *et al.*, 1980; 1985; Chen *et al.*, 1987; 1988a; Collins *et al.*, 1989).

In a follow-up to Swaen *et al.* (1992), the same group of 6,803 workers (2,842 exposed and 3,961 unexposed controls in eight chemical companies) were followed through January 1, 1996 to assess mortality (Swaen *et al.*, 1998). Workers were exposed to AN for at least six months prior to July 1, 1979. The 'control' group consisted of workers at a nitrogen fixation plant employed over the same time interval as the study cohort. All 6,803 workers were followed-up for mortality with causes of death obtained from the existing Dutch Central Bureau of Statistics. The follow-up was 99.6% complete and 99.3% of the deaths by cause were ascertained.

AN exposure at the plants began in 1959 to 1973. A job-exposure matrix was constructed for this study. The exposure assessment was carried out by an industrial hygienist who contacted the company industrial hygienist to ensure a uniform approach for all companies. An inventory of the available measurements in each plant formed the basis for the exposure assessment, together with temporal information on changes in the production process, task rotation, industrial hygiene, and total production. Information on the work environment and control measures was obtained through interviews with plant personnel.

The exposure matrix was created using detailed job history from information on the job held in a specific period and in a specific workplace. Within each department, job classes were constructed that included all the job titles believed to have had a similar exposure profile based on the exposure assessments. The eight-hour Time Weighted Average (TWA-8) exposure assessment results of all workers in an exposure class were grouped to determine the average exposure level of that job in that workplace for each calendar year. Based on this evaluation, it was decided in which exposure range each job class was placed for that specific

year. During the exposure assessment, it was found that exposure to AN in one plant only occurred 5% of the time worked. Therefore, the workers from this plant were excluded from further analyses.

Ranges used were 0 to 0.5 ppm, 0.5 to 1 ppm, 1 to 2 ppm and 2 to 5 ppm (there were no exposures thought to be greater than 5 ppm TWA-8). Respirator use and the potential for skin exposure, which were not taken into account, may have resulted in a different exposure than assessed. Various other exposure characteristics (*e.g.*, peak exposure, exposure to other carcinogens) were also studied. Peak exposures were defined as intervals with elevated exposure in the ranges of <10, 10 to 20, and >20 to 30 ppm occurring on a regular basis, at least once a week. An assessment of the occurrence of peak exposures could be made for all but one of the participating companies. In addition, an inventory was made of exposure to other agents considered to be potential human carcinogens.

Of the 6,803 study subjects, 6,774 could be completely followed, either until the end of the follow-up period, until the date of emigration, or until the date of death. This resulted in a completeness of follow-up of 99.6%. For nine (0.7%) deceased study subjects, it was not possible to trace the actual cause of death, either because the person had died abroad or because it was not possible to link the record with the cause of death file. Adjustments for differences in age distribution, follow-up period, and temporal changes in background mortality rates were made using SMRs for a range of separate causes of death. In the total study population, 1,273 deaths were observed (an approximate doubling of the observed number of deaths (n= 706) in the earlier study). The number of deaths in the exposed group increased from 134 to 290. In either group, the observed mortality is still lower than the expected, an indication of the 'healthy worker effect;' however, no direct comparisons were made between the exposed and unexposed group, which might have yielded larger relative risks (IARC, 1999). Stratification of the workers according to three levels of exposure and three latency periods (*i.e.*, latency was defined as time since the particular exposure group was entered) did not reveal any significant increases in terms of overall mortality.

A total of 97 cancer deaths were reported in the exposed group (110.78 expected). As in the earlier study, stratification of the workers according to three levels of exposure and three latency periods did not reveal any significant increases. As with total mortality, cumulative dose-effect relationships were investigated after classifying the exposed workers into three exposure categories and three latency periods. The results show that although there are some small fluctuations in cancer mortality, there does not appear to be any cancer excess related to occupational exposure to AN. The SMR for lung cancer overall was 110 (95% CI= 81 to 146). In the low, medium, and high exposure category, the SMR for lung cancer was 97 (95% CI = 3 to 227), 107 (95% CI = 68 to 159), and 118 (95% CI = 70 to 187), respectively. Although increasing exposure appeared to be associated with increasing SMR in the lowest exposure group, this trend was not observed in the two higher exposure group. Overall, no

excess lung cancer was observed in this cohort based on peak exposures, respirator use, or exposure to other carcinogens.

In a more recent follow-up of this cohort, which included up tp 1/1/2001, completeness of follow-up was 98.8% (Swaen *et al.*, 2004). In the total study population, the number of deaths observed increased from 1,273 to 1,775. The number of deaths in the exposed group increased from 290 to 432. Stratification of the workers according to three levels of exposure and three latency periods (i.e., latency was defined as time since the particular exposure group was entered) did not reveal any significant increases in terms of overall mortality. **Table 4-2** provides the exposure categories, latency periods, SMRs, and 95% CIs for lung cancer mortality in this cohort.

A total of 146 cancer deaths were reported in the exposed group (164.5 expected). The results show that although there are some small fluctuations in cancer mortality, there does not appear to be any cancer excess related to occupational exposure to AN. The SMR for trachea and lung cancer overall was 107.2 (95% CI= 83.1 to 136.1). In the low, medium, and high exposure category, the SMRs for lung cancer were 92.1 (95% CI = 36.9 to 189.0), 106.5 (95% CI = 74.6 to 147.4), and 114 (95% CI not reported), respectively. Overall, no excess lung cancer was observed in this cohort based on peak exposures, respirator use, or exposure to other carcinogens.

Table 4-2. Lung Cancer Mortality in Workers Exposed to AN Grouped into Three Cumulative Exposure and Latency Categories (Swaen *et al.*, 2004)

Exposure/Latency	Observed	SMR	95% CI
Low (<1 ppm/yr.)			
<10 years' latency	--	--	--
10 to 20 years' latency	3	103.4	20.8 to 296.8
≥20 years' latency	4	100	26.9 to253.1
Total	7	92.1	36.9 to 189.0
Moderate (1 to 10 ppm/yr.)			
<10 years' latency	1	31.3	0.4 – 157.9
10 to 20 years' latency	16	129.0	73.7 – 209.4
>=20 years' latency	19	104.4	62.8 – 162.9
Total	36	106.5	74.6 – 147.4
High (10 + ppm/yr.)			
<10 years' latency	3	120.0	24.1 – 344.2
10 to 20 years' latency	12	142.9	73.7 – 249.2
>=20 years' latency	9	90.0	41.1 – 170.4
Total	24	114.8	69.9 – 186.6

United Kingdom Cohort Studies

Cancer mortality was investigated in 1,111 male workers exposed to AN for at least one year between the years 1950 to 1968 at six UK facilities involved in the polymerization of AN and the spinning of acrylic fibers (Werner and Carter, 1981). Follow-up extended for ten years (1978) and workers were stratified by age. Total mortality was 79 deaths of which 68 occurred in those with more than one year of exposure (72.4 deaths were expected in this sub-group). In terms of cancer mortality, 21 deaths were observed with 18.6 expected overall, with nine lung cancer deaths versus 7.6 expected (this included three lung cancers among workers 15-44 with 0.7 expected, a significant elevation). A significant excess of stomach cancer was reported for men from all age groups (five observed versus 1.9 expected), but was particularly evident in workers of 55 to 64 years of age (three observed versus 0.7 expected). While the authors attempted to estimate historical exposure levels, they judged that the only available methods of estimation were so subjective as to be of little value, thus no quantitative data on the exposure levels to AN were given. The numbers of expected deaths were also too small to provide confidence in the results, but the relatively high SMR for total mortality is indicative of better case ascertainment and follow-up. Additionally, when considering the data and findings of this study, it should be noted that the cohort consisted of 40% Welshmen. In the vital statistics of the UK, the Welsh account for

only 4% of the data. It has been pointed out that since the incidence of stomach cancer was higher in the UK than Wales, the expected mortality rate for stomach cancer should be adjusted to account for the lower background stomach cancer mortality in Wales. This would likely make the expected stomach cancer incidence rate lower for this study.

An update of the mortality study conducted by Werner and Carter (1981) was conducted by Benn and Osborne (1998). The original study (Werner and Carter, 1981), looked at 1,111 male workers, employed in six UK plants between 1950 and 1978, and found significant excesses of stomach cancer overall, particularly in those workers aged 55 to 64 years, and excesses of lung cancer in those aged 15 to 44 years. As the results of this initial study were largely inconclusive, it was decided to extend the study by enlarging the population so as to include more recently exposed workers (*i.e*. those employed in polymerization or as spinners at some time between 1969 and 1978), and also to extend the follow-up period to 1991.

This study included 2,763 male workers exposed to AN for at least one year between 1950 and 1978 at one of the six UK factories involved in the polymerization of AN and the spinning of acrylic fibers (63,058 person-years, 72% from one plant - Factory 5). The follow-up period lasted until December 31, 1991. Mortality rates were compared to national rates for England and Wales (except one factory in Scotland, where Scottish rates were used). With regard to this study, no exposure measures were available for the earlier years covered. Up to 1979, AN exposure was subject to a threshold limit value of 20 ppm (TWA-8). In that year, the Health and Safety Commission, in view of concern about possible carcinogenicity of AN, issued a statement that exposure should be reduced to a level as low as reasonably practicable. A stated objective was to adopt a control limit of 2 ppm (TWA-8) by 1981. The statement provided for a staged reduction of limits in the interim period, with a limit of 5 ppm immediately, and 4 ppm from the first quarter of 1980. It seems reasonable to assume a general downward trend in AN exposure, but the lack of precise information and the fact that work histories were not updated after workers were recruited into the study meant that length of employment up to 1978 to 1980 was used as a proxy measure of exposure. Jobs were categorized as having any or no AN exposure. Following discussion with company personnel, a number of workers from Factory 5 who were classified as spinners in the original study with presumably high AN exposure were re-classified as "end-of-the-line" workers with minimal or no exposure. No explanation was provided as to the rationale for this change or its possible effect on the findings or interpretation of the original or current study (IARC, 1999).

Results were described as 'significant' when the calculated SMR value was outside the 95% CI for the national population. The results showed that, overall, there was a deficit in mortality for the combined analysis population, reflecting a significant deficit in circulatory disease deaths, and deficits in most other causes of death. Factory 5 showed deficits from various causes of death while the other plants combined showed non-significant increases.

All cancers combined showed a deficit (a total of 121 cancer deaths were observed, compared to 137.12 expected), and for most individual cancer sites (including lung and stomach), the observed numbers were close to the expected numbers (**Table 4-3**).

When the workers were stratified by age, lung cancer mortality showed an increased SMR in the 15 to 44 and 45 to 54 age groups (SMR = 284.4, 95% CI = 104.4 to 618.9; SMR = 148.7, 95% CI = 83.2 to 245.2) and a deficit for the older age groups. However, further analysis of these lung cancer excesses showed that the excess in the age group 45 to 54 was in Factory 5, while the excess in the 15 to 44 age group was in the other factories. While analysis by length of employment showed a significant tendency for increased all cause and circulatory disease mortality with time, there was no similar trend for cancer. Five lung cancer deaths (0.8 expected) were reported among those with highest exposure and under 45 years of age (SMR = 6.1, 95% CI = 2.0 to 14.6), and among those highly exposed first employed after 1969 (SMR = 2.7, 95% CI = 1.1 to 5.5).

With respect to mortality by job category, workers were counted in a category if they worked in it for one year or more; workers who changed jobs may thus appear in more than one category. In the two 'high exposure' categories (polymer or control room worker and spinner), cancer SMRs were raised but not significantly so. In the 'end of line' workers (a category used exclusively in Factory 5), the SMR for all cancers was significantly reduced.

On grouping the workers according to the level of AN exposure categories that they had worked in (*i.e*. high, possible, and little or no exposure), the SMR for each of the examined causes (other than respiratory disease) was higher in the high exposure group than in the other two groups (**Table 4-3**). The highest cancer SMRs were found in the high exposure group for total cancer (115.8), stomach cancer (166.3), and respiratory cancer (141.1); however, only stomach cancer showed a clear and statistically significant trend across the three groups. However, this finding was based on small numbers. For lung cancer, the SMR was lowest in the middle group. It should be noted, however, that information regarding the smoking habits of the workers was lacking. The authors concluded that the analysis does not provide consistent support for a causal association between AN exposure and cancer mortality.

TABLE 4-3. Mortality Related to Exposure Level (Benn and Osborne, 1998)

Cause of Death	Observed	Expected	SMR
HIGH AN EXPOSURE			
All causes	170	181.2	93.8
All cancers	58	50.1	115.8
- stomach	7	4.2	166.3
- respiratory	27	19.1	141.1
Circulatory disease	81	86.9	93.2
Respiratory disease	11	15.7	70.2
POSSIBLE AN EXPOSURE			
All causes	97	124.7	77.8*
All cancers	22	35.9	61.2*
- stomach	3	2.9	102.7
- respiratory	7	13.3	52.6
Circulatory disease	49	59.1	83.0
Respiratory disease	7	10.0	69.9
NO/LITTLE AN EXPOSURE			
All causes	142	179.6	79.1**
All cancers	41	51.12	80.2
- stomach	1	4.31	23.2
- respiratory	19	19.1	99.5
Circulatory disease	70	86.25	81.2
Respiratory disease	13	15.76	82.5

* significant at 5%; **significant at 1%

One particular factory involved in this study (Factory 5) influenced greatly the overall deficit in mortality observed in the total study population, and may explain some of the overall study results. At Factory 5, there were 246 observed deaths compare to 319.1 expected deaths (SMR= 77.1) while for all other factories, there were 163 observed deaths compared to 166.4 expected deaths (SMR= 97.9). This deficit carried over to the cancer mortality where for Factory 5 there were 70 observed cancer deaths compared to 91.7 expected cancer deaths (SMR = 76.3, 95% CI = 59.5 to 96.4), and in all other factories, there were 51 observed cancer deaths compared to 45.5 expected cancer deaths (SMR = 112.2, 95% CI = 83.5 to 147.5). These factories also showed non-significant excesses in lung cancer and stomach cancer, though it is difficult to interpret the figures at individual factories due to the smallness of the numbers involved. Four stomach cancer deaths were observed at Factory 5 compared to 7.5 expected (SMR = 53.4) while, for all other factories, there were seven stomach cancer deaths observed compared to four stomach cancer deaths expected (SMR = 177.2). Similarly, 30 lung cancer deaths were observed in Factory 5 compared to 33.8 expected (SMR = 88.8) while, in all other factories, 23 stomach cancer deaths were observed compared to 17.8 stomach cancer deaths expected (SMR = 129.4).

While there was no excess of cancer deaths above the expected, there were indications of excess cancer in those workers employed at jobs where there was the highest AN exposure, and in particular, there was an excess of lung cancer in workers aged under 55 years. When considering the results of this study, it should be noted that the study was hampered by certain limitations such as lack of exposure measurements and lack of information on smoking habits. Despite the shortcomings of this study, the overall findings indicate that UK AN workers did not appear to have increased mortality risk due to cancer or other causes.

NCI/NIOSH Cohort

A large mortality study of industrial workers exposed to AN was conducted by NCI/NIOSH using a cohort of 25,460 workers (18,079 white men; 4,293 white women; 2,191 non-white men; 897 non-white women) employed at eight AN producing or processing plants in the US. (Blair *et al.* 1998). Exposures at the eight plants started between 1952 and 1965. Two of the plants included those evaluated by Collins *et al.* (1989), as well as those evaluated in the unpublished studies by Zack (1980) and Gaffey and Strauss (1981). These workers, employed from the 1950s to 1983, were followed up until 1989 to determine their vital status and cause of death. Smoking habits were considered as a confounding factor. Mortality rates for exposed workers were compared with rates of unexposed workers in the cohort using Poisson regression to minimize the 'healthy worker effect' problem.

This investigation included a high quality and well-documented procedure to develop qualitative estimates of historical exposures that provided the basis for evaluating exposure-response relationships. In this study, a procedure was created to develop quantitative estimates of exposure by job, department, and time period. Personal monitoring of AN exposures was performed in all eight plants in 1986 by the study investigators using the recommended NIOSH method (1984). Seven of the plants also conducted their own monitoring simultaneously with the study monitoring. No major differences were found between the monitoring results. Seven of the eight plants had measurements dating back to 1977-78 and one plant started monitoring in 1960. Over 18,000 measurements were available from the companies between 1960 to 1989 and over 12,000 of these were personal samples (Stewart *et al.* 1998). The estimated time-weighted average for an eight-hour period (TWA-8), covering a minimum time period of one year, served as the primary index of historical exposure developed for this study. Estimates of median (and mean) AN exposure (TWA-8 in ppm) in the eight plants were 0.1 (1.88), 0.39 (2.17), 3.46 (6.13), 0.34 (5.30), 0.42 (3.37), 0.54 (0.63), 0.36 (1.34), and 1.90 (1.45), respectively. In addition to TWA-8 estimates, other exposure assessments included: 1) the frequency of peaks (defined as 15-minute exposures that averaged 20 ppm or greater); 2) TWA-8 estimates taking into account respirator use; 3) a dermal score to account for skin contact; and 4) the total mass inhaled (based on a semi-quantitative estimated level of physical activity associated with the job, the

respiratory rate expected to be associated with that level of activity, the average tidal volume, and the estimated air concentration).

The entire cohort generated 545,369 person-years of follow-up. Of the total person-years of follow-up, 348,642 were assigned to workers after their first exposure to AN and 196,727 person-years were associated with individuals never exposed, or with the time period prior to first exposure among workers who started employment in unexposed jobs. Over 44,000 person-years occurred among workers after their cumulative exposure exceeded 8.0 ppm-years. Exposure to chemicals other than AN also occurred, including exposure to methylmethacrylate, sodium thiocyanate, dimethylformamide, styrene, benzene, formaldehyde, ammonia, propylene, hydrogen cyanide, butadiene, vinyl bromide, vinyl acetate, vinyl chloride hexamethylene-diamine, zinc chloride, acetylene, and sulphuric acid. Worker-years of exposure totaled approximately 55,000 for benzene, 8,000 for butadiene, 50,000 for formaldehyde, 45,000 for styrene, 10,000 for sulphuric acid and 54,000 for vinyl chloride. Workers were stratified by exposure into quintiles and deciles, using a variety of exposure measures (*e.g.*, cumulative, average, peak, intensity, and duration) in order to evaluate different aspects of the exposure-response relationship.

Analyses by various indicators of exposure including cumulative (ppm-years), average, peak, intensity, duration, and lagged exposure revealed no elevated risk of cancers of the stomach, brain, breast, prostate, or lymphatic and hematopoietic system. Relative risks (and 95% confidence intervals) for lung cancer from lowest to highest quintile for cumulative exposure were 1.1 (0.7 to 1.7), 1.3 (0.8 to 2.1), 1.2 (0.7 to 1.9), 1.0 (0.6 to 1.6), and 1.5 (0.9 to 2.4), respectively. When the same data were restricted to workers with more than 20 years since their first exposure to AN, the relative risks (and 95% confidence intervals) were 1.1 (0.6 to 2.2), 1.0 (0.5 to 0.1), 1.2 (0.6 to 2.2), 1.2 (0.6 to 2.1) and 2.1 (1.2 to 3.8), respectively, achieving statistical significance for the highest quintile, compared to the unexposed group of workers. No increased risk was observed for lung cancer when external comparisons are used (Marsh *et al.*, 2001).

To evaluate RRs at a wider range of cumulative exposure, analyses were also conducted for decile categories. The RR did not continue to increase in a dose-response fashion, and actually decreased from 1.7 at the ninth decile to 1.3 at the tenth decile. When confounding from tobacco use was accounted for, the RR for lung cancer was reduced in the upper quintile slightly (from 1.5 to 1.4). Despite finding a significant increase in lung cancer mortality in workers from the highest quintile, analyses of the exposure-response data did not provide a strong or consistent evidence for a causal relationship. Separate analyses for wage and salaried workers, long-term and short-term workers, fiber and non-fiber plants, and by individual plants revealed no clear exposure response patterns and tests for trend were not statistically significant.

This study provided no evidence to indicate that exposure to AN at the levels experienced by these workers could be associated with any significant increased relative risk for most cancers of interest (*i.e.*, stomach, brain, breast, prostate, or lymphatic and hematopoietic system cancers). The excess of lung cancer seen in the highest quintile, particularly when time since first exposure was more than 20 years, could be taken as providing evidence for a carcinogenic effect at the highest exposure. However, the analyses of exposure-response do not provide strong or consistent evidence for a causal association between AN exposure and lung cancer. No dose-response effect was identified and the risk of lung cancer did not increase with increasing exposure.

In the Blair *et al.* study (1998), the relation between cumulative exposure between 0.01 and more than 8 ppm years and specific cancer mortality was extensively studied. No consistent significant trend with cumulative exposure could be detected in comparison with the cancer mortality data from the general US vital statistics.

In a re-evaluation of the lung cancer mortality data for the NCI/NIOSH cohort (Blair *et al.*, 1998), Marsh *et al.* (2001) reported deficits in lung cancer mortality when compared to external rates, even for the most exposed group of workers (SMR = 0.92). The authors concluded that the study provides little evidence that AN exposure increases the risk of lung or other cancers.

The data from the Blair *et al.* provided an excellent opportunity to update quantitative risk assessments for this widely used commodity chemical (Starr *et al.*, 2004) employed the semiparametric Cox relative risk regression model with a cumulative exposure metric to model cause-specific mortality from lung cancer and all other causes. The separately estimated cause-specific cumulative hazards were then combined to provide an overall estimate of age-specific mortality risk. Age-specific estimates of the additional risk of lung cancer mortality associated with several plausible occupational exposure scenarios were then obtained. For age 70, risk estimates were all markedly lower than those generated with the cancer potency estimate provided in USEPA's (1983) risk assessment for AN based upon O'Berg (1980). The authors concluded that the results are consistent with the failure of recent occupational studies to confirm elevated lung cancer mortality among acrylonitrile-exposed workers as was originally reported by O'Berg (1980), and it calls attention to the importance of using high quality epidemiology data in the risk assessment process.

Monsanto Cohort

Zack *et al.* (1980, unpublished) conducted a retrospective cohort study of 352 male workers at two Monsanto facilities in Texas and Alabama. Workers were exposed to AN for at least six months prior to 1968. Mortality follow-up extended through 1977 for a total of 5,837

person years. Although vital status ascertainment was stated to be complete, no details on the conduct of the follow-up were provided. Total mortality was less than expected (observed = 15, expected = 18.1). No excess cancer mortality was reported (observed = 3, expected = 2.8) and only one lung cancer death (0.8 expected) and one prostate cancer death (0.03 expected) was reported. The size of the cohort is too small to convey much useful information and, while the excess prostate cancer mortality rate is interesting in light of similar associations in other studies, it is based on only one case.

The mortality of 325 male workers potentially exposed to AN during the start-up operations of the Monsanto Alabama plant (study subjects had to have at least six months potential exposure during the period of April 1952 to December 1953 to be included). This cohort had an overlap of 21 people with the group studied by Zack *et al.* (1980, unpublished). Vital status was ascertained through company and a retail credit bureau records with follow-up extending through 1977 (7,531 person-years of follow-up). Total mortality experience consisted of 26 deaths with 53.3 expected based on US rates (SMR = 0.47, 95% CI = 0.31 to 0.68). Cancer deaths totaled four with 11.0 expected. There were two lung cancer deaths with 3.7 expected; for hourly workers (those with presumably higher exposure), no lung cancer deaths were reported (1.3 expected). Two deaths from kidney cancer were also reported (0.3 expected). Aside from the small size of the cohort and number of deaths, there were also questions as to the completeness of ascertainment in this and similar small studies that primarily relied on company records.

Individual Cohort Studies

EU (2001) and an earlier Environ report (Ward and Starr, 1993) both cite an unpublished study by Monson (1978, unpublished), which was referenced in the 1983 EPA AN Health Assessment Document (USEPA-660/8-82-007F). This study reported a statistically non-significant increase in deaths from cancer from exposure to AN, but indicates that workers were also exposed to other carcinogens.

Kiesselbach *et al.* (1979) examined the mortality rate, cancer rate, and cancer type in relation to the period of exposure (1950 to 1965) to AN in 884 workers at 16 Bayer plants for workers exposed for 12 months or more. Follow-up was carried out through 1977. The general mortality of the exposed group was markedly lower than that of the normal population (*i.e.*, 58 cases as opposed to an expected 104.2 based on state rates), possibly due to the 'healthy worker effect' although the deficit is higher than typically observed in blue-collar workers, suggesting some problem with the follow-up. However, the mortality rate for all malignant tumors was at expected levels, 20 observed and 20.4 expected, for lung cancer, six observed and 6.9 expected, and for stomach cancer, four observed and three expected. Cardiovascular, brain, respiratory, and gastrointestinal diseases, suicide, and other causes of death were also not different than expected and, in fact, displayed a deficit. No

relationship was found between length of exposure and mortality from tumors, but uncertainties exist about the quality of the follow-up employed in this study. The cancer rates for this study could have been underestimated.

Thiess *et al.* (1980) studied the mortality of 1,469 BASF workers from 12 factories employed six months or more in AN processing (15,350 person-years of follow-up). Their exposure was not quantified. Follow-up through 1978 was reportedly 98% complete for the 1,081 German workers in the study, but only 56% complete for the remaining 388 study subjects. The observed deaths numbered 89 compared to 92 or 99 expected (depending on national, local, or regional comparisons). The number of observed cancer deaths (27) was higher than the number expected (20.5). The increase in the number of lung cancer deaths (11 observed versus 5.6 expected) was statistically significant. Lymphatopoietic cancers (four observed versus 1.7 expected) also were increased. The workers in this study were exposed to polycyclic aromatic hydrocarbons, vinyl chloride, beta-naphthylamine and other compounds in addition to AN, and all cancer cases were known smokers. When workers without exposure to other carcinogens were considered alone, the lung and lymphatic cancer deaths were nine and four observed compared to 4.37 and 1.38 expected, respectively.

A retrospective cohort mortality study was conducted for 1,077 Uniroyal workers who worked for at least one year between 1951 and 1977, and followed through 1977 (Herman, 1981, unpublished). Workers were identified through company records, but no exposure information was developed. Vital status was determined through Social Security records and state motor vehicle records. Workers' mortality rates were compared to US rates. There were 59 deaths observed versus 92.0 expected. For hourly workers, total mortality was 42 observed versus 67.2 expected. Overall and among hourly workers, there was a deficit in cancer deaths (11 observed versus 16.1 expected overall, and four observed versus 11.2 expected for hourly workers). One lung cancer was observed (3.7 expected) and no data on prostate cancer was reported. The low overall SMR suggests under-ascertainment of deaths might have been a problem with this small study and thus the cancer rates may be underestimated.

Delzell and Monson (1982) reported an increase in lung cancer deaths among workers employed two or more years between January 1, 1940, and July 1, 1971, at an Ohio rubber chemicals plant (n = 327). Workers were stratified by duration of exposure and follow-up was carried out to 1978 using company and Social Security records. Mortality rates were compared to local and national rates. In an analysis of mortality among the 327 rubber chemical workers, there were 74 deaths compared to 89.5 expected. A total of 22 cancer deaths were observed compared to 17.9 expected. Nine deaths from lung cancer also were observed compared to 5.9 expected deaths based on US general population rates or 4.7 expected based on the rates of other rubber chemical workers. The highest lung cancer increase (four observed compared to 0.8 expected) was found in males who worked five to

14 years and had more than 15 years of follow-up since their first exposure. The observed and expected numbers of deaths were small and the study did not evaluate cigarette smoking as a confounding factor contributing to excess death.

A retrospective cohort mortality study was conducted for 419 male AN workers at an Ohio chemical plant during the period 1960 to 1980, with follow-up for the same time period (Stallard, 1982, unpublished). Workers were classified as exposed or unexposed based on job title, and any employee who held a job title classified as exposed for any length of time was included in the cohort. Vital status was ascertained from Social Security records and death benefit claims. A total of 4,577 person years of follow-up was included in the study, most for workers under 50. Seven deaths occurred among the cohort during the study period with 25.5 expected. Four cancer deaths (five expected) and two respiratory system deaths (1.6 expected) occurred in this cohort. The large deficit of overall deaths suggests underascertainment, and thus cancer rates among exposed workers may be underestimated. The lack of exposure estimates is also a limiting factor in interpreting the results.

Collins *et al.* (1989) studied cancer mortality in 1,774 American Cyanamid acrylic fiber workers exposed to AN between 1951 and 1973 at a Louisiana plant and between 1957 and 1973 at a Florida plant. The observation period for mortality follow-up ended on December 31, 1983. The mean observation time for latency was about 20 years. Mortality rates were compared to those 897 referents as well as the U.S. general male population. Exposures to AN were estimated based upon industrial hygiene data from 1977, which the authors indicated was representative of exposures going back to the beginning of plant operations. The workers in manufacturing plants were stratified into one of four exposure groups (<0.01, 0.01 to 0.7, 0.7 to 7.0, and >7.0 ppm-year). Smoking histories were taken into consideration but were available for only 58% of the cohort. In this study, as in the previously discussed studies (O'Berg *et al.*, 1980; O'Berg *et al.,* 1985), the subjects were relatively young, and only 9% of the cohort had died.

The total number of deaths among exposed workers was 145, giving an overall SMR of 0.67. Despite the sizeable deficit relative to the expected number for total deaths, the number of cancer deaths (n=43) was about equal to the expected number (SMR = 1.01). There was no significant difference between observed and expected cases of cancer or between the sub-groups, stratified by cumulative exposure to AN and by latency time. No significant relationship between lung cancer and AN exposure was established. The 15 lung cancer deaths observed were also about the same as the number expected (SMR = 0.95) according to the general population comparison, although the authors found some evidence in this study of confounding between smoking status and cumulative exposure to AN. There were two deaths from prostate cancer (SMR = 1.49). The relative risk of respiratory cancer for the four exposure categories were 1.1 for exposure under 0.01 ppm-year, 0.7 for exposure in the range of 0.01 to 0.7 ppm-year, 0.7 for exposure of 0.7 to 7.0 ppm-year and 1.2 for exposure greater

than 7.0 ppm-year. No internal comparison was presented outside of the analysis for lung cancer.

A retrospective cohort mortality study of 1,811 Chinese workers in a chemical fiber plant in Fushan was carried out by Zhou and Wang (1991). In addition to AN, workers were exposed to thiocyanate and methyl acrylate. The average AN exposure was reported as 0.57 ± 0.53 ppm (range = 0.026 to 1.9 ppm). The cohort includes all workers from the plant's opening in 1971 continuing through 1988. Retired or former workers were contacted by mail survey. Workers lost to follow-up (n = 115) were dropped from the study.

Total deaths (n = 42) were compared to expected deaths based on provincial and national rates. The analysis suggests that total deaths were 27% higher than expected and cancer deaths were 25% higher than expected. An analysis of death rates by number of years worked showed marked positive trends for total deaths, cancer mortality and death from cerebrovascular disease. Rothman (1994) considered that there were too many uncertainties in this study regarding methodology, ascertainment, and follow-up to infer much useful information from it. The fact that the results are so different from all other similar studies of AN workers make it suspicious as well. No serious weight was given to this study in Rothman's 1994 meta-analysis of AN studies because of these flaws although it was included in the meta-analysis of Collins and Acquavella (1998). These authors also pointed out the potential weaknesses in this study.

A mortality study was conducted in 894 workers at a BP facility that manufactured AN. Of the workers, 428 were actually exposed to AN. The study period covered from 1960 to 1988 (Ives *et al.*, 1993, unpublished). Information on AN exposure and smoking status was available. No statistically significant increase in mortality was reported for total cohort mortality (SMR = 0.64) or any cancer type. Mortality from lung cancer consisted of nine deaths (SMR = 1.1; 95% CI = 0.5 to 2.09) while six deaths from digestive system cancers (*i.e.*, stomach, colon, rectal, and gall bladder) were reported (SMR = 1.08; 95% CI = 0.4 to 2.35). Among exposed workers, four digestive system cancers (SMR = 1.63; 95% CI = 0.44 to 4.17) and six lung cancers (SMR = 1.68; 95% CI = 0.62 to 3.66) occurred. When the workers were stratified by level of exposure (0, <0.2, 0.2 to 2.0, 2.1 to 20, and >20 ppm), an inverse relationship was found for lung cancer rates. The authors concluded that, despite the small size of the cohort, the result provide evidence against a large increased risk of lung cancer in workers exposed to AN.

A retrospective epidemiological cohort study of mortality was undertaken in 671 workers with at least 12 months of exposure to AN in an acrylic fiber factory at Porto Marghera, Italy, between 1959 and 1988, and followed through 1990 (Mastrangelo *et al.*, 1993). A majority of subjects (n = 571) had simultaneous exposure to dimethylacetamide. Observed mortality in the cohort was compared with expected mortality, calculated on the basis of the mortality

rates of the general population in the region. SMRs were 1.0 for all causes of death (95% CI = 0.7 to 1.4), 3.4 for stomach cancer (95% CI = 0.4 to 12.3), 0.8 for lung cancer (95% CI = 0.1 to 2.9), and 2.6 for brain cancer (95% CI = 0.1 to 14.7). A statistically significant excess was found in the mortality rate from intestinal and colon tumors (four observed; SMR =10.5). However, this finding was significant only in subgroups with one to four years of exposure or one to nine years latency. Data are not adequate to determine if a relationship exists between AN or dimethylacetamide exposure and mortality from tumors of the colon and intestine.

Supporting Cohort and Case-Control Studies

Waxweiller *et al.* (1981) studied 4,806 vinyl chloride monomer workers who also were exposed to AN (among other chemicals) between 1942 and the end of 1973. The mortality of the cohort was followed through 1973 with only 1.5% lost to follow-up. Mortality was compared to the general US population adjusting for age and calender year. Exposure was estimated by company personnel on an ordinal scale (1 to 5) for 20 chemicals including AN and a technique known as the serially additive expected dose (SAED) model was also used (Smith *et al.*, 1980 cited in IARC, 1999). Cumulative exposure among cases was compared with that of other employees under follow-up when the case occurred. Forty-five deaths from lung cancer were observed with medical and histological records reviewed for 27. The observed dose of AN was lower among the lung cancer cases than among other employees; however, IARC (1999) observed that details on the work situation resulting in AN exposure were not available and results from this study were not directly comparable to other studies. Collins and Acquavella (1998) also excluded this study from their meta-analysis because the authors did not report their results in terms of relative risk estimates.

Although not specific for AN exposure, a study by Marsh (1983) was used in the AN meta-analysis carried out by Collins and Acquavella (1998). A retrospective cohort study of 2,490 male workers employed for at least one year in a plastics producing plant between 1949 and 1966 was conducted to compare cancer mortality experience with that of the local cancer registry for males. Vital status was determined as of December 31, 1976, for 99.7% of the cohort and death certificates were obtained for 98.0% of 603 observed deaths. Comparison with the local county white males revealed an SMR of 102 for digestive system cancer and a statistically significant excess ($P < 0.05$) in genitourinary cancer (SMR = 153.6). A relationship was suggested between cancer of the rectum, liver and pancreas and both the duration and interval from onset of exposure. A secondary matched case-control study was conducted to determine if particular jobs or work areas were related to the excesses found in the primary study. This analysis did not support the hypothesis that digestive or genitourinary cancer was related to a general plant exposure or date of hire. Possible associations warranting continued surveillance were found between rectal cancer and cellulose nitrate production and between prostatic cancer and polystyrene processing.

Digestive and genitourinary cancers other than rectal and prostate were not related to employment in any of 21 occupational exposure categories examined.

Although not specific for AN exposure, Ott *et al.* (1980) was used in the AN meta-analysis carried out by Collins and Acquavella (1998). Ott *et al.* examined the mortality of 2,490 Dow Chemical employees involved in the development or production of styrene-based products. Of these workers, 100 were exposed to AN. A multiple agent approach to exposure categorization was adopted to assess potential relationships between mortality and work exposure to multiple chemicals. The subjects' mortality rate was less than that of the corresponding USA white male population and was consistent with that of an industrial comparison group. Although deaths due to malignant neoplasms were fewer than expected for the total cohort, an increase in lymphatic leukemia was observed among a subgroup of employees who had exposure to polymer extrusion fumes, solvents, and coloring agents. Among the AN sub-group, one case of lung cancer (0.5 expected) and three cases of leukemia (1.25 expected) were reported. No relationship was found with duration or intensity of exposures experienced by these employees and their disease, and the etiology of the lymphatic leukemia cases was not established.

Although not specifically examining AN exposure, a possible association between occupational exposures and the development of bladder cancer was evaluated by re-examining the results of a study of cancer and occupational exposures (Siemiatycki *et al.,* 1994). This study was included in the AN meta-analysis carried out by Collins and Acquavella (1998), although it was subsequently excluded from a re-evaluation conducted by Collins (EU, 2001) because of insignificant exposure potential among cases and controls in the study. Data were evaluated from a population-based case-control study of men, 35 to 70 years old, conducted in Canada between 1979 and 1986 to examine the relationship between cancer and occupational exposures. There were 484 patients with bladder cancer, 1,879 control subjects with cancer at other sites, and 533 population control subjects. Odds ratios were estimated for bladder cancer and 19 occupations, 11 industries, and 23 substances. Several substances including natural gas combustion products, acrylic fibers, aromatic amines, cadmium compounds, photographic products, polyethylene, titanium-dioxide, and chlorine demonstrated weak associations. Occupations which showed an association with bladder cancer included motor vehicle drivers, production managers, teachers and professors, and textile dyers and finishers. Results for AN exposure were not reported.

Although not specific for AN exposure, the study of Thomas *et al.* (1987) was used in the AN meta-analysis conducted by Collins and Acquavella (1998). A case-referent study was conducted on 300 brain tumor cases among workers exposed to organic chemicals in petroleum refining and chemical manufacturing (including presumed exposure to acrylonitirle in some cases) and 386 referent cases who had died from causes other than brain

tumor, epilepsy, cerebrovascular disease, suicide, or homicide (Thomas *et al.* 1987). Case and controls were frequency-matched with the cases on age at death, year of death, and study area. The odds ratio was 0.9 (95 % CI = 0.5 to 1.6) for employment in the chemical industry. The risk of astrocytic tumors was elevated among the subjects with production or maintenance jobs in petroleum refining; however, it decreased with length of employment. There were non-significant excess risk of astrocytic tumors among the men exposed to cutting fluids or organic solvents and also among the subjects exposed to lubricating oils, organic solvents, or cutting fluids for $\geq$ 20 years. Despite the large number of cases, the study had a low power to detect an association of AN with astrocytic tumors because AN exposure was rare among these workers. Exposure information was based on next-of-kin data and consequently may not be very reliable. The overall indication is that no excess of astrocytic brain tumors occurred among these workers.

The study of Ott *et al.* (1989) was included in the AN meta-analysis of Collins and Acquavella (1998). Ott *et al.* (1989) conducted a nested case-control study on Union Carbide workers exposed to AN and several other chemicals at two chemical manufacturing facilities or a research and development center. Relative risks from AN exposure were not specifically reported. Non-Hodgkin's lymphoma (52 cases), multiple myeloma (20 cases), non-lymphocytic leukemia (39 cases), and lymphocytic leukemia (18 cases) were studied. Exposure odds ratios were examined in relation to 11 work areas, 21 specific chemicals, and 52 chemical activity groups. Associations were observed for a maintenance and construction subgroup and a chlorohydrin production unit. The former had an increased expectance of non-Hodgkin's lymphoma and the latter for non-lymphocytic leukemia. The odds ratio from the association of foremen and others with non-Hodgkin's lymphoma was 3.2 based on 11 cases. A duration response trend was noted for the chlorohydrin unit with three of four cases assigned more than five years to that unit. An association was also noted between non-Hodgkin's lymphoma and assignment to strong acid alcohol production units but this was not supported by a duration response trend. Two highly correlated chemical groups, antioxidants, and nitriles, were over-represented among multiple myeloma groups. A duration effect was observed. However, examination of work histories did not reveal common jobs or departments among these cases.

4.1.2.2 Meta-Analyses of Occupational Studies

A review of selected epidemiology studies on AN occupational exposure was performed by Rothman (1994). From the 12 published studies, eight studies were included in a meta-analysis on the basis of the quality of the studies. Four studies (O'Berg, 1980; Chen *et al.*, 1988a, 1988b; and Zhou and Wang, 1991) were not included in the meta-analysis for various short-comings including small numbers of AN exposed workers or methodological ascertainment, or follow-up inconsistencies or because they had been superceded by more recent studies (O'Berg *et al.*, 1985; Wood *et al.*, 1998). A summary of observed and

expected numbers of total cancer and respiratory cancer deaths in the studies selected for this meta-analysis is presented in **Table 4-4** below.

Table 4-4. Meta-Analysis of Cancer Mortality in Workers Exposed to AN (Rothman, 1994)

Study	Cohort Size	Obs. Cancer Mortality	Exp. Cancer Mortality	Obs. Lung Cancer Mortality	Exp. Lung Cancer Mortality
Kiesselbach *et al.* (1979)	884	20	20.4	6	6.9
Theiss *et al.* (1980)	1469	27	20.5	11	5.7
Werner and Carter (1981)	1111	21	18.6	9	7.6
Delzell and Monson (1982)	327	22	17.9	9	5.9
O'Berg *et al.* (1985)	1345	31	27 (wage)*	12	10.2 (wage)*
Chen *et al.* (1987)	1083	18	20.4 (wage)*	7	7.9 (wage)*
Collins *et al.* (1989)	1774	43	42.6	15	15.7
Swaen *et al.* (1992)	2842	42	50.8	16	19.5
TOTAL	**10835**	**224**	**218.2**	**85**	**79.4**

* "Wage" refers to job status, exposure is expected to be higher for 'wage' earners than for "salaried" (mostly office workers)

A total of 224 cancer deaths were observed (218.2 expected), of which 85 were respiratory cancers (79.4 expected). The author concluded that workers exposed to AN face no striking increases in cancer mortality. The weighted total for the observed number of deaths was close to the total for the expected number of deaths for all cancers (SMR = 1.03; 90% CI = 0.92 to 1.15) and for respiratory cancers (SMR = 1.07; 90% CI = 0.89 to 1.28). These findings are a quantitative indication that in the aggregate these studies do not show a strong relation between work in an environment in which there is exposure to AN and subsequent death from cancer, respiratory cancer in particular.

More recently, an analysis was conducted using the information available from 25 epidemiology studies (published and unpublished) of workers exposed to AN. The data were analyzed using meta-analysis techniques to assess the findings for 10 cancer sites (Collins and Acquavella, 1998). The predominant focus in the available studies was on worker mortality rates. All but four of the studies assessed were industrial cohort studies, with the remaining four being two nested industrial case-control studies (Marsh, 1983; Ott *et al.*,

1989) and two general population case-control studies (Thomas *et al.*, 1987; Siemiatycki *et al.*, 1994).

As a part of this meta-analysis, various parameters were examined, such as study design, country of study, acrylic fiber plants versus others, publication bias, and other exposures present at the plants involved. Only publication bias, country of study, and other plant exposures showed substantive findings. The analysis presented specific results for total mortality, lung cancer, prostate cancer, bladder cancer, and brain cancer, since these cancer sites were of particular interest given the results of human or animal studies. The average duration of follow-up for the study groups was 30.2 years for cohort mortality studies and 28.6 years for cohort incidence studies. The percentage lost to follow-up ranged from 0 to 12 percent in the cohort mortality studies with a mean of 4%. Losses to follow-up were not reported in the incidence studies. The percentage of death certificates not obtained in these studies ranged from 0 to 6% with a mean of 3%. **Table 4-5** below provides a summary description of the studies considered.

Table 4-5. Summary Description of Studies Included in the Meta-Analysis of AN Exposure (Collins and Acquavella, 1998)

Study	AN Use	Study Design	Study Period	Cohort Size
Keisselbach *et al.* 1977 published	monomer prod.& resin	cohort mortality	1950-77	884
O'Berg, 1980 published	fibers	cohort mortality & incidence	1950-76 (mortality & incidence)	1345
Thiess *et al.* 1980 published	resins	cohort mortality	1955-78	1469
Ott, 1980 published	styrene copolymers	cohort mortality	1950-75	100
Zack, 1980 unpublished	monomer prod. & fibers	cohort mortality	1952-77	352
Werner and Carter, 1981 published	fibers & resins	cohort mortality	1950-78	1111
Herman, 1981 unpublished	nitrile rubber & resins	cohort mortality	1951-77	not reported
Gaffey and Strauss, 1981 unpublished	fibers	cohort mortality	1952-77	1077
Delzell and Monson, 1982 published	nitrile rubbers	cohort mortality	1940-78	327
Marsh, 1983 published	styrene copolymers	nested case-control (digestive & genitourinary)	1949-76	13 cases, 52 control

O'Berg *et al.* 1985 published	fibers	cohort mortality & incidence	1950-81 (mortality), 1980 (incidence)	1345
Burke, 1985b unpublished	monomer prod.	cohort mortality & incidence	1957-80(mortality), 1956-83 incidence	700
Burke, 1985a unpublished	monomer prod.	cohort mortality & incidence	1962-82 (mortality), 1962-83 (incidence)	472
Thomas *et al.*, 1987	plastics and rubber	case-control (brain)	1978-1981	27 cases 43 controls
Chen *et al.*, 1987 published	fibers	cohort mortality & incidence	1957-81 (mortality), 1956-83 (incidence)	1083
Ott *et al.*, 1989	resins	nested case control (lymphoma, multiple myeloma, leukemia)	1940-1978	8 cases
Collins *et al.*, 1989	Fiber, monomer, other	cohort mortality	1950-1981	1774
Zhou and Wang, 1991 published	fibers	cohort mortality	1971-88	1811
Swaen *et al.* 1992 published	fibers & others	cohort mortality	1956-88	2842
Mastrangelo *et al.*, 1993 published	fibers	cohort mortality	1959-90	671
Siemiatycki *et al.*, 1994 published	tailors using acrylic fibers	case-control (bladder)	1979-86	484 cases, 1,879 controls
Wood *et al.*, 1998 published	fibers	cohort mortality & incidence	1947-91 (mortality), 1956-91 (incidence)	2559
Benn and Osborne, 1998 published	fibers & resins	cohort mortality	1950-91	2763
Swaen *et al.*, 1998 published	fibers & others	cohort mortality	1956-96	2842
Blair *et al.*, 1998 published	fibers & others	cohort mortality w/ case cohort	1950-89	25460

The evaluation of heterogeneity was a critical indicator of the factors necessary to make a proper causal inference about AN and cancer. The absence of heterogeneity indicates consistency of results and is important for making generalizations about the meta-relative risk (mRR). The heterogeneity of the data sets was evaluated using both graphical and statistical methods. The relative risk was calculated as an inverse variance weighted average of relative risks from the individual studies. There was considerable heterogeneity in the data, which was in part due to one obvious outlier study, that of Zhou and Wang (1991). This study showed a very high mortality rate. However, it is possible that local mortality variation or absence of the healthy worker effect was responsible for this result, or, more likely, there was a problem with vital status follow-up or non-comparability of the control

group as previously discussed. There was no description of the method of follow-up in this study, and the authors stated that the death information might not be comparable to the national population. Even when this study is omitted from the analysis, there was still considerable heterogeneity in the results for all causes of death ($p = 0.00004$), but not for individual cancer causes of death ($p \geq 0.11$).

Based on the results from the 14 unique study groups, mortality from all causes was about 20% less than general population rates and the results were heterogeneous ($p < 0.00001$). A total of 783 cancer deaths were observed. Cancer mortality rates were less than expected for total cancers (mRR= 0.9, 95% CI= 0.8 to 0.9). No significant increases were noted for the mortality rates by specific cancer type. All specific forms of cancer mortality were at or below expected levels with the exception of bladder cancer (mRR = 1.4, 95% CI = 0.9 to 2.0). All specific causes of cancer death were homogeneous across studies with the exception of colon cancer ($p = 0.00062$). The cancer incidence studies gave similar results to the mortality studies. Most cancer incidence rates were at expected levels with the possible exception of prostate cancer (mRR = 1.4, 95% CI = 0.8 to 2.6). The incidence rates from the three studies for all cancers were homogeneous. The mRRs representing the upper bound of cancer risk that could be detected in the meta-analysis and are presented in **Table 4-6** below.

Table 4-6. Relative Risk Detectable from the Expected Number of Cases in Collins and Acquavella (1998) Meta-Analysis

Cancer mortality	Observed	Expected	RR detectable at 5% significance w/ 80% power
All Cancers	783	922.8	1.083
Stomach	37	48.2	1.38
Colon	55	65.4	1.33
Liver	9	13.8	1.75
Lung	314	336.4	1.14
Prostate	33	32.9	1.47
Bladder	14	8.8	1.95
Brain	58	59.4	1.34
Lymphatic and Hematopoietic	52	68.6	1.32
Hodgkin's Disease	7	9.7	1.9
Leukemia	23	32.6	1.46
Non-Hodgkin's Lymphoma	22	26.3	1.53

For lung cancer mortality, cumulative relative risk by date of the study before 1992 among AN workers was slightly greater than 1.0. This could be a chance finding or reflect an early preference for publication of positive findings. However, after 1992, the cumulative lung cancer rates are at expected levels. The early studies were smaller than the four more recent studies as evidenced by the wide confidence intervals. The 1998 studies of Blair *et al.*, Wood *et al.*, Swaen *et al.*, and Benn and Osborne all have narrow confidence intervals and the SMRs are close to 1.0. The lung cancer mRR for these recent studies is 0.9 (95% CI= 0.9 to 1.1).

Several of the AN studies used in this meta-analysis examined worker mortality rates by level of exposure. It would be useful to separate workers with low and brief exposures from more highly exposed workers to assess the risk of chronic exposure on mortality and cancer risk. However, only seven studies (Thiess *et al.*, Delzell and Monson, Mastrangelo *et al.*, Blair *et al.*, Wood *et al.*, Swaen *et al.*, and Benn and Osborne) examined cancer risk by level of exposure and most of these evaluations are for lung cancer. These seven studies, which present data for highly exposed workers, had null rates for lung cancer overall (mRR = 1.0, 95% CI = 0.9 to 1.1) compared to studies that did not specifically examine workers with higher exposure (mRR = 0.7, 95% CI = 0.4 to 1.4). The highest exposed workers in the seven studies produced a mRR of 1.2 (95% CI = 1.0 to 1.5). None of the studies, however, found a lung cancer mortality trend with exposure level. Three of the studies (Blair *et al.*, Wood *et al.*, and Swaen *et al.*) made semi-quantitative estimates of likely exposure that allowed for the examination of workers with comparable high exposures. These three studies, which estimated the likely exposure, had an mRR for lung cancer of 0.9 (95% CI = 0.8 to 1.0) compared to an mRR of 1.1 (95% CI = 0.9 to 1.4) for the studies that did not include an estimation of likely levels of exposure. On combining the greater than 8 ppm-years category in the Blair *et al.* study, the 10 to 50, 50 to 100, and 100+ ppm-years categories in the Wood *et al.* study, and the 10+ ppm-years category in the Swaen *et al.* study, the mRR for these studies for lung cancer was 1.1 (95% CI= 0.9 to 1.4).

Eight studies considered latency periods of 15 years or longer for lung cancer. Studies that considered latency had an mRR of 1.0 (95% CI= 0.9 to 1.1) compared to an mRR of 0.9 (95% CI = 0.7 to 1.1) for those studies that did not. Only the studies of Blair *et al.* (RR= 1.3, 95% CI = 1.0 to 1.63) and Delzell and Monson (SMR = 1.7, 95% CI = 0.7 to 3.5) give any indication of elevated rates in workers with longer follow-up time. The six other studies report SMRs equal to or less than 1.0 for this category. The mRR for this category is 1.2 (95% CI = 1.0 to 1.4).

As in the previous meta-analysis of Rothman (1994), no excess mortality from all cancer or lung cancer among AN workers was identified. The 1998 Blair *et al.* study has almost five times more person-years of exposure than does the 1998 Wood *et al.* study, but the latter study has considerably more expected deaths from lung cancer than the Blair *et al.* study in

the highest exposure category (46.5 in Wood *et al.* study versus 17.3 in the Blair *et al.* study). It is possible that this difference in the highest exposure category was caused by different methods in conducting the exposure assessment. Also the larger number of expected deaths in the higher exposure categories of the Wood *et al.* study relative to the Blair *et al.* study may have resulted from older workers with longer durations of exposure to higher levels of AN. Therefore, the Wood *et al.* study may provide more information about higher cumulative exposure to AN than the Blair *et al.* study.

There was some indication of excess bladder cancer in three studies (*e.g.*, Kiesselbach *et al.*, 1979, Thiess *et al.*, 1980, and Delzell and Monson, 1982), a finding not reported elsewhere. However, the excess seems to be restricted to plants with potential exposure to aromatic amines and, therefore, seems unlikely to be related to AN exposure. The excess prostate cancer incidence reported by O'Berg *et al.* (1985), Chen *et al.* (1987), and confirmed by Wood *et al.* (1998) has raised concern that exposure to AN may increase prostate cancer incidence risk. However, there was no increase in cancer rates with increasing exposure and this finding has not been seen in the other mortality or incidence studies. Also, the excess of prostate cancer in the Wood *et al.* (1998) study was limited to a narrow reporting period (1978 to 1983), when improved diagnostic procedures were introduced. A deficit was observed (SIR = 0.3, 95% CI = 0.0 to 1.4) from 1983 to 1991. Accordingly, the evidence does not support an association between prostate cancer and AN exposure.

There is little evidence that AN workers have increased cancer rates even though exposures in some groups of workers were at or approach the levels that cause tumors in rats. All cancer sites examined in these workers show null or near null findings when studies are considered together. For lung cancer, Collins and Acquavella (1998) were able to evaluate consistency across studies, strength of the association, and some aspects of internal consistency within studies such as dose-response and latency. The excess risk of lung cancer from AN exposure, if any, is small. For less common cancers such as brain and prostate cancer, the authors were only able to evaluate consistency across studies. They found a relatively imprecise estimate of risk for prostate and brain cancers in AN workers, where AN cannot be completely ruled out as the cause. In the authors' opinion, based on the available studies, a causal relationship between AN exposure and cancer is not supported.

For the purpose of a recent AN risk assessment (EU, 2001), Collins, at the request of the EU authors, re-analyzed the data from the major meta-analysis reported in Collins and Acquavella (1998) after excluding the studies of Kiesselbach *et al.* (1979), and Siemiatycki *et al.* (1994), which were also considered to be outlier studies upon re-consideration. On completing this re-analysis, the only major difference noticed from the original findings of the meta-analyses was that the bladder cancer meta-relative risk (mRR) was reduced overall from 1.4 (95% CI = 0.9 to 2.0) from the 10 studies reporting bladder cancer relative risk in the original report to 1.1 (95% CI = 0.7 to 1.7) in the more recent analysis. Excluding

Kiesselbach *et al.* (1979), and Siemiatycki *et al.* (1994) also reduced the heterogeneity for the bladder cancer results as evidenced by the change in p-values from 0.18 in the original analysis to 0.45 in the later analysis. This finding indicates that the studies of Kiesselbach *et al.* (1979) and Siemiatycki *et al.* (1994) may have had a significant influence on the overall mRR for bladder cancer in the original analysis and so may be outliers as suspected.

4.2 Studies in Animals

4.2.1 Sub-Acute Studies

4.2.1.1 Sub-Acute Oral Studies

Barnes (1970) administered 15 daily oral doses of 30 mg/kg to groups of six rats, followed by seven doses of 50 mg/kg-day and then 13 doses of 75 mg/kg-day over a period of seven weeks. No effects on body weight and no neurotoxic effects (*i.e.*, gait, hindlimb activity, etc.) were observed.

Szabo *et al.* (1984) carried out a number of studies in order to elucidate the sub-acute and chronic actions of AN on the adrenals, stomach, and duodenum of experimental animals by correlation of biochemical, functional, and morphological findings, and to gain insight into AN's mechanisms of action. In the first experiment, AN was given to adult female rats (three to five animals per dose, strain unspecified) in drinking water over 14 days at levels of 0.0% (0 ppm), 0.01% (100 ppm), 0.05% (500 ppm), and 0.2% (2,000 ppm). Water and food intake were monitored continuously and body weights were recorded every four days. A group of pair-fed rats (with food restriction) was also included, with the aim of keeping body weights parallel with the 2,000 ppm dose group. A further group of rats was given 100 mg/kg of AN by gavage twice daily, this dose being thought equivalent to the daily intake of the 2,000 ppm in drinking water group. In the second experiment, AN was given to rats in drinking water at concentrations of 0, 1, 20, 100, and 500 ppm over a period of 60 days. Food and water intake and body weight were monitored as above. Additional groups were administered AN by gavage in distilled water once daily at levels corresponding to the intake of the drinking water animals, namely 0, 0.2 (1 ppm), 4.0 (20 ppm), 20 (100 ppm) or 60 (500 ppm) mg/kg. A third experiment was undertaken to determine if age-dependency was a factor in the sensitivity of the adrenals in rats exposed to AN. Weanling rats with an initial body weight of 35-40 grams and adult rats of 200-210 grams were given water containing 0, 20, 100, or 500 ppm AN or given the corresponding amount of AN by gavage daily for 21 days.

The final experiment was designed to assess the recovery of the adrenals from damage (including production of steroid hormones) and to characterize further the changes in the adrenal ultrastructure noted previously. Groups of young female rats were placed on

drinking water containing 0, 100, or 500 ppm of AN. One week later, one control and two treated groups (*i.e.*, one dosed at 100 ppm and one dosed at 500 ppm) were unilaterally adrenalectomized. Three weeks later the adrenalectomized and certain control and AN-treated groups were sacrificed. The adrenals were rapidly removed, weighed, and processed for electron microscopy. The remaining (non-adrenalectomized) control and experimental groups were kept for 60 days, when the animals were given ACTH. Four hours later, these were sacrificed and trunk blood was collected for plasma corticosterone determination.

In general, no overt signs of intoxication from AN exposure was noted and mortality only occurred in the 2,000 ppm dose group, in which 2/18 rats died from severe bilateral adrenal hemorrhage and necrosis. Decreased water and food intake was observed in both the 2,000 and the 500 ppm drinking water groups and following 100 mg/kg twice daily by gavage.

Adrenal weights were decreased in 7, 14, and 21 day studies in rats receiving 500 and 2,000 ppm AN in drinking water, accompanied in the 2,000 ppm group by polyuria. Pair-fed controls to the 2,000 ppm group also showed a decreased relative adrenal weight, but urinary output was normal. However, animals given the equivalent of 2,000 ppm (100 mg/kg twice daily) by gavage showed an enlargement of the adrenals, again accompanied by polyuria. Following 60 days administration of AN in drinking water, there was also a significant increase in adrenal weight that was particularly prominent in the group given 60 mg/kg-day (equivalent to 500 ppm) daily.

Histological examination of the adrenals from rats administered 500 and 2,000 ppm in drinking water for 7, 14, or 21 days revealed atrophy in the adrenal cortex (especially the zona fasciculata). In contrast, cellular hyperplasia with normal size or slightly shrunken cells was seen in the adrenals from rats given equivalent amounts of AN by gavage and in animals administered 500 ppm in drinking water for 60 days. The results suggest that the effects of AN on the adrenals were in part attributable to its inherent toxicity and the consequences of decreased food and especially water intake (probably due to its unpalatability in drinking water even at 20 ppm). When unpalatability was removed as a factor by gavage dosing, the adrenals responded with hypertrophy and hyperplasia of the cortex.

Plasma levels of corticosterone showed a dose-dependent decrease in rats administered 100, 500 or 2,000 ppm AN in drinking water, with larger decreases being seen when AN was administered by gavage. The decrease noted in the 2,000 ppm group (14 days administration) was even more marked in pair-fed controls. Plasma aldosterone levels were less affected by administration of AN. Effects were only seen at high levels and after prolonged exposure. A significant decrease was observed only after administration by gavage of 60 mg/kg-day for 60 days.

Other effects reported in these studies were increased liver weights following a 21-day administration period, with a decrease being reported after 60 days. Kidneys were enlarged in the 100 ppm group after 60 days of administration and in the 500 ppm group after 21 days. Hyperplasia was observed in regions of the gastric mucosa of rats receiving 100 and 500 ppm AN in drinking water for at least 21 days. Treatment-related effects occurred consistently at the 100 ppm level via drinking water, with 20 ppm representing a NOAEL (equivalent to an intake of 4 mg/kg-d).

Working *et al.* (1987) administered AN via gavage to groups of 10 male rats at levels of 45, 60, 68, 75, and 90 mg/kg-day for five days. Some treated animals died during or immediately after dosing at all dose levels above 60 mg/kg. All rats treated with 60 mg/kg survived the entire 42-day observation period. While no deaths were observed at the 60 mg/kg/day dose level, decreases in body weight were present for three to four weeks after dosing. Four animals receiving 68 mg/kg died on the first day after dosing.

4.2.1.2 Sub-Acute Inhalation Studies

Dudley *et al.* (1942) investigated the effects of daily inhalation exposure to AN in a number of animal species using a wide range of exposure levels and durations. The sex of the animals used in these studies was not specified and control animals were not included in the experiments. These studies also did not include quantitative data or statistical analyses. Target organs identified in the Dudley *et al.* studies, were the nervous system (transitory limb weakness and paralysis in dogs and cats), the kidney (histopathological changes in rats and rabbits), the upper respiratory tract (nasal irritation in all species studied), and the lung (bronchopneumonia in all species except cats).

Two dogs and four rhesus monkeys were exposed for four hours/day, five days/week for four weeks to an average concentration of 56 ppm (126 mg/m^3) of AN in air (Dudley *et al.,* 1942). The four rhesus monkeys survived and showed no evidence of toxicity during the four week exposure period. After the first four hour exposure, one dog died in convulsions while the second dog developed a transient paralysis of the hind legs after the 5th, 13th and 14th exposures. Subsequent exposures were well tolerated.

Sixteen rats, sixteen guinea pigs, three rabbits, and four cats were exposed for four hours/day, five days/week for eight weeks to an average concentration of 100 ppm (225 mg/m^3) of AN in air (Dudley *et al.,* 1942). Exposure to this level caused only a slight lethargy in the rats during exposure. Three of the seven females gave birth to and raised normal litters. Guinea pigs gained weight moderately and also showed a slight lethargy during the exposure. Otherwise no adverse effects were seen. Rabbits survived for the eight weeks, but were drowsy and listless during the exposure and showed no weight gain. Cats showed occasional vomiting, were lethargic, and lost weight. One animal developing transitory weakness of the

hind legs after the 3rd exposure and dying after the 11th exposure. The remaining three cats survived the entire exposure period with few adverse effects. In these experiments there was no evidence of cumulative action of AN.

Sixteen rats (eight adult and eight young animals), sixteen guinea pigs, four rabbits, four cats and two rhesus monkeys, were exposed for four hours/day, five days/week for eight weeks to an average concentration of 153 ppm (344 mg/m^3) of AN in air (Dudley *et al.,* 1942). Rats lost weight, their coats became rough, and their general physical condition was poor. Fifty percent of the animals died during the 3rd and 4th week of exposure. The eight young rats showed definite impairment of growth and marked irritation of the eyes and nose. One of these eight died during the 3rd week of exposure. All of the eight adult rats showed irritation of the eyes and nose. Four died by the end of the 5th week of exposure.

Guinea pigs showed irritation of the eyes and nose and salivation during the 1st week of exposure (Dudley *et al.,* 1942). Three of the 16 animals died during the 5th week of exposure. The remainder of the animals gained weight slightly and were in fair condition at the end of the study. Rabbits showed moderate irritation of the eyes and nose. One of the four animals died during the 5th week of exposure.

Cats were severely affected (Dudley *et al.,* 1942). All showed severe distress with each exposure and were frequently in collapse at the end of the exposure period. They suffered from marked nasal and conjunctival irritation and they all developed transitory weakness of the hind legs. One animal died after the 2nd exposure. Monkeys showed sleepiness, weakness, loss of appetite, and frequent salivation and vomiting. One animal died after six weeks of exposure and the second animal was in complete collapse after each exposure during the last two weeks of the study. Repeated exposure to 153 ppm (344 mg/m^3) of AN in air was definitely toxic to guinea pigs, rats, and rabbits, and were much more toxic to monkeys and cats. These exposures produced irritation of eyes and nose, loss of appetite, gastrointestinal disturbances, and an incapacitating weakness of hind legs that the animals recovered from relatively rapidly. Even with exposure to such high concentrations, no definite evidence of cumulative action was observed.

Paraffin sections were made from the spleen, kidneys, liver, lung, heart, pancreas, lymph nodes, stomach, duodenum, jejunum, ileum, and large intestine, from a representative number of animals in the above studies (Dudley *et al.,* 1942). A total of 680 sections from eighteen rats, six rabbits, six cats, sixteen guinea pigs, and one monkey were examined. A slight amount of hemosiderosis indicative of blood destruction was seen in the spleens of rats. This increased in degree and occurred in a greater number of animals with the higher concentrations. Negligible amounts were noted in the spleens of cats, guinea pigs, and rabbits.

Evidence of renal irritation was noted in most animals exposed. Hyaline casts were present in the straight collecting tubules of all of the animals exposed in the 2nd and 3rd studies, except for one cat and one rabbit receiving 100 ppm (225 mg/m^3), and the monkey exposed to 153 ppm (344 mg/m^3) (Dudley *et al.,* 1942). Subacute interstitial nephritis (*i.e*., focal lymphocyte collections, few polymorphonuclear leukocytes, small fibrotic areas with occasional tubule distension) was found in a significant number of animals, but it was never extensive. The monkey and all the rats exposed to 100 ppm (225 mg/m^3) failed to show these changes. The species difference in relation to kidney effects was not significant, although the guinea pig and rabbit appeared to be the most sensitive. As these animals were symptomatically the least susceptible, the greater renal damage may indicate either more active excretion or a difference in metabolism.

Subacute bronchopneumonia (*i.e*., congestion and edema of the alveolar walls, RBC and serum extravasation into the alveoli, focal lymphocytes and polymorphonuclear leukocytes collections) was present in all but one guinea pig and rabbit, respectively, in the monkey, and about one-third of the rats (Dudley *et al.,* 1942). No pneumonia occurred in cats, but liver damage was observed in cats alone. Weekly blood counts including red blood count, white blood count, hemoglobin, and differential counts were made on four rats and four rabbits during the eight weeks exposure to 153 ppm (344 mg/m^3). The red and white blood counts and hemoglobin determinations remained within normal limits. The differential counts revealed an increase in eosinophils in both rats and rabbits, ranging from no eosinophils at the end of the 1st week to a maximum of 35, 42, 36, and 25% in the rabbits, and from 1% to a maximum of 21% in the rats. The reason for this marked increase in eosinophils is not known.

Hopper *et al.*, (1981) observed that the formation of fibrin networks on the surfaces of stimulated peritoneal macrophages impaired their mobility and that macrophage-associated procoagulant activity (PCA) appeared to promote the formation of these fibrin networks. The Bhooma et al. (1992) study also demonstrated fibrin network formation in the lung following exposure to AN. The elevated macrophage PCA level up to day 14 and the decrease on day 28 shows the dynamic interplay between PCA and fibrinolytic factors. However, it should be noted that, since AN is a known irritant, the high level of exposure in the Bhooma experiment (100 ppm, five hours/d for five continuous days) would be anticipated to cause lung irritation that would be associated with an elevation of the macrophage PCA level.

Gut *et al.* (1985) repeatedly exposed male Wistar rats to AN via inhalation at a concentration of 130 ppm (280mg/m^3) eight hours/d for five days. The goal of this experiment was to study AN's effect on intermediary metabolism. Body weights gradually decreased over the five days of exposure, and inspection of the abdominal cavity revealed a marked decrease of intra-abdominal fat. The weight of the liver decreased, but the weight of the brain did not.

There were no changes in the absolute nor relative weights of the kidneys, lungs, and adrenals. The relative weight of the liver significantly decreased ($P < 0.05$), but that of the brain increased due to the body weight decrease ($P < 0.05$).

Clinical chemistry and biochemical measurements showed a significantly decreased serum concentration of cholesterol and triglycerides ($P < 0.05$), but the liver concentrations of phospholipids and esterified fatty acids were unchanged. The liver microsomal protein and cytochrome P-450 content decreased significantly ($P < 0.001$), while the levels of glucose, lactate, and pyruvate in the blood and brain increased significantly (up to 250% compared to controls). Microscopic examination of the lungs, liver, kidneys, and adrenals showed no histopathological changes, and the numbers and enzyme activities of alveolar macrophages were also unaffected. Based on these results, it was hypothesized that blood glucose could be a good marker as an indicator of exposure to AN in rats. Its relevance to man is uncertain.

Following the observation of Sitrin *et al.* (1983) that there was a close association between acute lung injury and abnormalities of the coagulant system, Bhooma *et al.*, (1992) studied the effect of AN on the PCA of rat lung. Six rats (strain and gender unspecified) were exposed to AN levels of 100 ppm (225 mg/m^3) for five hours/d for five days. The lungs and other organs were removed. The lungs were lavaged six times with 5 ml cold, sterile isotonic saline and the lavage fluid collected. Cells were counted in a hemocytometer chamber and viability was determined. Levels of PCA in macrophages and bronchoalveolar lavage (BAL) fluid were measured on days 1, 3, 5, 7, 14, and 28 days after AN exposure. An increased coagulation capability of alveolar macrophages of the lung detected in this study was indicative of a lung-damaging effect. The elevation of alveolar macrophage PCA occurred from day one to 14 post-exposure. On the 28th day, the levels returned to normal. BAL-PCA increased at this time, possibly due to the release of macrophagic PCA into BAL facilitated by fibrin degradation products.

4.2.1.3 Sub-Acute Studies by Other Routes

Daily subcutaneous (*sc*) administration of 40 mg/kg over four weeks or daily intraperitoneal (*ip*) injections of 20 mg/kg of AN for six weeks were not fatal to rats (Krysiak and Knobloch, 1971). Animals receiving AN at either 40 or 20 mg/kg via *sc* or *ip* injection for four and six weeks, respectively, showed a significant lengthening of the time to perform correctly in a conditioned food reflex test and a significant decrease in the number of correct reactions achieved, compared to the controls or pre-treatment observations. Performance improved when the treatment was discontinued.

Heart and liver weights were significantly increased in adult rats receiving daily *ip* injections of 50 mg/kg of AN over a three week period. Relative spleen and kidneys weights were also

increased. Liver and kidney parenchymal degeneration and vacuolation of neuronal cells of the brain cortex was observed in this experiment (Knobloch *et al.*, 1971).

4.2.2 Subchronic Studies

4.2.2.1 Subchronic Oral Studies

Adult Sprague-Dawley rats received AN in drinking water up to a dose equivalent of 42 mg/kg-day weight for 90 days (Humiston and Frauson, 1975). Reduced water consumption was observed at dose levels above 10 mg/kg-d, while growth retardation occurred at levels of about 22 mg/kg-day and higher in female rats and at 42 mg/kg-day in males. Mean weekly food consumption was reduced for the first seven weeks of the study at a dose level of 38 mg/kg-d. At a dose level of 17 mg/kg-day , it was reduced in the first two weeks. Increased relative liver weight was observed at AN levels of 10 mg/kg-day and higher.

$B_6C_3F_1$ male and female mice were administered AN by gavage at doses of 1.2, 2.4, 4.8, 9.6, and 12.0 mg/kg-day for 13 weeks to set dose levels for a subsequent carcinogenicity study (Serota *et al.*, 1996). A distilled water vehicle control was run concurrently. Parameters used to determine toxicity included survival, clinical observations, body weights, clinical pathology, sperm morphology and vaginal cytology, gross pathology, and organ weights. Ten animals of each sex were assigned to each of the six dose groups (including the controls). Each mouse received an oral gavage dose of vehicle or AN formulation for five days/week for 13 weeks. Additional animals (for a special study) were included in each group (41 to 71 male mice/dose) for the collection of blood and tissue samples to examine AN-associated cellular proliferation, apoptosis, hemoglobin adduct formation, and production of CEO. No treatment-related effects on survival, clinical observations, body weights, clinical pathology, sperm morphology and vaginal cytology, gross or microscopic pathology, or organ weights were observed. Isolated statistically significant findings in several toxicological parameters were noted but considered to be unrelated to treatment. All core animals survived the 13 weeks of treatment. With the exception of a single mouse in the group dosed at 4.8 mg/kg-day that was sacrificed in a moribund condition, all special study males also survived through all scheduled sacrifices. Regarding clinical observations, no treatment-related findings were reported. Alopecia was seen sporadically in several mice, but is a common background finding and was not considered to be treatment-related. Normal body weight gains were achieved except on once due to a lack of water supply overnight.

Biologically significant alterations were not detected in any of the hematological parameters evaluated in mice of either sex. Statistically significant declines in white blood cell (WBC) values were evident in the 2.4 and 9.6 mg/kg-day male treatment groups, but were not believed to be biologically significant since no dose-response relationship was evident. Statistically significant elevations in WBC values occurred in the 4.8 and 12.0 mg/kg-day

female treatment groups, but were also not considered dose-related and were also within the normal range for historical controls. A statistically significant increase occurred in the mean hematocrit (HCT) value in the 9.6 mg/kg-day female treatment group, but was within normal range. Statistically significant declines in lymphocyte counts present in the 1.2, 2.4, and 9.6 mg/kg-day male treatment groups were not believed to be of biological significance since a dose-response relationship was again not identified and the values reported were within normal range. Similarly, significant elevations in lymphocyte counts evident in the 4.8 and 12.0 mg/kg-day female treatment groups and an elevation in neutrophils in the 12.0 mg/kg-day female group were within the normal biological variation range.

No treatment-related gross lesions were noted. Isolated findings of pre-putial gland cysts and enlarged inguinal lymph nodes in males, and ovarian cysts and foci of ovarian tissue in females were considered to be unrelated to the treatment since these lesions are normal findings in mice of this strain and age. A single ovarian tumor (*e.g.*, an ovarian choriocarcinoma, a germ cell tumor with trophoblastic differentiation) occurred in a control mouse in this study. Although these tumors are rare, several have been reported in $B_6C_3F_1$ mice in studies conducted by the National Cancer Institute (NCI) and National Toxicology Program (NTP) (Alison *et al.* 1987).

Histopathological findings in tissues also indicated no treatment-related effects in this 90-day study. In investigations of sperm morphology and vaginal cytology, male mice epididymal sperm motility was significantly decreased at both the 1.2 (lowest) and 12.0 (highest) mg/kg-day dose levels (but not at the intervening dose levels of 2.4, 4.8, and 9.6 mg/kg-d). This change was statistically significant compared to the control animals, but no dose-response relationship existed. No other male or female mouse parameters were significantly affected at any dose level.

Since male and female $B_6C_3F_1$ mice administered AN by gavage, five days/week for 13 weeks, at dose levels up to 12.0 mg/kg-day exhibited no signs of toxicity, the NOAEL for this study was determined to be greater than 12.0 mg/kg-day for mice.

In a follow-up study, male and female B6C3F1 mice (10/sex/dose) received AN (>99% pure) in deionized water by gavage, five days/week for 14 weeks (NTP, 2001). The dose rate administered was 0, 5, 10, 20 , 40 and 60 mg/kg. All male and 9/10 females in the 60 mg/kg died the first day of treatment as did 3/10 of the females in the 40 mg/kg group. Clinical findings included lethargy and abnormal breathing in the 60 mg/kg group. The mean body weight gain of the 20 mg/kg males was less than the controls and the 20 mg/kg females. Leukocyte and lymphocyte counts were decreased in 20 mg/kg males and 40 mg/kg females, and a minimal hemolytic anemia was observed in the 40 mg/kg females. Heart weights of 20 mg/kg males were significantly greater than the controls and left cauda epididymis weights of 10 and 20 mg/kg males were significantly increased as well. The incidences of

chronic active inflammation and hyperplasia of the forestomach of 40 mg/kg females were significantly increased.

Quast *et al.* (1975), administered AN in drinking water at concentrations of 100, 200, or 300 mg/l to groups of four male and four female beagle dogs for 180 days. Average intakes of AN were the following for males: 10 mg/kg body weight at 100 mg/l, 16 mg/kg at 200 mg/l, and 17 mg/kg at 300 mg/l. For females, the corresponding average intakes were 8 mg/kg body weight at 100 mg/l, 17 mg/kg at 200 mg/l, and 18 mg/kg at 300 mg/l. At 100 mg/l, a slight decrease in water and food intake and a slight increase in relative kidney weight was observed. Five dogs died, or were sacrificed because of debilitation, in each of the two higher dosage groups. In the dogs receiving AN at 100 to 300 mg/l in the drinking water, early signs of toxicity included roughening of the coat and, later, retching, and vomiting. Terminal signs of lethargy, weakness, emaciation, and respiratory distress were noted.

4.2.2.2 Subchronic Inhalation Studies

A series of 90 day inhalation studies were carried out in groups of six beagle dogs (three male and three female) exposed to mean atmospheric concentrations of 0, 24 (54 mg/m^3), and 54 ppm (121.5 mg/m^3) AN, and in 40 albino rats and 30 albino CD-1 mice exposed to 0, 24 (54 mg/m^3), 54 ppm (121.5 mg/m^3), and 108 (243 mg/m^3) by Brewer (1976). The exposure regime was six hours/day, five days/week for a total of 57 exposures. A third of the male dogs and two-thirds of the females died at 121.5 mg/m^3. All animals showed decrease in weight gain, but organ weights of the liver, kidney, spleen, adrenal gland, lungs, gonads, thyroid gland, heart, and brain were similar to those of the control animals. Symptoms included rhinitis, ataxia, and increased diuresis. The hematological and clinical chemistry findings were similar to those in controls except for a slight increase in serum alkaline phosphatase. Histopathological examination revealed focal macrophage infiltration, focal fibrosis, and multifocal bronchopneumonia in a third of the males and all of the females. Dogs exposed to 54 mg/m^3 AN showed no mortality, but signs of lung irritation, comprising focal alveolar macrophage infiltration and multifocal bronchopneumonia were observed in two-thirds of female dogs only. Serum alkaline phosphatase was also slightly increased at this dose level. The NOAEL for AN in dogs is below 54 mg/m^3 based on this experiment.

Mortality in mice and rats was unaffected by exposure to 54 mg/m^3 and 121.5 mg/m^3 AN. In this experiment, 5/40 control rats, 5/40 at 54 mg/m^3, 5/40 at 121.5 mg/m^3, and 18/40 at 243 mg/m^3 died during the study, while the comparable figures in mice were 23/30 controls, 21/30 at 54 mg/m^3, 15/30 at 121.5 mg/m^3, and 27/30 at 243 mg/m^3. However, increased lethality was seen in rats exposed to a level of 243 mg/m^3, with some increase in deaths also seen in mice at this level. As in dogs, clinical symptoms included body weight retardation, rhinitis, ataxia, and increased diuresis. Organ weights, hematological, clinical chemistry findings were similar to those in control animals.

Microscopic examinations of brain (*i.e*., cerebrum, cerebellum, and pons), bronchi, small intestine, gonads, gall bladder (dogs only), heart, kidney, liver, lungs, lymph nodes, spleen, trachea, and thyroid were carried out in untreated controls (dogs) and dogs dosed at 54 mg/m^3 and 121.5 mg/m^3, untreated controls (rats and mice), and rats and mice at 243 mg/m^3. Histopathological examination of tissues in dogs revealed treatment-related changes in the lungs of some dogs at dose levels of 54 mg/m^3 and 121.5 mg/m^3. These changes were exposure-related with regard to incidence and relative severity. The changes consisted of focal to multi-focal suppurative bronchopneumonia, and focal aggregates of alveolar macrophages in the alveolar lumina, and considered indicative of mild irritation. There were three mortalities at the 121.5 mg/m^3 dose level. The focal hemorrhages described in the lung of sacrificed animals were agonal lesions related to the method of sacrifice. Any other changes seen in other tissues were lesions of naturally occurring diseases and they were present among both control and test animals. The histopathological examination of the tissues in rats resulted in findings confined to the lung, but only in rats exposed to 243 mg/m^3, which again were indicative of irritation. These changes consisted of a slight to mild increase in the number of alveolar macrophages in the lumina of alveoli and a suppurative bronchopneumonia. No other histopathological alterations were noted among the test mice. Asphyxiation secondary to the sub-acute bronchopneumonia in affected animals was the major cause of death; however, chronic respiratory disease was present in the lung and trachea of all the control and most of the test animals.

The presence of chronic respiratory disease in the rodents and the high mortality in all exposure groups makes the value of these experiments unclear in understanding the toxicity of AN. The results suggest that the main effect of AN inhalation in dogs was irritation of the lungs, to which the females seemed to be more sensitive than males. Dogs were more sensitive to AN than the equally sensitive rats or mice. The subchronic NOAEL is less than 54 mg/m^3 in the most sensitive species (dogs) since lung irritation was present at this dose level. This exposure level may be considered to be an LOAEL.

4.2.3 Chronic Studies

4.2.3.1 Chronic Oral Studies

Adult Sprague-Dawley rats were administered 5 mg/kg AN three times a week via olive oil gavage for 52 weeks (Maltoni *et al.*, 1977). The study used 40 male and 40 female, and 75 male and female controls. The animals were examined weekly and weighed every two weeks during the period of treatment and monthly after treatment was over, until spontaneous death occurred. A complete necropsy was carried out on each animal. Histological examination of the Zymbal's glands, interscapular brown fat, salivary glands, tongue, lungs, liver, kidney, spleen, stomach, different segments of the intestine, bladder, brain, and any other organs with pathological lesions was performed. Under these experimental conditions, AN administered

by gavage did not show effects on the survival and body weight of the test animals. No treatment-related histological changes were observed in liver, kidneys, or lungs.

AN was administered orally via drinking water to groups of 100 male and 100 female Fisher 344 rats at dose levels of 1, 3, 10, 30, and 100 ppm (equivalent to average daily doses of 0.08, 0.25, 0.84, 2.49, and 8.36 mg/kg-day in males and 0.12, 0.36, 1.25, 3.65, and 10.89 mg/kg-day in females, respectively) (Johanssen and Levinskas, 2002; Biodynamics, 1980a). The control group consisted of 200 male and 200 female. Thirty animals/sex/dose group and 60 controls were used for interim sacrifices. Interim necropsies were performed at 6, 12, and 18 months (10/sex/dose group and 20/sex/control). The study was terminated early because of the low survival rate. While this study was performed as a long-term carcinogenicity study on AN, the results of the study are relevant to the chronic toxicity of AN. Effects on survival and body weight were seen at relatively low doses and a NOAEL was established.

During this study animals were observed twice daily for mortality and gross signs of toxicological or pharmacological effects. The general physical observations noted throughout the study were variable in incidence and did not occur in a pattern suggestive of an adverse effect due to treatment. Mortality in the 100 ppm males and females was markedly greater than controls, while mortality in the 10 ppm males and the three and 30 ppm females was somewhat greater than controls. Due to the low survival in the 100 ppm females, all surviving females were sacrificed at 23 months. The males, however, were exposed for an additional three months, when survival in the 100 ppm group reached low levels. All surviving males were then sacrificed.

Body weights for the 100 ppm males and females were consistently lower ($P < 0.01$) than the controls, while body weights for the 30 ppm males only were significantly lower ($P < 0.01$) than the controls. The body weights for the animals in the other treatment groups were generally comparable to controls throughout the study. Food consumption for the 100 ppm females was consistently slightly lower than controls on a g/week basis, while this pattern was notable for the males of this group only after the first year of the study. On a g/kg-d basis, however, food consumption for both 100 ppm males and females was generally comparable to or slightly greater than controls as a result of the lower body weights for these animals. Differences from controls in food consumption for the other groups were sporadic and not indicative of a relationship to treatment. Water consumption for the 100 ppm males and females was generally lower ($P < 0.01$) than controls on a ml-3 d basis; however, on a ml/kg-d basis, differences from the controls were less marked for the females and comparable to or greater than controls for the males. Sporadic differences from controls noted for the other groups were not considered to be treatment related.

An increased incidence of subcutaneous and narcotizing or purulent masses associated with the ear canals (*i.e.*, Zymbal's gland) in male and female rats at 30 and 100 ppm that died or

were sacrificed after 12 months on the study. Other gross lesions occurring in control and treated groups were considered to be incidental findings and not uncommon in rats of similar age and strain. The number of malignant tumor-bearing rats was increased in the male and female rats at 10, 30, and 100 ppm when also compared to control. This was due to an increased incidence of CNS astrocytomas (*i.e.*, brain and spinal cord) and squamous cell carcinomas of the ear canal (*i.e.*, Zymbal's gland) as well as mammary gland carcinomas in the 100 ppm females. The increases in the incidence of neoplasms were noted predominantly in animals dying, sacrificed in a moribund state, or sacrificed at scheduled intervals after the first year of the study. The incidence of neoplasms in the 1 or 3 ppm rats was considered comparable to controls. Other neoplastic and non-neoplastic lesions occurred sporadically in various tissues and organs, but were not considered treatment-related.

Consistent, but not always statistically significant, elevations in the mean relative (to body weight) liver and kidney weights were noted for 100 ppm animals ($P < 0.01$) at most necropsy intervals. The mean relative heart weights were also elevated ($P < 0.05$) for this group at 18 months and termination. The mean absolute weights for these organs were generally comparable to the controls or slightly elevated. The increases in the mean relative weights of the liver, kidney and heart at most necropsy intervals in 100 ppm animals were considered treatment-related effects. In addition, at the terminal sacrifice the mean absolute and relative liver and heart weights were elevated for 30 ppm females ($P < 0.05$). Mean body weight was comparable to controls. Other organ weight differences were noted, but were considered attributable to body weight differences or else they did not occur in a treatment-related pattern. Elevated ($P < 0.05$) mean organ/brain weight ratios were noted for heart and liver in the 30 ppm females at termination. Other differences were sporadic and again not considered treatment-related. For 100 ppm animals, the mean absolute weights of the liver, kidney, and heart as well as the brain were not markedly different from the control animals.

Small but generally consistent reductions in hemoglobin (occasionally achieving statistical significance of $P < 0.05$), hematocrit, and erythrocyte counts were noted for the 100 ppm females throughout the study. These parameters were considered comparable to controls for males at this dose level. Alkaline phosphatase was also slightly elevated in 100 ppm females receiving from 12 months to termination ($P < 0.05$), while values for the males in this group were elevated from 18 months to termination ($P < 0.01$). Slight elevations in alkaline phosphatase activity were also noted in 10 and 30 ppm females at termination only ($P < 0.05$). Increased specific gravity of the urine was noted in 100 ppm males at 18 months and termination.

The clinical observations from this study were variable in incidence and did not occur in a pattern suggestive of treatment-related adverse effects. Most neoplastic and non-neoplastic lesions occurred sporadically in various tissues and organs, but were not considered treatment-related There was an increased incidence of masses in the area of the ear (*i.e.*,

Zymbal's gland) and an increased incidence of astrocytomas of the brain and spinal cord. Other than the increased number of malignant tumor-bearing animals in the groups receiving 10, 30, and 100 ppm, histopathological evaluation revealed no treatment related changes. The majority of non-neoplastic treatment-related effects seen in this study occurred at 10 ppm and upwards. There was an increase in mortality in males at 10 ppm and in females an increase was observed at 3 and 30 ppm. Salsburg (1990) derived a dose-response relationship from the mortality data from the Biodynamics (1980a) study. Since mortality in 3 ppm female rats was somewhat different from controls, a dose of 1 ppm was proposed as the NOAEL, using multivariate statistical procedures. It should be noted, however, that the number of deaths in the 10 ppm females was actually lower than that observed at 3 ppm, and that the statistical significance achieved by the 3 ppm females for this endpoint was likely due to the low mortality in the female controls. Since this increase is not reflected in the 10 ppm dose level, it suggests that a true dose-response relationship has not been established for the 3 ppm level in females. Also the time of death in the female test animals was not significantly different than that of the male test animals. A re-evaluation of this data by EU (2001) concluded that 3 ppm represented a NOAEL in the Biodynamics (1980a) study since there was a lack of a true dose-related trend in females at the lower dose levels. This level in drinking water is equivalent to an average daily dose of 0.25 mg/kg-day in males and 0.36 mg/kg-day in females via the oral exposure in rats.

AN was administered in the drinking water at doses of 0, 1, and, 100 ppm to 100 Sprague-Dawley rats/sex/dose for 19 to 22 months in a second Biodynamics study (Johannsen and Levinskas, 2002b; Biodynamics 1980b). These concentrations corresponded to oral doses of 0.093 and 7.98 for male rats, and 0.146 and 10.69 mg/kg-day for female rats. Interim necropsies were performed at 6, 12, and 18 months (10/sex/group). Rats were sacrificed at 6, 12, 18 months, and at the end of the study. Control and high-dose animals from both studies also received opthalmoscopic, hematological, clinical biochemistry, urinalysis, and full histopathological exams. Similar tests were performed on low dose animals to establish a dose-response relationship for observed effects. All animals were necropsied and underwent microscopic examination of target tissues (*i.e.*, brain, ear canal, stomach, spinal cord, and any observable tissue masses).

High dose animals had significant reductions in body weight, but food and water consumption were reduced. The increased number of deaths among the high dose groups animals prompted the early end of the study after 20 months. The surviving males and females were sacrificed after 22 and 19 months, respectively. Small, sometimes significant, reductions in hemoglobin, hematocrit, and erythrocyte counts were observed in high dose animals. Absolute kidney weights were increased in high dose females.

An increase in the degree of severity of forestomach hyperplasia was observed in all high dose animals. These effects were less pronounced, observed later, and were less correlated

with forestomach tumors than noted in the gavage study (see below). Elevations in epidermal cysts in high dose gavage animals and renal hyperplasia in high dose animals may have been treatment-related. Otherwise all clinical and microscopic findings in these studies were considered unremarkable and within normal bounds. There were no observable non-neoplastic effects attributable to treatment in the low concentration group (1 ppm in water). A NOAEL of 1 ppm in water is suggested.

In a third Biodynamics study, groups of 100 Sprague-Dawley rats of each sex were administered AN doses of 0, 0.1, or 10 mg/kg-day via de-ionized water gavage for five days/week for 19 to 20 months (Johannsen and Levinskas, 2002a/Biodynamics, 1980c). Rats were sacrificed at six, 12, 18 months, and at the end of the study. Control and high-dose animals from also received opthalmoscopic, hematological, clinical biochemistry, urinalysis, and full histopathological exams. Similar tests were performed on low dose animals to establish a dose-response relationship for observed effects. All animals were necropsied and underwent microscopic examination of target tissues (*i.e*., brain, ear canal, stomach, spinal cord, and any observable tissue masses).

High dose animals had significant reductions in body weight, and food and water consumption was not reduced. Small, sometimes significant, reductions in hemoglobin, hematocrit, and erythrocyte counts were observed in high dose animals. There were increases in absolute and relative organ weight ratios for liver and adrenal glands in the high dose gavage animals, but this could not be correlated with AN toxicity since adverse clinical biochemistry and histopathological changes were lacking. Absolute kidney weights were increased in the high animals of the gavage study.

An increase in the degree of severity of forestomach hyperplasia was observed in all high dose animals. Compared to the drinking water study (see above) these effects were more pronounced, observed earlier, and were better correlated to forestomach tumors in the gavage study, in which direct contact occurred to more concentrated AN solutions. Elevations in epidermal cysts in high dose gavage animals and renal hyperplasia in high dose animals may have been treatment-related. Otherwise all clinical and microscopic findings were considered unremarkable and within normal bounds. There were no observable non-neoplastic effects attributable to treatment in the low dose (0.1 mg/kg-d) animals. A NOAEL of 0.1 mg/kg-day is suggested.

Quast *et al.* (1980a) conducted a two-year study in male and female Sprague-Dawley rats (48 rats/sex and 80 controls/sex). Rats were exposed to nominal drinking water concentrations of AN of 0, 35, 85, or 210 ppm for the first 21 days and to levels of 0, 35, 100, or 300 ppm thereafter, until the study's end. The equivalent mean dosages of AN converted to mg/kg-day were estimated to be 3.4, 8.5, and 21.2 in male rats and 4.4, 10.8, and 25.0 in female rats, respectively, based on the assumption that a level of 10 ppm in drinking water is equivalent

to 1 mg/kg, and assuming a drinking water consumption of approximately 10% body weight, with female rats drinking slightly more than males.

The cumulative mortality of females in all treatment groups was statistically significantly higher than their controls. The increased early mortality rate was directly correlated to increasing concentrations of AN in the water. Insufficient information is available in this study to determine the contribution of tumorigenicity to mortality. Mortality occurred on a time scale similar to tumor development, and treatment-related histopathological changes were limited to tumor-bearing tissues. Increased mortality was observed only in males in the 300 ppm group as compared to their respective controls. A NOAEL could not be identified from this study.

During the course of this two-year study, hematology, urinalysis, and clinical chemistry determinations were performed at periodic intervals. The results of these determinations indicated that ingestion of AN did not have an adverse effect on bone marrow, kidney, or liver function in either male or female rats. However, the presence of AN in drinking water resulted in a variety of toxic effects in both male and female rats. There was a dose-related decrease in water and food consumption and a resulting reduced body weight gain in all treatment groups with females more severely affected than males. Clinical observations showed that AN-treated animals were unthrifty at the two highest doses, died earlier compared to controls, and had an earlier onset of tumors, many of which were detectable on external examination and palpation. While these observations were initially noted in the highest dose level rats, the same observations occurred at the lower doses as time went on.

Gross and microscopic examination of tissues revealed various pathologies in treated rats that occurred with statistically significant increased or decreased frequency compared to control animals. Certain non-neoplastic, age-related changes like chronic nephropathy were less frequent in the treated animals compared to controls. This may be due to the early mortality and decreased food and water consumption in treated animals. An increased incidence of endocardial fibrosis was noted only in males at the 300 ppm level.

In this chronic lifetime study, Bigner *et al.* (1986) exposed 600 Fischer 344 rats to AN in drinking water to study the effects of AN on the central nervous system, specifically nervous system cancers. Except for neurological and oncogenic effects, the incidence and severity of other effects are not presented quantitatively. Animals were six weeks old at the start of the study and were randomly assigned to four groups, as follows:

(1) 153 females and 147 males exposed to 500 ppm AN. This group were used to study tumor morphology, biology, and karyotype with complete necropsies performed on all animals that died spontaneously or were sacrificed;

(2) comparative survival and clinical symptomatology studies were made on this group of 50 females and 50 males also exposed to 500 ppm AN;

(3) comparative survival and clinical symptomatology studies were made on this group of 50 female and 50 male rats exposed to 100 ppm AN; and

(4) a control group of 49 females and 51 males that received no AN used in comparative survival and clinical symptomatology studies.

Within three weeks of administration of AN at 500 ppm to male rats, there was a significant decrease in mean weight. Females showed a similar pattern at 500 ppm but took longer for the mean weight to diverge from the controls. Throughout the study, a mean weight difference was observed in both sexes at the 500 ppm dose level. At 100 ppm, the divergence of the mean weight curves from those of the controls began about two months after the start of the test in males but was not apparent in females until well into the second year. A clear-cut dose-response effect in mortality was also observed in both sexes. Females at both 500 and 100 ppm dose levels died slightly earlier than males, whereas only a few controls of either sex died during the first 18 months of the study.

Animals from all groups were observed daily, and in greater detail during weekly weighing, for neurological signs. The neurological effects frequently seen included paralysis, head tilt, circling, and seizures. Other more non-specific signs, sometimes associated with brain tumors but also seen in their absence, included precipitate weight loss and huddling in a corner with decreased activity. The incidence of neurological signs (observed within18 months) was closely related to AN dose. The proportion of animals affected was 20/300 (6.7%) and 16/100 (16%) in the two groups dosed at 500 ppm AN compared to 4/100 (4%) in the 100 ppm dose level group and 0/100 (0%) in the controls. Brains from 215 animals from those exposed to 500 ppm AN were examined. Most of the animals died or were sacrificed between 12 and 18 months after the beginning of the study. In these 215 rats, 49 primary brain tumors were found. Additional tumors found in animals exposed to the highest concentration included Zymbal's gland tumors, lymphoid tumors, and skin papillomas.

Sprague-Dawley derived CD male rats (20 rats/dose) were administered AN in drinking water at levels of 0, 20, 100, and 500 ppm for a two year period (Gallagher *et al.,* 1988). Animals receiving the highest concentration of AN (0.05% or 500 ppm) had accelerated mortality, and the last rats from this group died just before the two-year terminal sacrifice. Survival in the control group and the remaining groups (20 and 100 ppm) was similar.

Animals were weighed at weekly intervals. The average body weight of the controls and the low dose group was virtually identical throughout the study. The animals receiving 100 ppm or 500 ppm of AN showed a slower body weight gain than the controls in the first year of the

study and a greater decrease in body weight gain than the controls during the second year. At intervals of one month, for periods of one week, food and water consumption was measured daily, with mean consumption calculated for each group of animals. No statistically significant differences in food and water intake were observed, but a trend towards decreased water consumption in animals ingesting 500 ppm of AN was reported. A significant increase in Zymbal's gland tumors, an increasing trend for forestomach tumors, and a decreasing trend for pituitary tumors were found. However, no brain tumors were observed. There were no histopathological changes reported in this study that were indicative of chronic toxicity, aside from the neoplastic effects noted.

4.2.3.2 Chronic Inhalation Studies

Male and female rats (30 each, strain unspecified) were exposed via inhalation to 5, 10, 20, and 40 ppm of AN, four hours/day, five days/week for 12 months to assess carcinogenicity (Maltoni *et al.*, 1977). One group of untreated rats acted as a control group for the study. After this period, the animals were given no further exposure to AN and kept under observation until spontaneous death. Slight increases in tumor incidences were observed in the mammary gland (males and females), forestomach (males), and skin (females), but none of these were statistically significant. The results were considered by the authors to indicate a "borderline carcinogenic effect."

No excess in mortality related to AN treatment was observed in any of the animals. A slightly lower survival rate (not significant) was noted in the control male rats. A statistically significant increase in malignant and total number of tumors occurred only in females at 5 ppm ($P < 0.01$). This study provides little information for the assessment of chronic toxicity of AN. No effect on body weight was observed. There was no excess in mortality nor body weight changes and the borderline effects were seen in females only at 5 ppm. This study could be used as an indicator that a NOAEL is between 5 and 10 ppm.

Sprague-Dawley (Spartan sub-strain) rats (100/sex/concentration) were exposed for six hours/day, five days/week for two years, to concentrations of 0, 20, and 80 ppm AN (0, 45, and 180 mg/m^3) (Quast *et al.*, 1980b). The control group was exposed to air alone. Additional animals were included for interim sacrifices at six months (seven/sex/concentration) and 12 months (13/sex/concentration).

Clinical observations detected a variety of adverse effects including body weight decreases, early mortality, unthrifty appearance, earlier onset of tumors, and more frequently observed palpable tumors. These observations were most apparent and occurred earliest in the high dose group (180 mg/m^3). A significant decrease in mean body weight was observed in rats exposed to 180 mg/m^3 AN. Less significant, but similar weight decreases, were also noted in the 45 mg/m^3 females after approximately one month. A treatment-related effect on mean

body weight was not observed in males at 45 mg/m^3. During the first six months, exposed rats drank more water and appeared to excrete lower specific gravity urine than control animals.

In the 180 mg/m^3 group of males, a significantly increased relative organ to body weight ratio was observed for the brain, heart, and testes ($P < 0.05$). However, since body weight of fasted animals was significantly decreased in this group ($P < 0.05$), the relative increase in these organ to body weight ratios may be due to the effect on body weight. In addition, the absolute kidney weight in the 180 mg/m^3 group of males was significantly decreased ($P < 0.05$). This observation was consistent with the decreased body weight and a decrease in the severity of chronic renal disease that was observed grossly and microscopically. The increased relative organ weights were likely a manifestation of the decreased body weight gain and do not indicate a specific toxic effect. In the few surviving females, there was a significantly increased liver to body weight ratio in the 45 mg/m^3 group ($P < 0.05$). The increased liver to body weight ratio and the slight increase in the absolute liver weight in these rats, as well as in the single surviving rat in the 180 mg/m^3 group, were considered to be the result of increased extramedullary hematopoiesis in the liver. This was a result of the greater number of bleeding tumors in these rats and was not indicative of a primary hepatotoxic effect due to AN.

Hematology, urinalysis, and clinical chemistry was performed at periodic intervals during this experiment. AN exposure did not have a primary adverse effect on bone marrow, kidney, or liver function in either male or female rats. Occasional significant reduction of the packed cell volume (PCV), hemoglobin, and in the RBC and WBC counts were noted, but these were interpreted as being secondary changes associated with decreased growth, tumor induction, hemorrhage, generalized stress, and inflammatory reactions resulting from AN exposure.

A statistically significant increase in mortality was observed within the first year in both male and female rats administered 180 mg/m^3 AN and in the females of the 45 mg/m^3 group during the last 10 weeks of the study ($P < 0.05$). The apparent increase in the reported mortality for the 45 mg/m^3 females was principally due to early sacrifice of rats with large, benign mammary gland tumors (Quast et al., 1980a). In Sprague-Dawley rats, the tumors are known to occur spontaneously at a high rate, but in this experiment the tumors were observed earlier and more frequently than in controls, and became larger in exposed animals. Statistically significant early mortality is indicated in both males and females at 180 mg/m^3. However the onset of early mortality begins much earlier into the study for male rats (*i.e.*, days 211-240), compared to females in which a significant increase in mortality was only seen at days 361-390.

Complete histopathological examinations were done on 40 organs of the rats in the control and 180 mg/m^3 groups. At least 23 selected organs were examined in 80% of the rats in the 45 mg/m^3 group. Histopathological examination revealed increased pathological changes in the heart and lungs of male rats of both treatment groups. The changes seen were identical to effects seen in the control animals and are usually associated with chronic renal disease. Microscopic examination of the kidneys indicated a slight, non-statistically significant increase in the incidence of spontaneously occurring advanced chronic renal disease. However, this slight increase could have been due to increased demand on the kidneys, resulting from increased water consumption seen earlier in the study.

A treatment-related increase in extramedullary hemopoiesis in the liver and the spleen, and an increase in focal liver cell necrosis was observed primarily at the 13-18 month and the 19-24 month intervals. Those found in treated rats were generally observed at the earlier time intervals compared to controls. The finding of extramedullary hemopoiesis was considered to be secondary to the presence of large, benign mammary tumors, which also occurred earlier in treated animals than controls (Quast, 2001, personal communication cited in EU, 2001). The presence of these tumors was frequently associated with hemorrhage and tissue damage or pressure necrosis due to contact with the wire mesh cage, the hemorrhage and blood loss in turn resulting in compensatory extramedullary hemopoiesis. The development of large, frequently ulcerated, necrotic, and hemorrhagic ear canal (*i.e.*, Zymbal's gland) tumors in AN treated rats also contributed to this compensatory response. The presence of increased focal hepatic necrosis in these rats was also considered to be a secondary effect due to repeated episodes of blood loss and associated anemia and hypoxia (Quast, 2001, personal communication cited in EU, 2001). It was concluded, therefore, that these findings were not indicative of a primary hepatotoxic effect of AN. This is supported by the fact that the six- and 12-month interim pathology data did not indicate any primary hemopoietic or liver toxicity attributable to AN exposure, nor was any such effect demonstrated in other chronic toxicity studies in rats and dogs exposed to AN by different routes (Quast, 2001, personal communication cited in EU, 2001).

A treatment-related effect was observed in the nasal turbinate mucosa of all rats examined in the180 mg/m^3 group as well as in some of the rats in the 45 mg/m^3 group. At the six- and 12-month interim pathology evaluations, a grading system was used to demonstrate the concentration-related effect in the nasal turbinates. In general, the changes were confined to the respiratory epithelium. These changes were considered minimal in degree and reflected the known irritant effects of AN. The changes in both exposure groups were qualitatively similar but much less severe in the 45 mg/m^3 group than in the 180 mg/m^3 group. In months 19-24, more pronounced changes were observed in males exposed to180 mg/m^3. The changes observed included suppurative rhinitis, hyperplasia in the region of the nasal turbinate mucosa lined by the respiratory epithelium, focal erosion of mucosa lining the respiratory epithelium, and squamous metaplasia of the respiratory epithelium. Similar

occurrences (though fewer in number) were noted for the 45 mg/m^3 males only at the terminal sacrifice. A similar pattern was noted for female test animals. Effects on the nasal turbinate mucosa were first observed at month 19 for the 180 mg/m^3 group, with similar though less frequent effects only being observed at terminal kill in the 45 mg/m^3 group. Some of these changes were also noted in the female control animals either at 19 months or at the terminal kill (no tissues from the male control group were examined for these specific effects). These changes were confined to the turbinate region extending from the external nares into the region lined by respiratory epithelium, and were considered to be a result of irritation due to AN exposure.

In addition, in two of the 180 mg/m^3 female rats, there was a microscopic metaplastic proliferation of the respiratory epithelium. Although the incidence of this lesion was not increased significantly, it was considered treatment-related, in view of its location in the same region of the nasal mucosa showing the degenerative and inflammatory changes and because of the historically low spontaneous incidence of this change. Dose-response data for nasal lesions in rats are summarized in **Table 4-7**.

Table 4-7. Incidence of Nasal Lesions in Rats Following Chronic Exposure to AN via Inhalation (Quast *et al.*, 1980a)

	Hyperplasia of nasal turbinate mucosa		Metaplasia of nasal turbinate mucosa		Hyperplasia of mucous secreting cells	
Concentration (ppm)	Male	Female	Male	Female	Male	Female
0	0/11	0/11	0/11	0/11	0/11	0/11
20	4/12	2/10	1/12	2/10	7/12	2/10
80	10/10	5/10	7/10	5/10	8/10	8/10

In addition to the changes observed in the nasal passages, treatment-related non-neoplastic lesions were also detected in the brain, characterized by focal perivascular cuffing and gliosis. In males at 45 and 180 mg/m^3, the incidence was 2/99 and 7/99 ($p < 0.05$, one-sided), respectively, and, for females exposed to the same concentrations, the incidence was 2/100 and 8/100 ($p < 0.05$, one-sided), respectively.

Quast *et al.*, (1980a) thus demonstrated treatment-related non-neoplastic changes in Sprague-Dawley rats exposed to 45 or 180 mg/m^3 AN six hours/day, five days/week for 104 weeks. These consisted of effects on body weight and early mortality in both sexes in the 180 mg/m^3 group and in females at 45 mg/m^3. As a result of irritation, inflammatory and degenerative changes (hyperplasia and metaplasia of the respiratory epithelium) were present in the nasal

turbinates of both 45 and 180 mg/m^3. A significantly increased number of rats in the 180 mg/m^3 exposure group also showed focal gliosis and perivascular cuffing in the brain.

The key toxicological findings were local irritant effects in the nasal epithelium comprising suppurative rhinitis, hyperplasia, focal erosions, and squamous metaplasia of the respiratory epithelium, with hyperplasia of the mucous secreting cells. Effects were seen at the lowest exposure level (45 mg/m^3) used in the study and represents a LOAEL for this outcome. EU (2001) suggested that, since this effect was due to local irritancy and the other systemic, non-neoplastic findings in treated rats were secondary to the tumorigenic effects of AN, rather than direct systemic toxicity, application of an uncertainty factor of five to the level of 45 mg/m^3 could be used to derive a suggested NOAEL (9 mg/m^3). The suggested NOAEL is supported by the evidence from the study of Sakurai *et al.* (1978) that levels below 3 ppm (6.5 mg/m^3) did not cause notable irritancy in humans.

4.2.4 Carcinogenicity

A number of long-term studies have been carried in rats exposed to AN orally via drinking water and by gavage, and also by inhalation. The animals were shown to develop tumors of the central nervous system, forestomach, intestines, Zymbal's gland (a sebaceous tissue associated with the ear duct of rodent species), and the mammary glands.

4.2.4.1 Oral Carcinogenicity Studies

Maltoni *et al.* (1977) conducted a study to evaluate the effects on adult Sprague-Dawley rats of AN, administered by gavage in olive oil at a single daily dose of 5 mg/kg, three times a week for 52 weeks. The study used 40 male and 40 female treated rats, and 75 male and female controls. The animals were examined weekly and weighed every two weeks during the period of treatment and monthly after treatment was over, until spontaneous death. A complete necropsy was carried out on each animal. Histological examination of the Zymbal's glands, interscapular brown fat, salivary glands, tongue, lungs, liver, kidney, spleen, stomach, different segments of the intestine, bladder, brain, and any other organs with pathological lesions was performed. Under these experimental conditions, AN administered by ingestion did not show effects on the survival and body weight of the test animals. No treatment-related histological changes were observed in liver, kidneys and lung.

In this study, AN did not affect the percentage of animals bearing benign and malignant tumors, the number of animals bearing malignant tumours only, the number of total malignant tumors per 100 animals or the incidence of Zymbal's gland carcinomas, extrahepatic angiosarcomas, hepatomas and encephalic gliomas. The only increase in incidence of tumors were in the mammary gland and forestomach of female rats.

Quast *et al.* (1980b) conducted a two-year study in male and female Sprague-Dawley rats (48 rats/sex and 80 controls/sex). Rats were exposed to nominal concentrations of AN in drinking water at dose levels of 0, 35, 85, or 210 ppm for the first 21 days and thereafter, for the remaining duration of the study, to levels of 0, 35, 100 or 300 ppm. The equivalent mean dosages of AN converted to mg/kg/day were estimated to be 3.4, 8.5 and 21.2 in male rats and 4.4, 10.8 and 25.0 in female rats. This is based on the assumption that a level of 10 ppm in drinking water is equivalent to 1 mg/kg, assuming a drinking water consumption of approximately 10% of body weight, with female rats drinking slightly more than males.

The first death in this study occurred during the 4th month and by the end of the first year losses amounted to 33 (14 males and 19 females). The mortality of females in all treatment groups was considerably higher than their controls. The increased early mortality rate was directly correlated to increasing concentrations of AN in the water. Early mortality was observed only in the 300 ppm group of males when compared to their respective controls. The total number of animals dead or removed from the study prior to the time of necropsy on day 746 was 206 males and 199 females (405 total = 90.4 %).

During the course of this two-year study, hematology, urinalysis, and clinical chemistry determinations were performed at periodic intervals. The results of these determinations indicated that ingestion of AN did not have a primary adverse effect on bone marrow, kidney or liver function in either male or female rats. However, the presence of AN in drinking water resulted in a variety of toxic effects in both male and female rats. There was a dose-related decrease in water and food consumption, and reduced body weight gain in all treatment groups, with females being more severely affected than males. Clinical observations showed that AN-treated animals were unthrifty, exhibited early mortality compared to controls, and had an earlier onset of tumors, many of which were detectable on external examination and palpation. While these observations were initially noted in the highest dose level rats, the same observations occurred at the lower doses as the study progressed.

Gross and microscopic examination of tissues revealed a variety of pathological findings in treated rats which occurred with statistically significant increased or decreased frequency compared to the respective control animals. Certain non-neoplastic age-related changes, for example chronic nephropathy, were less frequent in the treated animals compared to controls. This can be interpreted to be due to the early mortality and decreased food and water consumption in treated animals. An increased incidence of endocardial fibrosis was noted only in males at the 300 ppm level.

Both male and female AN-treated rats exhibited a statistically significant increased incidence of various tumor types. A statistically significant increase was seen in the incidence of tumors of the CNS and ear canal (Zymbal) gland. The occurrence of the various tumor types

(Zymbal's gland, forestomach, tongue, small intestine, mammary gland and multifocal glial cell tumors (astrocytoma)), are summarized in **Table 4-8**. These effects were detected first at the highest dose level (300 ppm) and later in the lower dose groups (100-35 ppm). Tumors of the subcutaneous tissue, mammary region, and pinna of the ear were not significantly different in treated and control rats.

Table 4-8. Tumors Observed in Rats Following Administration of AN in Drinking Water for up to Two Years (Quast *et al.*, 1980b)

Organs affected by tumor	Dose levels showing elevations in tumor incidence
Central nervous system	35, 100 and 300 * ppm (male & female)
Zymbal's gland	35, 100 and 300 * ppm (female) 300 * ppm (male)
Stomach (non-glandular)	35, 100 * and 300 * ppm (male) 100 and 300 * ppm (female)
Tongue	300 * ppm (male and female)
Small intestine	35 and 300 * ppm (male) 100 * and 300 * ppm (female)
Mammary gland:-	
Malignant	300 ppm (female)
Total no. of rats with mammary gland tumor, malignant and benign combined	35 and 300 ppm (female)

*Statistically significant compared to controls ($p < 0.05$)

Of the various organs in male and female rats that exhibited an oncogenic response at all treatment levels of AN, as shown in **Table 4-8**, the central nervous system (brain) appeared to be the most affected. In the intestinal tract of male and female rats the total number of tumors in locations other than the non-glandular gastric mucosa was increased with statistical significance only in the 300 ppm group. Carcinoma of the small intestine was the most frequently observed tumor in male and female rats bearing an intestinal tumor. There were no tumors in the large intestine of female rats and those identified in males were not significantly increased. Tumors of the endocrine glands involving the pituitary, thyroid and adrenals were generally decreased in male and female rats at all treatment levels. Similarly there was a decrease in pancreatic exocrine adenomas in males at 300 ppm and in uterine endometrial polyps in females at all treatment levels, which may partially be due to the earlier mortality in these groups.

Histopathological observations revealed that a significantly increased incidence of CNS tumors, characterized as astrocytomas, was observed in rats in all dose groups. In addition,

a significantly increased incidence of a focal or multifocal glial cell proliferation suggestive of an early tumor of the same cell type was observed in the 35 and 300 ppm groups. In each category of the two identified proliferative changes in the CNS, it was observed most frequently in the cerebral cortex, followed by the brain stem in the region of the cerebellum, and less frequently in the cerebellum and the thoracic spinal cord. In general, the changes of a proliferative type in the cerebral cortex sections were most frequently observed in the section obtained from the middle of the cerebral hemisphere.

Histopathological examination of the tongue showed a statistically significant increase in incidence of squamous cell tumors and for the non-glandular portion of the stomach (forestomach) the increase in incidence was in squamous epithelial tumors. On gross examination there were many rats with multiple papillomas present in this region of the stomach. Upon microscopic examination of these stomach tumors some were found to be papillomas only, others were carcinomas only, and yet other rats had both a papilloma and a carcinoma present. The earliest tumors were papillomas, while later in the study carcinomas were also frequently observed. Stages of the lesion progressed from hyperplasia and hyperkeratosis, to papilloma, and ultimately carcinoma (papillary and ulcerating) formation, with some overlap in the sequence of lesion development. These observations were dose related in severity at the 100 and 300 ppm groups. There were greater numbers of rats with a carcinoma in the stomach at the highest dose level, and they also showed a decreased latency period compared to the lower dose groups. The carcinomas present in the non-glandular stomach were predominantly papillary in type, with only a small proportion of the rats with a carcinoma having the ulcerating type. Only a single ulcerating carcinoma of the non-glandular stomach invaded through the wall of the stomach and extended locally into the mesentery.

In general, rats ingesting the highest dose of AN (300 ppm) showed the earliest onset and greatest number of tumors which infrequently metastasized. Female rats exhibited a slightly greater toxic and tumorigenic response than males, which was concluded to be the result of the higher dose of AN consumed by the females than males.

AN was administered in the drinking water at doses of 0, 1, and 100 ppm to 100 Sprague-Dawley rats/sex/group (Biodynamics, 1980a; Johannsen and Levinskas, 2002a). The water concentrations were designed to approximate the same daily intake of AN used in the gavage portion of the study (see below). The actual intake was calculated to be 0.093 or 7.98 mg/kg/day in males, and 0.146 or 10.69 mg/kg/day in females. Interim necropsies were performed at six, 12, and 18 months (10/sex/group). Control and high-dose animals from both study also received opthalmoscopic, hematological, clinical biochemistry, urinalysis, and full histopathological exams. Similar tests were performed on low dose animals to establish a dose-response relationship for observed effects. All animals were necropsied and

underwent microscopic examination of target tissues (*i.e.*, brain, ear canal, stomach, spinal cord, and any observable tissue masses).

High dose animals in both studies had significant reductions in body weight, and food and water consumption were reduced. The surviving males and females were sacrificed after 22 and 19 months, respectively. Small, sometimes significant, reductions in hemoglobin, hematocrit, and erythrocyte counts were observed in high dose animals. High dose animals had a higher incidence of palpable masses of the head, forestomach, and mammary glands (females only). In both sexes, dose-related CNS, Zymbal's gland, and gastrointestinal tumors were observed in high dose animals. Mammary gland tumors were also observed in high-dose females in the gavage study. Astrocytomas of the brain and spinal cord were found at a higher incidence in the drinking water study than in the bolus gavage dose (see below). On the other hand, a higher rate of squamous cell papillomas and carcinomas of the forestomach, intestinal adenomas, and mammary carcinomas (females only) were observed in the high dose gavage animals.

Groups of male and female Spartan Sprague-Dawley rats (100/sex/dose) were administered lifetime oral doses of AN by bolus gavage dose of either 0.1 or 10 mg/kg-d, seven days/week (Biodynamics, 1980c; Johannsen and Levinskas, 2002a). Exposure groups consisted of 100 rats/sex. Rats from either were sacrificed at six, 12, 18 months, and at the end of the study. Control and high-dose animals from both study also received opthalmoscopic, hematological, clinical biochemistry, urinalysis, and full histopathological exams. Similar tests were performed on low dose animals to establish a dose-response relationship for observed effects. All animals were necropsied and underwent microscopic examination of target tissues (*i.e.*, brain, ear canal, stomach, spinal cord, and any observable tissue masses).

High dose animals had significant reductions in body weight, but food and water consumption were not reduced. The increased number of deaths among the high dose groups in both studies prompted the early end of the gavage study after 20 months. High dose animals had a higher incidence of palpable masses of the head, forestomach, and mammary glands (females only). In both sexes, dose-related CNS, Zymbal's gland, and gastrointestinal tumors were observed in high dose animals. Mammary gland tumors were also observed in high-dose females. Animals treated by gavage had a much higher incidence of AN-related site-specific tumors than did animals in the drinking water study. There were some differences in organ-specific tumor incidences based on dosing regimens. Astrocytomas of the brain and spinal cord were found at a lower incidence in the gavage study than in the drinking water study. On the other hand, a higher rate of squamous cell papillomas and carcinomas of the forestomach, intestinal adenomas, and mammary carcinomas (females only) were observed in the high dose gavage animals.

An increase in the degree of severity of forestomach hyperplasia was observed in all high dose animals, regardless of dosing regimen employed. These effects were more pronounced, observed earlier, and were better correlated to forestomach tumors in the gavage study in which direct tissue contact with a more concentrated AN solution. Elevations in epidermal cysts in high dose gavage animals and renal hyperplasia in high dose animals of both studies may have been treatment-related. Otherwise all clinical and microscopic findings in these studies were considered unremarkable and within normal bounds.

A consistent spectrum of neoplastic and non-neoplastic lesions were produced by AN by either dosing regimen employed in this study. While the types of tumor and target organ toxicities are qualitatively similar for both dosing regimens, gavage dosing clearly increased the tumors associated with the gastrointestinal tract. Neoplasms found in other tissues were more prominent in the continuously dosed rats. Tumor incidence data from both the drinking water and gavage components of this study are summarized in **Table 4-9**.

Table 4-9. Summary of Tumors in Rats Exposed to AN via Drinking Water or Gavage (Johannsen and Levinskas, 2002b)

				Zymbals Gland			Foresomach	
Sex	**Exposure**	**Dose (mg/kg-day)**	**Brain Astro-cytoma**	**Papilloma**	**Adenoma**	**Carcinoma**	**Papilloma**	**Carcinoma**
Male	Gavage	0	2/100	0/96	0/96	1/96	2/99	0/99
		0.1	0/99	0/93	1/93	0/93	6/97	0/97
		10	16/97*	3/96	5/96*	10/96*	22/99*	18/99*
	Drinking water	0	2/98	0/100	0/100	1/100	3/98	0/98
		0.09	3/95	0/91	0/91	0/91	2/98	1/98
		7.98	23/97	0/93	5/93*	14/93*	8/98	4/97
Female	Gavage	0	1/100	0/85	1/85	0/85	2/99	0/99
		0.1	2/98	0/94	0/94	0/94	4/99	0/99
		10	17/100*	1/94	5/94	9/94*	16/99*	1/99
	Drinking water	0	0/99	0/99	1/99	0/99	1/100	0/100
		0.15	1/100	0/95	0/95	0/95	4/99	0/99
		10.7	32/97*	0/98	5/98	7/98*	7/99*	0/99

*significantly different from control incidence

The most informative drinking water study was performed by Biodynamics (1980b; Johannsen and Levinskas, 2002b). AN was administered orally via drinking water to groups of 100 male and 100 female Fisher 344 rats at dose levels of 1, 3, 10, 30, and 100 ppm. These dose levels are estimated to be equivalent to average daily doses of 0.08, 0.25, 0.84, 2.49 and 8.36 mg/kg/day in males and 0.12, 0.36, 1.25, 3.65 and 10.89 mg/kg/day in females respectively. The control group comprised 200 male and 200 female animals. Interim necropsies were performed at six, 12, and 18 months (10/sex/exposed group and 20/sex/control group). The study was originally designed to have a duration of 24 months, however, to ensure at least 10 animals/sex/group for histopathological evaluation at termination, the females were sacrificed early (*i.e.*, 23 months). The males were continued on the study until the 26th month when similar survival levels were reached at which time all remaining animals were sacrificed.

In this study, mortality in the males and females receiving 100 ppm was markedly greater than controls, while mortality in the males receiving 10 ppm and the females receiving 3 and 30 ppm was also significantly greater than control.

Body weights for the males and females receiving 100 ppm were consistently lower ($P < 0.01$) than the controls, while body weights for the males only receiving 30 ppm were significantly lower ($P < 0.01$) than the controls. The body weights for the animals in the other treatment groups were generally comparable to controls throughout the study.

Food consumption for the females at 100 ppm was consistently slightly lower than controls on a grams/week basis, while this pattern was notable for the males of this group only following the first year of the study. On a g/kg-d basis, however, food consumption for both males and females at 100 ppm was considered generally comparable to or slightly greater than controls as a result of the lower body weights for these animals. Differences from controls in food consumption for the other groups were sporadic and not indicative of a relationship to treatment. Water consumption for the males and females at 100 ppm was generally lower ($P < 0.01$) than controls on a ml/day basis; however, on a ml/kg-d basis, differences from the controls were less marked for the females and comparable to or greater than controls for the males. Sporadic differences from controls noted for the other groups were not considered to be treatment related.

A dose-related increased incidence of palpable masses on the head, (*i.e.*, in the area about the ears and eyes and in the cervical region) was noted in the males and females receiving 30 and 100 ppm that died or were sacrificed after 12 months. The masses observed in the area of the ear were characterized as subcutaneous and necrotising or purulent, and were associated with the ear canals. An increased incidence of masses in the mammary region was also noted in females receiving 100 ppm and in males receiving 3 and 10 ppm.

Histopathology showed that the number of malignant tumor-bearing rats was increased in the male and female rats at 10, 30, and 100 ppm, when compared to controls. This was due to an increased incidence of astrocytomas of the central nervous system (brain and/or spinal cord) and squamous cell carcinomas of the Zymbal's gland, as well as mammary gland carcinomas in the female at 100 ppm. The increases in the incidences of these neoplasms were noted predominantly in animals dying, killed in a moribund condition or sacrificed at scheduled intervals after the first year of the study. The incidence of neoplasms in the rats at 1 and 3 ppm was considered comparable to controls. Other neoplastic and non-neoplastic lesions occurred sporadically in various tissues and organs but were not considered attributable to treatment. The number of rats with specific tumors per dose level are summarized in **Table 4-10**.

Table 4-10. Incidence of Tumors Observed in Male and Female Rats Following Administration of AN in Drinking Water for up to Two Years (Biodynamics, 1980b/Johannsen and Levinskas, 2002b)

Sex	Concentration (ppm)	Brain Astrocytoma	Zymbals Gland papilloma, adenoma, carcinoma	Forestomach papilloma, carcinoma
Male	0	2/200	2/189	0/199
	1	2/100	1/97	1/100
	3	1/100	0/93	4/97*
	10	2/100	2/88	4/100*
	30	10/99	7/94*	4/100*
	100	21/99	16/93*	1/101
Female	0	1/199	0/193	1/199
	1	1/100	0/94	1/100
	3	2/201	2/92	2/100
	10	4/95	4/90*	2/97
	30	6/100*	5/94*	4/100*
	100	23/98*	10/86*	2/97

*significantly different from control incidence

It should be noted that the table above reflects the number of animals as per study design. However, in this Biodynamics study, 30 animals were taken out for interim sacrifices, and the actual incidence should, therefore, be related to at least a total population of 70 for treatment groups and 140 for controls.

In general, the physical observations noted throughout this study were variable in incidence and did not occur in a pattern suggestive of adverse effects or toxicity following long-term exposure to AN via drinking water, other than those relating to neoplastic effects. Non-neoplastic lesions occurred sporadically in various tissues and organs, but was not considered attributable to treatment. Therefore, other than the increased number of malignant tumor-bearing animals in the groups receiving 10, 30, and 100 ppm, histopathological evaluation revealed no treatment- related changes. The main tumors observed in rats exposed to AN are microscopic brain tumors and Zymbal's gland tumors. This is a consistent finding in chronic oral and inhalation studies on exposure of rats to AN.

In a chronic lifetime study Bigner *et al.* (1986) exposed 600 Fischer 344 rats to AN in drinking water, the primary aim being to examine the neuro-oncogenic effects of AN on the central nervous system. Other than for neurological and oncogenic effects the incidence and severity of effects is not presented quantitatively in the study report. Animals were six weeks old at the start of the study and were randomly assigned to four groups, as follows:

- Group I contained 153 females and 147 males exposed to 500 ppm AN. The animals from this group were used for studies of tumor morphology, biology and karyotype. Complete necropsies were performed on all animals that died spontaneously or were killed for tumor examination.

- Group II consisted of 50 females and 50 males exposed to 500 ppm AN and comparative survival and clinical symptomology studies were made on this group.

- Group III consisted of 50 female and 50 male rat exposed to 100 ppm AN and was also used to determine comparative survival and clinical symptomology.

- Group IV was the control group, consisting of 49 females and 51 males, which received no AN and was used in comparative survival and clinical symptomology studies.

There was a significant decrease in mean body weight within two to three weeks after the commencement of administration of AN at 500 ppm to male rats. Females showed a similar pattern at 500 ppm but with a slightly longer period before the mean weight clearly diverged from that of the controls. Throughout chronic administration of AN, this mean weight difference was observed in both sexes at the 500 ppm dose level. At 100 ppm (Group III) the divergence of the mean weight curves from those of the controls began about two months after the start of administration in males but was not apparent in females until well into the second year of administration. A clear-cut dose-response effect in mortality was observed in both sexes. Females at both 500 and 100 ppm dose levels died slightly earlier than males, whereas only a few controls (Group IV) of either sex died during the first 18 months of the study. However, it was not determined whether these differential effects between the sexes were due to greater ingestion of AN-containing water or to other sex-related factors.

Animals from all groups were observed daily, and in greater detail during weekly weighing, for neurological signs. The neurological effects frequently seen included paralysis, head tilt, circling, and seizures. Other more non-specific signs, sometimes associated with brain tumors but also seen in their absence, included precipitate weight loss and huddling in a cage corner with decreased activity. The incidence of neurological signs (observed within 12-18 months) was closely related to AN dose. The proportion of animals affected were 20/300 and 16/100 in the two groups (Group I and II) dosed at 500 ppm AN, compared to 4/100 in the 100 ppm dose level group and 0/100 in the controls.

There were no reported histopathological changes due to chronic toxicity reported in this study. The incidences of Zymbal's gland tumors, stomach and skin papillomas and of brain tumors were higher in AN exposed animals than in controls. While an increased incidence of tumors, other than brain tumors is noted, this study specifically deals with the question of biological significance and histogenesis of neuro-oncogenic effects in rats chronically exposed to AN.

A total of 215 brains were examined from the rats (Group I) exposed to 500 ppm AN. Most of these animals died or were killed for tumor examination between 12 and 18 months after the beginning of exposure. Out of these 215 rats, 49 primary brain tumors were found. Tumors were observed in the cortex (approximately 75%), brain stem and cerebellum. When the tumors were differentiated according to size, 10/49 (20%) were found to be larger than 5 mm in greatest diameter, 28/49 (58%) were between 1 and 5 mm in diameter and were detectable by visual examination of a stained slide without a microscope, leaving 11/49 (22%) that could only be detected microscopically.

Despite the variation in their size and regardless of their location in the brain, all 49 primary tumors were remarkably similar in their cellular and architectural features. The lesions were densely cellular in the center with diffusely infiltrative margins. The cells were consistently uniform in size with round or oval nuclei and moderate amounts of pink or clear cytoplasm. Multinucleated giant cells were not seen. Very rarely, tumors contained focal necrosis surrounded by palisading nuclei, but endothelial proliferation was not present in any of the 49 brain tumors. Infiltrating cells at the periphery of lesions often accumulated around small blood vessels, forming perivascular cuffs. Neuronal stellitosis by tumor cells was also observed frequently. Tumor cells gathered in the subpial regions and invaded the ventricles and subarachnoid space in lesions where these spaces were accessible.

The tumors found proved to be similar to, and probably indistinguishable from, a subset of spontaneously occurring rat brain tumors that have been generally classified as astrocytomas or anaplastic astrocytomas by light microscopic evaluation of stained slides. Despite this superficial similarity to astrocytomas, karyotypic analysis did not provide definite evidence to identify any of the neoplastic cells as astrocytic. No glial fibrillary acidic protein (GFAP) was detectable in the tumor cells, despite prominent staining of reactive and normal astrocytes in the same section. Electron microscopy revealed no distinctive intermediate filaments or junctions, nor was there evidence of differentiation of the neoplastic cells. This is in conflict with the hypothesis that the neoplastic cells found in this study are astrocytic in origin.

Regardless of the classification of the primary brain tumors, the occurrence of neurological signs in a dose-related manner and the ability to detect most of the brain tumors macroscopically in the Group I animals (500 ppm) in this study suggest that the lesions are

biologically significant and are capable of causing death. With respect to brain tumor occurrence in long-term toxicity studies in rats, Koestner (1986) noted that some of these microscopic tumors are transplantable, and so it would be prudent to take them into account in an assessment of carcinogenic activity. However, it was stressed that these lesions are difficult to distinguish from reactive gliosis. Final results for tumor incidence in this study were not published.

Gallagher *et al.* (1988) studied the carcinogenic effects in rats resulting from the ingestion of AN in drinking water for a two-year period. Eighty male Sprague-Dawley derived CD rats were divided randomly into four experimental groups (20 rats/group) and were administered AN in their drinking water at levels of 0, 20, 100, and 500 ppm. Animals receiving the highest concentration of AN (0.05% or 500 ppm) had accelerated mortality, and the last rats from this group died just before the two-year terminal sacrifice. Survival in the control group and the remaining groups (20 and 100 ppm) was similar.

Animals were weighed at weekly intervals. The average body weight of the controls and the 20 ppm group was virtually identical throughout the course of this study. The animals receiving 100 ppm or 500 ppm of AN showed a slower body weight gain than the controls in the first year of the study and a greater decrease in body weight gain than the controls during the second year. At intervals of one month, for periods of one week, food and water consumption was measured daily, with mean consumption calculated for each group of animals. No statistically significant differences in food and water intake were observed, but a trend towards decreased water consumption in animals ingesting 500 ppm of AN was noted. There were no histopathological changes reported in this study which were indicative of chronic toxicity, as opposed to the neoplastic effect, following exposure to AN.

The necroscopy results revealed no tumors in the heart, brain, liver, lungs, kidneys, adrenals, or testes of experimental animals or controls, with the exception of a few primary or metastatic tumors as outlined in **Table 4-11**.

Table 4-11. Tumors Observed in Rats Following Administration of AN in Drinking Water for up to Two Years (Gallagher *et al.*, 1988)

Site of tumor	Concentration (ppm)			
	0	20	100	500
Blood	1	0	0	0
Soft tissue	1	1	5	1
Forestomach	0	0	0	4
Zymbal's gland	0	0	1	9
Pituitary	5	3	1	0
Pancreas	1	0	2	0
Kidney	0	0	0	1
Parathyroid	1	1	0	1
Skin	0	0	2	1

[a] = 20 rats per group per concentration of AN

The only conclusive dose-related lesions were those found in the forestomach, pituitary and Zymbal's gland. Papillomatous proliferation of the squamous epithelium of the forestomach was observed in four animals receiving 500 ppm AN. Although one of these pre-neoplastic/neoplastic lesions (from a single animal) showed cytological atypia, invasion of the submucosa by proliferating epithelium was not seen. Zymbal's gland tumors were associated with AN exposure in a dose-related manner in animals exposed to 100 or 500 ppm AN. All of these lesions were centered around the ear canal, and most were locally destructive and histologically poorly-differentiated squamous carcinomas with numerous abnormal mitotic figures. One metastatic lesion which morphologically resembled the primary tumor was observed in the lung. In some cases growth of the tumors restricted mouth opening and contributed to the death of the animal.

Pituitary adenomas were found in five of 18 control animals (28%). The incidence decreased among the animals receiving increasing concentrations of AN. These tumors expanded locally but were not noted to be invasive or metastatic; however, they were a major cause of mortality among the control and low dose groups. Although cytological atypia was often pronounced, in the absence of other features of malignancy, it was clear that these lesions represented benign neoplasms. In some adenomas, multinucleated giant cells were seen. Immunocytological staining revealed the presence of prolactin in the cytoplasm of several scattered adenoma cells. This lower incidence of pituitary tumors in AN-treated rats is interesting. Increased mortality among the high-dose animals possibly contributes to the apparent protective effect noted in that group by prematurely reducing the number of animals at risk. Most of the animals with pituitary adenomas in the other groups died between 16 and 22 months. This, however, together with the dose-response relationship, suggests that the

effects observed were not simply due to attrition among the AN rats. The high-dose rats dying during that period were examined microscopically for tumors, and none were found.

The results of this study revealed that in rats ingesting 0, 20, 100, or 500 ppm AN in drinking water for two years, body weight gain was consistently retarded and mortality was slightly accelerated in the high dose group (500 ppm). There was a dose-dependent increase in the incidence of tumors in the Zymbal's gland. The occurrence of age-dependent pituitary adenomas was, on the other hand, dose-dependently suppressed. Tumors in other systems (*e.g.*, the central nervous system, respiratory tract, or urogenital tract) were not related to chronic exposure to AN. Papillomas of the forestomach were increased, however, indicating a trend towards the development of forestomach papillomas following chronic exposure to AN at the highest concentration (500 ppm), almost certainly due to the irritant action of AN. The overall incidence of tumors in control and treated rats was not however statistically significant.

Groups of male and female B6C3F1 mice (50/sex/dose) were administered AN (>99% pure) in deionized water by gavage at doses of 0, 2.5, 10, or 20 mg/kg, five days/week for 105 weeks (NTP, 2001); Ghanayem *et al.*, 2002). Urine was collected from five mice of each sex at two weeks and 1, 12, and 18 months and analyzed for thiocyanate and N-acetyl-S-(2cyanoethyl)-L-cysteine as markers of exposure.

Survival of the high dose group was significantly less than controls, and mean body weights of high dose animals was generally less than those of the controls throughout most of the study. Dose-related increases in urinary biomarkers occurred in all dosed groups at each sampling. The incidences of mild focal or multifocal epithelial hyperplasia (combined) of the forestomach in high dose mice, mild diffuse or focal hyperkertosis (combined) in high dose males were increased, while Haderian gland hyperplasia in 10 mg/kg males and atrophy and ovarian cysts in 10 and 20 mg/kg females were significantly increased.

The incidences of squamous cell papilloma and carcinoma, and squamous cell papilloma or carcinoma (combined) of the forestomach occurred with a positive trend in males and females, and the incidences in 10 and 20 mg/kg mice were generally significantly greater than controls. The incidences of Haderian gland adenoma and adenoma and carcinoma (combined) occurred with positive trends in males and females and were significantly increased in all dosed groups of males and in 10 and 20 mg/kg females. The incidence of benign or malignant granulosa cell tumor (combined) in the ovary of 10 mg/kg was greater than controls as was the incidence of alveolar/bronchiolar adenoma or carcinoma (combined), but the high dose group did not display an increase in these tumors. Tumor incidence data from this study are summarized in **Table 4-12**.

Table 4-12. Summary of Tumors in Mice Exposed to AN via Oral Gavage for Two Years (Ghanayem *et al.*, 2002)

		Forestomach			Harderian Gland				
Sex	**Dose (mg/kg-day)**	Papilloma	Carcinoma	Combined	Adenoma	Carcinoma	Combined	Lung**	Ovary**
Male	0	3/50	0/50	3/50	5/50	1/50	6/50	--	--
	2.5	4/50	0/50	4/50	16/50*	1/50	16/50*	--	--
	10	19/50*	8/50*	26/50*	24/50*	4/50	27/50*	--	--
	20	25/50*	9/50*	32/50*	27/50*	3/50	30/50*	--	--
Female	0	3/50	0/50	3/50	10/50	1/50	11/50	6/50	0/50
	2.5	6/50	1/50	7/50	10/50	0/50	10/50	6/50	0/50
	10	24/50*	1/50	25/50*	25/50*	3/50	26/50*	14/50*	4/50
	20	19/50*	11/50*	29/50*	23/50*	2/50	25/50*	9/50	1/50

*significantly different from control incidence
**increased judged to be equivocal by study authors (Ghanayem *et al.*, 2002)

NTP concluded that AN was associated with increased forestomach and Harderian gland non-neoplastic lesions and tumors in B6C3F1 mice as well as non-neoplastic ovarian lesions in females, and may have been associated with ovarian and lung neoplasms as well, although lack of a dose-response relationship made this conclusion an equivocal finding.

Although not designed as a carcinogenicity bioassay, a three generation reproductive toxicity test of AN in drinking water study did find a tumor response as well (Friedman and Beliles, 2002). Sprague-Dawley rats were administered AN in drinking water at 0, 100, or 500 ppm (0, 11±5, or 37±10 mg/kg and 0, 20±3 or 40±8 mg/kg for males and females, respectively) and two matings per generation were conducted. Water consumption was reduced in F_0 rats at both AN doses and food intake and body weight gain was reduced in the high dose F_0 rats (these parameters were not investigated subsequently). The possibility that subsequent generations might have higher cancer risks as a consequence of *in utero* or neonatal (*i.e.*, nursing) exposure or genetic damage was explored. There was a dose-related effect of AN on gross masses in female rats at each parental generation held 20 weeks after weaning of the second litter (an exposure period of approximately one year). Histopathological evaluation of these dams also showed an increase in astrocytomas and Zymbal's gland tumors. There was an increase in tumor between F_0 and F_1 and the tumor incidence was significant in the F_1 generation and for the three generations as a whole as shown in the

following table. The F_2 generation, however, had a similar tumor incidence to that of the F_0. Tumor incidence results from this study are summarized in **Table 4-13**.

Table 4-13. Tumor Incidence Results from a Multigeneration Reproduction Study in Rats (Friedman and Beliles, 2002)

	Astrocytoma				Zymbals Gland			
Concentration (ppm)	**F0**	**F1**	**F2**	**Total**	**F0**	**F1**	**F2**	**Total**
0	0/19	0/20	0/20	0/59	0/19	0/20	0/20	0/59
100	1/20	1/19	1/20	3/59	0/20	2/19	0/20	2/59
500	2/25	4/17*	1/20	7/62*	2/24	3/17*	3/20	8/61*

*significantly different from control incidence

4.2.4.2 Inhalation carcinogenicity studies

As with the oral route of administration a number of inhalation studies in rats have been performed in order to evaluate effects caused by long-term exposure to AN, in particular any carcinogenic effects that may result from such exposure. Some of these were previously discussed under chronic toxicity.

Maltoni *et al.* (1977) studied the effects on groups of 30 male and 30 female rats of inhalation exposure to 5, 10, 20, and 40 ppm of AN, four hours daily, five days weekly, for a 12 month period. One group of untreated rats acted as a control group for the study. The animals were kept under observation until spontaneous death. No effect on body weight was noted.

A statistically significant increase in the percentage of animals bearing benign and malignant tumors ($P < 0.01$), malignant tumors alone ($P < 0.01$), and in the number of total malignant tumors per 100 animals was found in several treated groups, although a strong dose-response relationship was not established. No increase in Zymbal's gland tumors, or extrahepatic angiosarcomas and hepatomas was observed. Encephalic gliomas (3/60 and 2/60) were observed in animals exposed to the two highest concentrations of AN. While this finding did not achieve statistical significance, it is biologically significant given that the brain was clearly shown to be the target organ in rats following oral administration.

The long-term inhalation study of Quast *et al.* (1980a) exposed Sprague-Dawley (Spartan substrain) rats (100/sex/concentration) six h/day, five days/week for two years to concentrations of 0 (control), 20 (44 mg/m^3), and 80 (176 mg/m^3) ppm. The control group

was only exposed to air. Additional animals were included for interim sacrifices at six months (n=7/sex/dose) and 12 months (n=13/sex/dose). During the course of this study, hematology, urinalysis, and clinical chemistry determinations were performed at periodic intervals. In this study, clinical observations detected a variety of toxic effects characterized by decreases in body weight, early mortality, unthrifty clinical appearance, earlier onset of tumors, and more frequently observed palpable tumors. These observations were most apparent and occurred earliest in the high dose group (80 ppm).

A statistically significant increase in mortality ($p < 0.05$) was observed within the first year in both male and female rats administered 80 ppm AN and in the females of the 20 ppm group during the last 10 weeks of the study. The apparent increase in the reported mortality for the 20 ppm females was principally due to early sacrifice of rats with large, benign, mammary gland tumors (Quast, *et al.,* 1980a). In the Sprague-Dawley rat, these tumors are known to occur spontaneously at a high rate, but in this experiment the tumors were observed earlier and more frequently, and became larger in exposed animals.

Primary treatment-related effects were observed in the nasal turbinate mucosa of all rats examined in the 80 ppm group as well as in most of the rats in the 20 ppm group. The changes in both groups were qualitatively similar but much less severe in the 20 ppm group than in the 80 ppm group. These changes were confined to the turbinate region extending from the external nares into the region lined by respiratory epithelium. The inflammatory and degenerative changes present in the nasal turbinates were characterized by suppurative rhinitis, hyperplasia, focal erosions, and squamous metaplasia of the respiratory epithelium, with hyperplasia of the mucous secreting cells. These changes were interpreted to be a result of irritation due to AN exposure. In addition, in two of the 80 ppm female rats there was a microscopic metaplastic proliferation of the respiratory epithelium. Although the incidence of this lesion was not statistically significantly increased, it was considered treatment-related in view of its location in the same region of the nasal mucosa showing the degenerative and inflammatory changes and because of the historically low spontaneous incidence of this finding.

Focal perivascular cuffing and gliosis was reported in the brain. In males at 20 and 80 ppm, the incidence was 2/99 and 7/99 ($p < 0.05$, one-sided), respectively, and, for females, the incidence was 2/100 and 8/100 ($p < 0.05$, one-sided), respectively. A treatment-related increase in extramedullary hemopoiesis in the liver and the spleen and an increase in focal liver cell necrosis was observed primarily at the 13-18 month and the 19-24 month intervals, with findings in treated rats generally being observed at the earlier time intervals when compared with the controls. The finding of extramedullary hemopoiesis was considered (Quast, 2001, personal communication cited in EU, 2001) to be secondary to the presence of large, benign mammary tumors and ear canal (Zymbal's gland) tumors in the animals,

which occurred earlier in treated animals than controls. It was concluded, therefore, that these findings were not indicative of a primary hepatotoxic effect of AN.

An increased incidence of brain tumors was observed, although they were rarely the cause of death. Many of the tumors could not be detected by gross pathology, but were identified histopathologically as focal or multifocal glial cell tumors (astrocytomas). The incidence was significantly increased for both male and females at the 80 ppm exposure level compared to the controls. The incidence of proliferative glial cell lesions, suggestive of early tumors, was significantly increased in the 80 ppm males, but not in the females at any dose level. Collectively, proliferative changes in the glial cells (*i.e.*, tumors and early proliferation suggestive of tumors) were significantly increased in the 20 ppm and 80 ppm females, but only in the 80 ppm males, compared to controls.

Recorded deaths were often attributable to severe ulceration of the Zymbal's gland or mammary tissue tumors, and at the highest dose level (80 ppm) were also due to suppurative pneumonia due to the irritant effects of AN on the lungs. The occurrence of Zymbal's gland tumors was observed to be significantly increased in both male and female animals in the 80 ppm group (11/100 in both male and female, $p < 0.05$). For females the highest incidence occurred during the 13 to 18 month interval. An incidence of 3/100 was also seen in males exposed to 20 ppm, compared with 1/100 for control males, but no Zymbal's gland tumors were seen in females at this exposure level. These tumors showed an increased incidence and a decreased latency period that was consistent with the life records of the palpable masses in this region. The type of tumor observed in both males and females was sebaceous squamous cell carcinoma of the external auditory canal gland, without metastasis. The tumor incidence data reported for this study are summarized in **Table 4-14**.

Table 4-14. Summary of Tumors in Rats Exposed to AN via Inhalation (Quast *et al.*, 1980a)

Sex	Concentration (ppm)	Zymbals Gland carcinoma	Tongue papilloma, carcinoma	Mammary Gland fibroadenoma	Small Intestines cystadenocarcinoma	Brain astrocytoma
Male	0	1/100	1/96	--	2/99	0/100
	20	3/100	0/14	--	2/20	4/99
	80	11/100*	7/89*	--	14/98*	15/99*
Female	0	0/100	--	79/100	--	0/100
	20	0/100	--	95/100*	--	4/100*
	80	10/100*	--	75/100	--	17/100*

*significantly different from control incidence

The main tumors observed in rats exposed to AN were microscopic brain tumors and Zymbal's gland tumors. This is again a consistent finding in chronic oral and inhalation studies on AN in rats and is supported in particular by the key study above (Quast *et al.*, 1980a).

4.2.5 Reproductive and Developmental Studies

4.2.5.1 Oral Studies

Oral Reproductive Studies

A three-generation reproduction study in rats dosed with AN was carried out by Litton Bionetics (Beliles *et al.*, 1980/Friedman and Beliles, 2002). Sprague-Dawley rats were administered AN in drinking water at 0, 100, or 500 ppm (0, 11±5, or 37±10 mg/kg and 0, 20±3 or 40±8 mg/kg for males and females, respectively). Water consumption was reduced in F_0 rats at both AN doses and food intake and body weight gain was reduced in the high dose F_0 rats. These parameters were not investigated in subsequent generations.

Groups of 15 male and 30 female rats (post-weanling) comprised the F_0 parental generation. These animals were administered AN for 100 days before mating. During this period, observations were limited to daily clinical observations (emphasis on signs of neurotoxicity), measurement of body weight, and food and water consumption. Limited histopathology was performed on the F_0, F_{1b}, and F_{2b} parents to assess any neoplastic changes. At the end of the 100 day dosing period, 20 females and 10 males were paired for mating over a six day

period, and any females not bred at the end of six days, as evidenced by the absence of vaginal plugs, were mated to another proven male. Exposure to AN continued throughout the mating period and the subsequent gestation and lactation phases in females.

The results of the first and second matings of the F_0 generation were analyzed for the following: 1) male fertility index (#males producing a litter/#mated); 2) female fertility index (#pregnant females/#mated); 3) gestation index (#litters born/#females pregnant); 4) viability index(#live pups at 4 days/#pups born alive); 5) lactation index (#pups weaned/#live pups at 4 days); 6) duration of mating and gestation; 7) pup weight; and 8) live pups per litter.

F_{1a} offspring were examined on post-natal days (PNDs) 0, 4, and 21, with body weights being recorded on day 4 (litter) and day 21 (individual). Litters were reduced to 10 pups per litter on PND 4, with equal numbers of males and females being retained. Although the original study design called for discarding of the F_{1a} pups at weaning, due to pup mortality at the 500 ppm level, surviving pups were retained beyond weaning to assure adequate numbers for subsequent testing.

The female F_0 rats were then re-mated to produce the F_{1b} offspring, the previously unmated F_0 females also being mated at this time in order to ensure sufficient numbers of offspring to be selected for the F_2 generation. Half of these pups were fostered at birth on to untreated females, while at weaning (PND21) one male and female from each un-fostered litter were selected as breeders for the F_2 generation.

F_2 breeders were administered AN in drinking water for 100 days and then mated with production of an F_{2a} and an F_{2b} litter as described for the F_0 generation. Reproductive performance was assessed using the parameters described above for F_0. Due to high pup mortality in the 500 ppm F_{1b} offspring, some F_{1a} animals were used as replacements to ensure a sufficient number of parental animals. Similarly, F_3 breeders were selected from the F_{2b} litters, with additional animals being used from surviving litters in order to achieve required breeding numbers. Following production of the F_{3a} and F_{3b} litters, reproductive performance was assessed using the parameters described above for F_0. Ten weanlings of each sex in the control and 500 ppm dose groups were selected for histopathological examination.

The report did not include results of the clinical investigations, and it is not clear therefore whether any clinical signs of toxicity were observed during the study. The study showed no effect of AN on male or female fertility in the F_1, F_2, or F_3 generations, as assessed by the male or female fertility index. Fertility in some experimental groups was occasionally low (*e.g.*, 50-60% in F_2 generation for controls and the 100 ppm group); however, slightly higher fertility was consistently recorded in the 500 ppm groups. There was also no indication of an embryotoxic effect since the gestation index was similar across all groups and the numbers of live pups per litter was also reasonably consistent across the dose groups.

The viability index was reduced in the 500 ppm litters, with statistical significance being attained in the F_{1a} (94%, $p < 0.05$), F_{1b} (91% $p < 0.05$), and F_{3a} (95%, $p < 0.05$) generations. There was a trend towards reduced viability also at 100 ppm, but it was only statistically significant in the F_{1b} generation (90%, $p < 0.05$). This effect on pup viability was confirmed by reductions in the lactation index at both the 500 ppm (66% in F_{1a}, $p < 0.05$, 88% in F_{1b}, 94% in F_{2a}, $p < 0.05$, and 99% in F_{3a}) and 100 ppm level. Body weights of pups at 500 ppm in all three generations at PND21 were also reduced (mean control pup weight was 10.5 ± 0.55 gm at day 4 for all generations, while mean pup weight at 500 ppm was 9.0 ± 1, $p < 0.05$).

Although pup survival rate at 500 ppm was reduced, fostering of pups to untreated mothers lessened their mortality rate suggesting that the effect was due to maternal toxicity. This was confirmed by findings in the F_0 parental generation, in which AN at 500 ppm caused reduced body weight gain in the first generation parent rats (not investigated in F_2 or F_3 parents). Evidence of a possible neurotoxic effect, as evidenced by abnormal gait was also reported for some rats in the F_0 and F_1 generations. A dose-related tumorigenic effect occurred in female rats held 20 weeks after weaning of the second litter and histopathological examination of these dams showed an increase in astrocytomas and Zymbal's gland tumors. Overall, the results indicate no obvious effect of AN on fertility in the rat at an average level of 552 ppm in drinking water (approximately 35 mg/kg-d). No histopathological examination of gonads in the male rats, other than in the F_{3b} offspring in which no abnormality was reported was conducted. Sperm parameters were also not investigated.

Working *et al.* (1987) conducted a dominant lethal study in groups of 50 male Fisher 344 rats. AN in saline administered by gavage at 60 mg/kg-day for five days. Beginning on day one after dosing, each male was caged with one untreated female weekly for 10 weeks. Females were removed after six days and replaced by a new female one day later. Females were sacrificed 13 days after the end of each respective mating week and examined for numbers of viable fetuses, early fetal deaths (resorptions), late fetal deaths, and corpora lutea. Pre-implantation losses were calculated from the number of corpora lutea minus the total number of implants, while post-implantation losses were considered to be the sum of both early and late fetal deaths.

The results of the study showed that AN was toxic to the male rats under study, as evidenced by significant reductions in body weight during the exposure period. The body weights did not return to normal until five weeks after dosing. However no effects on male fertility were seen, and there was no increase in either pre- or post-implantation loss in the females mated with the treated males indicative of a dominant lethal effect. In respect to possible DNA-damaging effects of AN, Hurtt *et al.* (1987) and Butterworth *et al.* (1992) used an autoradiographic method for determination of unscheduled DNA synthesis (UDS) in the spermatocytes of rats exposed to AN via a single gavage dose of 75 mg/kg or to a repeated

gavage dose of 60 mg/kg-day for five days. There was no significant difference in thymidine incorporation into spermatocyte DNA in treated and control animals, and the authors concluded that UDS in spermatocytes was apparently not induced by AN.

The results of this study suggest that AN may be able to alkylate testicular DNA and induce DNA repair. It should be noted, however, that the dose level used in this study is again very high, and approaches the oral LD_{50} for the rat. It also represents a single bolus dose, as opposed to the three-generation fertility study (Beliles *et al.*, 1980) where *ad lib* administration in drinking water will provide lower plasma levels sustained over a longer period. Additionally, the incorporation of radio-label into DNA in this study is not definitive proof of DNA alkylation since AN reacts preferentially with thiol groupings in proteins to give stable protein adducts. Even slight contamination of isolated DNA with protein may lead to erroneous results in quantifying possible DNA adduct formation.

Tandon *et al.* (1988) reported testicular damage and a decrease in epididymal spermatozoa in CD-1 mice following oral administration of AN at 10 mg/kg-day in saline for 60 days. This dose is approximately 30% of the LD_{50} in mice. The testicular damage consisted of tubular atrophy and degeneration in approximately 40% of seminiferous tubules, with cytolysis and nuclear pyknosis of spermatids, formation of multinucleate giant cells, and interstitial edema. These changes were accompanied by a decrease in testicular sorbitol dehydrogenase (22% decrease, $p < 0.05$) and acid phosphatase (16% decrease, $p < 0.05$), and an increase in lactate dehydrogenase (12% increase, $p < 0.05$) and B-glucuronidase (36.7% increase, $p < 0.05$). Glucose-6-phosphatase was unaffected. These changes were seen in the absence of overt signs of toxicity or any effect on body weight or testicular weight. A dose of 1 mg/kg-day AN did not produce any biochemical changes or histopathological evidence of damage in the testis.

The results of the Tandon *et al.* study indicated that AN may have an effect on the testis in the mouse; however, testicular damage was not observed in the 90-day study in $B_6C_3F_1$ mice administered 12 mg/kg-day (Serota *et al.*, 1996). The significance of the epididymal sperm motility finding in the latter study is unclear, but the absence of a dose-response relationship should be noted.

Abdel Naim and co-workers (Abdel Naim *et al.*, 1994; Abdel Naim, 1995) administered AN by gavage to rats (strain and number unspecified) at dose levels of 11.5, 23, and 46 mg/kg in saline daily over a period of two and four weeks. A dose-dependent decrease in body weight gain and in testicular weight was observed. Decreases in testicular weight were paralleled by decreases in weight of the cauda epididymis and caput epididymis, however, there was no significant effect on the weights of ventral prostate and seminal vesicles.

Histopathological examination of testes in the studies of Abdel Naim *et al.* indicated that spermatogenesis was affected after four weeks treatment with 23 or 46 mg/kg AN, based on a decreased number of spermatocytes and spermatids. Sperm count and sperm motility were significantly decreased at all dose levels, and testicular LDH-X, a marker of pachytene spermatocytes, was inhibited at dose levels of 23 and 46 mg/kg. Flow cytometric analysis of testicular aspirates from rats treated with 46 mg/kg showed a reduction in the proportion of haploid cells (22% reduction after four weeks) and tetraploid cells (65% reduction), while diploid cells were increased (83%). This suggests that repeated administration of AN can produce testicular damage in the rat. The dose levels are very high and approach the acute oral toxicity dose so the effects seen may have been secondary to systemic toxicity. No details on the condition of the animals was provided. The dose level of 11.5 mg/kg-day was a LOAEL in this study, since effects on sperm count and sperm motility were present at this dose level.

A 90-day gavage study in $B_6C_3F_1$ mice administered AN in saline to groups of 10 mice per dose level at dose levels of 0, 1.2, 2.4, 4.8, 9.6, and 12.0 mg/kg-day AN in saline (Serota *et al*, 1996). The study included a reproductive sub-study with evaluation of testicular weights, epididymal weights, epididymal sperm density, motility, and testicular spermatid counts. The left testis and accessory gonads being used in this study while the right testis and accessory gonads were processed for histopathological examination.

All reproductive parameters assessed were unaffected by AN administration with the exception of epididymal sperm motility, which was significantly decreased at both the 1.2 and 12.0 mg/kg dose levels. No effect was detected at the intervening dose levels so a dose-response relationship was lacking. Histopathological examination of the testis in the control and 12 mg/kg-day groups did not reveal any difference between treated and control mice.

Oral Developmental Studies

Murray *et al.* (1978) administered doses of 0, 10, 25, and 65 mg/kg AN in water daily to groups of 29-39 mated Sprague Dawley female rats on days 6-15 of gestation, with daily clinical examination and periodic determination of body weight, and food and water consumption. The animals were sacrificed on day 21 and the numbers and positions of implantation sites, live, dead and re-sorbed fetuses were recorded. All fetuses were examined macroscopically for external abnormalities and cleft plate, one-third were then examined for visceral abnormalities under a dissecting stereo-microscope and the heads were examined by razor-section. All remaining fetuses were examined for skeletal alterations.

Animals receiving 65 mg/kg-day showed hyper-excitability and excessive salivation. Their body weights were significantly decreased compared with controls between days six and nine of the study and between days 10 and 15. Food consumption was decreased in the early

stages of the study while water consumption was increased in the later stages. One dam at this dose level died on day one of the study. Body weight was unaffected by AN administration at the lower dose levels. Thickening of the glandular forestomach was observed in the majority of animals receiving 65 mg/kg-day and in 3/39 animals receiving 25 mg/kg-d. Swollen salivary glands (*e.g.*, sialodacryadenitis) was seen in many animals in the study, including controls.

The incidence of pregnancy was significantly decreased in rats given 65 mg/kg-day (69% compared with 88% in controls, $p < 0.05$) and implantation sites were detected in four apparently non-pregnant dams at this dose level (14%). No effect on the incidence of pregnancy was seen at lower dose levels, and no effect was detected on numbers of implantations per dam, live fetuses per litter or resorptions per litter at any dose level. Fetal body weight was significantly decreased at 65 mg/kg-day (7.4% decrease, $p < 0.05$), indicative of a fetotoxic effect and crown-rump length was also decreased (1.9% decrease, $p < 0.05$).

In fetuses examined for skeletal and visceral abnormalities, short tail occurred significantly more often among the litters of dams given 65 mg/kg-day than in control litters (in 8/212 fetuses examined at 65 mg/kg-d, compared with 1/443 in controls, $p < 0.05$). Short-tailed fetuses also had missing vertebrae, ranging from lack of one lumbar vertebra to lack of all sacral, lumber and most thoracic vertebrae, with associated ribs. Additional malformations in these fetuses included short trunk (3/212 fetuses verus 0/443 in controls) imperforate anus (2/212), right-sided aortic arch (1/212), missing kidney (1/212) and anteriorly-placed ovaries (1/212). There was also an increased incidence of minor skeletal abnormalities in the 65 mg/kg-day offspring compared with controls, these included delayed ossification of sternebrae, split sternebrae, and delayed ossification of cervical vertebrae. At 25 mg/kg-d, no single malformation occurred with an incidence statistically different to that in the controls, although a number of the same malformations seen in the 65 mg/kg-day group also occurred at this dose level.

AN has the potential to interfere with embryonic and fetal development, but apparently only at doses producing significant maternal toxicity. The developmental effects seen in the gavage study (Murray *et al.*, 1978) were only seen at a dose level approaching the LD_{50} in rats, which produced significant maternal toxicity including death of one dam. Interpretation of the results is also clouded by the outbreak of sialodacryadenitis in the study.

Mehrotra *et al.* (1988) investigated the prenatal effects of AN on early morphological and neurobehavioural development in rats. Groups of 30 pregnant Charles-Wistar rats were administered oral doses of 0, 1, or 5 mg/kg-day AN on gestational days five to 21. Dams were weighed daily, and food and water consumption was recorded. At parturition, litters were culled to eight, with equal numbers of males and females. Pups were evaluated post-

partum for morphological development and functional teratology using a screening protocol suggested by Vorhees (1979). On PND 21, the pups were sacrificed and a range of neurochemical analyses were carried out on the brain.

No effect of AN was detected on maternal body weight, length of gestation, numbers of litters, sex within litters, or in pup weight at parturition and post-partum. Nor was any effect detected on post-natal development of neonates and behavior and reflexes appeared normal. However, there were alterations in brain levels of noradrenaline, 5-hydroxytryptamine, and monoamine oxidase, which the authors suggested could be indicative of a derangement in synaptic transmission. The dose levels used in this study were low and the significance of the brain chemistry changes reported is unclear.

4.2.5.2 Inhalation Studies

Inhalation Reproductive Studies

Histopathological examination of the testis and epididymis indicated no treatment-related testicular degeneration in the Quast *et al.* (1980a) carcinogenicity study in Sprague Dawley rats. One hundred rats/sex/concentration were exposed six hours/day, five days/week for two years to concentrations of 0, 20 and 80 ppm AN (0, 45 and 180 mg/m^3). This was equivalent to an oral uptake 0, 4.3 or 17 mg/kg-d. The control group was exposed to air alone. Additional animals were included for interim sacrifices at six months (n = 7/sex/dose) and 12 months (n = 13/sex/dose). Clinical observations detected a variety of toxic effects characterized by decreases in body weight, early mortality, unthrifty clinical appearance, earlier onset of tumors, and more frequently observed palpable tumors. These observations were most apparent and occurred earliest in the high dose group (80 ppm; 180 mg/m^3). The incidence of testicular changes was similar across all groups. The sperm content of the epididymis in treated rats was also comparable to controls.

Inhalation Developmental Studies

Murray *et al.* (1978) exposed groups of 30 pregnant rats to dose levels of 0, 40, or 80 ppm AN by inhalation for six hours/d on gestational days six to 15. The 80 ppm/6-hour exposure was stated to be equivalent to a single gavage administration of 23 mg/kg-d. The experimental parameters assessed were for the gavage study described above. Dams in this study showed little clinical evidence of the toxicity observed in the high dose group in the gavage study, although maternal body weight was significantly decreased in both the 40 ppm and the 80 ppm groups compared with control between days six to nine of the study and between days 10 to 15. Food consumption was decreased in the early stages of the study while water consumption was increased in the later stages.

No effects on incidence of pregnancy, numbers of implantations per dam, live fetuses per litter, resorptions per litter, fetal body weight, or crown-rump length was detected at any dose level when AN was given by inhalation. The incidence of total major malformations was slightly increased (from 8/421 in controls to 11/416 at 80 ppm, p=0.06), however, no single major abnormality occurred at an incidence significantly different than that in the controls. There was a decrease in the incidence of delayed ossification of skull bones at 80 ppm, but not at 40 ppm.

Saillenfait *et al.* (1993) examined the development toxicity of AN as one of a series of eight aliphatic mononitriles. Groups of 20 to 23 previously mated Sprague Dawley rats were exposed to dose levels of 0, 12, 25, 50 or 100 ppm AN by inhalation for six hours/day from gestational day six to 20. Clinical examination was carried out daily and periodic determination of body weight was made. The animals were sacrificed on day 21 and the numbers and positions of implantation sites, live, dead, and resorbed fetuses were recorded. Live fetuses were sexed and examined macroscopically for external abnormalities. One-half of them were then examined microscopically following fixation in Bouins, while the other half were examined for skeletal alterations following clearance and staining with alizarin red S.

Body weights of dams exposed to 25, 50, or 100 ppm AN were significantly depressed compared with control from the commencement of the AN exposure period (13% decrease at 100 ppm, $p < 0.01$, 4.3% at 50 ppm, $p < 0.01$, 1.8% at 25 ppm, $p < 0.01$). No adverse effects on the pregnancy rate, average number of implantations, live fetuses, incidences of non-surviving implants, or resorptions per litter were noted in any of the groups exposed to AN. There was a dose-dependent reduction in fetal weight in the litters from dams exposed to 25, 50, or 100 ppm AN. A 5% decrease was seen at 25 ppm, which reached 13 to 15% at 100 ppm. Evaluation of the incidences of external, visceral, and skeletal variations among fetuses from AN-exposed dams gave no indication of any effect. One fetus from a 25 ppm litter a had missing thoracic center, but there was no other evidence of major malformation in any AN-exposed litter.

The results of this study indicate that, although AN was fetoxic at exposure levels that were also maternally toxic, there was no evidence of a developmental effect. The dose level of 12 ppm represented a No Observed Adverse Effect Level (NOAEL) for the fetotoxic effect.

4.2.5.3 Other Dose Route Studies

Other Dose Route Reproductive Studies

A single dose of AN administered intraperitoneally to AB Jena-Halle and DBA mice at a dose level of 32 mg/kg on days five, seven, or nine of pregnancy caused a significant

decrease in post-implantation losses in the Jena-Halle mouse (46.2% after administration on day five, $p < 0.01$ and 24.4% after administration on day seven, $p < 0.05$, no effect following administration on day nine) (Scheufler, 1980). However, no similar effect was seen in the DBA mouse. Schuefler also examined the effect of repeat dose intraperitoneal administration of AN at levels of up to 26 mg/kg-day on days one to 14 of pregnancy in Jena-Halle mice or levels of up to 16 to 32 mg/kg-d, respectively, on days seven to14 in DBA or C57B1 mice. The author observed no effects in any of these experimental groups.

Other Dose Route Developmental Studies

Willhite *et al.* (1981) administered doses of 4.8, 10, 25, 65, 80, or 120 mg/kg of AN in saline via intraperitoneal injection to pregnant golden hamsters on day eight of gestation. Separate groups of animals received intraperitoneal injections of 1 g/kg sodium thiosulphate 20 minutes before and 80 minutes after administration of AN. Dams were sacrificed on day 14 of gestation and numbers of live fetuses, implantation sites and resorptions were recorded. Fetuses were examined macroscopically and after fixation for evidence of malformations.

No clinical signs of toxicity or developmental effects were seen in the offspring of dams administered up to 65 mg/kg AN. Animals receiving 80 mg/kg showed dyspnea, gasping, uncoordination, hypothermia, salivation, and convulsions one to five hours after the injection. Those administered 120 mg/kg all died. The dose of 80 mg/kg resulted in encephaloceles (7/51 fetuses), rib fusions, and bifurcations in many of the offspring. The percentage of abnormal fetuses was 15.7%, compared with 0.8% in controls. Administration of sodium thiosulphate prevented overt signs of maternal toxicity but developmental effects were still seen in the offspring, indicating that the effects of AN seen in this study may be due to the metabolic release of cyanide.

The study suggests that AN may have developmental effects in the hamster, but once again only at dose levels which are maternally toxic.

Saillenfait *et al.* (1992) cultured 10 day old rat embryos in rat serum for 26 hours in the presence of AN at concentrations of 76 to 760 µM. Survival of embryos was unaffected at any concentration. Normal growth was observed at the lowest concentration tested, 76 µM. However, at higher concentrations, there were decreases in growth parameters such as yolk sac diameter, crown-rump length, head length, and number of somite pairs. At concentrations above 152 µM, there were significant increases in the number of malformations observed. The predominant malformations seen were shortened caudal extremity and a reduction of brain and head length. When a hepatic microsomal preparation (S9) was added, an increase in malformations was observed, suggesting that maternal mono-oxygenase metabolism may contribute to the developmental toxicity of AN. The results of

this study again indicate a potential for AN to interfere with embryonal and fetal development.

4.2.6 Genotoxicity

Data regarding the genotoxicity of AN in humans and human cell lines were summarized earlier in **Section 4.1.1.3**. Data regarding the genotoxicity of AN in animals and nonmammalian cell systems are summarized below. Interpretation of the genotoxicity data available for AN is provided in **Sections 4.3** and **4.4.**

4.2.6.1 DNA Adducts

In Vitro Studies

In vitro studies have reported that AN binds covalently to isolated DNA. An initial binding rate of 1.5 nmol/mg DNA/hour was reported for AN binding to bovine DNA (Peter *et al.*, 1983). However, most of the AN was not bound as adducts, and the rapid saturation of the binding suggests that the binding to contaminating protein contributed to this association. Although AN binding to calf thymus DNA was barely detectable in the absence of metabolic activation, the inclusion of NADPH and a microsomal fraction resulted in measurable adduct formation, which was enhanced using phenobarbital-induced microsomes (Guengerich *et al.*, 1981). In addition, CEO was observed to bind to calf thymus DNA without metabolic activation (Guengerich *et al.*, 1981). A much slower rate of binding for AN to calf thymus DNA, forming cyanoethyl adducts to guanine and thymine and carboxyethyl adducts to adenine and cytosine was reported over a 40-day period using extremely high concentration (1.4 M) which cannot be achieved *in vivo* (Solomon *et al.*, 1984). The formation of 7-(2-cyanoethyl)guanine and O6-2-cyanoethyl)guanine was observed following exposure nucleotide with 0.068 M (Prokopczyk *et al.*, 1988). *In vitro* studies have reported that CEO can bind to DNA at concentrations of 1.4 M (Solomon and Segal, 1989; Solomon *et al.*, 1993). This concentration is approximately 10,000-fold higher than achieved in rat blood following acute exposure to 10 mg/kg AN (Kedderis *et al.*, 1993), making it more than 1,000,000-fold higher than the levels associated with carcinogenic doses in rats (Whysner *et al.*, 1998).

Yates *et al.* (1993) characterized the formation of N3-(2-cyano-2-hydroxyethyl)deoxythymidine and its degradation product following reaction of calf thymus DNA with 0.15 M CEO. CEO was also found to form phosphodiester adducts with nucleotides following exposure to 0.15 M CEO (Yates *et al.*, 1994). These levels are approximately 1,000-fold than achieved in rat blood following acute exposure to 10 mg/kg AN (Kedderis *et al.*, 1993), making it more than 100,000-fold higher than the levels associated with carcinogenic doses in rats.

In Vivo Studies

In rats exposed to radiolabeled AN via ip injection, some of the radiolabel was found to be associated with DNA obtained from liver tissue (Peter *et al.*, 1983). Similarly, in rats exposed to 50 mg/kg AN or 6 mg/kg CEO via ip injection, low levels of DNA adducts (7-OEG) was reported in liver tissue, but not in brain tissue (Hogy and Guengerich, 1986). Other types of DNA or RNA adducts were not detected. In rats exposed to [2,3-14C]AN via inhalation, no radiolabel was detected in an organ, including brain and liver, however, the detection limit for this assay may not have been sufficiently sensitive to detect low level binding (Pilon *et al.*, 1988). Radiolabel was detected in RNA from several tissues, but was highest in the brain. This concentration of radiolabel was reduced by approximately 50% in GSH-depleted rats (Pilon *et al.*, 1988). It is important to note that some adducts, such as the phosphodiester adducts reported in the *in vitro* study (Yates et al., 1994), have not been specifically evaluated in *in vivo* studies.

4.2.6.2 Oxidative DNA Damage

In Vitro Studies

Recent studies have shown that AN induced oxidative stress selectively in rat brain in a dose-responsive manner (Kamendulis *et al.* 1999a). In this study, glial cells and hepatocytes were treated for 1-24 hours with sublethal concentrations of AN. AN induced an increase in the production of 8-hydroxy-2'-deoxyguanosine (8-OH-dG) in glial cells but not in rat hepatocytes. Hydroxyl radical formation following AN treatment was also selectively increased in glial cells. Following 1 and 4 hours of AN exposure, the levels of the non-enzymatic antioxidant glutathione, as well as the activities of the enzymatic antioxidants catalase and superoxide dismutase were significantly decreased in the rat glial cells. Lipid peroxidation and the activity of glutathione peroxidase were not affected by AN treatment in rat glial cells. No changes in any of these biomarkers of oxidative stress were observed in hepatocytes treated with AN. The results of this study indicate that AN selectively induced oxidative stress in rat glial cells.

An increased production 8-oxo-dG was reported in rat glial cells (Murata *et al.* 2001). AN enhanced the formation of 8-oxo-dG induced by hydrogen peroxide and Cu(II), whereas AN itself did not cause DNA damage. The enhancing effect of AN was much more efficient in the double-stranded DNA than that in the single-stranded DNA. Experiments with 32P-labeled DNA revealed that addition of AN enhanced the site-specific DNA damage at guanines, particularly at 5'-site of the GG and GGG sequences. An electron spin resonance spectroscopy showed that a nitrogen-centered radical was generated from AN in the presence of hydrogen peroxide and Cu(II).

In Vivo Studies

A study was conducted to examine the ability of AN to induce oxidative stress in male Sprague-Dawley rats (Jiang *et al.*, 1998). Rats were administered AN at concentrations of 0, 5, 50, 100, or 200 ppm in the drinking water and sampled after 14, 28, or 90 days of continuous treatment. Increased formation of 8-oxo-dG was observed in rats exposed to 100 and 200 ppm after 14 days of treatment. Rats receiving 50 ppm AN showed elevated levels of 8-oxo-dG in the brain after 28 and 90 days. No statistically significant increase in 8-oxo-dG was observed in rats exposed to 5 ppm. Decreased levels of GSH and activities of catalase and superoxide dismutase were also observed in the brains of AN-treated rats compared to the control group. There were no changes of these indicators of oxidative stress in the livers of AN-treated rats. These results indicate that AN selectively induces oxidative stress in rat brain at doses that produce brain tumors in chronic studies.

Male Sprague-Dawley rats were exposed to 0, 3, 30, and 300 ppm AN in drinking water for 21 days, a range that includes doses associated with brain tumorigenesis (Whysner *et al.*, 1998a). In the 30 and 300 ppm AN groups, 8-oxo-dG levels were two fold greater than in the controls. Levels of 8-oxo-dG in brain nuclear DNA of rats exposed to 3 ppm were not significantly different from controls. Measures of glutathione levels, glutathione peroxidase and catalase were not significantly changed, but cysteine was somewhat increased. No changes were found in brain cytochrome oxidase activity, which indicates a lack of metabolic hypoxia. Also, no effects on thiobarbituric acid reactive substances were found, indicating a lack of lipid peroxidation. In an additional experiment, male Sprague-Dawley rats were exposed to 0 or 100 ppm AN in drinking water for 94 days; interim sacrifices were conducted at three, 10, and 31 days. Levels of liver nuclear DNA 8-oxo-dG were significantly increased in rats exposed to 10 days, but not in rats exposed to 3 or 94 days. These studies suggest the possibility that AN-induced tumors may be produced by a mode of action involving 8-oxo-dG.

In an unpublished study, rats were exposed to 3 to 500 ppm AN in drinking water for 105 days (Walker, 1994; personal communication). DNA from liver, spleen, stomach, and brain were analyzed for the presence of 7-(2-oxoethyl)-guanine (7-OEG) adducts. No accumulation of these adducts were in all tissues except for liver, in which very low levels (3 pmol/mg DNA) were observed. The levels of adducts found in the liver were much lower than observed in the livers from rats exposed to vinyl chloride (positive control).

4.2.6.3 DNA Strand Breaks

In Vitro Studies

In vitro studies indicate that CEO can produce strand breaks in purified DNA and in mammalian cells. Using purified DNA, strand breaks were not observed with AN, but were observed with CEO (Peter *et al.*, 1983). Similarly, single and double-stranded breaks were observed in plasmid DNA exposed to CEO concentrations greater than 50 mM (Yates *et al.*, 1994). In rodent cell lines, AN induced single-strand DNA breaks in F344 rat hepatocytes (Bradley, 1985), hamster embryo and Chinese hamster ovary (CHO) cells (Parent and Casto, 1979; Douglas *et al.*, 1985). In contrast, Lathanisky and Hendriks (1985) did not demonstrate DNA single-strand breaks in CHO cells. AN produced strand breaks in human bronchus epithelial cells at concentrations of 200 and 500 ug/mL (Chang *et al.*, 1990).

In Vivo Studies

In *in vivo* studies in mice, DNA strand breaks were not induced in bone marrow following exposure to 20 mg/kg AN i.p. (Hachiya *et al.*, 1984; 1986).

4.2.6.4 Unscheduled DNA Synthesis

In Vitro Studies

In vitro studies are mixed for AN in producing unscheduled DNA synthesis (UDS). An increase in UDS was reported in cultured human lymphocytes exposed to AN at high concentrations (0.5 M) (Perocco *et al.*, 1982). UDS was not observed in rat hepatocytes exposed to AN (Williams *et al.*, 1985; Probst and Hill, 1985, or in HeLa cells exposed to AN with or without metabolic activation (Martin and Campbell, 1985). Concentrations of 1 mM AN or 0.1 mM CEO did not result in increased UDS in rat hepatocytes (Butterworth *et al.*, 1992). Although AN was negative for UDS in cultured human mammary epithelial cells, CEO produced a positive response.

In Vivo Studies

In rats exposed to a single oral dose of 50 mg/kg AN UDS was increased three-fold in liver tissue but was unchanged in brain (Hogy and Guengerich, 1986). Conversely, replicative DNA synthesis was decreased in brain but not liver. Following exposure to a single oral dose of 46.5 mg/kg AN, UDS was increased in rats in the testes and stomach (Abdel-Rahman *et al.*, 1994; Ahmed *et al.*, 1992a,b,1996). UDS in the stomach was inhibited by pretreatment of rats with SKF 525-A, indicating a role for oxidative metabolism (CEO formation) in UDS (Ahmed *et al.*, 1996).

4.2.6.5 Mutations

In Vitro Studies

In bacterial test systems, AN was consistently mutagenic to *Salmonella typhimurium* TA1530 and TA1535 as long as a metabolic activation system (S9) was used, mixed results with in TA100 with S9, and negative results in TA98, TA102, TA1537, and TA1538, and generally negative results in all strains in the absence of metabolic activation (IARC, 1999). Positive results for AN mutagenicity were reported in several strains of *S. typhimurium* (Milvy and Wolf, 1977). In 23 strains of *S. typhimurium*, the results for AN were largely negative (Venitt *et al.*, 1985). The general requirement for metabolic activation to yield a positive response indicates a role for oxidative metabolites in the mutagenic responses observed.

Unlike *S. typhimurium*, AN tested positive in three out of four strains of *Escherichia coli* (Venitt *et al.*, 1977). Variable results were reported for the mutagenic effects of AN on fungi (*Saccharomyces cerevisiae*). Both positive and negative results have been obtained for gene conversion, forward and reverse mutation, and aneuploidy with and without metabolic activation using this test system. AN also did not induce sex-linked recessive mutations or genetic crossing over in *Drosophila melanogaster* (Vogel, 1985; Wuergler *et al.*, 1985; Foureman *et al.*, 1994).

In mammalian test systems, positive results for AN-induced gene mutation in mouse L5178Y lymphoma cells (with and without activation) have been reported (Brooks *et al.*, 1985; Myhr *et al.*, 1985; Amacher and Turner, 1985; Lee and Webber, 1985; Garner and Campbell, 1985; Rudd, 1983; Oberly *et al.*, 1985; Matthews *et al.*, 1985). In contrast, Garner and Campbell (1985) obtained negative results when testing mouse lymphoma (oubain resistant) cells for gene mutation. Styles and Clay (1985) obtained negative results when testing AN in the mouse L5178Y lymphoma cells while Anderson and Cross (1985) obtained negative results for AN in mouse P388F lymphoma cells without activation and positive results with activation.

In human lymphoblastoid cells, AN produced a positive mutagenic response in the presence (3.5-fold increase) and absence (2-fold increase) of metabolic activation (Crespi *et al.*, 1985). In the absence of S9, the increased mutational frequency was associated with marked cytotoxicity at this exposure level (18% survival). Recio and Skopec (1988) reported that AN produces a positive mutagenic response but required metabolic activation. CEO, on the other hand, did not require activation to increase mutation frequency. The authors concluded that CEO was 15-fold more potent that AN in producing mutations, and that the amount of CEO produced by S9 could account for the positive response for AN.

In Vivo Studies

The capacity of AN to produce dominant lethal mutations in somatic and germ cells of male NMRI mice was investigated (Leonard *et al.*, 1981). Bone marrow cells were evaluated six, 18, 24, 48 and 72 hours after a single ip injection of 20 or 30 mg/kg AN. The dominant lethal test yielded negative results for AN.

In this study, the ability of AN to induce dominant lethal mutations in the germ cells of male Fischer 344 rats was evaluated. Three groups of 50 males were gavaged daily for five days with AN (60 mg/kg in normal saline) or the vehicle only. Starting one day after exposure, each male was bred to one female per week for 10 weeks. AN treatment of male rats induced no increases in dominant lethal mutations.

A single study reported a significant increase in the frequency of HPRT mutations in splenic T-cells of mice exposed to 20 mg/kg AN, but no increase in P4502E1 null mice exposed to the same dose (Walker and Ghanayem, 2003). Negative results for lacZ mutations were reported for a number of tissue sites from the same animals, including bone marrow, lung, splenic lymphocytes, male germ cells, and brain (data unpublished).

4.2.6.6 Chromosomal Endpoints

In Vitro Studies

In vitro test systems using mammalian cell lines (*i.e.*, rat liver and CHO cells) exposed to AN gave negative results for SCE without metabolic activation and positive results with activation (Priston and Dean, 1985; VedBrat and Williams, 1982; Nataragan *et al.*, 1985). Chromosomal aberrations were induced *in vivo* in Chinese hamster lung, liver, and ovary cells in the absence of metabolic activation (Sasaki *et al.*, 1980; Ishidate *et al.*, 1981; Danford, 1985; Ishidate and Sofuni, 1985; Natarajan *et al.*, 1985; Priston and Dean, 1985).

In Vivo Studies

In laboratory animals, chromosomal aberrations were not induced in mouse bone marrow or rat cells exposed to AN *in vivo* (Leonard *et al.*, 1984; Rabello-Gay, 1980; Ahmed, 1980; Leonard *et al.*, 1981; Zhurkov *et al.*, 1983). Sharief *et al.* (1986) reported weakly positive results for sister chromatid exchange (SCE) in mouse bone marrow.

4.2.6.7 Cell Transformation

Exposure to AN has resulted in cell transformations in Syrian hamster embryo cells (Sanner and Rividal, 1985; Parent and Casto, 1979; Barrett and Lamb, 1985), as well as the

C3H/10T1/2 and NIH/3T3 mouse cell systems (Banerjee and Segal, 1986). Matthews *et al.* (1985) and Lawrence and McGregor (1985) obtained negative results for this endpoint in the absence of activation and positive results with activation in BALB/C-3T3 and C3H/10T1/2 mouse cell systems.

More recently, the ability of AN to induce morphological transformation and oxidative damage was examined in SHE cells (Zhang *et al.*, 2000). AN induced an increase in morphological transformation at doses of 50, 62.5, and 75 microg/ml (maximum sub-toxic dose tested) following seven days of continuous treatment. However, SHE cells exposed to AN for 24 hours failed to increase morphological transformation. Morphological transformation by AN was inhibited by co-treatment with the antioxidants. Treatment of SHE cells with 75 microg/ml AN produced a significant increase in 8-oxo-dG that was also inhibited by co-treatment with antioxidants. These results support the proposal that oxidative stress and the resulting oxidative damage is involved in AN-induced carcinogenicity.

An *in vitro* study was conducted to examine the effects of AN on enzymatic and nonenzymatic antioxidants in SHE cells (Zhang *et al.*, 2002). SHE cells were treated with subcytolethal doses of AN (0, 25, 50, and 75 microg/ml) for four, 24, and 48 hours. AN (50 microg/ml and 75 microg/ml) increased the amount of reactive oxygen species in SHE cells at all time points. GSH was depleted and catalase and superoxide dismutase activities were significantly decreased in SHE cells after four hours of treatment. The inhibition of these antioxidants was temporal, returning to control values or higher after 24 and 48 hours. Xanthine oxidase activity was increased following 24 and 48 hours treatment with AN. 1-Aminobenzotriazole (a suicidal P450 enzyme inhibitor) attenuated the effects of AN on catalase and xanthine oxidase in SHE cells, suggesting that P450 metabolism is required for AN to produce its effects on these enzymes. Additional studies showed that in the absence of metabolic sources AN had no effect on either catalase or superoxide dismutase activity. These results suggest that the induction of oxidative stress by AN involves a temporal decrease in antioxidants and increase in xanthine oxidase activity that is mediated by oxidative metabolism of AN.

4.2.7 Other Studies

4.2.7.1 Acute Toxicity

The clinical signs resulting from acute AN administration have been examined in a number of different species and have been to found to vary little between species. The clinical signs following acute exposure to AN has been divided into four stages (Nerland *et al.* 1989), based on the work of other investigators. Immediately after administration, the animal goes through an excitatory phase; the eyes water, and the animal becomes agitated. A tranquil phase follows and cholinergic symptoms, such as salivation, lachrymation, urination, and

defecation occur, and may be a true cholinergic response since atropine was reported to block the effect. Application of 10-20 ng of AN to an isolated guinea pig ileum caused vigorous contractions that were blocked by atropine (Abreu and Ahmed, 1980). Next, there is a convulsive phase in which the animal undergoes clonic seizures. The terminal stage preceding death is a paralytic phase in which the animal is immobile. These clinical signs indicate that the action of AN is that of a typical nitrile, with toxic action probably due to a cleavage of the molecule to produce hydrogen cyanide, which is one of the key mediators of the toxicity. However, as for any nitrile, there is a complex interplay of a number of factors that affect the outcome of AN toxicity. These include the rate of cyanide liberation and detoxification, the dose of cyanogen, the route of administration, the species of animal, and the presence of other bioreactive sites within the molecule (Nerland *et al.* 1989).

Lethality

With regard to the acute lethality of AN in animals, dogs appeared to be the most sensitive species following exposure via inhalation. However, as outlined previously, the acute toxicity of AN is for the greater part caused by the release of cyanide, to which dogs are much more sensitive. Dogs are more susceptible to the toxicity of cyanide because they have lower levels of the detoxifying enzyme rhodanase and/or sulfane sulfur in the liver than other mammals.

Reported oral LD_{50} values for AN for various species lie in the range of 25 to 186 mg/kg (BUA, 1995). Vernon *et al.*, in a study carried out in 1969 but reported in the Journal of the American College of Toxicology in 1990, orally dosed four groups of five young adult male CF Nelson rats with 50, 100, 200, and 400 mg/kg and observed them for 14 days. All deaths occurred during the first 24 hours with no significant clinical signs being observed. The acute oral LD_{50} (in males) was 81 mg/kg, with 95% confidence limits of 62 to107 mg/kg. The oral LD_{50} in mice was reported by Tullar (1947) to lie between 25to 48 mg/kg, as summarized in WHO (1983).

Following oral dosing, the mouse appeared to be the most sensitive species, with an oral LD_{50} ranging from 28 to 48 mg/kg body weight. The reported range in the guinea pig was 50 to 85 mg/kg, an oral LD_{50} of 93 mg/kg was reported in the rabbit, while in the rat the range was 72 to 186 mg/kg. No oral toxicity data exist for the dog.

The reported dermal LD_{50} for the rat lay between 148 and 282 mg/kg body weight, the dermal LD_{50} in the rabbit was 226 mg/kg and that in the guinea pig was between 260 to 690 mg/kg. The percutaneous LD_{50} in the rabbit was only three times higher than the intravenous LD_{50}, and was approximately three to 10 times higher in guinea pigs, indicating that AN can readily penetrate the skin. Acute administration of AN produced pathological findings in the gastrointestinal tract, gastrointestinal bleeding apparently being independent of the route of

administration since it was reported after oral or subcutaneous dosing, and changes have also been reported in the kidney, the liver and in hematological and clinical chemistry parameters.

Dermal LD_{50} values for various species were in the range of 148 to 693 mg/kg with the rat reacting most sensitively (BUA, 1995). In a study by Vernon *et al.* (1969), a single dose of 200 mg/kg was applied occlusively to the intact skin of 15 young adult male rabbits for an exposure period of 24 hours. This study resulted in death of all animals within the first 24 hours, with no clinical signs being noted. The acute dermal LD_{50} of AN in this study was <200 mg/kg. This indicates that AN can readily penetrate the skin.

The LC_{50} values reported for a range of species following a four hour inhalation exposure was in the concentration range of 300 to 1,210 mg/m^3. Inhalation studies provided an approximate LC_{50} of 200 mg/m^3/4 hr in the dog, 300 mg/m^3/4 hr in the mouse, and 990 mg/m^3/4 hr in the guinea pig. In rats, the data of Dudley *et al.* (1942) and those of Appel *et al.* (1981) provided a figure of between 1,030 and 1,210 mg/m^3/4 hr, although a lower value of 470 mg/m^3/4 hr was reported by Knobloch *et al.* (1971).

Dudley and Neal (1942) investigated the individual susceptibility of a range of species (*i.e.*, rats, guinea pigs, rabbits, cats, dogs, and monkeys) to a single four-hour exposure to varying concentrations of AN. The results indicated that rabbits were moderately susceptible to AN with exposure to a level of 260 ppm (560 mg/m^3) for four hours causing 100% mortality in four to five hours, while a level of 135 ppm produced marked, but transistory effects. Rats and cats were of about equal susceptibility, 100% mortality being observed in rats within two to six hours of exposure to 635 ppm (1,380 mg/m^3) AN and in cats within 1.5 hours of exposure to 600 ppm (130 mg/m^3). Exposure of two monkeys to 90 ppm (196 mg/m^3) produced only slight transitory redness of the face, genitalia, and extremities with full recovery in 12 hours.

Delayed mortality (25%) was observed in guinea pigs exposed to a level of 575 ppm (1,250 mg/m^3), mortality occurring as a result of lung edema three to five days following exposure. In general, guinea pigs appeared to be less sensitive to AN than rats following inhalation exposure, but yet the lethality in both species after administration by other routes is comparable. This could be due to the lower respiratory volume per kg body weight of guinea pigs (*i.e.*, 320 l/kg/8 hour compared to 150 l/kg/8 hour at rest for rats and guinea pigs, respectively) (Zielhuis and Van der Kreek, 1979).

In the experiments of Dudley and Neal (1942), the dog was shown to be the most sensitive species. Exposure to 110 ppm (240 mg/m^3) AN was fatal in two out of three dogs exposed, while a four-hour exposure to a level of 100 ppm resulted in convulsions followed by coma in two out of three dogs. One of these dogs recovered completely within 48 hours while the other showed partial paralysis of the hind legs for three days. The third dog exposed to 100

ppm showed severe salivation during the test, but recovered fully withing 24 hours. At an exposure level of 29 ppm (63 mg/m^3) for four hours, signs of toxicity in dogs were confined to slight salivation at the end of the test.

Dudley and Neal (1942) also investigated the protective effects of sodium nitrite as one of three known antidotes to cyanide poisoning, the others investigated being thiosulfate and methylene blue. They showed that sodium nitrite was the most effective of the three in delaying the onset of symptoms of AN toxicity and reducing the severity of the effects in rats and rabbits, although it had no such protective effect in guinea pigs. They suggested that it was likely that AN was metabolized to hydrogen cyanide, as had been postulated for other nitriles.

The same authors also investigated the effect of increasing exposure levels of AN and increasing duration of exposure, from 0.5 to 8 hours, in rats. Their results are summarized in **Table 4-15** below.

Table 4-15. Acute Toxicity of AN in the Rat Following Inhalation Exposure (Dudley and Neal, 1942)

mg/m^3 AN	Exposure period (minutes)	No. of rats exposed	No. of deaths
5300	30	16	0
3230	30	16	0
2750	30	16	0
1440	30	16	0
5300	60	16	12
3230	60	16	4
2750	60	16	0
1440	60	16	0
2730	120	16	16
1440	120	16	1
660	120	16	0
1380	240	16	16
680	240	16	5
280	240	16	0
690	480	16	15
590	480	16	7
460	480	16	1
290	480	16	0
200	480	16	0

Appel *et al.* (1981) administered lethal doses of AN to male Wistar rats by different routes of application (*ip*, gavage and inhalation) in order to observe the effect of potential antidotes

on the acute toxicity of AN. Inhalation exposure for 30 minutes to 3,000 ppm (6,490 mg/m^3) of AN proved to be lethal in all six rats examined, as shown in **Table 4-16**.

Table 4-16. Acute Toxicity of AN in the Rat Following Inhalation Exposure (Appel *et al.*, 1981)

mg/m^3 AN	Exposure period (minutes)	No. of rats exposed	No. of deaths
1406 (650)	180	3	1
2055 (950)	120	3	1
2380 (1100)	120	3	3
3461 (1600)	30	3	0
5192 (2400)	10	3	0
5624 (2600)	30	3	1
6490 (3000)	30	6	6

The results from the Dudley and Neal (1942) and Appel *et al.* (1981) experiments above were used to establish LC_{50} values for the rat, using the method of Probit Analysis (Finney, 1971) in the EU, 2001 report. Results are shown in **Table 4-17**. The LC_{50} value for inhalation of AN obviously decreases with increasing exposure time, the LC_{50} values after four hours of exposure being in the concentration range 1,030 to 1,210 mg/m^3, as shown by these two independent studies. The consistency between the results for the two studies should be noted, particularly considering the time gap of approximately 40 years between them.

Table 4-17. LC_{50} Values for AN in the Rat, Derived from the Data of Dudley and Neal (1942) and Appel *et al.* (1981)

Exposure (minutes)	LC_{50} mg/m^3 Dudley *et al.*, 1942	LC_{50} mg/m^3 Appel *et al.*, 1981
30	7880	5740
60	4000	3410
120	2030	2030
240	1030	1210
360	690	890

Vernon *et al.*, in a study carried out in 1985 and reported in the Journal of the American College of Toxicology in 1990, exposed a group of ten young adult Sprague-Dawley rats (five of each sex) to a AN concentration of 1,008 ppm (2,240 mg/m^3) for one hour. There was no mortality. Clinical signs noted included shallow and rapid breathing, decreased activity, nasal discharge, salivation, lacrimation and coma (3/10 animals). Based on these observed clinical signs, the CNS is indicated to be a target organ. The extremities of all animals appeared red after 37 minutes of exposure. However, animals recovered fully within five minutes when

exposed to fresh air. The acute inhalation (rat) LC_{50} was calculated to be >1,008 ppm (2,240 mg/m^3).

With respect to lethality in other species following inhalation exposure, the LC_{50} for dogs following a four hour exposure has been estimated from the data of Dudley and Neal (1942) to be 200 mg/m^3 (90 ppm), while exposure to 580 to 670 mg/m^3 (267 to 309 ppm) was fatal for three rabbits within two to three hours. **Table 4-18** summarizes the lethality data for AN in a range of species following different routes of administration.

Based on the information in **Table 4-18**, the oral LD_{50} values for the various species range from 28 to 186 mg/kg. The sensitivity decreases in the order mouse, guinea pig, rabbit, and rat. The LD_{50} values for *iv*, *ip* or *sc* administration are similar to those reported for oral administration. Dermal LD_{50} values ranged from 148 to 690 mg/kg for the rat, guinea pig and rabbit, with the rat being the most sensitive species. The LC_{50} values after a four hour exposure lie in the concentration range of 300 to 1,210 mg AN/m^3. The sensitivity decreases in order from mouse to guinea pig to rat.

Table 4-18. Acute Toxicity of AN by Different Routes of Administration in a Range of Species (WHO, 1983 unless otherwise specified)

Species	Route	Toxicity
Mouse	inhalation	LC_{50} 300 mg/m^3 /4 hour
Rat	inhalation	LC_{50} 470 mg/m^3/4 hour
Rat	inhalation	LC_{50} 1030 mg/m^3/4 hour[a]
Rat	inhalation	LC_{50} 1210 mg/m^3/4 hour[b]
Guinea pig	inhalation	LC_{50} 990 mg/m^3/4 hour
Mouse	oral	LD_{50} 28-48 mg/kg
Guinea pig	oral	LD_{50} 50-85 mg/kg
Rat	oral	LD_{50} 72-186 mg/kg
Rabbit	oral	LD_{50} 93 mg/kg
Mouse	intraperitoneal	LD_{50} 47-5-0 mg/kg
Rat	intraperitoneal	LD_{50} 65-100 mg/kg
Mouse	subcutaneous	LD_{50} 25-50 mg/kg
Mouse	subcutaneous	LD_{50} 35 mg/kg[c]
Hamster	subcutaneous	LD_{50} 60 mg/kg[c]
Rat	subcutaneous	LD_{50} 80-96 mgkg[c]
Rat	subcutaneous	LD_{50} 100 mg/kg
Guinea pig	subcutaneous	LD_{50} 130 mg/kg
Guinea pig	percutaneous(dermal)	LD_{50} 260-690 mg/kg
Rat	percutaneous(dermal)	LD_{50} 148-282 mg/kg
Rabbit	percutaneous(dermal)	LD_{50} 226 mg/kg
Rabbit	intravenous	LD_{50} 69 mg/kg
Guinea pig	intravenous	LD_{50} 72 mg/kg

[a] Dudley and Neal (1942)
[b] Appel *et al.*, (1981)
[c] Cote *et al.*, (1984)

Acute AN Target Organ Toxicity

Target organs following acute oral AN exposure include the endocrine system, lung, brain, stomach, and duodenum (Szabo *et al.*, 1976, 1980, 1982a, 1982b; Jaeger *et al.* 1982). In the early studies of Dudley and Neal (1942), exposure of rats, guinea pigs, rabbits, cats, dogs, and monkeys to high (acute) levels of AN indicated marked flushing and reddening of the skin, particularly in rats and rabbits. This was considered to be due to either a dilatation of the blood capillaries or to some change in the respiratory cycle, which rendered the blood more highly oxygenated. Gross pathological examination showed marked lung congestion in all species except guinea pigs, in which the lungs were pale in color and gave a frothy exudate on sectioning. Subsequent work in guinea pigs (Jedlicka *et al.*, 1958) showed that lethal doses of AN (50 or 100 mg/kg) caused dilatation of the right ventricle, congestion of the coronary blood vessels, hepatic and splenic congestion, and inflammation of the intestinal mucosa.

The adrenal has been reported by Szabo and co-workers to be a primary target organ following acute administration of AN. These workers showed rapidly developing adrenocortical hemorrhagic necrosis ("apoplexy") following either *iv* or oral administration (Szabo *et al.*, 1976, 1980), and suggested that the effects seen could be due to peroxidative damage induced by AN in the adrenal (Silver and Szabo, 1982).

Szabo and co-workers (1982a, 1982b,1983) also reported that acute administration of AN to the rat by the oral route produced pathological findings in the gastrointestinal tract. They reported a duodenal ulcerogenic effect that was markedly enhanced by pretreatment of the rats with the mixed function oxidase inducers polychlorinated biphenyl (PCB), Arochlor 1254, or phenobarbital. Ghanayem *et al.* (1985) also reported gastrointestinal hemorrhage. The severity of the gastrointestinal bleeding was independent of the route and was dose and time dependent. The gastric lesions were associated with a decrease in glutathione content of the stomach.

Other organs affected by acute administration of AN have included liver, kidney, and blood. Focal superficial necrosis of the liver in association with hemorrhagic gastritis was reported by Silver *et al.* (1982), following administration of AN at 150 mg/kg in drinking water. As with AN-induced adrenal necrosis, Silver and Szabo (1982) suggested that this could be due to peroxidative damage induced by AN, while Ivanov *et al.* (1989) also reported increased peroxidation in the liver. Intraperitoneal administration of 20 mg/kg (and higher) of AN produced nephrotoxic effects including glucosuria (Rouisse *et al.*, 1986). Electron microscopic examination revealed a slight increase in dense bodies and moderate vesiculation of endoplasmic reticulum membranes in proximal tubular epithelium at dose levels of 40 mg/kg and higher. Rouisse and co-workers (1986) also identified altered hematological and clinical-chemical parameters that supported the observation of dose-dependent kidney damage, while Farooqui and Ahmed (1983a) and Gut *et al.* (1984) observed an effect on blood count and glucose metabolism. Although the blood is not believed to be the primary target organ in AN toxicity, some hematological effects were observed in rats and guinea pigs administered lethal doses of AN (WHO, 1983; VROM. 1984). Glutathione depletion has been reported in liver, kidney, and lung (Szabo *et al.*, 1977).

Neurotoxicity

The central nervous system has been identified as a target organ in various animal species (Buchter and Peter, 1984; Ghanayem *et al.*, 1991). AN has been shown to induce dose- and time-dependent cholinomimetic neurotoxicity in rats, regardless of the route of administration. Clinical symptomology indicates an effect on cholinergic transmission at dose levels in excess of 20, 40, and 80 mg/kg in rat (Ghanayem *et al.*, 1991), although no clinical symptoms of neurotoxicity were identified in a mouse study performed by Tanii *et al.* (1989) in which a single oral dose of AN was administered to four animals at four dose levels of between 23 and 78 mg/kg (0.44 and 1.48 mmol). Of the 16 animals used in this study, only seven survived.

Neurotoxic effects reported following exposure to AN may be mediated by cyanide, liberated *in vivo* as a result of metabolism. Hashimoto and Kanai (1965) suggested, however, that the toxicity of AN was due not only to the liberated hydrogen cyanide, but to AN itself. This conclusion was based on the observation that a reduction of the AN concentration in the blood by L-cysteine (which may have been caused by cyanoethylation of AN with L-cysteine) was very effective in protecting animals from AN poisoning. Benesh and Cerna (1959) and Hashimoto and Kanai (1965) applied lethal doses of AN to rats and mice, pre-treated with an intravenous dose of 0.5 to 1 gram thiosulphate. During these experiments, the cyanide blood level remained far below the level of specific cyanide symptoms, and yet the animals still died. These results indicate that, in addition to release of cyanide, another mode of action plays a role in the acute toxicity of AN.

The complexity of the neurotoxicity resulting from acute exposure to AN is reflected in the evidence that classic cyanide antidotes are effective in preventing the acute toxicity in some animal models, but are totally ineffective in others (Nerland *et al.*, 1989a). The acute toxicity of nitriles in general apparently depends on the complex interplay of a number of factors such as the rate of cyanide liberation and detoxification, the dose of cyanogen, the route of administration, the species, and the presence of other bioreactive sites within the nitrile molecule. While AN is cyanogenic, it is also metabolized to a reactive epoxide, 2-CEO, and the parent molecule is also capable of non-enzymatically cyanoethylating essential functional groups in the body. All these factors contribute to the overall toxicity of AN (as summarized by Nerland *et al.*, 1989a,b, based on studies from Dudley and Neal, 1942, Dudley *et al.*, 1942, Abreu and Ahmed, 1980; Szabo *et al.*, 1984; Tanii *et al.*, 1986; Gut *et al.*, 1985).

Effects on cholinergic transmission in the rat may result from an inactivation of acetylcholinesterase by cyanoethylation of the hydroxyl group of one serine residue (Buchter *et al.*, 1984) or may be due to damage to acetylcholine muscarinic receptors by AN or its metabolites (Ghanayem and Ahmed, 1986). These effects are particularly marked in glutathione-depleted animals.

Experimental evidence also suggests the involvement of acetylcholine muscarinic receptors in AN-induced toxicity. AN produces 'cholinomimetic' actions, such as salivation, diarrhea, and increased acidic gastric secretions, which are prevented by prior treatment with atropine (Ahmed *et al.*, 1986). Burhan *et al.* (1991) demonstrated that AN causes acute gastric hemorrhage and mucosal erosions. A possible mechanism of this AN-induced GI bleeding may involve the interaction of AN with critical sulfhydryl groups that in turn causes alteration of acetylcholine muscarinic receptors and leads to gastric hemorrhagic lesions. Pre-treatment of rats with atropine sulphate (1 mg/kg) 30 minutes before administration of 40 mg/kg of AN significantly protected animals against the AN-induced neurotoxicity seen in animals dosed at the same level (40 mg/kg) without any atropine. In addition, treatment of rats with the same

dose of atropine sulphate after the first appearance of neurotoxic signs, prevented further progress of toxicity (Burhan *et al.*, 1991).

Studies by Burhan *et al.* (1991) were designed to quantitatively characterize the acute phase of AN-induced cholinomimetic neurotoxicity, and to determine the effects of dose, route of administration, and atropine on such toxicity. Groups of three to four male Sprague-Dawley rats were administered doses of 20, 40, or 80 mg/kg AN in distilled water by gavage, or in sterile saline subcutaneously. Control groups received only the vehicle. Two distinctive phases of acute neurotoxic effects were observed in the treated animals. Early after treatment with AN, rats exhibited salivation, lacrimation, miosis, diarrhea, polyuria, and peripheral vasodilation, reaching a maximum within 60 minutes of dosing. Other signs of toxicity observed, but not quantified, included flushing of the face, ears, and extremities. This early phase was followed by a delayed phase (> 4 hours) that included central nervous system abnormalities such as respiratory depression, convulsions and, at high doses, death. The neurotoxic signs observed were dose-dependent, regardless of the route of administration. The intensity of the early clinical symptomatology, which reached a maximum in about 0.5 to 1 hour, was relatively similar after subcutaneous or oral administration, although oral administration produced more severe salivation and gastric secretion.

Brain slices prepared from male guinea pigs and frogs were used to study the potential neurotoxicological effects of AN. The oxygen consumption of normal or potassium-stimulated slices was measured using a Warburg manometer in the presence or absence of AN, cyanide, or a range of narcotic agents. The results of these studies indicated that AN caused inhibition of the respiration of brain slices, the effect being similar to that produced by the narcotic agents also tested in the system. The inhibitory effect was modulated by sodium thiosulphate, which completely suppressed the inhibition caused by cyanide. Acrylamide and acrylic acid were also tested and had no effect on brain respiration. These results suggest that the effects of AN on the brain could be attributable to AN itself rather than its metabolites. AN has also been reported to have a strong blocking effect on peripheral nerves similar to the effects of various local anaesthetics and general narcotics, although the active concentration *in vitro* was much higher than that estimated to be possible *in vivo*.

From these observations it seems likely that AN acts both on the central and peripheral nervous system. The symptoms of acute AN poisoning, such as general convulsions and paralysis of the lower limbs, seem to support this conclusion.

Gagnaire *et al.* (1998) exposed male Sprague-Dawley rats to 12.5, 25, and 50 mg/kg AN via olive oil gavage (2 ml) once a day, five days/week for 12 weeks to assess its neurotoxic effects as determined by changes in motor and sensory conduction velocities and amplitudes of sensory and motor action potentials in the tail nerves. Neurophysiological evaluations were also carried out in rats exposed by inhalation to 25, 50, or 100 ppm AN, six hours/day, five

days/week for 24 weeks. Mean body weights were below controls in the mid and high dose animals given AN by gavage and in the high dose group exposed via inhalation. Rats given AN orally developed behavioral sensitization to the subsequent administration of AN. One week after onset of treatment, the rats developed salivation, locomotor hyperactivity, and moderately intense sterotypies associated with fur wetting. These effects started shortly after gavage dosing, lasted two to three hours and become more pronounces as treatment continued. High dose animals developed hindlimb weakness associated with decreases in sensory conduction velocities and amplitudes of sensory action potentials and could not rear from the 9th week on, but the weakness abated during the recovery period. Dose-response data were collected for several measures of peripheral nerve function, including motor nerve conduction velocity (MCV), sensory nerve conduction velocity (SCV), amplitude for motor nerve action potential (AMAP), and amplitude for sensory nerve action potential (ASAP). Rats exposed to AN vapors exhibited time and concentration dependent decreases in motor and sensory conduction velocities and amplitudes of sensory action potentials. These changes were only partially reversible after an eight week recovery period. Related nitriles (*i.e.*, methAN, trans-3-pentenenitrile, 3-methyl-2-butenenitrile, and 4-penetenitrile) did not cause abnormal behavioral or electrophysiologcial changes in spite of an obvious general toxicity. For oral exposures, this study identifies NOAEL and LOAEL values of 25 and 50 mg/kg-day for hindlimb weakness and changes in peripheral nerve conduction velocity and action potential. For inhalation exposures, this study identifies a LOAEL value of 25 ppm for changes in nerve conduction velocity and action potential. Dose-response data for the four measures of peripheral nerve function are provided in **Table 4-19** for oral exposure and **Table 4-20** for inhalation exposure.

Table 4-19. Effect of AN on Nerve Conduction Velocities and Action Potentials in Rats Following Oral Exposure (Gagnaire *et al.*, 1998)

	End of Exposure (12 weeks)				End of Recovery (20 weeks)			
Dose (mg/kg-day)	**MCV (m/s)**	**SCV (m/s)**	**AMAP (mV)**	**ASAP (uV)**	**MCV (m/s)**	**SCV (m/s)**	**AMAP (mV)**	**ASAP (uV)**
0	36 (0.8)*	46.6 (0.8)	10.5 (0.9)	146 (8)	41.7 (0.6)	53.8 (1.5)	10.8 (0.7)	167 (10)
12.5	35.5 (0.8)	46.7 (0.9)	11.0 (0.9)	127 (9)	40.4 (0.9)	51.8 (0.9)	11.9 (1.0)	157 (9)
25	35.1 (0.8)	44.4 (0.9)	11.0 (0.9)	134 (6)	41.2 (0.6)	51.3 (0.7)	11.9 (1.0)	158 (9)
50	33.5 (0.8)	39.8 (0.6)**	10.9 (0.7)	135 (12)	39.9 (0.7)	48.1 (0.7)**	10.7 (1.1)	126 (12)**

*values depict the mean (with the standard error in the mean in parentheses) based on 12 samples
**significantly different from control values

Table 4-20. Effect of AN on Nerve Conduction Velocities and Action Potentials in Rats Following Inhalation Exposure (Gagnaire *et al.*, 1998)

	End of Exposure (24 weeks)				End of Recovery (20 weeks)			
Concentration (ppm)	**MCV (m/s)**	**SCV (m/s)**	**AMAP (mV)**	**ASAP (uV)**	**MCV (m/s)**	**SCV (m/s)**	**AMAP (mV)**	**ASAP (uV)**
0	42.9 (0.9)*	53.3 (1.0)	17.8 (1.2)	185 (8)	44.3 (0.9)	53.4 (0.6)	17.2 (1.1)	189 (11)
25	41.5 (0.8)	50.5 (0.8)**	15.1 (0.8)	164 (11)	40.1 (0.8)	51.8 (0.8)	17.4 (1.7)	167 (11)
50	38.1 (0.9)**	49.1 (0.6)**	15.7 (1.0)	159 (5)**	40.0 (0.8)	51.3 (1.0)	16.6 (0.7)	159 (8)
100	38.5 (1.2)**	48.4 (1.0)**	17.4 (0.9)	133 (11)**	42.1 (1.6)	50.4 (0.8)	16.0 (1.5)	148 (12)**

*values depict the mean (with the standard error in the mean in parentheses) based on 12 samples
**significantly different from control values

In a three-generation study (with two matings per generation) of Friedman and Beliles (2002), AN was tested for reproductive and neurotoxicological effects in a drinking water study. Sprague-Dawley rats were administered AN in drinking water at 0, 100, or 500 ppm (0, 11±5, or 37±10 mg/kg and 0, 20±3 or 40±8 mg/kg for males and females, respectively). Water consumption was reduced in F_0 rats at both AN doses and food intake and body weight gain was reduced in the high dose F_0 rats. These parameters were not investigated in subsequent

generations. The pup survival based on both viability and lactation indices were reduced at the high dose in both matings of all three generations. Fostering the high dose pups on untreated mothers following the second mating reduced mortality, suggesting a maternal effect consistent with decreased water consumption. No adverse effects were observed in the tissues of a limited number of third generation weanlings (F_{3b}) upon gross and microscopic examination. No effect on the sciatic nerve was evident among adult female rats held for 20 weeks after weaning of the second litter. AN did not induce neurotoxicity in this study. Animal behavior as measured by cage side observation (*i.e.*, abnormal gait, etc.) appeared normal. AN had no cumulative neurotoxic effects. Exposure to AN throughout the entire developmental cycle did not result in observable neurotoxic effects.

The acute and persistent effects of AN (50 mg/kg sc) on auditory sensitivity were assessed in rats (Fechter et al., 2003). Acute ACN administration produces a loss in auditory threshold sensitivity that reached a maximum 10-20 min following sc injection. Auditory thresholds returned to control levels 75-100 minutes following exposure. In the study of permanent auditory threshold shifts, ACN plus noise increased auditory threshold impairment relative to rats receiving noise only when thresholds were assessed 3 weeks following exposure. ACN by itself did not produce permanent threshold impairment 3 weeks following administration. Assays were undertaken in separate groups of rats to track the elevation in blood CN and the depletion of total glutathione in cochlea, brain, and liver following ACN treatment. Systemic blood CN levels were not significantly elevated until 60-120 min following injection, and cochlear glutathione levels showed significant depletion as little as 15 min after injection and remained depressed for about 4 h. The results confirm the prediction that ACN is acutely ototoxic and can enhance noise-induced hearing loss.

4.2.7.2 Irritation/Corrosivity/Sensitization

Skin

Vernon *et al.* (1969) as reported in the Journal of the American College of Toxicology (1990), applied 0.5 ml of AN occlusively to the shaved skin of six young adult New Zealand White rabbits for a period of 24 hours. Evaluation of skin irritancy was made at 24 and at 72 hours, when the study was terminated. The scores at 24 and 72 hours after administration were averaged to produce six individual animal scores of 0 to 4 for both erythema and edema. The 0 to 72 hour mean of the six individual animal scores for both erythema and edema was 3.6, with slightly higher scores being obtained for abraded skin. This study indicates that AN is strongly irritating to the skin.

Zeller *et al.* (1969) applied liquid AN on a cotton pad for 15 minutes or 20 hours to shaved rabbit skin (2.5 × 2.5 cm). The skin was exposed for 15 minutes and then washed with concentrated polyethylene glycol and water. Skin exposed for 20 hours remained unwashed.

The skin exposed for 15 minutes showed edema only, however, increasing the duration of exposure to 20 hours produced clear necrosis of the tissue. No further details are available.

Reports in the literature have indicated blistering of the skin and scab formation in humans following accidental contact (*e.g*. Dudley and Neal, 1942; Wilson *et al.*, 1948). However, in the case report where a ship worker was sprayed with AN while off-loading the chemical, no corrosive effect or skin burns were observed even though his face, eyes and body were covered in the chemical. Isolated reports of a corrosive effect of AN exist, as indicated above, relating in the main to exposure of humans in an accident situation. However, overall the available studies on both skin and eye irritation in animals and more recent human experience indicate that while AN is irritant to skin, eye and respiratory tract, it should not be considered as corrosive.

Koopmans and Daamen (1989) carried out a Guinea Pig Maximization Test in compliance with EC and OECD guidelines. Sensitization was induced by an intradermal injection of 2.5% AN and an epidermal application of 2% AN seven days later. Animals challenged with AN concentrations of 0.5% and 1.0% AN showed a 95% positive sensitization rate. Exposure to 0.2% on challenge caused an 80% sensitization rate. It can be concluded that AN has marked sensitizing properties.

Eye

In a BASF study (1963), approximately 0.05 ml of AN was applied undiluted to the left eye of two rabbits. The right (control) eye was treated with NaCl solution. No findings were observed in the control eyes. The treated eyes at one hour both exhibited slight conjunctival redness (score of one), diffuse corneal opacity, edema (severe in one animal, score of three), and miosis and secretion occurred in one animal. At 24 hours, conjunctival redness (score of two) and corneal opacity remained with edema in one animal (score of two) and ciliary injection. At 48 hours, conjunctival redness had reduced with some corneal opacity still remaining in one animal. At 72 hours, the eye of one animal was clear of all effects, while the second animal still had conjunctival redness and petechiae, and diffuse milky corneal opacity. After seven days, the eye of the second animal had returned to normal.

Vernon *et al.* (1969) also carried out an eye irritancy study in rabbits. A dose of approximately 0.1 ml of AN was instilled in one eye, the other eye serving as control. The eyes were examined and the grade of ocular reaction recorded at 24, 48, and 72 hours, when the study was terminated. The scoring system used was the original system proposed by Draize, and results from this study were presented in accordance with the Draize scoring system, taking into account both the intensity and the area of involvement. AN gave a maximum Draize score of 35 out of 110 at 24 hours, falling to 31 at 48 hours, and 22 at 72 hours. It has not been possible to deduce accurately the scores for intensity alone, other than for iritis, where a mean score of

1.0 was obtained over 24 to 72 hours. Scores for corneal opacity are estimated to have been in the range of one to two, with little reversibility of the damage over the period of the study. Scores for conjunctival redness and chemosis appear to have been in the range of two to three, with some reversibility shown over the three days of the test.

In a study by Zeller *et al.* (1969), edema and slight necrosis of the conjunctiva after eight days were observed in rabbits. No further details are available.

In another low-volume study, following application of 0.05 ml of undiluted AN, mild irritation was observed in the eye of the test animal (rabbit), and after one hour, mild conjunctivitis had developed. By 24 hours, however, the eye had returned to normal (McOmie, 1949). Other investigators found a severe burn of the cornea after application of 0.02 ml of undiluted AN in the rabbit eye (VROM, 1984).

In a study performed by Haskell Laboratories of DuPont (1975), 0.1 ml of undiluted AN was placed in the right conjunctival sac of each of two albino rabbits. After 20 seconds, the treated eye of one rabbit was washed with tap water for one minute. The treated eye of the other rabbit was not washed. Observations of the cornea, iris, and conjunctiva were made with a hand-slit lamp at one and four hours, and at one, two, seven, 14, and 21 days. Fluorescein stain and a biomicroscope were used at examinations after the day of treatment. In this study, AN produced moderate corneal opacity, moderate iritis, and severe conjunctival irritation in the unwashed treated eye. The eye treated with AN and promptly washed showed slight temporary corneal opacity, transient, moderate iritic congestion, and moderate conjunctival irritation. The washed eye was normal within three days. The unwashed eye showed signs of healing by day three with partial circumcorneal vascularization. By days 14 and 21, mild opacity remained with traces of vascularization. Both rabbits showed possible systemic effects with pupil dilation at four hours. In conclusion, therefore, severe to moderate ocular effects occurred by treatment with AN. These effects were not completely reversible in the unwashed eye, while by washing the eye, the effects were considerably lessened as was the duration of these ocular effects. AN should be regarded as a serious eye irritant.

Respiratory Tract

No specific animal studies of respiratory irritancy have been carried out for AN, but both long-term and short term toxicity studies in a range of species have indicated that AN has irritant effects on the upper respiratory tract (Quast *et al.*, 1980a). Effects have included rhinitis, nasal discharge and hyperplastic changes in the nasal mucosa. The irritation of the throat and the respiratory tract is a delayed effect and provides no warning of exposure to AN in the first period of exposure.

4.3 Synthesis and Evaluation of Major Effects and Mode of Action

Within this section, the information presented above in **Sections 4.1** and **4.2** regarding the major noncancer and cancer effects of AN are summarized and interpreted. In addition, information regarding the potential mode(s) of action by which AN produces these effects are presented. Consistent with USEPA guidelines (USEPA, 1999, 2003), the term "mode of action" is defined here as a series of key events that start with exposure to an agent which then leads to interaction of the agent with key macromolecules in the target cell, and ultimately results in operational and anatomical changes associated with the primary effects. "Mode" of action is contrasted with "mechanism" of action, which implies a more detailed understanding and description of events, often at the molecular level, than is meant by mode of action.

4.3.1 Noncancer Effects

4.3.1.1 Major Effects

As indicated by studies in humans (**Section 4.1**) and laboratory animals (**Section 4.2**), the principal noncancer endpoints for AN include neurological, irritation, hematological, reproductive/developmental, and effects on survival. Other effects (*e.g.,* kidney, adrenal toxicity) are generally associated with higher exposures to AN, or are not consistently identified as an effect for AN. Available information from human and animal studies is summarized for each of these endpoints below.

Neurological Effects

Information regarding the potential neurological effects of AN obtained from human and animal studies are summarized below.

- *Human Studies* - Information from reports indicate that neurological effects are associated with exposures to high concentrations/doses of AN. Only limited data exist in respect of the acute effects of AN in humans, based mainly on reports of specific incidents or accidents. In humans, acute exposure to sufficiently high concentrations of AN results in characteristics of cyanide-type toxicity. Neurological symptoms in humans associated with AN poisoning include limb weakness, dizziness and impaired judgment, nausea, collapse, and convulsions (Wilson and McCormick, 1949; Zeller *et al.*, 1969; Buchter and Peter, 1984; WHO, 1983; Baxter, 1979). However, the doses that produce these effects were not clearly defined. The human data are difficult to assess for purposes of establishing a dose-response relationship, but the findings and approximate dose levels thought to be involved in these human experiences are consistent with the information obtained from animal studies. This data indicates that AN is toxic by the oral, inhalation, and dermal routes, and causes neurotoxic effects (which relates both to the AN itself and

also to the release of cyanide). At sub-lethal dose levels, AN neurotoxicity may exhibit reversible effects. More serious acute exposures have resulted in tremors, convulsions, unconsciousness, respiratory and cardiac arrest, and even death (Buchter and Peter, 1984). In general, chronic exposures to AN in workers caused, among other symptoms, nausea, vomiting, general weakness, and symptoms of neurasthenia. In humans, specific case reports and workplace surveys indicate that chronic exposure to AN is associated with neuropathological effects following exposure to AN via inhalation or by physical contact with the substance.

- *Animal Studies* - In rats, the acute neurotoxicity of AN has been described as having two distinctive phases: (1) an early phase, which has a rapid onset, with signs that are cholinomimetic in nature, and included salivation, lacrimation, chromodacryorrhea, polyuria, miosis, vasodilatation in face, ears and extremities, increased gastric secretion, and diarrhea; and (2) a late phase, which developed hours after dosing, and included depression, convulsions, and respiratory failure followed by death at high doses (Ghanayem *et al.*, 1991; Burnham *et al.*, 1991). With respect to neurotoxicity following repeated exposures to AN, hind limb weakness and alterations in nerve cell conduction velocities have also been observed in rats following oral and inhalation exposure to AN (Gagnaire *et al.*, 1998). For oral exposures, NOAEL and LOAEL values of 25 and 50 mg/kg-day have been reported. For inhalation exposures, a LOAEL values of 25 ppm was reported. In addition to noncancer effects, the central nervous system has also been identified as a primary target for the carcinogenic effects of AN in rats (see Section 4.3.2).

In summary, human and animal studies are consistent in identifying neurological effects as an endpoint of concern following acute and repeated exposures to AN. Although data regarding the exposure levels producing neurological effects in humans are generally lacking, adequate information are available from rat studies (Gagnaire *et al.*, 1998) to provide a characterization of the dose-response relationship for oral and inhalation exposures to AN.

Irritation Effects

Information regarding the potential irritating effects of AN obtained from human and animal studies are summarized below

- *Human Studies* - Limited information from case studies indicate that worker exposures to high levels of AN are associated with irritation of the nose, eyes, and skin (Dudley and Neal, 1942; Wilson *et al.*, 1948; Hashimoto and Kobayashi, 1961; Davis *et al.*, 1973; Bakker *et al.*, 1991). While early reports indicated signs of irritation at concentrations as low as 0.3-5 ppm (Sakurai and Kusumoto, 1972; Babanoov *et al.*, 1959), a more careful consideration of the actual exposures experienced by workers from six AN plants suggests that air concentrations less than 3 ppm (6.5 mg/m^3) do not produce notable

irritancy (Sakurai *et al.*, 1978). Another study of exposed workers reported an increased frequency of complaints of irritation in workers exposed to 4-31 mg/m^3 AN (Kaneko and Omae, 1992), although this did not demonstrate an increase with concentration.

- *Animal Studies* - Studies in laboratory animals indicate that high levels of AN can produce irritation. Gastrointestinal lesions seen following oral dosing may be due in part to the local irritant effect of AN. The respiratory tract is also affected following inhalation of AN primarily as a result of the irritancy of AN. Hyperplasia of the forestomach has been observed in rats and mice oral exposed to AN (Szabo *et al.*, 1984; Ghanayem *et al.*, 1997; Johannsen and Levinskas, 2002a,b). Similarly, signs of nasal irritation, including hyperplasia/metaplasia of the nasal turbinate, and hyperplasia of mucus secreting cells have been reported in rats following chronic inhalation exposures to AN (Quast et al., 1980a). The Quast *et al.* (1980a) study is considered to be a key study for exposure to AN via inhalation. As a result of irritation due to AN exposure, inflammatory and degenerative changes (hyperplasia and metaplasia of the respiratory epithelium) were present in the nasal turbinates of both exposed groups (20 and 80 ppm). The NOAEL for this study is less than 20 ppm, based on the nasal changes (local effect) that occurred at 20 ppm. This value of 20 ppm can be considered the LOAEL. Quast (2001, personal communication cited in EU, 2001) has indicated that a No Observed Effect Level for the local effects of AN on nasal respiratory epithelium probably lies in the region of 10 ppm based on experience in numerous acute to chronic inhalation studies and studies of nasal irritation in the rat.

In summary, human and animal studies are consistent in identifying irritation effects following acute and repeated exposures to AN. Based on the available animal data and limited human experience, the latter resulting mainly from accidental exposures, AN is considered to be both a skin irritant and a severe eye irritant, and it also has irritant effects on the respiratory tract. In humans, the irritation of the throat and the respiratory tract appears to have a delayed action, with no sensation of irritation being felt in the initial period following exposure. Limited data are available regarding the dose-response relationship for irritation effects of AN and inhalation exposures in humans (Sakurai *et al.*, 1978). Adequate information is available to characterize the dose-response relationship for the irritation effects of AN in rodents following oral (Johannsen and Levinskas, 2002a,b; Ghanayem *et al.*, 2002) and inhalation (Quast *et al.*, 1980a) exposures.

Hematological Effects

Information regarding the potential hematological effects of AN obtained from human and animal studies are summarized below.

- *Human Studies* - Occupational exposures to AN have been associated with reports of a variety of hematological effects, including decreased hemoglobin, erythrocyte counts and leukocyte counts (WHO, 1983). Humans exposed to AN at concentrations where nausea, vomiting and weakness occurred (16 to 100 ppm for 20 to 45 minutes), were also reported to have low grade anemia and leucocytosis among other symptoms. However, complete recovery after cessation of exposure was reported (Wilson, 1944; Wilson *et al.* 1948). However, exposure to other chemicals can be assumed for at least some of these workers and generally the exposures conditions are inadequately characterized for direct causation to be determined (BUA, 1995). Furthermore, other studies have reported no changes in hematological parameters in workers exposed to AN (Ginceva *et al.*, 1977; Sakurai *et al.* 1978).

- *Animal Studies* - In rats, hematological effects (extramedullary haemopoeisis in liver and spleen) have been reported following chronic inhalation exposures to AN (Quast et al., 1980a). Additionally, hematological effects, including decreased hemoglobin, erythrocyte counts, and leukocyte counts were reported in rats exposed to high doses of AN in drinking water (Johannsen and Levinskas, 2002a,b). GSH depletion and hemolysis has been demonstrated in rat erythrocytes exposed to AN *in vitro* (Farooqui and Ahmed, 1983a).

In summary, data from human and animal studies do not consistently identify hematological effects as a key endpoint following exposure to AN.

Reproductive/Developmental Effects

Information regarding the potential reproductive/developmental effects of AN obtained from human and animal studies are summarized below.

- *Human Studies* - Prior to 1990, no studies of reproductive or developmental effects in humans exposed occupationally or environmentally to AN by any route were located by ATSDR (1990); however, few specific studies have been carried out since that time.

- *Animal Studies* - Studies in laboratory animals indicate that fetotoxicity and developmental effects are only observed at AN doses that are maternally toxic (Saillenfait *et al.*, 1993; Friedman and Beliles, 2002). The results of a three generation reproduction study (Friedman and Beliles, 2002), despite some methodological deficiencies, did not show any effects on fertility, although effects were seen on pup viability following oral exposures to AN. Body weights of pups in all three generations at PND21 were also reduced. These effects could be attributed to maternal toxicity. A number of other studies have also indicated that AN is fetoxic based on dose-dependent reductions in pup weight at exposure levels that are also maternally toxic. A NOAEL of 12 ppm for the

fetotoxic effect was established in the study of Saillenfait *et al.* (1993). Other studies have reported that AN causes testicular toxicity in the rat (at doses approaching the LD_{50}), although no such effect was seen in a 90-day study in mice or in other repeat dose toxicity studies. A gavage study in rats and a study in hamsters using intraperitoneal administration indicated some developmental toxicity potential of AN, and this was supported by the findings of an *in vitro* study in 10-day rat embryos. However, developmental effects *in vivo* were only seen in the presence of significant maternal toxicity, and there was little evidence for a developmental effect following exposure of rats by inhalation (Murray *et al.*, 1978). An absence of developmental effects following inhalation exposure was confirmed by another group of researchers using comparable exposure levels (Saillenfait *et al.*, 1993). Existing animal data do not show any clear indication of fertility, dominant lethal, reproductive, or teratogenic effects of AN at doses below those producing parental toxicity.

A single study reported testicular effects in mice exposed to 10 mg/kg-day AN via gavage for 60 days (Tandon *et al.*, 1988). However, these data are in sharp contrast to the lack of testicular effects in mice exposed to 12 mg/kg-day in the drinking water for 90 days (Serota *et al.*, 1996), in mice receiving up to 60 mg/kg-day vai gavage for 90 days (NTP, 2001; Ghanayem *et al.*, 2002), or in mice receiving up to 20 mg/kg-day via gavage for two years (Ghanayem *et al.*, 2002). Additionally, testicular effects have not been observed in rats chronically exposed to AN via gavage, drinking water, or inhalation (Maltoni *et al.*, 1977, 1988; Quast et al., 1980a,b; Gallagher, 1980; Bigner, 1986; Johannsen and Levinskas, 2002a, b). Accordingly, the weight-of-evidence do not support the testes as a target organ in rodents exposed to AN.

In summary, data from human and animal studies are not consistent in identifying reproductive effects as an endpoint of concern following exposure to AN.

Survival Effects

Information regarding the potential effects of AN on survival obtained from human and animal studies are summarized below.

- *Human Studies* - Information from a large body of epidemiology data do not support a relationship between AN exposure and increased mortality from any cause. In general, no increase in overall or specific mortality has been reported for AN workers in numerous epidemiological studies. No consistent relationship has been noted in terms of increasing dose or duration of exposure (Collins and Acquavella, 1998; Blair *et al.*, 1998; Benn and Osbourne, 1998; Wood *et al.*, 1998).

- *Animal Studies* - Repeat dose exposure by both the oral and inhalation route has also been associated with lethality in various animal species. Dogs appear to be the most sensitive species to exposure to AN by inhalation, with mortalities being seen at exposure levels causing no deaths in other species (however, no long-term oral study has been carried out in the dog). For example, a dose-dependent increase in mortality was reported in rats chronically exposed to AN in the drinking water (Johannsen and Levinskas, 2002). Survival was significantly reduced in mice orally exposed to 20 mg/kg-day AN for life (Ghanayem *et al.*, 2002). The most relevant study from assessing chronic oral exposure to AN was performed by Biodynamics, (1980a), in which AN was administered orally via drinking water to 100 Fisher 344 rats/sex/group at dose levels of 1, 3, 10, 30, and 100 ppm and to a control group of 200/sex. Treatment-related non-neoplastic changes were seen at 10 ppm and upwards. Mortality was increased in males at 10 ppm and in females an increase was observed at 3 and 30 ppm. However, the increase at 3 ppm was small and, overall, there was not a dose-related trend in the range 0 to10 ppm in females. In addition, it was noted that in this study the female control animals had a low mortality rate that would directly affect the comparison with mortality in the female test animals. The first true indication of a dose-response relationship for mortality in females began at the 10 ppm dose level. This study can be used to establish a NOAEL of 3 ppm (equivalent to an average daily dose of 0.25 mg/kg/day in males and 0.36 mg/kg/day in females) for the oral exposure in rats. An assumed NOAEL of 3 ppm derived from the Biodynamics (1980a) study is considerably lower than the apparent NOAELs that can be derived from the long-term oral studies in rats of Gallagher *et al.* (1988) (20 ppm) or Maltoni *et al.* (1977) (5 mg/kg).

In summary, data from human and animal studies are not consistent in identifying survival effects as an endpoint of concern following exposure to AN.

4.3.1.2 Mode of Action

Information regarding the modes of action for AN in producing noncancer effects can be used to support decisions made in the dose-response assessments for AN in which RfD and RfC values are derived (**Section 5**). Mode of action information, where available, is summarized below for each of the major noncancer effects of AN.

Neurological Effects

The mode of action by which AN produces neurological effects in animals has been well studied, and appears to involve both the parent chemical and to the release of cyanide during metabolism. Neurological disturbances appear to be the main effect of AN at sublethal dose levels and these may be reversible. In the case of lethal dose levels, there is also a direct effect on the central nervous system, that cannot be counteracted by cyanide antidotes. Irreversible

damage occurs possibly by cyanoethylation of vital structures in the central nervous system. While AN is cyanogenic, it is also metabolised to a reactive epoxide, CEO, and the parent molecule is also capable of non-enzymatically cyanoethylating essential functional groups in the body. All of these factors may contribute to the neurotoxicity of AN. The effects of the early, cholinomimetic phase are generally attributed to AN. Since the convulsive effects of AN are typical of other nitriles, the release of cyanide during AN metabolism appears to be associated with the late phase (Ghanayem *et al.*, 1991; Nerland *et al.*, 1989). More recently, AN was reported to inhibit the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase, by binding to critical cysteine residues (Campian *et al.*, 2002). Acute toxicity to the brain may result by inhibition of ATP production from glycolysis by AN, together with the inhibition of mitochondrial ATP production by its metabolite cyanide. Other factors, such as the binding of CEO to key macromolecules may also play a role in neurotoxicity. Oxidative stress, possibly as a result of glutathione depletion, has been suggested as a possible role for some of the neurological effects of AN (Fechter *et al.*, 2003).

Irritation Effects

The precise mode of action by which AN produces irritation is not known, but may include the binding of AN or CEO to cellular macromolecules or depletion of tissue GSH levels. Mucosal lesions (gastric necrosis) observed in rats following oral exposure to AN were diminished by pretreatment with sulfhydryl compounds or with atropine, suggesting an important role for GSH levels and for cholinergic receptors (Ghanayem *et al.*, 1985). GSH depletion may result from the metabolism of AN and CEO, both of which react with GSH nonenzymatically and via GST (Kedderis *et al.*, 1995). Because nasal tissue in rats has shown fairly high activity for metabolizing AN to cyanide (Dahl and Waruszewski, 1989), nasal lesions may involve the parent chemical or one of its key metabolites, CEO and/or cyanide.

Hematological Effects

Both AN and CEO are capable of forming adducts with hemoglobin (Bergmark, 1997; Fennell *et al.*, 2000), which may play a role in some of the mild hematological effects observed. GSH depletion in erythrocytes can result in oxidation of hemoglobin to methemoglobin (Farooqui and Ahmed, 1983a). Extramedullary hematopoiesis observed in rats following chronic exposure to AN was judged to be secondary to the carcinogenic effects of AN (Quast, 2001, personal communication), and therefore are not appropriate endpoints for noncancer risk assessment.

Survival Effects

The most plausible mode of action for effects of AN on survival rodents is that these effects are secondary to the carcinogenic effects of AN. This mode of action is consistent with the

observations for decreased survival noted only at exposure levels that produce an increased tumor burden in rats and mice (Johannsen and Levinskas, 2002; Ghanayem *et al.*, 2002), and no significant noncancer histopathology was observed in animals exposed orally to doses affecting survival (Johannsen and Levinskas, 2002). Quast *et al.* (1980) reported that a number of recorded deaths were attributed to severe ulceration of the Zymbal's gland or mammary tissue tumors. In addition, a role for rat brain tumors in the increased mortality is supported by observations that mortality was increased in rats chronically exposed to low concentrations of AN in drinking water (30-100 ppm) when brain tumors were observed (Johanssen and Levinskas, 2002a,b; Quast *et al.*, 1980a; Bigner *et al.*, 1986), while mortality was not increased in rats chronically exposed to 100 ppm AN in water when brain tumors were not observed (Gallagher *et al.*, 1988).

Reproductive/developmental Effects

The developmental effects of AN observed in animals may be associated with the release of cyanide during maternal metabolism of AN. In a study of several aliphatic nitriles, all the chemicals investigated produced the characteristic defects developed by embryos exposed to sodium cyanide *in utero* or in culture, suggesting that maternal formation of cyanide plays an important role in the developmental effects of AN (Saillenfait and Sabate, 2000).

A comparison of the major effects of AN with respect to weight-of-evidence and relevance to noncancer risk assessment is provided in **Table 4-21**.

Table 4-21. Summary of the Weight-of-Evidence from Animal and Human Studies Regarding the Major Noncancer Endpoints for AN

Noncancer Endpoint	Supported by Weight-of-Evidence from Animal Studies	Supported by Weight-of-Evidence from Human Studies	Mode of Action Relevant to Noncancer Risk Assessment
Neurological	Yes	Yes	Yes
Irritation	Yes	Yes	Yes
Hematological	Yes	Mixed	No
Survival	Yes	No	No

Based upon this comparison, the neurological and irritation effects of AN serve as the most appropriate basis for noncancer risk assessment (**Section 5.3.1**).

4.3.2 Cancer Effects

4.3.2.1 Major Effects

A large number of epidemiological studies have been carried out on populations exposed or potentially exposed to AN (often in combination with other potential or known carcinogenic compounds). The results from the major epidemiology studies for AN are summarized in **Table 4-1**. Some of these studies have reported slight excesses in certain cancers, but the results are not consistent from study to study. Additionally, these studies suffer to some degree from many of the same deficiencies in design or methodology: small cohort or small numbers of observed and expected events or of exposed cases, questionable comparison groups, uncertain or unquantified exposure estimates, exposure to multiple chemicals and carcinogens, lack of control for other potential confounders (*e.g.*, smoking), and incomplete follow-up or relatively short observation time for latency. Attempts have been made to correct some of these weaknesses by conducting meta-analyses. The primary cancer sites of concern suggested by these studies have been lung and prostate, although excesses of bladder, colon, and brain cancer have also been reported in some studies.

In animals, a number of chronic bioassays have been conducted for AN, either through ingestion (drinking water or gavage) or by inhalation. Most of the studies to date have been performed using the rat, although different strains of rat were tested, and a broad range of dose levels has been used. The results of these studies have shown that AN is carcinogenic to rats following either ingestion or inhalation. Common target organs identified were the central nervous system (brain and spinal cord), Zymbal's gland, gastrointestinal tract (tongue, non-glandular stomach and, small intestine), and mammary gland. A single study has been conducted in mice following chronic exposure via oral gavage. Although brain tumors were not observed in mice, a number of other tumors were found including forestomach and Harderian gland. The results of the carcinogenicity studies carried out in laboratory rodents for AN are summarized in **Table 4-22**.

Table 4-22. Summary of Tumor Findings in Cancer Bioassays Conducted for AN

Source	Test Species	Dose/ Concentration	Route of Exposure	Tumor Sites
Biodynamics, 1980b; Johannsen and Levinskas, 2002	Rat	0,1,3,10,30,100 ppm	Ingestion (drinking water)	CNS Astrocytomas. Ear & mammary gland carcinomas
Bigner *et al.*, 1986	Rat	0,100,500 ppm	Ingestion (drinking water)	Brain tumors
Quast *et al.*, 1980a	Rat	0,35,85,210 ppm for 21 d; 0,35,100,300 ppm thereafter	Ingestion (drinking water)	CNS, Zymbal's gland, forestomach, tongue, small intestine, mammary gland
Gallagher *et al.*, 1988	Rat	0,20,100,500 ppm	Ingestion (drinking water)	Zymbal's gland, forestomach papillomas.
Maltoni *et al.*, 1977	Rat	5 mg/kg-day	Ingestion (gavage)	Forestomach & mammary tumors.
Quast *et al.*, 1980b	Rat	0,20,80 ppm	Inhalation	CNS, Zymbal's gland, tongue, small intestine, mammary gland.
Maltoni *et al.*, 1977	Rat	0,5,10,20,40, 60 ppm	Inhalation	Mammary & Zymbal's glands, angiosarcomas, encephalic gliomas
Biodynamics, 1980a; Johannsen and Levinskas, 2002	Rat	0,1,100 ppm; 0, 0.1, 10 mg/kg-day	Ingestion (drinking water and gavage)	Astrocytomas of the brain and spinal cord, Zymbal's gland and forestomach tumors
Ghanayem *et al.*, 2002; NTP, 2001	Mouse	0, 2.5, 10, 20 mg/kg-day	Ingestion (gavage)	Forestomach and Harderian gland

The weight-of-evidence from human and animal studies with respect to the carcinogenicity in a number of tissue sites is discussed below.

Lung Cancer

Information regarding the potential carcinogenic effects of AN on the lung obtained from human and animal studies are summarized below.

- *Human Studies* - While some early studies reported small excesses of lung cancer mortality (Thiess *et al.*, 1980; Werner and Carter 1981; Delzell and Monson, 1982; O'Berg *et al.*, 1985), other studies have reported no such excess (Collins *et al.*, 1989) or even small deficits (Kiesselbach *et al.*, 1979; Swaen *et al.*, 1998; Blair *et al.*, 1998; Mastrangelo *et al.*, 1993). In general, workers with less than one year exposure to AN have shown no excess of lung cancer. However, in the O'Berg (1980) study, exposure

from six months onwards appeared to increase the risk of cancer at all sites studied (*e.g.*, lung, colon, prostate, bladder, etc.) with increasing exposure. Wage earners (maintenance mechanics) were generally more at risk by virtue of their greater potential for exposure than salaried workers. The original database was subsequently extended (O'Berg *et al.*, 1985) and again in 1998 (Wood *et al.*, 1998). The most recent study reports no increase in lung cancer risk with increasing cumulative exposure. Two lung cancer cases were inadvertently excluded from the O'Berg study, raising concern regarding completeness of cohort ascertainment in the early studies. Thiess *et al.* (1980) reported lung cancer rates by duration of exposure. The SMRs were elevated for each duration of exposure category, but showed no trend. Delzell and Monson (1982) examined duration of employment and years since first employed and also showed no trend with duration of exposure; however, an increased SMR after 15 years since starting employment was reported. Collins *et al.* (1989) examined cumulative AN exposure and found a modest U-shaped curve in the effect estimates for lung, but this did not show a linear trend overall. Swaen *et al.* (1992) reported only a small (non-significant) increase in lung cancer mortality with increasing dose and latency for the highest exposure group (*i.e.*, 10+ ppm). However, this observation may well reflect the fading out of the healthy worker effect in an aging work force rather than an actual increased risk from lung cancer following exposure to AN. Overall, Swaen *et al.* could not identify a trend in lung cancer mortality data by cumulative exposure and no increased risk with increasing latency. Mastrangelo *et al.* (1993) also found no trend with duration of exposure. In the Blair *et al.* (1998) study, there was no evidence to indicate that exposure to AN at the levels experienced by these workers was associated with any significant increased relative risk for most cancers. The elevated lung cancer rate ratio seen in the highest exposure quintile, particularly when exposure began 20 years ago or more may indicate some risk at high exposure. However, no dose-response effect was identified when exposure was divided into deciles and the rate of lung cancer did not increase consistently with increasing exposure in an analysis that considered deciles of exposure. In the Benn and Osborne study (1998), lung cancer mortality showed an increased SMR in the 15 to 44 and 45 to 54 age groups and a deficit for the older age groups. However, this study was hampered by certain limitations (*e.g.*, lack of exposure/measured data and lack of information on smoking habits) that make drawing conclusions difficult. Overall, the results of the Swaen *et al.* (1998, 2004) studies did not indicate any cancer excess related to occupational exposure to AN. Similarly, the Wood *et al.* (1998) study showed no significant increases in cancer, nor was any dose-response relationship established regarding cancer mortality or morbidity. The meta-analyses carried out by Rothman (1994), Collins and Acquavella (1998), and EU (2001) also found no excess of lung cancer among AN workers. These conclusions held even when considering the more recent epidemiological studies and when analyses were conducted with respect to exposure level and induction or latency periods. For lung cancer, Collins and Acquavella (1998) evaluated consistency across studies, strength of the association, and some aspects

of internal consistency within the studies such as dose-response and latency. Overall, their conclusion was that, if any excess lung cancer is related to AN exposure, the risk is small. Considering the results of the more recent studies (including the meta-analyses) and the limitations of the older studies, the overall conclusion drawn is that there is no consistent trend in the lung cancer data.

- *Animal Studies* - Lung tumors have not been observed in rats exposed to AN via inhalation, drinking water, or gavage (Maltoni *et al.*, 1977, 1988; Quast et al., 1980a,b; Gallagher, 1980; Bigner, 1986; Johannsen and Levinskas, 2002a,b). A significant increased in lung tumors was reported for mice exposed to AN via gavage, however, based upon the magnitude of the increase and the lack of a consistent dose-response trend, the authors determined the evidence for carcinogenicity in the mouse lung to be equivocal (NTP, 2001; Ghanayem *et al.*, 2002).

The lung cancer mortality data have greater statistical power than other cancer mortality data sets (based upon the observed number of deaths), and lung cancer mortality was used previously by USEPA to estimate cancer potency (USEPA, 1983). Although not significantly increased, lung cancer SMR values and corresponding exposure estimates are available for a large cohort of exposed workers (Blair *et al.*, 1998; Stewart *et al.*, 1998), and can be used to place bounds upon the cancer potency of AN derived from animals studies. A comparison of the epidemiology lung cancer mortality data to the dose-response data for brain tumors in rats is presented in **Appendix G**.

Prostate Cancer

Information regarding the potential carcinogenic effects of AN on the prostate obtained from human and animal studies are summarized below.

- *Human Studies* - An increased incidence of prostate cancer morbidity was reported by O'Berg *et al.* (1985) and by Chen *et al.* (1987). In the O'Berg *et al.* (1985) study, a trend analysis did not reveal a significant relation between cumulative exposure to AN and prostate cancer morbidity. All six cases occurred at least 20 years after first exposure to AN in the workplace. Two cases were found in the lowest exposure group (0.3 expected), 0 cases in the middle exposure group (0.4 expected) and four in the high exposure group (0.7 expected). The SMR for prostate cancer between the lowest and highest exposure group was not different despite the greater than six times difference in estimated exposure. A dose-dependent trend could not be observed and, therefore, a causal relationship between AN and prostate cancer morbidity cannot be established based on the O'Berg *et al.* (1985) data. Chen *et al.* (1987) stated that three of the prostate cancer cases had a latency time of more than 20 years. Both O'Berg and Chen used the internal DuPont Cancer Registry for estimating the expected prostate cancer morbidity. A major

drawback with using this Registry as a reference is that workers leaving DuPont for other jobs or retiring are no longer followed with respect to morbidity and mortality for the internal DuPont Cancer Registry. In the most recent update of these DuPont workers, no significantly increased prostate cancer risk or dose-response relationship was seen (Wood *et al.*, 1998). In the more recent studies of Blair *et al.* (1998) and Swaen *et al.* (1998, 2004), no excess of prostate cancer was reported as well. Blair *et al.* (1998) studied over 25,000 workers and found no excess of prostate cancer mortality. Swaen *et al.* (1998, 2004) also failed to find any effects in a large, well-designed Dutch mortality study. According to the meta-analysis of Collins and Acquavella (1998), the studies of Thiess *et al.* (1980), Delzell and Monson (1982), and Burke (1985b) reported no prostate cancers, but confidence intervals were wide. Keisselbach *et al.*, and Wood *et al.* reported small excesses, and Burke (1985a) reported an excess based on a single case. On the other hand, the large studies of Blair *et al.* and Swaen *et al.* report slight deficits of prostate cancer. The mRR for the prostate cancer mortality from Collins and Acquavella (1998) is 1.0 (95% CI = 0.7 to 1.5). Only the Blair *et al.*, Swaen *et al.*, and Wood *et al.*, studies report exposure level analyses for prostate cancer risks. None of these studies show any increasing risk with increasing exposure. The single nested case-control study of Marsh (1993) does report duration of exposure for the prostate cancer cases and controls. There were no cases in the highest category of 10 or more years of exposure, but numbers were small overall. The two unpublished studies of Burke (Beaumont plant, 1985a) and Burke (Memphis plant, 1985b), which included prostate cancer mortality, reported one death from prostate cancer (mRRs = 3.9) compared to a mRR of 1.0 for the published studies. Overall, the mRR for prostate cancer incidence was 1.4 (95% CI = 0.8 to 2.6). The Wood *et al.* study found 12 prostate cancer cases versus 7.6 expected. The other two studies that examined incidence found no prostate cancer cases with 0.8 and 0.1 expected, respectively. The Wood *et al.* update of the earlier DuPont studies (Chen *et al.*, 1987 and O'Berg *et al.*, 1985) found only one new case versus 3.89 expected (SIR = 0.3, 95% CI = 0.0 to1.4) in the update period. Therefore, the cases of prostate cancer are limited in time. In addition, the non-significant excess of prostate cancer in the Wood *et al.* study was limited to a narrow reporting period (*i.e.*, 1978 to 83) when improved diagnostic procedures were being introduced. A deficit is observed (SIR = 0.3, 95% CI = 0.0 to 1.4) from 1983 to 1991 (Collins and Acquavella, 1998). This indicates the potential for diagnostic bias as cases may have been detected early (EU, 2001). No trend with exposure level was observed and there was no accompanying increase in prostate cancer deaths (Collins and Acquavella, 1998). The excess prostate incidence reported by O'Berg *et al.* (1985) and Chen *et al.* (1987) (confirmed in the analysis of Wood *et al.*, 1998) raised the concern that exposure to AN may increase prostate cancer incidence risk. However, there is no increase in prostate cancer rates with increasing exposure and this finding has not been confirmed in the mortality studies. Considering the results of the more recent studies (including the meta-analyses) and the limitations of the older studies,

the overall conclusion drawn is that there is no association between AN and prostate cancer.

- *Animal studies* - No evidence of an increased incidence of prostate tumors has been reported in rats exposed orally or via inhalation (Maltoni *et al.*, 1977, 1988; Quast et al., 1980a,b; Gallagher, 1980; Bigner, 1986; Johannsen and Levinskas, 2002a, b), or in mice exposed orally to AN (NTP, 2001; Ghanayem *et al.*, 2002).

In summary, data from human and animal studies are consistent in yielding negative results for the carcinogenic effects of AN on the prostate.

Bladder Cancer

Information regarding the potential carcinogenic effects of AN on the bladder obtained from human and animal studies are summarized below.

- *Human Studies* - Collins and Acquavella (1998) presented data in their meta-analysis that suggested bladder cancer risk may be increased among AN workers, but the excess was not dose-related. It was associated with the plants where exposure to aromatic amines was also possible. The plants with no aromatic amine exposure had a bladder cancer mRR of 0.9 (95% CI = 0.5 to 1.5) while the plants with aromatic amine exposure had a mRR of 4.5 (95% CI = 1.8 to 10.9). Further the finding of increased bladder cancer risk is due mainly to the inclusion of results from Kiesselbach *et al.* (1979), and Siemiatycki *et al.* (1994). Collins has since re-evaluated the data excluding the two studies mentioned (EU, 2001). This resulted in the bladder cancer mRR being reduced from 1.4 (95% CI = 0.9 to 2.0) from the 10 studies reporting a bladder cancer relative risk in the original meta-analysis report to 1.1 (95% CI = 0.7 to 1.7). Excluding these two studies also reduced the heterogeneity as evidenced by the change in p-values from 0.18 in the original analysis to 0.45. This provides some evidence that both the Kiesselbach and Siemiatycki studies are outliers and may not be representative of the bladder cancer risk in the remaining studies. However, it is possible that the change (*i.e.*, increase) in p-value is partially due to the decrease in the amount of data analyzed when these two studies are excluded. Blair *et al.* (1998) was the only study to report exposure levels for bladder cancer, and it was found that there was no increased risk with increasing exposure levels. It should also be noted that the most potent occupational cause of bladder cancer is aromatic amines and that three of the studies reported the presence of aromatic amines in the plant environment (Keisselbach *et al.*, 1979; Thiess *et al.,* 1980; and Delzell and Monson, 1982). Their data are consistent with the possibility of these substances being confounding factors for this endpoint. Considering the results of the re-analysis of Collins and Acquavella (1998), the limitations of the older studies, and the findings of the

more recent studies, it also seems unlikely that there is any association between AN and bladder cancer.

- *Animal studies* - No evidence of an increased incidence of bladder tumors has been reported in rats exposed to AN orally or via inhalation (Maltoni *et al.*, 1977, 1988; Quast et al., 1980a,b; Gallagher, 1980; Bigner, 1986; Johannsen and Levinskas, 2002a, b), or in mice exposed orally to AN (NTP, 2001; Ghanayem *et al.*, 2002).

In summary, data from human and animal studies are consistent in yielding negative results for the carcinogenic effects of AN on the bladder.

Brain Cancer

Information regarding the potential carcinogenic effects of AN on the brain obtained from human and animal studies are summarized below.

- *Human Studies* - The epidemiological record has been examined to determine if there is any increased risk of nervous system tumors in AN workers. Case-referent studies offer the possibility of increased efficiency when studying rare endpoints, such as occurrence of astrocytic brain tumors. The only case-referent study conducted on brain tumor cases in chemical workers was by Thomas *et al. (*1987). This study found no meaningful excess of astrocytic brain tumors occurred among these workers (n = 300). The studies of Kiesselbach *et al.* (1979), Herman (1981, unpublished), Burke (1985b, unpublished), Mastrangelo *et al.* (1993), Swaen *et al.* (1998, 2004), and Wood *et al.* (1998) report RRs for brain and central nervous system cancers in excess of 1.0. On the other hand, Thiess *et al.* (1980), Delzell and Monson (1982), Burke (1985a, unpublished), and Blair *et al.* (1998) report RRs for brain and central nervous system cancers less than 1.0. From the meta-analysis carried out by Collins and Acquavella (1998), the mRR for brain cancer is 1.2 (95% CI = 0.8 to 1.7). Only studies on two cohorts (Blair *et al.*, 1998; Swaen *et al.*,1998, 2004) report brain cancer rates by exposure level. There is no apparent increase in risk with increasing level of exposure in either study. Overall, there was no excess of brain cancers in the cohort studies. Although the estimates were imprecise, the RR for nervous system tumors were higher in the unpublished studies (mRR = 1.1, 95% CI = 0.6 to 9.3) than in the published studies (mR = 1.1, 95% CI = 0.7 to 1.5) cited by Collins and Acquavella (1998); however, there also was a tendency to not report SMR less than 1.0. Studies reporting expected deaths had an mRR of 2.2 (95% CI = 0.7 to 6.4) compared to an mRR of 1.1 (95% CI = 0.7 to 1.6) for studies not reporting expected deaths (Collins and Acquavella, 1998). The authors of the meta-analysis concluded that, based on the available studies, there is little support for a causal relationship between AN exposure and brain cancers.

- *Animal studies* - Laboratory studies have identified the brain as a target organ for the carcinogenic effects of AN in rats. An increased incidence of brain tumors has been reported in rats exposed to AN orally or via inhalation (Maltoni *et al.*, 1977, 1988; Quast et al., 1980,b; Bigner, 1986; Johannsen and Levinskas, 2002a, b). However, a single study failed to find an increased incidence of brain tumors in rats chronically exposed to AN (Gallagher, 1980). An explanation for the lack of brain tumors observed in this study is not readily apparent. Furthermore, the incidence of brain tumors was not increased in mice exposed orally to AN, and therefore the brain does not appear to be a target organ in this species (NTP, 2001; Ghanayem *et al.*, 2002).

In summary, data from human and animal studies are not consistent in identifying the brain as a target tissue for AN carcinogenicity. A number of data sets are available to characterize the dose-response relationship for brain tumors in rats (Maltoni *et al.*, 1977, 1988; Quast et al., 1980a,b; Bigner, 1986; Johannsen and Levinskas, 2002a, b). However, excess mortality from brain cancer has not been observed in human studies.

Other Tumors

Information regarding the potential carcinogenic effects of AN on other tumors obtained from human and animal studies are summarized below.

- *Human Studies* - Information from a large body of epidemiology studies of workers exposed to AN do not lend support for the hypothesis that AN is a human carcinogen (Blair *et al.*, 1998; Wood *et al.*, 1998; Benn and Osbourne, 1998; Collins and Acquavella, 1998).

- *Animal Studies* - Information from animal studies indicate that AN exposure produces an increased incidence of tumors in a number of tissue sites other than brain in rats (Zymbal's gland, forestomach, tongue, small intestine, and mammary gland) following inhalation exposures (Maltoni *et al.*, 1977, 1988; Quast et al., 1980a) or oral exposures (Gallagher, 1980; Bigner, 1986; Johannsen and Levinskas, 2002a, b). In addition to lung tumors, mice chronically exposed to AN via oral gavage have developed an increased incidence of a number of other tumors, including forestomach and Harderian gland (Ghanayem *et al.*, 2002).

In summary, data from human and animal studies are not consistent in identifying other tissues as a targets for AN carcinogenicity.

Given the negative results supported by a large database of epidemiology studies, the relevance of the tumors reported in laboratory rodents is not fully understood. However, because many of these tumor sites lack human tissue homologues, they are not useful for quantitative dose-

response assessment. For example, there is no human tissue counterpart that corresponds to the Zymbal's gland in rodents. Similarly, the forestomach is a portion of the gastrointestinal tract of some animals with a squamous cell epithelia located between the esophagus and glandular stomach (Wester and Kroes, 1988; Kroes and Wester, 1986). The forestomach serves as an organ for storage and predigestion of food material. Although the forestomach was the only organ in which both rats and mice developed tumors, there are two reasons that the relevance of forestomach tumors to human health is not fully understood, the first pertaining to biology and the second pertaining to mechanism. With respect to biology, there is no tissue homologue in humans that corresponds directly to the rodent forestomach (Wester and Kroes, 1988; Kroes and Wester, 1986). With respect to mechanism, the forestomach typically achieves high concentrations of the chemical following gavage, which occurs over an extended period of exposure time since it serves as a storage organ. In a number of cases, these two factors combine to result in forestomach hyperplasia and inflammation (as observed in AN-exposed mice), which in turn contribute to the carcinogenic process through a tumor promotional mechanism. Nongenotoxic chemicals, such as butylated hydroxyanisole and sodium chloride produce forestomach tumors at high concentrations by such a mechanism (Ito *et al.* 1983; Furihata *et al.* 1996). In animals with forestomach tumors, additional tumors of surrounding tissues (esophagus, glandular stomach) are generally lacking. Therefore, the presence of forestomach tumors generally fails as a predictor of other GI tumors even within the same species. The relatively high sensitivity of the forestomach to tumors under specific conditions suggests its reliance in quantitative risk assessment would result in an overestimation of potential risks. Another example tumor site is the Harderian gland. The harderian gland is an ocular gland with tubular alveoli present in some animals (rodents, amphibians, reptiles, birds) but absent in others (dogs, cats, sheep, goats) (Albert *et al.*, 1986; Sheldon, 1994). In rodents, the gland opens on the outer surface of the third eyelid, where it secretes lipids and porphyrins. Like the forestomach, the harderian gland has no tissue homologue in humans (Albert *et al.*, 1986; Sheldon, 1994). Incidence of harderian gland tumors in rodents has been correlated with porphyrin content (Figge *et al.*, 1942). The harderian gland, like the forestomach, also appears to be a tissue in which significant noncancer effects (toxicity, hyperplasia) were observed. Given the lack of tissue homologues in humans for the Zymbal's gland, forestomach, and Harderian gland, the presence of tumors in these tissues cannot be used to support a quantitative dose-response assessment for AN. Rather, these data can only be used qualitatively to support the conclusion that AN is a multi-site carcinogen in laboratory rodents.

A comparison of the weight-of-evidence from human and animal studies for the carcinogenicity AN in various tissue sites is provided in **Table 4-23**.

Table 4-23. Tissue-Specific Weight-of-Evidence for the Carcinogenicity of AN

Tissue Site	Supported by Weight-of-Evidence from Human Studies	Supported by Weight-of-Evidence from Animal Studies	Magnitude of Dose-Response Relationship Observed in Animal Studies	Human Tissue Homologue
Lung	No*	Negative in multiple rat studies; Equivocal in a single mouse study	Weak	Yes
Prostate	No	No	--	Yes
Bladder	No	No	--	Yes
Brain	No	Positive in multiple rat studies; Negative in a single mouse study	Strong	Yes
Mammary Gland	No	Mixed results in multiple rat studies; Negative in a single mouse study	Weak	Yes
Tongue	No	Mixed results in multiple rat studies; Negative in a single mouse study	Weak	Yes
Intestines	No	Mixed in multiple rat studies; Negative in a single mouse study	Weak	Yes
Forestomach	--	Yes	Weak	No
Zymbal's gland	--	Positive in multiple rat studies; Negative in a single mouse study	Strong	No
Harderian gland	--	Positive in a single mouse study; Negative in multiple rat studies	Strong	No

*Although no significant increases in lung cancer mortality were reported in epidemiology studies, these data can be used to place bounds on cancer potency estimates from animal data (see **Appendix G**).

The selection of a data set to serve as the basis for an oral unit risk value for AN should include a careful consideration of the relevance of the data to human health as well as the degree of

protectiveness (conservatism) it offers. Because the epidemiology data for AN do not provide evidence for carcinogenicity, the relevance of all rodent data sets is not fully understood. There are two possible explanations for the apparent discrepancy between rodent and human studies: (1) there is a qualitative difference between rodents and humans such that AN is carcinogenic to rodents but not to humans; and (2) there is a quantitative difference between rodents and humans, such that the degree of exposure in human studies was not sufficient to result in a large number of cancer deaths, or such that differences in kinetics/dynamics result in a lower sensitivity to the carcinogenic effects in humans than in rodents. In the absence of specific information to support the former explanation, the latter explanation was conservatively adopted for this assessment. Of the rodent tissue sites demonstrating a strong dose-response relationship (brain, Zymbal's gland, Harderian gland), only the brain is also present in humans. Accordingly, the brain was selected for use in the quantitative dose-response assessment for AN based upon animal data (**Section 5.3.2**)

4.3.2.2 Mode of Action

The mode of action information presented in this section for AN draws upon information presented earlier in this document regarding its metabolism (**Section 3.3**) and genotoxicity (**Sections 4.1.1.3** and **4.2.6**). Information on the mode of action for AN is of primary importance to human health risk assessment in that it impacts several decision points including the selection of an appropriate dose measure, the selection of a method for extrapolating to low doses, and potentially other decision points in the dose-response assessment (**Section 5.0**).

With respect to the carcinogenic effects of AN, there is considerable information available regarding the mode of action by which AN produces brain tumors in rats. Research into the mode of action for AN is ongoing. Some critical pieces of information regarding the mode of action for AN have yet to be published, but are included in reviews written by scientists who have made significant contributions to the mechanistic research for AN. These include reviews written by Dr. James Klaunig (2002; **Appendix A**) and Dr. John Whysner (2002; **Appendix B**). Where possible, issues which impact the dose-response assessment with respect to relevance to human health, important species differences, and sources of nonlinearity have been identified.

There is little information regarding the potential modes of action for tumor types other than rat brain tumors. For this reason, mode of action information is presented separately below for brain and other tumor types.

Brain Tumors

AN is a small, water-soluble molecule, which once absorbed into the blood, is capable of distributing throughout the body. The brain does not possess a large capacity for metabolizing

AN, therefore it is proposed that the bulk of AN metabolites reaching the brain are formed in other tissues (*e.g.,* liver, lung) and transported by the blood. AN and its stable metabolites CEO and cyanide (CN^-) are capable of crossing the blood-brain barrier. Although the precise molecular mechanism of action by which AN or its metabolites produce brain tumors in rodents is not known, potential modes of action include both genetic (direct genotoxicity) and epigenetic (indirect genotoxicity, nongenoxic) mechanisms. Data regarding these potential modes of action are discussed below within the context of the Hill criteria for causation.

Direct Genotoxicity

Under a direct genotoxic mode of action, it is hypothesized that upon reaching brain cells in the rats, AN or one of its metabolites forms adducts with DNA. Such adducts would be capable of producing mutations, which in turn can alter gene expression, resulting in loss of cell growth control, and ultimately tumor formation. An evaluation of this potential mode of action for AN is provided below with respect to satisfying the Hill criteria for causation.

- *Strength of Association* - Data regarding the strength of an association between direct genotoxicity and brain tumor formation are mixed, with positive results from *in vitro* studies contrasting sharply with negative results obtained from *in vivo* studies. Initial support for a genotoxic mechanism for AN comes from structure activity relationship (SAR) considerations for its epoxide metabolite, CEO. Reactive epoxides (ethylene oxide) or chemicals that are metabolized to reactive epoxides (vinyl chloride, acrylamide, 1,3-butadiene) have been suggestedto produce brain tumors in rats. Data from *in vitro* studies indicate that at high concentrations CEO reacts with DNA to form N7-(oxoethyl)-guanine, N3-(2-hydroxy-2-carboxyethyl)-uracil, and 1,N6-ethenoadenine (Whysner *et al.*, 1998). In *in vitro* studies, AN produces a statistically significant increase in mutation frequency in test systems particularly when metabolic activation is included (Lijinsky and Andrews, 1980; Zeiger and Haworth, 1985; Venitt *et al.* 1977). AN is considerably cytotoxic, and in some cases this effect may have contributed to a positive response. *In vitro* studies also show that CEO is considerably more potent that AN in producing mutations, suggesting its formation may be responsible for the positive results observed for AN (Guengerich *et al.*, 1981; Soloman *et al.*, 1984, 1993). Other metabolites of AN, such as cyanide do not produce an increase in mutation frequency (Owais *et al.*, 1985).

  In sharp contrast to the results observed from *in vitro* studies, results from *in vivo* studies are largely negative for DNA adduct formation. Attempts to identify specific DNA adducts (7-OEG) in the rat brain following exposure to AN have been unsuccessful (Hogy and Guengerich, 1986; Whysner et al., 1998). None of the three types of adducts observed *in vitro* (N7-(oxoethyl)-guanine, N3-(2-hydroxy-2-carboxyethyl)-uracil, and 1,N6-ethenoadenine) were found in the brain tissue from rats exposed to 500 ppm AN in drinking water for 15 months (Walker et al., 1994). Other adducts (*e.g.,* 2-cyano-2-

hydroxyethyl phosphodiester adducts) were observed in an *in vitro* study using high concentrations of CEO (Yates *et al.*, 1994). Although a single study reported the binding of radiolabel to rat brain DNA following exposure to radiolabeled AN (Farooqui and Ahmed, 1983b), these results were not confirmed by more recent studies using more sensitive methods (Pilon *et al.*, 1988a,b; Whysner *et al.*, 1998). Because AN binds to protein sulfhydryls, any contamination of DNA with protein would be confounding. Some investigators have attributed the DNA binding reported in the early study to contamination of the DNA fraction with protein-bound AN (Geiger *et al.*, 1983; Guengerich *et al.*, 1986).

In vivo studies for the genotoxicity of AN have largely returned negative results. A single study reported a significant increase in the frequency of HPRT mutations in splenic T-cells of mice exposed to 20 mg/kg AN, but no increase in P4502E1 null mice exposed to the same dose (Walker and Ghanayem, 2003). Despite achieving statistical significance, the relevance of these findings to rat brain tumors is complicated by the fact that the results were reported in a tissue remote from the target organ, and were collected in a species that does not develop AN-induced brain tumors. Additionally, the possibility remains that the phenotypic changes could result from an indirect genotoxic mechanism (see below). These positive results for the T-cell HPRT mutations are contrasted with the negative results obtained for lacZ mutations in a variety of tissues, including brain, collected from the same animals.

- *Consistency of Association* - Given the disparity between the positive results observed for the DNA reactivity and genotoxicity of AN and CEO *in vitro* compared to the largely negative results observed *in vivo*, the association between genotoxicity and brain tumors has not been consistently demonstrated.

- *Specificity of Association* - Evidence argue against a genotoxic mode of action with respect to the specificity of an association. Following exposure to AN, specific DNA adducts known to be formed by reactive epoxides were observed in rat hepatocytes, which are not target cells for carcinogenicity, but were not observed in brain cells, which are target cells (Whysner *et al.*, 1998; Hogy and Guengerich, 1986). The lack of specific measurable adducts in rat brain, using very sensitive analytical methods, indicates that a direct genotoxic mode of action for AN fails to explain the target organ specificity of AN carcinogenesis in rats. Other DNA adducts, which have been observed at high concentrations *in vitro* (Yates et al., 1994), have not been specifically evaluated in *in vivo* studies.

- *Dose-Response Concordance* - Although DNA adducts and mutation frequency increase as a function of concentration *in vitro*, the fact that DNA adducts in the rat brain do not increase with dose, even with water concentrations (500 ppm) that are higher than those

associated with brain tumor formation in rats (30 - 300 ppm) indicates a lack of dose-response concordance.

- *Temporal Relationship* - Because DNA adducts have not been observed in rat brain tissue following exposure to AN (Whysner *et al.*, 1998), a temporal relationship between DNA adducts and brain tumor formation cannot be inferred.

- *Biological Plausibility and Coherence* - The available evidence indicate that AN is not a direct acting genotoxic carcinogen. In addition, if a direct genotoxic mode of action is truly relevant for the epoxide metabolite, CEO, then the most sensitive tumor type would be expected to occur where the highest rates of metabolism are observed (*i.e.*, liver, as is the case for vinyl chloride). Since the liver is not a target organ and point-of-contact sites are less sensitive than the brain, a direct genotoxic mode of action is not supported for brain tumor formation in rats.

In summary, while there are some data, largely from *in vitro* studies, along with theoretical associations between mutation and tumor formation, that offer limited support for a direct genotoxic mode of action, the weight-of-evidence from *in vivo* studies do not support direct genotoxicity as the predominant mode of action for AN in producing brain tumors in rats.

Indirect Genotoxicity

Under an indirect genotoxic mode of action for AN, upon reaching brain cells in exposed rats AN or one of its metabolites produces oxidative stress, resulting in the formation of 8-oxo-dG, which in turn can alter gene expression resulting in loss of cell growth control and ultimately in tumor formation. An evaluation of this potential mode of action for AN is provided below with respect to meeting the Hill criteria for causation.

- *Strength of Association* - *In vitro* and *in vivo* studies demonstrate a statistically significant increase in the formation of 8-oxo-dG in rat brain tissue following exposure to AN (Jiang *et al.*, 1998; Kamendulis *et al.*, 1999a; Whysner *et al.*, 1998). Furthermore, *in vitro* studies using Syrian hamster embryo cells demonstrate that AN produces morphological transformations which were correlated with the formation of 8-oxo-dG (Zhang *et al.*, 2000), suggesting that this lesion can alter cell phenotype. Oxidative DNA damage has also been shown to alter gene expression by affecting the binding of transcription factors to promoter elements of DNA (Ghosh and Mitchell, 1999).

- *Consistency of Association* - The formation of 8-oxo-dG has been consistently observed in rat brain tissue using both *in vitro* and *in vivo* studies (Jiang *et al.*, 1998; Kamendulis *et al.*, 1999a; Whysner *et al.*, 1998). Evidence for lipid peroxidation in rat brain, however, has not been consistently observed (Jiang *et al.*, 1998; Kamendulis *et al.*,

1999a). One possible explanation is that the discrepancy reflects the use of different tissues with negative results reported for whole brain (Whysner *et al.*, 1998) and the positive results reported for the brain cortex (Jiang *et al.*, 1998). Because glial cells are present in greater abundance in brain cortex, the difference may be attributed to a dilutional effect of the target cells in whole brain. Another possible explanation is that the discrepancy reflects the use of different indices to measure lipid peroxidation (thiobarbituric acid reactive substances vs. malondialdehyde formation)

- *Specificity of Association* - A mode of action for AN involving oxidative damage to DNA is consistent with the differences observed across species and target organs. The formation of 8-oxo-dG in brain cell DNA has been observed in rats exposed to AN (Jiang *et al.*, 1998; Kamendulis *et al.*, 1999a; Whysner *et al.*, 1998), but was not observed in the brain cells of mice exposed to AN (personal communication Dr. Klaunig, 2002; **Appendix A**). In rats, *in vitro* and *in vivo* studies indicated that 8-oxo-dG is produced in brain cells, which are target cells, but is not produced in hepatocytes, which are not target cells, following exposure to AN (Jiang *et al.*, 1998; Kamendulis *et al.*, 1999a), indicating that this mode of action is consistent with the target-organ specificity of AN carcinogenicity. Additionally, a reanalysis of the brain tumor histopathology slides from the rat bioassays indicates that tumors were observed primarily in the anterior and mid portion of the brain, which corresponds to the regions where 8-oxo-dG formation is observed (Whysner, 2002; **Appendix B**).

- *Dose-Response Concordance* - Both oxidative stress to brain cell DNA and brain tumors increase in a dose-dependent manner in rats exposed to AN. The range of water concentrations of AN (5-200 ppm) producing a significant increase in oxidative stress to DNA (Jiang *et al.*, 1998; Whysner *et al.*, 1998) compares well to the range of concentrations of AN in drinking water (30-300 ppm) producing a significant increase in brain tumors in rats (Johansen and Levinskas, 2002).

- *Temporal Relationship* - Based upon a consideration that formation of 8-oxo-dG is observed in rats following subchronic exposures to AN (Jiang *et al.*, 1998; Whysner *et al.*, 1998), whereas brain tumors are observed in rats following chronic exposures (Johansen and Levinskas, 2002a,b), a temporal relationship between oxidative stress and rat brain tumors can be inferred.

- *Biological Plausibility and Coherence* - Overall, the formation of 8-oxo-dG in rat astrocytes provides a coherent mode of action that offers a biologically plausible explanation for the production of brain tumors in rats exposed to AN. Astrocytes play an important role in maintaining extracellular levels of glutathione in the brain, for which there are quantitative differences between rats and humans (Stewart *et al.*, 2002). Given the stresses that AN, CEO, and cyanide place on tissue sulfur levels (GSH and sulfane

sulfur), this protective role of astrocytes may be an important determinant of its relative susceptibility compared to other cell types. The precise mechanism by which AN produces oxidative stress in rat astrocytes is not known, and may include (1) an increase in the generation of reactive oxygen species; (2) a diminished capacity to defend against oxidative stress; and/or (3) a diminished capacity to repair 8-oxo-dG. Of these three possible mechanisms, an increase in reactive oxygen species is the best supported. However, it has not been conclusively determined whether AN or one of its metabolites is ultimately responsible for the increase in reactive oxygen species. Although the induction of oxidative stress by AN in rat astrocytes *in vitro* (Kamendulis *et al.*, 1999a) would suggest that AN is directly responsible for this effect, it should be noted that the brain cells possess some capacity to metabolize AN (Ahmed and Abreu, 1981). Furthermore, several studies lend support to the hypothesis that the metabolism of AN is important to oxidative stress. Pretreatment of with a cytochrome P-450 inhibitor (1-aminobenzotriazole) revealed that the induction of xanthine oxidase and catalase in Syrian hamster embryo (SHE) cells required the oxidative metabolism of AN by cytochrome P450 (Zhang *et al.*, 2002). *In vitro* studies in rat hepatocytes indicate that lipid peroxidation (production of thiobarbituric acid reactive substances) was produced to a much greater extent by CEO than by AN (Nerudova *et al.*, 1988). One possible role for metabolism in oxidative stress is that reactive oxygen species are generated by CYP2E1. Ethanol metabolism by this enzyme has produced oxidative stress in rat astrocytes (Montoliu *et al.*, 1995), however, brain tumors are not associated with exposures to ethanol at comparatively large doses. Furthermore, such a role for CYP2E1 would suggest that the liver would be a target organ for AN carcinogenicity, and yet this is not consistent with observations made for AN.

Another potential mechanism for oxidative stress is the formation of cyanide during the hydrolysis of CEO (Benz *et al.* 1997a; Mostafa *et al.*, 1999; Abdel-Aziz *et al.* 1997). Studies have shown that the release of cyanide from AN requires oxidation, either enzymatically (Wang *et al.*, 2002) or possibly nonenzymatically (Mohamadin, 2001). Because cyanide inhibits cytochrome a/a3, it affects mitochondrial oxygen utilization, and has been shown to produce oxidative stress (*i.e.*, lipid peroxidation) in brain but not in liver (Ardelt *et al.*, 1994). Pretreatment of rats with sodium thiosulfate, whose availability is the rate-limiting step for cyanide detoxification, protected against the formation of AN-induced brain glial fibrillary acidic protein (GFAP), a biomarker for astrogliosis (Enongene *et al.*, 2000). However, other cyanogenic chemicals such as methylacrylonitrile do not produce brain tumors in rats (Ghanayem, 2000), suggesting that some factor in addition to cyanide release may be required for brain tumor formation. One possible factor is that the cyanide release must be accompanied by an increased demand on cellular cysteine levels. As indicated in **Figure 3-1**, glutathione (which is conjugated with AN and CEO) and sulfane sulfur (which is used to detoxify cyanide) both require the transport of cysteine across the blood-brain barrier to maintain cellular

levels. *In vitro* studies indicate that lipid peroxidation in hepatocytes is produced by cyanide only in the presence of diethylmaleate, a chemical that depletes hepatic GSH (Nerudova *et al.*, 1988). In rats exposed to AN, cysteine levels were elevated and GSH levels were maintained in brain tissue (Whysner *et al.* 1998). However, sulfane sulfur status has not been assessed, but could be compromised for the sake of maintained cellular GSH levels. Another possible factor is that the cyanide-induced oxidative stress must be accompanied by the presence of AN, which has been shown to catalyze the formation of 8-oxo-dG in the presence of hydrogen peroxide and copper (Murata *et al.*, 2001).

The mechanism by which cyanide produces oxidative stress in brain cells is not known, but may involve cyclooxygenase activity. Cyanide-induced oxidative stress is inhibited by aspirin (Daya *et al.*, 2000), a well known inhibitor of cyclooxygenase activity. An inducible cyclooxygenase is present in rat brain (Knapp and Crews, 1999; Strauss *et al.*, 2000), and is associated with apoptotic cell death (Ho *et al.*, 1998). Co-oxidation of AN in the brain (thereby increasing cyanide release) and alterations in prostaglandin synthesis and resulting astrogliosis are two potential roles in which cyclooxygenase may contribute to the production of brain tumors in rodents. Although the metabolism of AN by brain cyclooxygenase has not been evaluated, the fact that it can be oxidized by a lung lipoxygenase (Roy and Kulkarni, 1999) suggests that such a pathway is at least feasible. The generation of reactive oxygen species may also contribute to the nonenzymatic release of cyanide from AN, as has been observed *in vitro* with a structurally similar chemical (dichloroacetonitrile), in which a four-fold increase in peroxide levels resulted in a 35-fold increase in cyanide release (Mohamadin, 2001). Because cyanide release from AN may contribute to oxidative stress, while reactive oxygen species may contribute to the nonenzymatic release of cyanide from AN, the potential exists for a positive feedback loop between cyanide release and oxidative stress in the rat brain.

In summary, the weight-of-evidence for AN, when put into the context of the Hill criteria for causation, support an indirect genotoxic mode of action for AN in producing brain tumors in rats.

Nongenotoxic

Under a nongenotoxic mode of action, it is hypothesized that upon reaching brain cells in the rats, AN or one of its metabolites produces effects which indirectly alters gene expression, resulting in loss of cell growth control, and ultimately in tumor formation. Two potential mechanisms by which this could occur involve intercellular communication and/or reparative hyperplasia due to the cytotoxic effects of AN. An evaluation of this potential mode of action for AN is provided below with respect to satisfying the Hill criteria for causation.

- *Strength of Association* - AN caused inhibition of gap junction intercellular communication (GJIC) in rat astrocytes (Kamendulis *et al.*, 1999b). AN-induced GJIC inhibition was reversible upon removal of AN from the test media. GJIC has been identified as an important factor in regulating cell growth, and has been implicated as a potential mechanism for other nongenotoxic carcinogens (Trosko, 2001).

  Another potential nongenotoxic mode of action for AN includes the potential involvement of cytotoxicity and reparative hyperplasia in tumor promotion. Cytolethality increases in a concentration-dependent manner in rats astrocytes exposed to AN (Kamendulis *et al.*, 1999a).

- *Consistency of Association* - Because only a single study has investigated the effects of AN on GJIC, the consistency of this mode of action cannot be evaluated.

- *Specificity of Association* - Because GJIC inhibition is observed in rat brain cells, which represents a critical target organ for carcinogenicity, but is not observed in hepatocytes, which does not represent a target organ, a mode of action for AN involving GJIC provides target organ specificity.

- *Dose-Response Concordance* - The lack of an *in vivo* experiment for AN in producing GJIC precludes an evaluation of concordance with the dose levels producing AN tumors. However, the range of concentrations producing GJIC inhibition (0.1-1 mM) are similar to those producing oxidative stress *in vitro* (0.01-1 mM) (Kamendulis *et al.*, 1999b). With respect to AN-induced cytoxicity in rat astrocytes (as indicated by LDH release), this effect appears to occur at concentrations that are much higher (1-10 mM) than those associated with oxidative stress (0.01-1 mM) (Kamendulis *et al.*, 1999a). As such, a role for cytotoxicity in AN-induced rat brain tumors is not supported.

- *Temporal Relationship* - Because GJIC inhibition occurs shortly after exposure of brain cells to AN *in vitro* (Kamendulis *et al.*, 1999b), and because brain tumors occur in rats following chronic exposure to AN (Johanssen and Levinskas, 2002), a temporal relationship can be inferred.

- *Biological Plausibility and Coherence* - While limited to the results of a single *in vitro* study, a role for GJIC inhibition in rat brain tumor formation is biologically plausible. However, because inhibition of this function was protected by cotreatment with vitamin E or a glutathione precursor, the involvement of oxidative stress is suggested.

A summary of potential modes of action for AN in producing brain tumors in rats is provided in **Table 4-24**. The implications of the modes of action on the dose-response assessment are identified.

Table 4-24. Comparison of Data Supporting Potential Modes of Action for AN in Producing Brain Tumors in Rats

Potential Mode of Action			Specificity					Implications for Dose-Response Assessment	
	Strength of Association	Consistency of Association	Species	Target Organ	Dose-Response Concordance	Temporal Relationship	Coherence and Plausibility	Internal Dose Measure	Low-Dose Extrapolation
Direct Genotoxicity	*in vitro*						weak	CEO	linear
Indirect Genotoxicity	*in vivo & in vitro*	*in vivo & in vitro*	*in vivo*	*in vivo & in vitro*	*in vivo & in vitro*	*in vivo & in vitro*	strong	CN^- (using CEO as a surrogate)	nonlinear or linear
Nongenotoxic	*in vitro*			*in vitro*	*in vitro*		limited	CEO	nonlinear
Other								Unknown	nonlinear

Of the potential modes of action listed above for AN in producing brain tumors in rats, indirect genotoxicity through an oxidative stress mechanism, with the involvement of cyanide release combined with an increased demand for cellular cysteine (repletion of GSH and thiosulfate), appears to be best supported by the available data. It is recognized that uncertainty remains in the mode of action for AN which impacts decisions made in the dose-response assessment, such as the selection of an internal dose measure and method for low-dose extrapolation. Fortunately, all of the mode(s) of action described here appear to implicate CEO as an appropriate dose measure.

Less clear, however, is the question regarding which measure of CEO in rat brain tissue (area-under-the-curve or AUC, average concentration, or peak concentration) would serve as an appropriate internal dose measure. A comparison of these internal dose measures for CEO and AN in brain tissue for both oral and inhalation exposures to AN (which offer different concentration-time profiles) revealed that peak CEO demonstrated a more consistent correlation with brain tumor response in rats than the other dose measures (Kirman *et al.*, 2000). It is recognized that this is an empirical approach rather than a mechanistic approach. With respect to mechanism, peak tissue concentrations can serve as a more appropriate dose measure than AUC when tissue thresholds are present, resulting in false-positives for AUC

when the threshold is not exceeded. However, mechanistic data to support the existence of such a threshold are not available.

Under an indirect genotoxic mode of action for AN, the key events are listed in **Table 4-25**. In addition, potentially important species differences and potential contributions to a nonlinear dose-response relationship are identified for each event.

Table 4-25. Description of the Key Events in the Proposed Mode of Action for AN in Producing Brain Tumors in Rats

Event	Description	Potential Species Differences (Rat vs. Human)	Potential Contributions to Nonlinearity in Dose-Response Relationship
1	Exposure to AN	Exposures to rats developing tumors are much higher than anticipated for human populations	None identified
2	Absorption	None identified	None identified
3	Distribution of parent chemical to metabolizing tissues (predominantly hepatic, but occurs in other tissues as well)	None identified	None identified
4	Oxidative metabolism	Based upon *in vitro* studies, the rate of AN oxidation is greater in humans than in rats (Kedderis *et al.*, 1993b).	Saturable metabolism for activation Depletion of cofactors (GSH) required for detoxification
5	Distribution of parent chemical (AN) and stable metabolite(s) (CEO, CN^-) across blood:brain barrier to brain	Although no specific data are available to suggest blood:brain barrier differences exists between rats and human for AN, significant species differences exist for some enzyme systems (*e*.g., monoamine oxidase) .	None identified
6	Increased demand for cellular cysteine due to increased demand for glutathione and thiosulfate	Although no specific differences have been identified for AN, any differences between rats and humans with respect to the transport of cysteine across the blood-brain barrier would impact relative susceptibility	Depletion of thiosulfate can prolong elevated CN^- levels in tissues. GSH and thiosulfate pathways compete for cellular cysteine
7	Release of CN^- from CEO and AN in glial cells (enzymatically or possibly nonenzymatically)	CN^- release from AN has been measured in micromal and nuclear fractions of rat brain; unknown if similar pathways exist for humans	Potential positive feedback loop between oxidative stress and nonenzymatic CN^- release

8	Oxidative stress, presumably through the formation of reactive oxygen species, but with potential contribution to decreased cellular defense capabilities	Potential species differences in susceptibility to oxidative stress	If cyclooxygenase activity is involved, there is a potential for induction of this enzyme at high doses that are not expected at low doses
9	DNA oxidation	Potential species differences in DNA repair capacity	Inhibition of DNA repair
10	Gene modification -Base miscoding, strand breaks, altered DNA methylation leading to alterations in genes controlling cell growth	None identified	None identified
11	Altered gene expression	None identified	None identified
12	Tumor formation/progression	Higher background rate for brain tumors in rats (>1%) than in humans (~0.007%; SEER, 2004)	None identified

The information presented above with respect to species differences and sources of nonlinearity are of potential use to the dose-response assessment for AN (**Section 5.0**), specifically regarding efforts for interspecies extrapolation and high-to-low dose extrapolation.

It should be noted that the mode of action information presented above applies to brain tumors in rats, whereas the applicability of this mode of action to humans is uncertain. As discussed in **Section 4.1.2**, given that the large body of epidemiology data do not indicate an increase in brain cancer mortality in exposed workers (Collins and Acquavella, 1998; Blair *et al.*, 1998; Wood *et al.*, 1998), there may be important species differences between rats and humans with respect to this mode of action.

Other Tumors

Mode of action information for tumors other than brain are very limited, and therefore do not warrant a comparison to the Hill criteria for causation.

With respect to a potential mode of action for AN in producing lung tumors, the lungs possess a substantial capacity for metabolizing AN, containing both cytochrome P-450 and GST enzyme systems. Additionally, human lung lipoxygenase demonstrated appreciable activity for metabolizing AN to cyanide *in vitro* (Roy and Kulkarni, 1999). As such, the burden of AN metabolites in the lung is comprised of a mixture of internally and externally (*i.e.*, transported from the liver) formed metabolites. Little is known about the potential mode of action by which AN or one of its metabolites may produce lung tumors. AN produced DNA strand breaks in human bronchial epithelial cells exposed *in vitro* (Chang *et al.*, 1990). *In vitro*

exposure of human lung fibroblasts to AN resulted in the induction of both p53 and p21(WAF1) proteins (Rossner *et al.*, 2002). However, no difference was found for the expression of these proteins in blood from exposed workers (0.05 - 0.3 mg/m^3) as compared to unexposed controls. Data regarding a potential role for reparative hyperplasia are not consistent. In rats, a single oral dose of radiolabeled AN (46.5 mg/kg) produced moderate to marked hyperplasia of the Clara cells lining the bronchioles (Ahmed *et al.*, 1992). Covalent binding of the radiolabel was detected in lung DNA. Replicative DNA synthesis was decreased, while DNA repair was increased. However, the incidence of alveolar epithelial hyperplasia was not increased in female mice in which the incidence of adenoma and carcinomas was slightly increased (Ghanayem *et al.*, 2002). Exposure to AN resulted in a potentiation of oxygen toxicity in rats, which was due in part (but not completely) to GSH depletion (Vilim *et al.*, 1988). In the absence of convincing evidence to support a plausible mode of action for lung tumors, a genotoxic mode of action is conservatively assumed for AN, presumably through the formation of its mutagenic metabolite, CEO.

With respect to a potential mode of action for AN in producing forestomach tumors, following oral exposure to a single dose of radiolabeled AN, radiolabel was found covalently bound to gastric DNA (Abdel-Rahman *et al.*, 1994). Unscheduled DNA repair synthesis (UDRS) was increased while gastric GSH levels were decreased following exposure to AN (Ahmed *et al.*, 1996). Pretreatment of rats with a cytochrome P-450 inhibitor protected against AN-induced URDS, while pretreatment with a GSH depleting agent resulted in a significant increase in AN-induced URDS. The authors concluded that GSH levels and AN metabolism may play an important role in gastric carcinogenesis.

With respect to a potential mode of action for AN in producing Harderian gland tumors in mice, the incidence of harderian gland tumors in rodents has been correlated with porphyrin content (Figge *et al.*, 1942). A plausible mode of action for this tissue site involves the accumulation of porphyrins. Cumulation of porphyrins in the secretory cells produces mitochondrial destruction and ultimately cell death (Antolin *et al.*, 1996). Porphyrins are very sensitive to oxidative stress. As described above for brain tumors in rats, the release of cyanide from AN may be associated with oxidative stress.

With regard to all other tumor, there is no clear evidence to support a specific mode of action. In the absence of convincing evidence, a genotoxic mode of action is conservatively assumed for AN, presumably through the formation of its mutagenic metabolite, CEO. Given that the large body of epidemiology data do not indicate an increase in cancer mortality of any type in exposed workers (Collins and Acquavella, 1998; Blair *et al.*, 1998; Wood *et al.*, 1998), there may be important species differences between rodents and humans with respect to the mode of action described in this section.

4.4 Cancer Weight-of-Evidence

The cancer weight-of-evidence for AN includes a consideration of the following provided by the following four types of information: (1) carcinogenicity in humans; (2) carcinogenicity in animals; (3) genotoxicity in humans; and (4) genotoxicity in animals.

Carcinogenicity in Humans

While a large number of older epidemiological studies carried out on workers exposed to AN have limitations due to methodological or design weaknesses (*e.g.*, small sample sizes, insufficient or incomplete follow-up, inadequate exposure assessment, uncontrolled potential confounding by other compounds or smoking, etc.), taken *in toto* they do not present convincing evidence that AN is carcinogenic to humans at current levels of occupational exposure or the even lower levels likely to be encountered in the environment. This conclusion is supported by the more recent, larger cohort studies of Wood *et al.* (1998), Blair *et al.* (1998), Swaen *et al.* (1998, 2004), and Benn and Osborne (1998), as well as the meta-analyses performed by Rothman (1994), Collins and Acquavella (1998), and EU (2001), all of which found that the cancer risk associated with AN exposure is likely to be nil or very low.

These studies reported no significant excess risk of cancer when all exposed workers were compared to unexposed workers or an external comparison population. There is typically no association with exposure that would suggest a dose-response relationship. When subdivided by exposure levels (or cumulative exposure where possible), there was no excess risk of cancer seen at any site except for an inconsistent and often weak association with lung cancer. Even results from studies of older groups that reflect much higher average levels of exposure did not detect cancer excesses, which reinforces the assessment that the current levels experienced are probably without significant cancer risk to workers or others incidentally exposed to low levels of AN. The relative risks from these various studies for the different cancer endpoints considered are typically around 1.0 and often less. When the relative risks did exceed 1.0, the excursion was usually small and the confidence intervals almost always included 1.0 in the range suggesting the results could be accounted for by chance alone. Considering specific cancers, the excess risk of lung cancer from AN exposure, if it exists at all, is small and associated with long-term exposure to levels that no longer occur in the workplace. The strongest recent evidence for an association was reported by Blair *et al.* (1998) who noted an increasing RR with increasing exposure in the group with the longest exposure ($\geq$ 20 years). Overall, however, recent studies provide negative or only weakly supportive evidence of an association between AN and lung cancer. Even in the Blair *et al.* study, the association with lung cancer was not consistently significant. The RR was between 1.2 and 2.1 in the two highest quintiles studied, but no coherent dose-response relationship existed in the second highest and highest decile (2.5 and 1.8, respectively). Relatively imprecise estimates of risk were found for prostate and brain cancers in AN workers, and AN cannot be completely ruled

out as associated with these cancers. The increases in bladder cancer reported in some studies may have been due to confounding from exposure to aromatic amines, smoking, or some other uncontrolled risk factor(s). However, given all the evidence available, in particular the recent studies, there is little or no evidence to support a causal relationship between AN exposure and human cancer under the conditions of occupational exposures.

While excess cancer at multiple sites has been observed in rats exposed to relatively low levels of AN, there is little evidence that AN workers have increased cancer rates even though exposures in some groups of workers were at levels that caused tumors in rats. Ward and Starr (1993) explored the discordance between laboratory animal and human study findings. According to the US EPA estimates derived from animal studies (based on USEPA's potency estimates from their 1983 assessment), lifetime exposure to 1 $\mu g/m^3$ AN translates into an increased cancer risk of 1 in 6,700 people (6.7×10^{-3}) and into an increased risk of brain cancer of 1 in 12,000 people (1.2×10^{-4}). Assuming that workers in older studies were exposed to an average level of 2 to 5 ppm AN during their working lifetime, they determined the statistical power of the AN epidemiological studies was high enough (>80%) to reliably detect the USEPA predicted increases of cancer due to occupational AN exposure. However, these predicted increases were not found in any of the epidemiological studies. The authors concluded that the upper bound estimate of the AN inhalation cancer potency as estimated by the USEPA was too high to be consistent with the human experience in occupational exposure situations.

Carcinogenicity in Animals

A number of chronic bioassays have been conducted with AN, either through ingestion (drinking water or gavage) or by inhalation. The majority of the studies to date have been performed using the rat, although different strains of rat were tested, and a broad range of dose levels has been used. The results of these studies have shown that AN is carcinogenic to rats following either ingestion or inhalation. Common target organs identified were the central nervous system (brain and spinal cord), Zymbal's gland, gastrointestinal tract (tongue, non-glandular stomach and, small intestine), and mammary gland. The most important finding arising from the studies is the general consistency of the findings of primary brain and Zymbal (ear canal) tumors, irrespective of route of administration. A single cancer bioassay was conducted in mice exposed to AN via gavage. Again, a number of tumor sites were identified (forestomach, Harderian gland, ovary), but with little concordance with sites identified in the rat studies. The results of the carcinogenicity studies carried out in laboratory rodents for AN are described in Section 4.2.4 and are summarized in **Table 4-22**.

Based upon these bioassays which cover both oral and inhalation routes of exposure, there is sufficient evidence of carcinogenicity in laboratory animals.

Genotoxicity in Humans

AN has been shown to be weakly mutagenic in *in vitro* systems, indicative of some genotoxic potential in human cell lines. However, these findings are not reliably reflected in the *in vivo* situation, suggesting that AN or its active metabolites do not reach target tissues *in vivo*, possibly due to the detoxification of the epoxide metabolite CEO via pathways that may not exist in *in vitro* test systems. Alternatively, the lack of concordance between *in vivo* and *in vitro* studies for the genotoxicity of AN may reflect the use of high (non-physiological) concentrations in some *in vitro* studies in which cytotoxicity may have contributed to the results. Nevertheless, the body of evidence leads to the conclusion that AN could be regarded as genotoxic most likely through the formation of CEO.

Positive results for mutagenicity have been obtained for AN in mutagenicity assays using the TK6 human lymphoblast cell line, generally in the presence of metabolic activation only and frequently only at cytotoxic concentrations. AN induces sister chromatid exchanges and chromosomal aberrations in *in vitro* studies; however, negative responses have generally been obtained in DNA repair assays using human mammary epithelial cells *in vitro*.

A number of *in vitro* assays have included the metabolite epoxide, CEO. The responses of the metabolite in several of the test systems described above indicate that it is a direct acting mutagen. Coupled with the observation that AN is mutagenic *in vitro* mainly in the presence of S9, indicating that metabolic activation is required to exert the mutagenic potential, it may be concluded that the DNA active compound is CEO and that AN itself has relatively low DNA reactivity. The epoxide has been shown to bind to DNA with a much greater affinity than AN.

Based upon the positive results for genotoxicity from *in vitro* studies, but largely negative results from *in vivo* studies, there is limited evidence of genotoxicity for AN in humans.

Genotoxicity in Animals

In *in vitro* studies, AN is weakly mutagenic in *Salmonella typhimurium* test systems, generally only when a metabolic activation system (liver S-9 fraction) is present. However, in *Escherichia coli* and in rodent test systems, metabolic activation is not required, but will generally increase a weak response elicited by AN when used. In contrast to the positive results obtained with acrylonitrile in numerous in vitro mutagenicity assays in bacteria, yeast, and mammalian cells, the majority of in vivo mammalian cell assays measuring a variety of endpoints gave negative results. These included tests for chromosome aberration induction in mouse and rat bone marrow micronucleus, and sister chromatid exchange induction in mice, and induction of dominant lethal mutations in rat and mouse sperm.

Although positive results were obtained in a broad array of acrylonitrile *in vitro* assays, *in vivo* clastogenicity has not been demonstrated. The contrast between in vitro and in vivo test results with acrylonitrile in chromosomal damage assays may be due to differences in the metabolism of acrylonitrile in intact animals compared to cultured cells. In animals, less efficient metabolism of 2-cyanoethylene oxide may occur, or there may be rapid detoxification and elimination of 2-cyanoethylene oxide, thereby reducing the chance for DNA interaction.

Based upon the positive results for genotoxicity from *in vitro* studies, but largely negative results from *in vivo* studies, there is limited evidence of genotoxicity for AN in animals.

Cancer Weight-of-Evidence

Epidemiology data do not support an increased cancer risk from acrylonitrile exposure in exposed workers. In contrast, the experimental animal data clearly support the conclusion that acrylonitrile is carcinogenic in rodents. The proposed cancer modes of action in rodents involve general processes (e.g., oxidative stress, GJIC, DNA damage) that are known to occur in humans, and so the data are presumed to support the use of the rodent data in establishing a quantitative cancer risk value. Although the data are insufficient to rule out any contribution due to direct DNA reactivity, an overall weight of evidence evaluation does not support this as a predominant contributor to rodent carcinogenesis. These modes of action may not be mutually exclusive, and multiple modes of action may be operating at different dose levels. Furthermore, linear extrapolation from the animal data is not supported by the available epidemiology data. Based on this information, the overall weight of the evidence suggests that the cancer risk associated with the levels to which humans have been exposed in occupational settings is negligible, but that acrylonitrile may be carcinogenic to humans at higher doses based on extrapolation from rat studies.

Cancer Classification by Other Agencies

In February, 1998, IARC downgraded AN from a category 2a to a category 2b (IARC, 1999). Their decision to do this was based mainly on the information and lack of carcinogenic evidence found in the more recent epidemiological studies. IARC now considers the data relating to potential carcinogenicity of AN to humans to be inadequate and no evidence of a causal association exists. This decision supports the conclusion that AN at historical and present exposure levels was and is probably not carcinogenic to man.

NTP concluded that AN is reasonably anticipated to be a human carcinogen based on sufficient evidence of carcinogenicity in experimental animals (NTP, 2002).

4.5 Susceptible Populations

Increased Susceptibility Due to Kinetic Factors

Although there is a considerable amount of information available regarding the kinetics of AN in both rats and humans, it is not certain what kinetic factors will determine susceptibility. Based upon the modes of action described in **Section 4.3**, cyanide appears to be important to a number of effects for AN, including its carcinogenicity in rats. Accordingly, the key kinetic factors are likely to be those which determine the rate of release of cyanide, as well as those which determine the rate of cyanide detoxification. With respect to cyanide release, since oxidative metabolism of AN is required prior to the release of cyanide, individuals with a higher capacity for oxidative metabolism, such as may occur with the induction of CYP2E1, may produce cyanide at a faster rate than individuals in a uninduced state. With respect to cyanide detoxification, since the availability of sulfane sulfur is the rate limiting step of cyanide metabolism, individuals with lower circulating levels of sulfane sulfur, as may occur with diets low in cysteine or possibly during peak demands on cellular sulfane sulfur supplies (*i.e*., GSH repletion), may detoxify cyanide at a slower rate than individuals with normal circulating levels of sulfane sulfur. Neither increased cyanide release nor decreased cyanide detoxification by themselves may result in increased tissue cyanide levels. Rather, both rates need to be considered together to determine their net effect on tissue cyanide levels.

A study was conducted for CYP2E1 polymorphisms in a cohort of 59 persons with industrial handling of low levels of AN that has been studied from 1994 through 1999 (Thier *et al.*, 2002). The individual means and medians of N-(cyanoethyl)valine levels over the entire observation period were compared with the CYP2E1 variants. No influences of the investigated CYP2E1 polymorphisms on the N-(cyanoethyl)valine levels appeared at the 5% level. However, there was a trend ($p \sim 0.1$) pointing to higher AN-specific adduct levels in persons with the A-316G mutation in CYP2E1. The higher adduct levels would be compatible with a slower CYP2E1-mediated metabolism of AN. Accordingly, individuals with this defect would be expected to be less susceptible to the adverse health affect associated with the formation of CEO and cyanide.

The same cohort of 59 persons was evaluated with respect to polymorphisms in GST (Thier *et al.*, 2001). Although a previous assessment of hemoglobin adducts (N-(cyanoethyl)valine and N-(hydroxyethyl)-valine) and glutathione transferases hGSTM1 and hGSTT1 polymorphisms indicated no influence of hGSTM1 or hGSTT1 polymorphisms on specific adduct levels, a re-evaluation of the cohort for glutathione transferases hGSTM3 and hGSTP1 point to a possible influence of a human enzyme polymorphism of the GSTP1 gene at codon 104 on the detoxication of AN. The impact of this polymorphism on susceptibility is not clear.

A study was conducted to examine the relationship between cigarette smoking and hemoglobin adducts (N-(2-cyanoethyl)valine) derived from AN and to investigate whether null genotypes for glutathione transferase (GSTM1 and GSTT1) alters the internal dose of AN (Fennell et al., 2000). GST genotypes were determined in blood samples obtained from 16 nonsmokers and 32 smokers (one to two packs/day). Hemoglobin adduct levels increased with increased cigarette smoking dose (both self-reported and cotinine-based). However, GSTM1 and GSTT1 genotypes had little effect on adduct levels concentrations. Accordingly, the results of this study suggest that GST polymorphisms may not have a significant impact on susceptibility to AN.

Sensitivity and variability analyses conducted using the PBPK model for AN indicate that, while the contributing parameters depend somewhat upon the route of exposure, variation in internal dose measures for AN and CEO is relatively small (Sweeney *et al.*, 2003). The primary contributor to predicted variance in human blood ACN concentrations in people exposed through drinking water was the Vmax for conversion of ACN to CEO. In contrast, the main contributors for variance in people exposed by inhalation were expected to be the rate of blood flow to the liver and alveolar ventilation rate, with the brain:blood partition coefficient also contributing to variability in predicted concentrations of ACN in the brain. Expected variability in blood CEO concentrations (peak or average) in humans exposed by inhalation or drinking water was modest, with a 95th- and 99th-percentile individuals expected to have respective blood concentrations 1.8-times and 2.2-times higher than an average individual.

Increased Susceptibility Due to Dynamic Factors

No data were located regarding the impact of dynamic factors on susceptibility to AN-induced effects.

4.5.1 Possible Childhood Susceptibility

Noncancer

No data were located to suggest that age affects susceptibility to the noncancer effects of AN.

Cancer

A three-generation reproductive toxicity study (Friedman and Beliles, 2002) provided limited data to suggest an increased susceptibility in young rats compared to adult rats. This hypothesis is suggested by an approximate two-fold increase in tumor incidence in F_1 animals (exposed to AN at all stages of life) compared to F_0 animals (exposed during adulthood only). However, the absence of an increased incidence of tumors in F_2 animals (also exposed to AN

at all life stages) suggests that the results in the F_1 may be attributable to chance rather than indicative of a true increase in susceptibility.

Regarding the ontogenesis of CYP2E1, activity is generally absent in fetal liver during the first trimester, but is seen at low levels during the second and third trimesters (McCarver *et al.*, 2003). Levels increase after birth, but generally remains low (compared to levels in adults) during the neonatal period. CYP2E1 levels show a clear trend gradually increase with age (concentration in pmol/mg protein indicated in parentheses) such that fetus (0.35-6.7) < neonate < older infants (8.8) < children (23.8) < young adults (41.4). Because AN oxidation primarily requires CYP2E1 activity, children are expected to be less susceptible than adults to the adverse effects of AN produced by CEO and cyanide.

4.5.2 Possible Gender Differences

Noncancer

No data were located to suggest that gender affects susceptibility to the noncancer effects of AN.

Cancer

No data were located to suggest that gender affects susceptibility to the carcinogenic effects of AN.

5. DOSE-RESPONSE ASSESSMENTS

The purpose of this section is to provide a quantitative characterization of the dose-response relationship for adverse health effects (noncancer and cancer endpoints) associated with exposure to AN. The assessments are based upon the major effects identified for AN in **Section 4.3**, and considers the mode(s) of action by which AN produces these effects. Within this section, efforts have been made to harmonize the non-cancer and cancer dose-response assessments for AN with respect to (1) the nomenclature used, (2) the units in which the values are presented, (3) the factors considered in the assessments, and (4) the decision points encountered in the assessments. Each of these points is discussed briefly below.

Nomenclature

Historically, the term benchmark dose (BMD) and its lower confidence limit (BMDL) has been reserved for non-cancer assessment, whereas the term effective dose (ED) and its lower confidence limit (LED) have been reserved for cancer assessment, despite the fact that their meaning is essentially the same. In this section, the terms ED and LED are used for oral and inhalation routes (rather than using EC and LEC for effective concentration and its lower confidence limit) and are used for both noncancer and cancer risk assessments for AN.

Units

1 ppm = 2.17 mg/m^3

A molecular weight of 53.06 g/mol was used to calculate this conversion factor (53.06/24.45 for an ideal gas at standard temperature and pressure). Cumulative occupational exposures were converted to lifetime continuous exposures using the following conversion factor:

1 ppm-year (occupational) = 4.9 ppb (continuous lifetime) = 10.6 $\mu g/m^3$

This conversion factor assumes a 70-year lifetime, relative inhalation rates of 10 and 20 m^3/day, and relative exposure frequencies of 250 and 365 days/year for occupational and continuous lifetime, respectively.

Factors Considered

A dose-response assessment can include characterizations of up to four factors or dimensions, the first of which is obligatory (dose), but may also include incidence (the number of animals or persons affected divided by the number examined), severity (degree of the adverse effect), and time (duration of exposure), and may also consider the influence of age, sex, and a variety individual factors on response. Historically, noncancer and cancer dose-response methods have approached these four factors differently. For example, noncancer risk assessments have characterized the relationship between dose and severity (no observed adverse effect level or NOAEL, lowest observed adverse effect level or LOAEL, Frank effect level or FEL).

However, with the advent of benchmark dose (BMD) methods, characterizations of the relationship between dose and incidence are appearing at an increasing frequency. Time in noncancer risk assessment is generally fixed to one of three levels (acute, subchronic, or chronic/lifetime). Although approaches are available for incorporating time in noncancer risk assessment (categorical regression, benchmark regression), their application is not widespread at present. Cancer risk assessment, on the other hand, has historically been an exercise in characterizing the relationship between dose and incidence. The importance of severity (tumors vs. tumor precursor lesions, treatability of different cancer types) is often ignored for the sake of simplicity. Approaches are available for addressing time in cancer risk assessment (*i.e.*, time-to-tumor analysis).

In the spirit of harmonizing noncancer and cancer risk assessment, it is important to define up front the dose-response factors to be characterized in the quantitative assessments for AN. The assessments presented in this section for the noncancer effects of AN focus primarily upon characterizing the relationship between dose and severity for lifetime oral exposures, and for dose and incidence for lifetime inhalation exposures, based upon a consideration of the specific data sets available for each route. The cancer assessment for AN focuses primarily upon a characterization of the relationship between dose and incidence for lifetime oral and inhalation exposures.

Decision Points

The process for deriving RfDs, RfCs, and cancer potency estimates includes a number of decision points, many of which are based on available data, some based on professional judgment, while others are made as a matter of policy. The first five decision points are common to both non-cancer and cancer dose-response assessments, and as such are termed "harmonized" decision points. The last two decision points are specific to either non-cancer or cancer dose-response assessment.

Harmonized Decision Points for Noncancer and Cancer Dose-Response Assessment

1. Identification of the Critical Effect/Data Set(s)
2. Identification of a Dose Measure(s)
3. Identification of a Response Measure(s)
4. Selection of a Dose-Response Model(s)
5. Selection of Response Level(s) (Point of Departure)

Decision Points Specific To Noncancer Dose-Response Assessment

6a. Selection of Uncertainty/Modifying Factors

7a. Calculation of RfD/RfC

Decision Points Specific To Cancer Dose-Response Assessment

6b. Selection of a Method for Extrapolating to Low Doses

7b. Calculation of Cancer Value

The remaining portions of this section are dedicated to discussing the specific decisions made in deriving the inhalation RfC (**Section 5.1**), oral RfD (**Section 5.2**), and cancer values (**Section 5.3**) for AN. This section was prepared following the general guidelines for risk assessment as set forth by the National Research Council (1994). EPA guidelines that were used in the development of this assessment may include the following: the Guidelines for Carcinogen Risk Assessment (U.S. EPA, 1986a), Guidelines for the Health Risk Assessment of Chemical Mixtures (U.S. EPA, 1986b), Guidelines for Mutagenicity Risk Assessment (U.S. EPA, 1986c), Guidelines for Developmental Toxicity Risk Assessment (U.S. EPA, 1991), Guidelines for Neurotoxicity Risk Assessment (U.S. EPA, 1998a), Proposed Guidelines for Carcinogen Risk Assessment (1999, 2003), and Reproductive Toxicity Risk Assessment Guidelines (U.S. EPA, 1996b); Recommendations for and Documentation of Biological Values for Use in Risk Assessment (U.S. EPA, 1988); Methods for Derivation of Inhalation Reference Concentrations and Application of Inhalation Dosimetry (U.S. EPA, 1994b); Peer Review and Peer Involvement at the U.S. Environmental Protection Agency (U.S. EPA, 1994c); Use of the Benchmark Dose Approach in Health Risk Assessment (U.S. EPA, 1995); Science Policy Council Handbook: Peer Review (U.S. EPA, 1998b); Guidance Document for the Use of Data in Development of Chemical-Specific Adjustment Factors (CSAFs) for Interspecies Differences and Human Variability in Dose/Concentration–Response Assessment (IPCS, 2001); A Review of the Reference Dose and Reference Concentration Process (USEPA, 2002); and memorandum from EPA Administrator, Carol Browner, dated March 21, 1995, Subject: Guidance on Risk Characterization.

5.1 Oral Reference Dose (RfD)

Traditionally, uncertainty and modifying factors of up to 10 each have been used to account for interspecies variability (UFa), intraspecies variability (UFh), LOAEL-to-NOAEL extrapolation (UFl), subchronic-to-chronic extrapolation (UFs), and deficiencies in the toxicity database (UFd). These factors are typically applied to a NOAEL, LOAEL, or LED as indicated in the equation below:

$$RfD = \frac{NOAEL or\ LOAEL or\ LED}{UF * MF}$$

Where,

RfD	=	reference dose (mg/kg-day);
NOAEL	=	No observed adverse effect level, adjusted for discontinuous exposure;

LOAEL	=	Lowest observed adverse effect level, adjusted for discontinuous exposure;
LED	=	Lower confidence limit of an effective dose required to produce a specified response level;
UF	=	Net uncertainty factor (UFa*UFh*UFl*UFs*UFd); and
MF	=	Modifying factor.

5.1.1 Identification of a Critical Effect/Data Set

As summarized in **Section 4.3.1.1**, a number of noncancer endpoints have been identified as major effects of AN by studies in animal and/or humans. These endpoints include neurological, irritation, hematological, reproductive/developmental, and survival effects. Hematological and reproductive endpoints were eliminated as a potential basis for the oral RfD AN since the effects reported by a few studies were not consistently supported by the weight-of-evidence of the database. The effects on survival, while reported at very low doses of AN, were eliminated as a potential basis for the oral RfD based upon a consideration of the mode action by which AN produces these effects (**Section 4.3.1.2**). Specifically, because the effects on survival in rats are secondary to the carcinogenic effects of AN, they are not suitable for noncancer risk assessment. The cancer potency estimates calculated for AN (**Section 5.3**), which address the carcinogenic effects of AN in rats, are considered to be protective for the survival effects as well. Since the neurological and irritation effects of AN have been reported in both human and animal studies, they are considered suitable endpoints for the RfD. However, because the irritation effects reported for AN in laboratory animals occur in a tissue for which there is no human homologue (forestomach), the neurological effects of AN were identified as the critical effect for deriving an RfD.

A single study was identified which addressed the dose-response relationship for the neurological effects of AN following oral exposure in rats (Gagnaire *et al.*, 1998). In this study, rats were exposed to 0, 12.5, 25, or 50 mg/kg-day AN via gavage for 12 weeks. Additional groups of animals were allowed to recover for eight weeks following the cessation of exposure. Hind limb weakness was reported in animals during exposure to the highest dose. In addition, dose-response data were collected for several measures of peripheral nerve function, including motor nerve conduction velocity (MCV), sensory nerve conduction velocity (SCV), amplitude for motor nerve action potential (AMAP), and amplitude for sensory nerve action potential (ASAP). These data are summarized **Table 4-19** and are presented in **Figure 5-1**. It is unclear as to whether or not decrements in these endpoints are adverse effects by themselves. However, for the purposes of this assessment, it was assumed that these endpoints may serve as precursors to the adverse effects observed at high doses (*i.e.,* hind limb weakness). To facilitate a determination as to which of the four measures of peripheral nerve function is the most sensitive to AN exposure, the data in **Figure 5-1** are expressed as a percentage of their respective control values. Based upon visual inspection of this figure,

ASAP is identified as a more sensitive measure than the other three endpoints. ASAP demonstrated the steepest decrease in slope for response between the control group and lowest exposure group, and exhibited a higher degree of variation in response than the other endpoints (**Table 4-19**), and therefore is expected to produce smaller (more conservative) BMDL values. Preliminary modeling runs for these endpoints characterized in terms of external dose indicate that this true. Although the data for ASAP reported post-exposure and post-recovery appear to be similar in severity, the fact that the post-recovery data describe a more consistent dose-response relationship (*i.e.,* decreasing with each increase in dose) along with the fact that these data demonstrate that there is little to no recovery, the post-recovery data for ASAP were selected as the basis for the oral RfD.

5.1.2 Identification of a Dose Measure

PBPK models are available which can be used to describe the kinetics of AN and CEO in rats (Kedderis *et al.*, 1996) and humans (Sweeney *et al.*, 2003). These models were used to assess the dose-response data for ASAP in terms of internal dose. Information on the mode of action described for the neurological effects of AN (**Section 4.3**) indicate the potential involvement of the parent chemical and one of its metabolites (cyanide). However, the authors of the key study evaluated a number of other cyanogenic chemicals (2-methylacrylonitrile, trans-3-pentenenitrile, 3-methyl-2-butenenitrile, and 4-pentenenitrile) which failed to affect the same neurological parameters and hind limb weakness as AN (Gagnaire et al., 1998). Therefore, cyanide is not considered to be the causative agent for the neurological effects observed in the key study. At this point, it is unclear as to whether the peak concentration or area-under-the-curve (AUC) serves as the better measure of internal dose for neurological effects. In the absence of specific information, the default dose measure, AUC, was selected for this assessment. Blood concentration for AN was considered to be a suitable surrogate for corresponding levels in peripheral nerves. Internal dose measures for the critical study are provided in **Table 5-1**.

Table 5-1. Dose Measures for Neurological Endpoints in Rats Exposed via Oral Gavage

Administered Dose (mg/kg-day)	Adjusted Dose (mg/kg-day)*	AUC AN Blood (mg/L-hr)
0	0	0
12.5	8.9	1.3
25	17.9	2.8
50	35.7	6.3

*Administered dose multiplied by 5/7.

5.1.3 Identification of a Response Measure

Because continuous data are not described in terms of risk (extra, added, or relative), this decision point does not apply.

5.1.4 Selection of a Dose-Response Model

For continuous data, several dose-response models are available in USEPA's BMD Software, including linear, polynomial, power, and Hill models. The Hill model was not considered since there are an insufficient number of dose groups (*i.e.,* degrees of freedom) to assess its goodness-of-fit. Output from the BMDS for these data provided in **Appendix C**. A comparison of the remaining continuous models applied to the neurological dose-response data is provided in **Table 5-2**.

Table 5-2. Goodness-of-Fit for Continuous Dose-Response Models Applied to Neurological Data in Rats

Model	Degrees of freedom	P-Value
linear	2	0.663
polynomial	1	0.521
power	1	0.506
Hill	0	NA

NA = not applicable, insufficient degrees of freedom

The linear model was considered appropriate for deriving an oral RfD, since it provided the best overall fit to the data (*i.e.,* highest p-value). The fit of the linear model to the dose response data is depicted in **Figure 5-2**. Visual inspection of the linear model also indicates that it fits the data well in the range of observation and for the control response.

5.1.5 Selection of a Response Level

It is not known what degree of reduction in the ASAP response constitutes an adverse or biologically significant effect such that normal nerve function is compromised. The dose corresponding to a 5% decrease in the mean response (ED05) and its lower confidence limit (LED05) were used for the RfD. The resulting ED and LED values are 1.3 and 0.9 mg*hr/L expressed in terms of internal dose. Human equivalent doses were determined using the human PBPK model for AN (Sweeney *et al.*, 2003) to calculate the continuous oral doses for humans exposed to AN which produce the corresponding internal doses. This was accomplished in an

iterative manner by manually adjusting the oral dose until the desired internal dose (LED05 of 0.9 mg*hr/L) was achieved. The internal ED and LED values correspond to human equivalent doses of 12 and 8.5 mg/kg-day, respectively.

5.1.6 Selection of Uncertainty/Modifying Factors

The selection of uncertainty factor values requires careful consideration to ensure that the resulting RfD value is protective without being unnecessarily restrictive. The UFs employed typically fall within a range of one to ten and are applied to various aspects of an experimental study that might critically bear on its extrapolation to human health. It is recognized by numerous authoritative bodies (*i.e.*, National Academy of Sciences (NAS/NRC), the World Health Organization (WHO), and USEPA) that UFs can and should accommodate a wide continuum of numerical expressions other than a single default value (most notably, 10). The NAS/NRC states, "*There is no strong scientific basis for using the same constant uncertainty factor for all situations...*" (NRC, 1994). USEPA in the Agency's draft report entitled "*A Review of the Reference Dose and Reference Concentration Process*" (USEPA, 2002), also stated "*... that rigid application of log or ½ log units for UFs could lead to an illogical set of reference values."* There is growing support for chemical-specific of data-driven uncertainty factors in non-cancer risk assessment, which incorporate toxicokinetic and toxicodynamic data, and as a consequence, the application of uncertainty factors other than three or 10 may become more frequent in human health risk assessment in the future (IPCS, 2001; USEPA, 2002). Uncertainty factor values used in deriving the RfD for AN, including those based upon chemical-specific information, are presented below.

- *UFa* - The default value of 10 for UFa can be treated as two specific factors of 3.2 for kinetic variation and 3.2 for dynamic variation. Because PBPK models (Kedderis *et al.*, 1996; Sweeney *et al.*, 2003) were used to account for kinetic differences between rats and humans, thereby improving the confidence in the interspecies extrapolation, the kinetic component of UFa was set equal to one. For the dynamic component of UFa, a value of 3.2 was used in deriving the RfD to account for potential dynamic differences between rats and humans.

- *UFh* - The default value of 10 can treated as two specific factors of 3.2 for kinetic variation and 3.2 for dynamic variation. Using a PBPK model and variability analysis to address human variation for AUC AN in blood, the model coefficient of variation for this internal dose was found to be 0.47 (Sweeney *et al.*, 2003). Accordingly, the 95th percentile for AUC AN in blood is predicted to be approximately 1.8-fold higher (1+1.64*0.47, assuming a normal distribution) than the mean value (Sweeney *et al.*, 2003). Use of the 99th percentile would result in a values that is essentially the same (2.1-fold, calculated as 1+2.32*0.47, assuming a normal distribution). Accordingly, a factor of 1.8 was combined with the default factor of 3.2 for human variation in toxicodynamics

to yield an UFh value of 5.8. This approach is consistent with guidelines for chemical-specific adjustment factors (IPCS, 2001; USEPA, 2002).

- *UFl* - Since BMD methods were used for an endpoint of minimal severity, an uncertainty factor for use of a LOAEL is not required (UFl=1). In BMD methods, the use of the lower confidence limit in the effect dose levels incorporates an added degree of conservatism compared to the maximum likelihood fit of the dose-response model.

- *UFs* - Since the exposure regimen in the key study was subchronic in nature (12 weeks), a default uncertainty factor of 10 was used to extrapolate from subchronic to chronic duration.

- *UFd* - Because the toxicological database for AN is robust, an uncertainty factor to account for deficiencies in the database is not required (UFd=1). The database includes the four of the five critical studies required for designating high confidence in the RfD, including: (1) chronic toxicity in Sprague-Dawley rats by the inhalation route (Maltoni *et al.*, 1977, 1988; Quast *et al*., 1980a); (2) chronic toxicity in B6C3F1 mice by the oral route (Ghanayem *et al.*, 2002); (3) developmental toxicity in Sprague-Dawley rats by the inhalation route (Murray *et al.*, 1978; Saillenfait *et al.*, 1993) and in Sprague-Dawley and Wistar rats by the oral route (Murray *et al.*, 1978; Mehrotra *et al.*, 1988); and (4) a three-generation reproduction study in rats by the oral route, which included additional evaluations for neurological and cancer effects (Friedman and Beliles, 2002). Although a developmental study in a second mammalian species was not identified for AN, available studies include two different strains of rat (Sprague-Dawley and Wistar). Furthermore, studies on aliphatic nitriles indicate the formation of cyanide may play an important role in developmental toxicity (Saillenfait *et al.*, 2000). Because developmental toxicity studies have been conducted for cyanide using several mammalian species, including rats, hamsters, and pigs (Singh, 1981; Willhite, 1982; Frakes *et al.*, 1985, 1986a, 1986b; Tewe and Maner, 1981a, 1981b), the lack of a developmental toxicity study in a second mammalian species does not constitute a data gap for AN.

- *MF* - An additional modifying factor was not considered necessary for the RfD (MF=1).

When combined, the individual uncertainty factors summarized above yield a net uncertainty factor of 180 (3.2x3.2x1.8x10) for the oral RfD.

5.1.7 Calculation of RfD

Based on LED05 value of 8.5 mg/kg-day and a net uncertainty factor of 180, an **oral RfD value of 0.05 mg/kg-day** was derived for AN. Because the derivation of an RfD involves a number of decision points, and because there are alternative options for each decision point, a discussion of the impact of alternative decisions on the resulting RfD value is warranted.

- *Alternative Data Sets* - Consideration was given to using mortality data as the basis for an RfD. Use of the dose-response data for mortality, which are likely related to the carcinogenic effects of AN, results in a point of departure that is slightly lower than calculated for neurological endpoints. Under an assumption that mortality is independent of carcinogenicity, the study of Quast et al. (1980) identifies a LOAEL of 35 ppm (4.4 mg/kg-day) in female rats, and the study of Johanssen and Levinskas (2002) identified a NOAEL of 10 ppm (1.3 mg/kg-day) in female rats. Application of an uncertainty factor of 100 (UFa=10; UFh=10; UFl=1; UFs=1; UFd=1) to the NOAEL value results in an alternative RfD of 0.01 mg/kg-day. Although this alternative RfD value is slightly lower than the value derived from Gagnaire et al. (1998), it is anticipated that with the inclusion of PBPK modeling, the net uncertainty factor value would decrease by approximately a factor of 5.5, which would then result in an RfD that is slightly higher than the value derived from Gagnaire *et al.* (1998). Reliance on forestomach irritation would also likely result in a lower point of departure. Becausethe mortality and forestomach data are derived from chronic study (which would require a UFs of 1.0) whereas the neurological data are derived from a subchronic study (which require a UFs of 10), the lower value for mortality does not translate to a lower RfD. Consideration was also given to using alternative neurological endpoints reported by Gagnaire et al. (1998). Based upon preliminary dose-response modeling efforts and inspection of **Figure 5-1**, consideration of other neurological endpoints, such as changes in nerve conduction velocity, observed in the same critical study generally resulted in larger (less conservative) points of departure.

- *Alternative Dose Measure* - Preliminary dose-response modeling efforts indicated that use of external dose results in fairly similar points of departure when compared to those obtained using internal dose.

- *Alternative Dose-Response Models* - Consideration was given to using one of several other continuous dose-response models. In general, these models predicted similar points of departure to those predicted by the linear model. Therefore, the selection of dose-response model does not have a significant impact upon the RfD.

- *Alternative Response Level* - Consideration was given to using variation in control response levels (ED1SD) instead of the LED05. Use of this level would result in slightly higherbenchmark doses.

Based upon this assessment, an **oral RfD of 0.05 mg/kg-day** is recommended for AN. The oral RfD is contrasted with the oral value derived for cancer endpoints in **Section 5.3.1.7**. Confidence in the oral RfD for AN is considered to be medium-to-high. Confidence in the key study is considered to be medium primarily because the critical study was subchronic in duration, and the exposure regimen (gavage) does not reflect typical human exposures. However, the appearance of similar effects in rats from the same study following inhalation exposure to AN allays some of this concern. Confidence in the database is considered high since there are a number of well-conducted, chronic bioassays in both rats and mice, and a multigeneration reproductive toxicity study has been conducted. Confidence in the mode of action is medium, since a potential role for cyanide is ruled out by the lack of an effect for other cyanogenic compounds in the critical study. Uncertainty remains regarding whether the neurological effects observed in rats are determined by peak or cumulative (AUC) tissue exposures, and whether or not there are important toxicodynamic differences between rats and humans. Confidence in the PBPK modeling is considered to be medium since the model has been validated in rats but not in humans, and because the model provides a good description of the kinetics of AN. Although blood concentrations are likely to be good surrogates for concentrations in peripheral nerves, confidence in the model predictions would be improved by the inclusion of a compartment for the target organ.

5.2 Inhalation Reference Concentration (RfC)

Like the RfD derivation process, uncertainty and modifying factors of up to 10 each have been used to account for interspecies variability (UFa), intraspecies variability (UFh), LOAEL-to-NOAEL extrapolation (UFl), subchronic-to-chronic extrapolation (UFs), and deficiencies in the toxicity database (UFd) in deriving an RfC. These factors are typically applied to a NOAEL, LOAEL, or LED as indicated in the equation below:

$$RfC = \frac{NOAEL\ or\ LOAEL\ or\ LEC}{UF * MF}$$

Where,

RfC	=	Reference concentration (mg/m^3);
NOAEL	=	No observed adverse effect level, adjusted for discontinuous exposure;
LOAEL	=	Lowest observed adverse effect level, adjusted for discontinuous exposure;

LEC	=	Lower confidence limit of an effective concentration required to produce a specified response level;
UF	=	Net uncertainty factor (UFa*UFh*UFl*UFs*UFd); and
MF	=	Modifying factor

5.2.1 Identification of a Critical Effect/Data Set

As summarized in **Section 4.3.1.1**, a number of noncancer endpoints have been identified as major effects of AN by studies in animal and/or humans. These endpoints include neurological, irritation, hematological, reproductive/developmental, and survival effects. Hematological and reproductive endpoints were eliminated as a potential basis for the inhalation RfC AN since the effects reported by a few studies were not consistently supported by the weight-of-evidence of the database. The effects on survival, while reported at very low doses of AN, were eliminated as a potential basis for the inhalation RfC based upon a consideration of the mode action by which AN produces these effects (**Section 4.3.1.2**). Specifically, because the effects on survival in rats are secondary to the carcinogenic effects of AN, they are not suitable for noncancer risk assessment. The cancer potency estimates calculated for AN (**Section 5.3**), which address the carcinogenic effects of AN in rats, are considered to be protective for the survival effects as well. Since the neurological and irritation effects of AN have been reported in both human and animal studies, they are considered suitable endpoints for the RfC.

A number of early studies reported on the presence or absence of clinical symptoms in workers exposed to AN (Babanov et al., 1959; Ageeva et al., 1970; Ginceva et al., 1977; Stamova et al., 1976; all as reported in WHO, 1983). Concentrations in these studies were reported to range from 0.3 to 12 ppm, but exposures to other chemicals can be assumed for at least some of these workers, and the exposures to AN were generally considered incompletely characterized (BUA, 1993). However, collectively these studies support a threshold for clinical symptoms in the range of 3 to 12 ppm. A NOAEL of 3 ppm is supported by the absence of clinical signs in exposed workers (Ginceva et al., 1977; as reported in WHO, 1983).

A larger, better characterized study by Sakurai et al., (1978) was used to estimate a NOAEL value of 3 ppm (6.5 mg/m^3) based on subjective symptoms, including irritation, in workers exposed to AN (EU, 2001). Concentrations above this level were associated with complaints of irritation. In this study, 102 workers whose exposure to AN exceeded five years and 62 matched controls, all randomly selected from six acrylic fiber factories in Japan were examined. In most of the factories, the concentrations of AN ranged from 1 to 10 ppm, with mean concentrations less than 1 ppm, while in a few factories concentrations as high as 100-200 ppm were recorded. Medical examinations including multiple clinical chemistry measurements failed to detect any health effects attributable to AN. A non-significant increase in the prevalence of irritative signs was reported in the factory with the highest exposure. In

factories in which the prevalence of irritative signs was not increased, a NOAEL of 3 ppm (6.5 mg/m^3) was considered to be representative of exposures ranging from 1 to 10 ppm.

Consideration was also given to using the rat data for nasal lesions from Quast *et al.* (1980), which can be used to support a LOAEL of 20 ppm. This study was used to derive an alternative RfC value for AN in **Appendix D**. Due to important species differences between nasal architecture between rodents and humans, these data were not selected as the primary basis for the RfC, but rather are included to provide support for the RfC derived from the human data.

5.2.2 Identification of a Dose Measure

Because irritation is attributed to peak exposure levels, rather than cumulative exposure, adjustment of the NOAEL of 6.5 mg/m^3 for irritation in humans (e.g.,breathing rates, exposure frequency) was not appropriate.. Accordingly, a NOAEL value of 6.5 mg/m^3 was used for the RfC.

5.2.3 Identification of a Response Measure

Several risk measures can be calculated for a given effect, including:

Added Risk	=	*P(d)-P(0)*
Relative Risk	=	*P(d)/P(0)*
Extra Risk	=	*[P(d)-P(0)]/[1-P(0)]*

Where,

P(d)	=	Probability of the adverse response at dose d; and
P(0)	=	Probability of the adverse response at zero (or background) dose.

Because the human data are categorical in nature, the decision point for response measure is not applicable.

5.2.4 Selection of a Dose-Response Model

Because the human data are categorical, the decision point for dose-response model is not applicable.

5.2.5 Selection of a Response Level

Because the human data are categorical, the decision point for selecting a response level is not applicable.

5.2.6 Selection of Uncertainty/Modifying Factors

The selection of the uncertainty factor values requires careful consideration to ensure that the resulting RfC value is protective without being unnecessarily restrictive. The UFs employed typically fall within a range of one to ten and are applied to various aspects of an experimental study that might critically bear on its extrapolation to human health. It is recognized by numerous authoritative bodies (*i.e.*, National Academy of Sciences (NAS/NRC), the World Health Organization (WHO), and USEPA) that UFs can and should accommodate a wide continuum of numerical expressions other than a single default value (most notably, 10). The NAS/NRC states, "*There is no strong scientific basis for using the same constant uncertainty factor for all situations...*" (NRC, 1994). USEPA in the Agency's draft report entitled "*A Review of the Reference Dose and Reference Concentration Process*" (USEPA, 2002), also stated "*... that rigid application of log or ½ log units for UFs could lead to an illogical set of reference values."* There is growing support for chemical-specific of data-driven uncertainty factors in non-cancer risk assessment, which incorporate toxicokinetic and toxicodynamic data, and as a consequence, the application of uncertainty factors other than three or 10 may become more frequent in human health risk assessment in the future (IPCS, 2001; USEPA, 2002). Uncertainty factor values used in deriving the RfC for AN, including those based upon chemical-specific information, are presented below.

A net uncertainty factor was determined based upon a consideration of the following uncertainty factors:

- *UFa* - Since human data were used, an uncertainty factor for interspecies variation is not required (UFa=1).

- *UFh* - Because the critical effect (irritation) occurs at the point of contact, variation in systemic factors (tissue volumes, hepatic metabolism, blood flow) are not likely to have an impact on sensitivity and therefore human variation is expected to be small. However, because the study population was healthy workers, a value of 10 was considered appropriate for UFh.

- *UFl* - Because a NOAEL was identified from the key study, an uncertainty factor for use of a LOAEL is not required (UFl=1).

- *UFs* - Because the critical study involved exposures to AN of five years or more, and because the critical endpoint (irritation) is more likely related to peak exposures than to cumulative exposures, an uncertainty factor for less-than-lifetime exposure durations is not required (UFs=1).

- *UFd* - Because the toxicological database for AN is robust, an uncertainty factor to account for deficiencies in the database is not required (UFd=1). The database includes the four of the five critical studies required for designating high confidence in the RfC, including: (1) chronic toxicity in Sprague-Dawley rats by the inhalation route (Maltoni *et al.*, 1977, 1988; Quast *et al*., 1980a); (2) chronic toxicity in B6C3F1 mice by the oral route (Ghanayem *et al.*, 2002); (3) developmental toxicity in Sprague-Dawley rats by the inhalation route (Murray *et al.*, 1978; Saillenfait *et al.*, 1993) and in Sprague-Dawley and Wistar rats by the oral route (Murray *et al.*, 1978; Mehrotra *et al.*, 1988); and (4) a three-generation reproduction study in rats by the oral route, which included additional evaluations for neurological and cancer effects (Friedman and Beliles, 2002). Although a developmental study in a second mammalian species was not identified for AN, available studies include two different strains of rat (Sprague-Dawley and Wistar). Furthermore, studies on aliphatic nitriles indicate the formation of cyanide may play an important role in developmental toxicity (Saillenfait *et al.*, 2000). Because developmental toxicity studies have been conducted for cyanide using several mammalian species, including rats, hamsters, and pigs (Singh, 1981; Willhite, 1982; Frakes *et al.*, 1985, 1986a, 1986b; Tewe and Maner, 1981a, 1981b), the lack of a developmental toxicity study in a second mammalian species does not constitute a data gap for AN. In addition, although the multigeneration reproduction study was conducted by the oral route, tools are available (i.e., PBPK modeling) for performing route-to-route and interspecies extrapolations for AN (Kedderis *et al.*, 1996; Sweeney *et al.*, 2003).

Based on the individual uncertainty factors defined above, a net uncertainty factor of 10 was considered appropriate for deriving an RfC for AN using human data.

5.2.7 Calculation of RfC

Based upon an NOAEL of 6.5 mg/m^3 and a net uncertainty factor of 10, an **inhalation RfC of 0.7 mg/m^3** was derived. This value represents an approximate 350-fold increase when compared to the RfC value of 0.002 mg/m^3 derived previously for AN based upon nasal lesions in rats (USEPA, 1983). This change largely reflects the use human data as well as alternative uncertainty factor values, particularly the fact that a uncertainty factor of 10 is no longer required for an incomplete database for AN.

Because the derivation of an RfC involves a number of decision points, and because there are alternative options for each decision point, a discussion of the impact of alternative decisions on the resulting RfC value is warranted.

- *Alternative Data Sets* - Consideration was given to using the results of Ginceva et al. (1977; as reported in WHO, 1983), which identifies a NOAEL value of 3 ppm for irritation, which is similar to the NOAEL determined from Sakurai et al. (1978). Use of this study results in the same RfC value as obtained from Sakurai et al. (1978). Consideration was also given to using data for nasal lesions in rats exposed to AN for a lifetime (Quast *et al.*, 1980a). An alternative RfC value of 0.2 mg/m^3 was derived for AN using this study in **Appendix D**. This value is approximately 3.5-fold lower than the value derived from human data, but was not used as the primary basis for the RfC due to important differences between nasal architecture between rodents and humans. Consideration was also given to using alternative endpoints from rodent studies for the RfC. Use of either the neurological (Gagnaire *et al.*, 1998) or maternal body weight (Saillenfait *et al.*, 1993) dose-response data results in subchronic points of departure that are higher than the chronic point of departure calculated for nasal lesions in rats.

Confidence in the **inhalation RfC value of 0.7 mg/m^3** is considered medium. This value is compared to the inhalation value derived for cancer effects in **Section 5.3.2.7**. Confidence in the critical study is considered to be medium since it is based upon a observations in humans, but there are limitations in the exposure estimates. Confidence in the database is considered high since there are a number of other well-conducted, chronic bioassays in both rats and mice, and a multigeneration reproductive toxicity study has been conducted by the oral route, and methods are available (PBPK modeling) to support route-to-route extrapolations. Although the proposed RfC value is 350-fold higher than derived previously for AN by USEPA, confidence is high that it remains protective of human health. The RfC is supported by an alternative RfC value of 0.2 mg/m^3 derived from rat nasal lesion data in **Appendix D**.

5.3 Cancer Assessment

The cancer assessment for AN includes the derivation of potency estimates for both oral and inhalation exposures.

5.3.1 Oral Cancer Dose-Response Assessment

5.3.1.1 Identification of a Critical Effect/Data Set

Although no human data are available regarding the carcinogenicity of AN following oral exposures, a large number of cancer bioassays have been conducted in laboratory animals. As summarized in **Section 4.3.2**, a number of tumor types have been reported (oral cavity,

Zymbal's gland, forestomach, small intestines, mammary gland), but brain tumors appear to be the predominant tumor type observed following inhalation (Maltoni *et al.* 1977, 1988; Quast et al., 19801), drinking water (Gallagher, 1980; Bigner *et al.* 1986; Johannsen and Levinskas, 2002a,b; Friedman and Beliles, 2002), and gavage (Quast *et al.*, 1980b; Johannsen and Levinskas, 2002a) exposures. More recently, a cancer bioassay was conducted in mice exposed to AN via gavage (Ghanayem *et al.*, 2002). Although no brain tumors were observed in mice, several tumor types were identified as being potentially increased in a treatment-related manner, including tumors of the forestomach, Harderian gland, ovary, and lung. As described in **Section 4.3.2**, tumor types other than brain tumors were considered to be less important for the purposes of quantifying potency estimates because they are either (1) observed at higher doses; (2) observed at lower incidence than brain; and/or (3) observed in tissues for which there is no human homologue. In addition, use of combined tumor incidence was not considered since the incidence of tumors for a number of tissue sites was lower in exposed animals, such that in some studies the number of tumor-bearing rats was lower than that observed for control animals (Quast *et al.*, 1980a). Accordingly, the brain tissue data were selected for determining the cancer potency of AN. Exposure-response data for AN and brain tumors in rats following oral and inhalation exposure are provided in **Table 5-3**.

Table 5-3. Summary of Exposure-Response Data for Brain Tumors in Rats Following Oral and Inhalation Exposures to AN

Study	Route	Dose (mg/kg-day) or Concentration (µg/m^3)	Brain Tumor Incidence	
			Females	Males
Maltoni *et al.* (1977)	Inhalation	0	0/30	0/30
		11,015	0/30	0/30
		22,030	0/30	0/30
		44,060	1/30	1/30
		88,120	1/30	2/30
Quast *et al.* (1980a)	Inhalation	0	0/100*	0/100*
		44,060	8/100*	4/99*
		176,240	21/100*	22/99*
Maltoni *et al.* (1988)	Inhalation	0	2/209	2/158
		208,440	10/54	11/67
Johannsen and Levinskas (2002b)	Drinking water	0	1/199	2/200
		0.12 (F), 0.08 (M)	1/100	2/100
		0.36 (F), 0.25 (M)	2/101	1/100
		1.25 (F), 0.83 (M)	4/95	2/100
		3.65 (F), 2.48 (M)	6/100	10/99

		10.9 (F), 8.37 (M)	23/98	21/99
Johannsen and Levinskas (2002a)	Drinking water	0	0/99	2/88
		0.15 (F), 0.09 (M)	1/100	3/85
		10.7 (F), 7.98 (M)	32/97	23/87
Quast et al. (1980b)	Drinking water	0	1/80*	1/80*
		4.36 (F), 3.42 (M)	20/48*	12/47*
		10.76 (F), 8.53 (M)	25/48*	22/48*
		24.97 (F), 21.18 (M)	31/48*	30/48*
Gallagher (1980)	Drinking water	0	NA	0/20
		1	NA	0/20
		5	NA	0/20
		25	NA	0/20
Bigner *et al.* (1986)	Drinking water	0	Not reported	
		5	Not reported	
		25	49/215**	
Johannsen and Levinskas (2002b)	Gavage	0	1/86	2/90
		0.1	2/90	0/87
		10	17/100	16/98
Maltoni *et al.* (1977)	Gavage	0	2/75	1/75
		5	1/40	0/40

*Incidence includes spinal tumors

**Incidence obtained at 18-months, incidence at study termination not reported.

The selection of a data set to serve as the basis for an oral potency estimate for AN should include a careful consideration of the relevance of the data to human health as well as the degree of protectiveness (conservatism) it offers. Because the epidemiology data for AN do not provide evidence for carcinogenicity, the relevance of all rodent data sets to human health is not fully understood. There are two possible explanations for the apparent discrepancy between rodent and human studies: (1) there is a qualitative difference between rodents and humans such that AN is carcinogenic to rodents but not to humans; and (2) there is a quantitative difference between rodents and humans, such that the degree of exposure in human studies was not sufficient to result in a large number of cancer deaths, or such that differences in kinetics/dynamics result in a lower sensitivity to the carcinogenic effects in humans than in rodents. In the absence of specific information to support the former explanation, the latter explanation was conservatively adopted for this assessment.

The brain tumor data for rats were selected as providing the most conservative basis for estimating potency. However, the relevance of rat brain tumor data to human health is not fully

understood, considering that brain cancer mortality was not found to be increased in a large body of epidemiological studies. In addition, the failure to confirm the brain as a target organ of the carcinogenic effects of AN in a second mammalian species (mouse) further suggests that this endpoint may be unique to the rat. Nevertheless, considering the possibility that the differences between rats and humans may be quantitative, rather than qualitative, in nature, the dose-response data for brain tumors in rats were used to assess cancer potency. These data were assessed by pooling the data contained in **Table 5-3**. Under USEPA's *Draft Guidelines for Carcinogen Risk Assessment* (USEPA, 1999, 2003), assessments based upon the combined results of different experiments are considered to be an appropriate option, however pooled analyses are not described specifically. By pooling the data from several studies, an assumption is made that the background rates for brain tumors in rats are similar across strains. Given the relatively low incidence of brain tumors in control rats from all data sets, this assumption appears to be reasonable. For the pooled data, some individual data sets were excluded, including: (1) data from gavage studies were excluded because of limitations in the relevance of bolus dosing to human health, and the use of intermittent exposure regimens (3x/week) in some cases; (2) data from Bigner *et al.* (1986) were excluded since the final results were not reported and reliance upon data from interim sacrifices may serve to underestimate response; (3) data from Gallagher (1980) were excluded because of the small number of animals tested per group and because, inexplicably, no brain tumors were observed in exposed rats. In all three cases, exclusion of these data sets from the pooled data set may be viewed as conservative, since their inclusion would only serve to decrease cancer potency estimates. For comparison purposes, oral dose-response assessments based upon individual data sets in terms of external and internal dose in **Appendix E**.

5.3.1.2 Identification of a Dose Measure

The selection of an internal dose measure for cancer dose-response assessment requires a careful consideration of the mode(s) of action by which the chemical produces its carcinogenic effect. A comparison of several plausible modes of action for AN to the Hill criteria was provided in **Table 4-24**. Although none of the modes of action considered could be ruled out with a high degree of confidence, the data are most consistent with a role for oxidative stress, as mediated by an oxidative metabolite of AN, in producing brain tumors in rats. More importantly, regardless of the mode of action, CEO concentration in brain tissue was considered to be an appropriate internal dose measure. Less clear, however, is whether peak tissue concentration or cumulative exposure (AUC) is more appropriate. An evaluation was conducted for the pooled data set for brain in rats for several internal dose measures, including peak and AUC for AN in blood, peak and AUC for AN in brain, peak and AUC for CEO in blood, and peak and CEO in brain (Kirman *et al.*, 2000). Of the internal dose measures considered, peak concentration of CEO in brain provided the most consistent dose-response relationship for the pooled data set. Peak tissue concentration is very sensitive to the dose-rate assumptions used for the drinking water exposures in rats because of its pulsatile nature (peak

tissue concentration is much less sensitive for inhalation exposures in which steady state was generally achieved for several hours). AUC, on the other hand, is relatively insensitive to dose-rate-timing assumptions for drinking water exposure (*i.e.,* AUC obtained for a single bolus drinking water event is very similar to that obtained assuming many drinking water events). In this way, any changes to the drinking water assumptions used in rat PBPK simulations cannot improve the performance of AUC as an internal dose measure with the pooled data set. One possible reason for the failure of AUC in providing a consistent dose-response relationship is the existence of a threshold, which when present will yield false positive response predictions for low level exposures using AUC as the dose measure. Although the evidence supporting peak tissue concentration over AUC is empirical in nature, empirical evidence has been used in selecting an internal dose measure past assessments, as was the case for methylene chloride (USEPA, 2003). Based upon the mechanistic and empirical support, peak CEO in brain was selected for use in this dose-response assessment.

Internal dose measures (peak CEO in brain) were calculated for both oral and inhalation studies using the PBPK model for AN in rats (Kedderis *et al.*, 1996). Steady-state is rapidly achieved for CEO dose measures, and therefore there was no need to run multiple-day exposures to account for tissue build-up from day-to-day. For the inhalation exposures, the exposure times reported for the individual studies (ranging from 4 hours/day to 7 hours/day) were used in the simulations. For drinking water exposures, exposure was assumed to occur during 6 drinking episodes per 24-hour period, with the majority of the intake (70%) split between 3 drinking episodes at night, and the remaining intake (30%) split between 3 drinking episodes during the day (Kirman *et al.*, 2000). The resulting internal dose measures are listed in **Table 5-4**.

Table 5-4. Internal Dose Measures Estimated Using PBPK Modeling

Study	Route	Dose (mg/kg-day) or Concentration ($\mu g/m^3$)	Peak CEO Concentration in Brain (mg/L)	
			Females	Males
Maltoni *et al.* (1977)	Inhalation	0	0	0
		11,015	0.0031	0.0031
		22,030	0.0061	0.0062
		44,060	0.012	0.012
		88,120	0.024	0.024
Quast *et al.* (1980a)	Inhalation	0	0	0
		44,060	0.012	0.012
		176,240	0.044	0.045
Maltoni *et al.* (1988)	Inhalation	0	0	0
		208,440	0.034	0.035
Biodynamics (1980b)	Drinking	0	0	0
		0.12 (F), 0.08 (M)	0.0016	0.0012
		0.36 (F), 0.25 (M)	0.0047	0.0035
		1.25 (F), 0.83 (M)	0.016	0.012
		3.65 (F), 2.48 (M)	0.042	0.032
		10.9 (F), 8.37 (M)	0.094	0.084
Biodynamics (1980c)	Drinking	0	0	0
		0.15 (F), 0.09 (M)	0.0020	0.0014
		10.7 (F), 7.98 (M)	0.097	0.085
Quast et al. (1980b)	Drinking	0	0	0
		4.36 (F), 3.42 (M)	0.052	0.046
		10.76 (F), 8.53 (M)	0.097	0.089
		24.97 (F), 21.18 (M)	0.141	0.137

5.3.1.3 Identification of a Response Measure

Several measures of cancer risk were considered for the oral dose-response assessment. These are as follows:

Relative Risk = [Observed Cancer Deaths]/[Expected Deaths]
Extra Risk = [P(d)-P(0)]/[1.0-P(0)]
Added Risk = P(d)-P(0)

Where,

P(d) = Probability of a cancer response at AN dose, d; and
P(0) = Probability of cancer response at zero (or background) dose.

For this assessment, the default response measure (extra risk) was considered appropriate.

5.3.1.4 Selection of a Dose-Response Model

A dose-response model was selected on the basis of the goodness of fit. Outputs from the BMDS model are provided in **Appendix E**. A comparison of goodness-of-fit statistics for the pooled data set is provided in **Table 5-5**.

Table 5-5. Comparison of Goodness-of-Fit Statistics for Dose-Response Models Applied to the Pooled Data for Brain Tumors in Rats Assessed in Terms of Internal Dose

				Low-Dose Residuals*	
Model	**Chi-square**	**Degrees of freedom**	**P-value**	**Mean**	**Sum**
gamma	79.07	43	0.0007**	-0.103	13.9
Weibull	78.93	43	0.0007	-0.107	14.0
quantal linear	83.78	44	0.0003	-0.112	16.4
quantal quadratic	130.25	44	0.0000	-0.450	14.7
probit	132.9	44	0.0000	-0.812	19.07
logistic	154.21	44	0.0000	-0.998	22.53
multistage	failed				

*Residuals below a peak CEO concentration of 0.01 mg/L
**Although statistically significant results were not obtained with the BMDS program, use of Microsoft Excel to include of additional parameters to account for species-, sex-, and laboratory differences resulted in a statistically significant fit (p=0.746) by the gamma model.

Although none of the models in the BMDS program (version 1.3.2) provided a statistically acceptable fit to the dose-response data ($p<0.05$), the gamma model was selected for the pooled data set since it provided the best overall fit to the data in the range of observation (p=0.0007). The gamma and Weibull models were found to yield nearly identical fits to the dose-response data. The multistage model of BMDS failed to provide a result. An analysis of residuals in the low-dose region (peak CEO<0.01 mg/L) was conducted to evaluate the fit of the models at low

doses. Again, the gamma model performed slightly better than the other models. The fit of this model to the pooled dose-response data is depicted in **Figure 5-3a** across the entire dose region, and in **Figure 5-3b** at low doses. Based upon visual inspection of this figure, the gamma model was determined to provide an acceptable fit to the dose-response data, suggesting that the lack of a statistically acceptable fit reflects the scatter in the data rather than the fit of the model. The relatively low p-values provided by the models reflect a limitation of the BMDS model in that it cannot be used to include model parameters to account for differences across sex, strain, or laboratory. When these additional model terms are included in the gamma model (conducted in Microsoft Excel, with optimal fits of the modified gamma model obtained using the Solver function to maximize the log-likelihood function), the degrees of freedom decreases from 43 to 40 while the p-value increases significantly from 0.0007 to 0.746.

5.3.1.5 Selection of a Point of Departure

A number of points of departure were considered, as provided in **Table 5-6**. Because there are many more data points in the range of observation, points of departure below the 10% response level were considered.

Table 5-6. Points of Departure Based Upon Pooled Data for Brain Tumors in Rats Assessed in Terms of Internal Dose

	Peak CEO in Brain (mg/L)	
Response Level	**ED**	**LED**
0.1	0.030	0.026
0.05	0.017	0.014
0.01	0.0049	0.0032
0.005	0.0029	0.0017
0.001	0.00087	0.00041

The dose-response points for the pooled data set in **Figure 5-3b** are essentially equivalent to the background response level below internal doses of 0.01 mg/L. The ED05 value corresponds to a region of the dose-response curve where an increased brain tumor risk becomes apparent (**Figure 5-3b**). Accordingly, the ED05 and LED05 of 0.017 and 0.014 mg/L, respectively, were considered to be appropriate points of departure for the pooled data set. The PBPK model was used to calculate the continuous oral doses for humans that correspond to the point of departure. This was accomplished in an iterative manner by

manually adjusting the oral dose until the internal dose predicted by the human PBPK model agrees with the internal points of departure. Accordingly, the ED05 and LED05 correspond to human equivalent doses of 2.1 mg/kg-day and 1.7 mg/kg-day, respectively.

5.3.1.6 Selection of a Method for Extrapolating to Low Doses

The database to support a cancer risk assessment for AN is unique in that it includes robust epidemiological data, bioassay data from two rodent species by two routes of exposure, a PBPK model, and extensive mechanistic data. It is important that all of these sources of data be considered in selecting a low-dose extrapolation method.

The selection of an appropriate method for extrapolating to low doses requires a careful consideration of the mode of action and supporting weight of evidence. The mode of action for tumor induction in laboratory rodents is likely complex and could include multiple mechanisms, each of which could predominate at different doses. These mechanisms include likely indirect DNA damage (*e.g.*, caused by oxidative stress), possible direct DNA damage (e.g., caused by the acrylonitrile metabolite CEO, or even by acrylonitrile itself), and epigenetic changes (e.g., as indicated by inhibition of gap junction intercellular communication [GJIC] in the target tissues). All of the MOAs proposed for the observed animal carcinogenicity are relevant to humans. It is important that the robust epidemiological database also be used to further inform this risk assessment. The robust epidemiology data on AN do not support a causal association between AN exposure in workers and increased cancer incidence. Taken together with the data from laboratory rodents and mechanistic studies, these data suggest that AN should not be considered as posing a cancer hazard at current and past occupational and environmental exposure levels. A comparison of the rodent data for brain tumors used here and epidemiology data for lung cancer mortality is provided in **Appendix G**. The comparison is sensitive to any adjustments made for the differences in exposure duration (lifetime for rats, less than lifetime for exposed workers). When duration adjustments are excluded, there is considerable overlap between the internal doses experienced by workers and those tested in rats, suggesting a lack of concordance in response between rodent and human data sets (**Figure G-1**). When internal doses are adjusted for duration, the internal doses experienced by workers approach those tested in rats. Regardless of the duration adjustment, the behavior of the epidemiological data across a broad range of internal doses do not support the existence of a dose-response relationship (**Figure G-2**). Uncertainty factors used for the nonlinear approach were defined as follows:

- *UFa* - Consistent with the UFa value used for the oral RfD, the default value of 10 for UFa can be treated as two specific factors of 3.2 for kinetic variation and 3.2 for dynamic variation. Because PBPK models (Kedderis *et al.*, 1996; Sweeney *et al.*, 2003) were used to account for kinetic differences between rats and humans, thereby improving the confidence in the interspecies extrapolation, the kinetic component of UFa was set equal to one. For the dynamic component of UFa, a value of 3.2 was used

nonlinear approach to account for potential dynamic differences between rats and humans.

- *UFh* - The default value of 10 can also be treated as two specific factors of 3.2 for kinetic variation and 3.2 for dynamic variation. Using a PBPK model and variability analysis to address human variation for peak CEO in brain following oral exposure, the model coefficient of variation for this internal dose was found to be 0.59 (Sweeney *et al.*, 2003). Accordingly, the 95th percentile for AUC AN in blood is predicted to be approximately 2.0-fold higher (1+1.64*0.59, assuming a normal distribution) than the mean value (Sweeney *et al.*, 2003). Use of the 99th percentile would result in a values that is essentially the same (2.4-fold, calculated as 1+2.32*0.59, assuming a normal distribution). Accordingly, a factor of 2.0 was combined with the default factor of 3.2 for human variation in toxicodynamics to yield an UFh value of 6.4. This approach is consistent with guidelines for chemical-specific adjustment factors (IPCS, 2001; USEPA, 2002).

- *UFl* - Although a dose-response model was used to characterize the dose-response relationship for exposure and brain tumors in rats, it is recognized that a 5% response levels reflects fairly significant response, and cannot be treated as a NOAEL for an effect of this severity. To account for the severity of the response used in this dose-response assessment, a value of 10 was used for UFl.

- *UFs* - Since the exposure regimen in the key studies are chronic in nature, UFs is not needed (UFs=1).

- *UFd* - Because the toxicological database for AN is robust, an uncertainty factor to account for deficiencies in the database is not required (UFd=1).

- *MF* - An additional modifying factor was not considered necessary for the RfD (MF=1).

When combined, the individual uncertainty factors summarized above yield a net uncertainty factor of 200 (3.2x3.2x2.0x10) for the oral dose-response assessment. For the purposes of comparison, a linear extrapolation from the point of departure was included in **Appendix E**.

5.3.1.7 Calculation of Oral Cancer Value

Based on a point of departure (LED05) of 1.7 mg/kg-day and an uncertainty factor of 200, **oral doses below 0.009 mg/kg-day are not expected to pose an appreciable risk of cancer**. A comparison of this value with the oral RfD derived in **Section 5.1** for AN based upon neurological effects (0.2 mg/kg-day) indicates that human health risk assessment for oral exposures to AN will be driven by its potential carcinogenic effects. Because the derivation

of a cancer potency estimate involves a number of decision points, and because there are alternative options for each decision point, a discussion of the impact of alternative decisions on the resulting potency estimate is warranted.

- *Alternative Data Sets* - Cancer potency estimates were also derived for AN using individual data sets in **Appendix E**. Nonlinear cancer values for AN based upon individual data sets using external dose range from 0.0002 to 0.0005 mg/kg-day, and without application of the PBPK model, using a net UF of 2,000. Similarly, nonlinear cancer values for AN based upon individual data sets using internal dose range from 0.003 to 0.01 mg/kg-day, with the application of the PBPK model and the use of an LED10 (vs. LED05), using a net UF of 400. Additionally, consideration was given to using the combined tumor incidence from multiple tissue sites in rats exposure to AN, as was done in USEPA's 1983 assessment. As indicated above, the cancer dose-response assessment was not based on combined tumor types due to potential differences in severity, latency, and mechanism. An evaluation of the points of departure expressed in terms of external dose for combined tumors in rats were generally within a factor of two of the values calculated in terms of using brain tumors only. As such, increasing the complexity of the assessment by using combined tumors does not appear to be warranted. A number of data sets for the mouse were also considered, but were not used. Comparison of the points of departure for mouse tumors expressed in terms of external dose were generally higher (less conservative) than the values calculated for the rat brain tumor data.

- *Alternative Dose Measure* - Despite uncertainty in the mode of action for AN in producing brain tumors in rats, all of the modes of action considered (**Table 4-24**) suggest that some measure of CEO is a useful internal dose measure. Consideration was given to use of other internal dose measures, including the peak AN concentration in brain tissue, as well as AUC for AN or CEO in brain tissue. An evaluation of these internal doses measures failed to provide a consistent dose-response relationship for the dose-response data set pooled across routes of exposure (Kirman *et al.*, 2000), and as such are not useful for providing a consistent estimate of cancer potency. Because of differences in oxidative metabolism between rats and humans (*i.e.*, humans>rats), the use of internal dose measures based on CEO is more generally conservative than use of internal dose measures based on AN.

- *Alternative Risk Measure* - Since the background rate for brain tumors is low in rats (approximately zero), added risk and extra risk are essentially equivalent. With low background rates, relative risk can become very sensitive to uncertainty in the background rate, and is generally reserved for use with epidemiology data. Therefore, relative risk is not recommended for use with the rat brain tumor data.

- *Alternative Dose-Response Models* - A number of dose-response models were considered for the pooled data set. The dose-response models which provided a good fit to the pooled dose-response data (gamma, Weibull, and multistage) models provided the same predictions at the ED10 level, while predictions at the ED001 were general within a factor of two. As such, the selection of the dose-response model did not have a significant impact on the potency estimates.

- *Alternative Response Level* - The gamma dose-response model predicts a nonlinear dose-response relationship between internal dose and rat brain tumor incidence, whose slope increases with increasing dose. Because a value of 10 was used for UFl, any changes in the point of departure would likely require a corresponding change in the UFl value, and therefore is not expected to have a significant effect on the resulting cancer value for oral exposures to AN.

- *Alternative Low-Dose Extrapolation* - Based on an assumption of linearity below the point of departure, a slope factor of 0.029 per mg/kg-day (0.05/1.7 mg/kg-day) could be derived for AN. A linear estimate of cancer potency was derived for AN due to uncertainty in the mode of action for brain tumors in rats. However, based upon the weight of evidence for the mode of action and from the epidemiology data, a linear extrapolation was not adopted as a final recommendation for AN.

Confidence in the **oral cancer value of 0.009 mg/kg-day** is considered medium-to-high. Confidence in the critical studies is high since there are a large number of studies conducted in the rat, each study was generally well designed and used an adequate number of test groups and animals, and the studies provide a consistent dose-response relationship for 46 groups of rats (34 exposure groups, 12 control groups) across both oral and inhalation routes of exposure, and allow for a more complete description of the dose-response relationship at low doses. Confidence in the database is high, since the carcinogenicity has been evaluated in rats and mice, as well as in exposed workers. Confidence in the PBPK modeling used to support this assessment is considered medium since the model has been validated in rats but not in humans, and because the model provides a good description of the kinetics of AN. The PBPK model for AN demonstrated difficulties in predicted CEO concentrations in rat brain shortly after bolus oral doses, which may be related to analytical difficulties associated with brain tissue, and the model does not yet include a description of cyanide kinetics (which may be an important determinant of oxidative stress) in either rats or humans. Although medium confidence is initially placed in the mode of action for AN (**Section 4.3.2**), confidence is increased by the large body of epidemiology data which do not support the carcinogenic results for AN reported in animal studies. A comparison of the rat and human cancer data in **Appendix G** demonstrates a lack of concordance between these two data sets. This comparison demonstrates that the exposure experienced by AN workers in the past are not orders of magnitude lower than the exposure administered to rats in cancer bioassays. Rather, past

worker exposures to AN are seen here as either overlapping the lower end of the rat exposures using unadjusted internal dose, or at least approximately equivalent with the lower end of the exposures administered to rats using duration-adjusted internal dose. Accordingly, based upon a nonlinear assessment for AN-induced brain tumors in the rat, oral doses of AN below 0.009 mg/kg-day are not expected to pose an appreciable risk to human populations.

5.3.2 Inhalation Cancer Dose-Response Assessment

5.3.2.1 Identification of a Critical Effect/Data Set

As summarized in **Section 4.3.1**, the potential carcinogenic effects of AN following inhalation exposures have been well studied in laboratory animals and in exposed human worker populations. Consideration was given to selecting the human data to serve as the primary basis for the inhalation dose-response assessment due to the uncertain relevance of brain tumors observed in rats. However, because the human data do not support a causal relationship for AN exposure and cancer mortality under the conditions of occupational exposures, it was not considered an appropriate application of the epidemiology data. A comparison of these data to the rodent dose-response data for brain tumors is provided in **Appendix G**. For this reason, the rodent data serve as the primary basis of the inhalation dose-response assessment, and the epidemiology data are used as a "reality check" to place bounds upon the rodent estimates of cancer potency.

Although a number of tumor types have been observed in rats following inhalation exposure to AN, the central nervous system appears to be the primary target of AN carcinogenicity (Maltoni *et al.*, 1977, 1988; Quast et al., 1980a). Tumor types other than brain tumors were considered to be less important for the purposes of quantifying potency estimates because they are either (1) observed at higher doses; (2) observed at lower incidence than brain; and/or (3) observed in tissues for which there is no human homologue. In addition, use of combined tumor incidence was not considered since the incidence of tumors for a number of tissue sites was lower in exposed animals, such that the number of tumor-bearing rats was lower than that observed for control animals (Quast *et al.*, 1980a). Accordingly, the brain tissue data were selected for determining the cancer potency of AN. Exposure-response data sets for brain tumors in rats following inhalation exposures are provided in **Table 5-3**. For this assessment, the rat dose-response data for inhalation exposure were pooled with the drinking water dose-response data. An alternative assessment using individual data sets is provided in **Appendix F**.

5.3.2.2 Identification of a Dose Measure

The selection of an internal dose measure for cancer dose-response assessment requires a careful consideration of the mode(s) of action by which the chemical produces its carcinogenic

effect. A comparison of several plausible modes of action for AN to the Hill criteria was provided in **Table 4-24**. Although none of the modes of action considered could be ruled out with a high degree of confidence, the weight of evidence is most consistent with a role for oxidative stress, as mediated by an oxidative metabolite of AN, in producing brain tumors in rats. More importantly, regardless of the mode of action, CEO concentration in brain tissue was considered to be an appropriate internal dose measure. Less clear, however, is whether peak tissue concentration or cumulative exposure (AUC) is more appropriate. An evaluation was conducted for the pooled data set for brain in rats for several internal dose measures, including peak and AUC for AN in blood, peak and AUC for AN in brain, peak and AUC for CEO in blood, and peak and CEO in brain (Kirman *et al.*, 2000). Of the internal dose measures considered, peak concentration of CEO in brain provided the most consistent dose-response relationship for the pooled data set. Although the evidence supporting peak tissue concentration over AUC is empirical in nature, empirical evidence has been used in selecting an internal dose measure past assessments, as was the case for methylene chloride (USEPA, 2003). Based upon the mechanistic and empirical support, peak CEO in brain was selected for use in this dose-response assessment.

Internal dose measures (peak CEO in brain) were calculated for both oral and inhalation studies using the PBPK model for AN in rats (Kedderis *et al.*, 1996). Steady-state is rapidly achieved for CEO dose measures, and therefore there was no need to run multiple-day exposures to account for tissue build-up from day-to-day. For the inhalation exposures, the exposure times reported for the individual studies (ranging from 4 hours/day to 7 hours/day) were used in the simulations. For drinking water exposures, exposure was assumed to occur during 6 drinking episodes per 24-hour period, with the majority of the intake (70%) split between 3 drinking episodes at night, and the remaining intake (30%) split between 3 drinking episodes during the day (Kirman *et al.*, 2000). The resulting internal dose measures are listed in **Table 5-4**.

5.3.2.3 Identification of a Response Measure

Quantal data on incidence were used from animal data sets. Several measures of cancer risk were considered, including:

Relative Risk	=	[Observed Cancer Deaths]/[Expected Deaths]
Extra Risk	=	[P(d)-P(0)]/[1.0-P(0)]
Added Risk	=	P(d)-P(0)

Where,

P(d)	=	Probability of a cancer response at AN dose d
P(0)	=	Probability of cancer response at zero (or background) dose

The brain tumor dose response data were assessed in terms of extra risk. Because the background risk for brain tumors in rats is near zero, extra risk and added risk are nearly identical.

5.3.2.4 Selection of a Dose-Response Model

A large number of models are available for assessing cancer dose-response data. For the pooled data set (oral and inhalation studies) expressed in terms of internal dose, the gamma model provided a better fit to the data than the other models (see **Table 5-5**).

5.3.2.5 Selection of a Point of Departure

The dose-response points for the pooled data set in **Figure 5-3b** are essentially equivalent to the background response level below internal doses of 0.01 mg/L. The ED05 value corresponds to a region of the dose-response curve where an increased brain tumor risk becomes apparent (**Figure 5-3b**). Accordingly, the ED05 and LED05 of 0.017 and 0.014 mg/L, respectively, were considered to be appropriate points of departure for the pooled data set. The PBPK model was used to calculate the continuous inhalation exposure for humans that correspond to the internal point of departure. This was accomplished in an iterative manner by manually adjusting the inhalation concentration until the internal dose predicted by the human PBPK model agrees with the internal points of departure. Accordingly, the ED05 and LED05 correspond to human equivalent exposures to 25.9 and 21.3 mg/m^3, respectively.

5.3.2.6 Selection of a Method for Extrapolating to Low Doses

The selection of an appropriate method for extrapolating to low concentrations requires a careful consideration of the mode of action and supporting weight of evidence. As discussed in **Section 4.3.2**, although none of the modes of action considered in **Table 4-24** can be ruled out with a high degree of confidence, a mode of action involving oxidative stress mediated by an oxidative metabolite (cyanide, CEO) is best supported by the weight of evidence. Together with the negative findings from a large database of epidemiology studies in workers exposed to AN, a nonlinear extrapolation, similar to the RfC approach used for noncancer endpoints, was considered the most appropriate method for extrapolating to low doses. For the purposes of comparison, a linear extrapolation from the point of departure was included in **Appendix F**. Uncertainty factors used for the nonlinear approach were defined as follows:

- *UFa* - Consistent with the UFa value used for the inhalation RfC, the default value of 10 for UFa can be treated as two specific factors of 3.2 for kinetic variation and 3.2 for dynamic variation. Because PBPK models (Kedderis *et al.*, 1996; Sweeney *et al.*, 2003) were used to account for kinetic differences between rats and humans, thereby improving the confidence in the interspecies extrapolation, the kinetic component of

UFa was set equal to one. For the dynamic component of UFa, a value of 3.2 was used nonlinear approach to account for potential dynamic differences between rats and humans.

- *UFh* - The default value of 10 can also be treated as two specific factors of 3.2 for kinetic variation and 3.2 for dynamic variation. Using a PBPK model and variability analysis to address human variation for CEO in brain following inhalation exposures, the model coefficient of variation for this internal dose was found to be 0.72 (Sweeney *et al.*, 2003). Accordingly, the 95th percentile for AUC AN in blood is predicted to be approximately 2.2-fold higher (1+1.64*0.72, assuming a normal distribution) than the mean value (Sweeney *et al.*, 2003). Use of the 99th percentile would result in a values that is essentially the same (2.7-fold, calculated as 1+2.32*0.72, assuming a normal distribution). Accordingly, a factor of 2.2 was combined with the default factor of 3.2 for human variation in toxicodynamics to yield an UFh value of 7.0. This approach is consistent with guidelines for chemical-specific adjustment factors (IPCS, 2001; USEPA, 2002).

- *UFl* - Although a dose-response model was used to characterize the dose-response relationship for exposure and brain tumors in rats, it is recognized that a 5% response levels reflects fairly significant response, and cannot be treated as a NOAEL. To account for the severity of the response used in this dose-response assessment, a value of 10 was used for UFl.

- *UFs* - Since the exposure regimen in the key studies are chronic in nature, UFs is not needed (UFs=1).

- *UFd* - Because the toxicological database for AN is robust, an uncertainty factor to account for deficiencies in the database is not required (UFd=1).

- *MF* - An additional modifying factor was not considered necessary for the RfD (MF=1).

When combined, the individual uncertainty factors summarized above yield a net uncertainty factor of 220 (3.2x3.2x2.2x10) for the inhalation dose-response assessment.

5.3.2.7 Calculation of Inhalation Cancer Value

Based upon a point of departure of 21.3 mg/m^3 and an uncertainty factor of 220, **inhalation exposures to concentrations less than 0.1 mg/m^3** are anticipated to be without appreciable risk of cancer in human populations. A comparison of this value with the inhalation RfC derived in **Section 5.2** for AN based upon irritation in humans (0.7 mg/m^3) indicates that human health risk assessment for inhalation exposures to AN will be driven by its carcingenic

effects. Because the inhalation dose-response assessment involves a number of decision points, and because there are alternative options for each decision point, a discussion of the impact of alternative decisions on the resulting potency estimate is warranted.

- *Alternative Data Sets* - Inhalation dose-response assessments were also conducted for AN using individual data sets in **Appendix F**. Nonlinear cancer values for AN based upon individual data sets using external concentration range from 0.03 to 0.04 mg/m^3, based upon a net UF of 2,000. Similarly, nonlinear cancer values for AN based upon individual data sets using internal dose range from 0.04 to 0.07 mg/m^3, based upon a net UF of 440.

- *Alternative Dose Measure* - Despite uncertainty in the mode of action for AN in producing brain tumors in rats, all of the modes of action considered suggest that some measure of CEO is a useful internal dose measure. Consideration was given to using other internal dose measures, including the peak AN concentration in brain tissue, as well as AUC for AN or CEO in brain tissue. An evaluation of these internal doses measures failed to provide a consistent dose-response relationship for the dose-response data set pooled across routes of exposure (Kirman *et al.*, 2000), and as such are not useful for providing a consistent estimate of cancer potency. Because of differences in oxidative metabolism between rats and humans (*i.e.*, humans>rats), the use of internal dose measures based on CEO is more generally conservative than use of internal dose measures based on AN.

- *Alternative Risk Measure* - Since the background rate for brain tumors is low in rats (approximately zero), added risk and extra risk are essentially equivalent. With low background rates, relative risk can become very sensitive to uncertainty in the background rate, and is generally reserved for use with epidemiology data. Therefore, relative risk is not recommended for use with the rat brain tumor data.

- *Alternative Dose-Response Models* - A number of dose-response models were considered for the pooled data set. The dose-response models which provided a good fit to the pooled dose-response data (gamma, Weibull, and multistage) models provided the same predictions at the ED10 level, while predictions at the ED001 were general within a factor of two. As such, the selection of the dose-response model did not have a significant impact on the potency estimates.

- *Alternative Response Level* - The gamma dose-response model predicts a nonlinear dose-response relationship between internal dose and rat brain tumor incidence, whose slope increases with increasing dose. Because a value of 10 was used for UFl, any changes in the point of departure would likely require a corresponding change in the

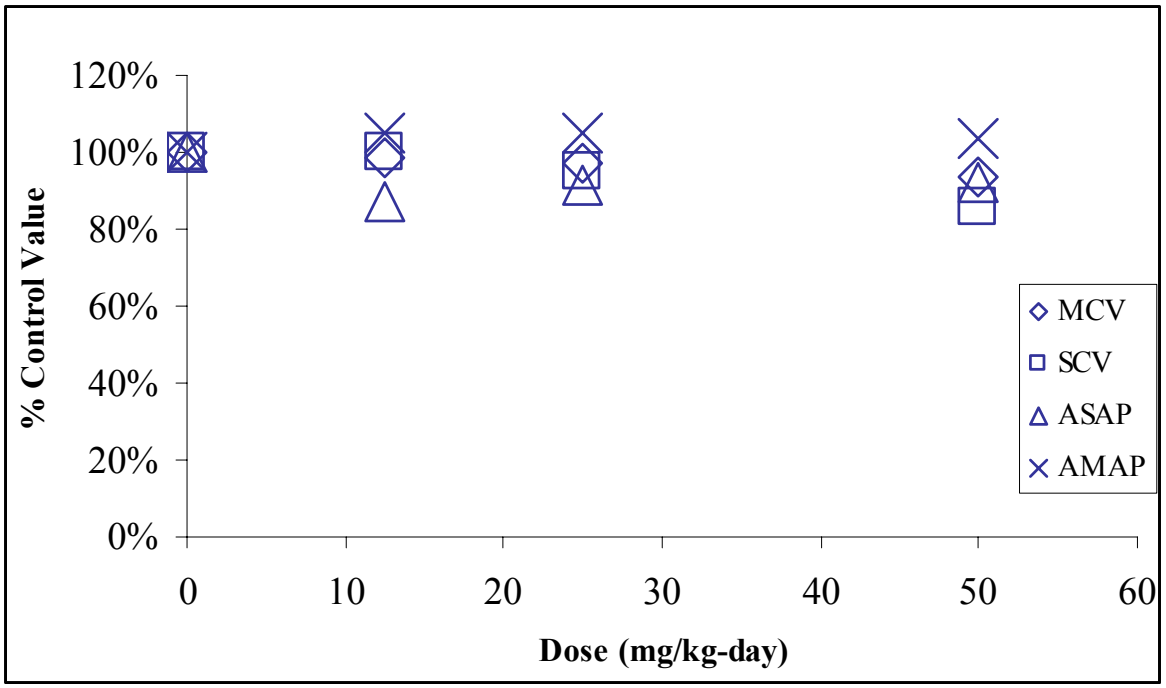
UFl value, and therefore is not expected to have a significant effect on the resulting cancer value for inhalation exposures to AN.

- *Alternative Low-Dose Extrapolation* - Based on an assumption of linearity below the point of departure, a unit risk of 0.0023 per mg/m^3 (0.05/21.3 mg/m^3) could be derived for AN. A linear estimate of cancer potency was derived for AN due to uncertainty in the mode of action for brain tumors in rats. However, based upon the weight of evidence for the mode of action and from the epidemiology data, a linear extrapolation was not adopted as a final recommendation for AN.

Confidence in the **inhalation cancer value of 0.1 mg/m^3** is considered medium-to-high. Confidence in the critical studies is high since there are a large number of studies conducted in the rat, each study was generally well designed and used an adequate number of test groups and animals. The pooled data provide a consistent dose-response relationship for 46 groups of rats (34 exposure groups, 12 control groups) across both oral and inhalation routes of exposure, and allow for a more complete description of the dose-response relationship at low concentrations. Confidence in the database is high, since the carcinogenicity has been evaluated in rats and mice, as well as in exposed workers. Confidence in the PBPK modeling used to support this assessment is considered medium since the model has been validated in rats but not in humans, and because the model provides a good description of the kinetics of AN. The PBPK model demonstrated difficulties in predicted CEO concentrations in rat brain shortly after bolus oral doses, which may be related to analytical difficulties associate with brain tissue, and the model does not include a description of cyanide kinetics (which may be an important determinant of oxidative stress) in rats or humans. Although medium confidence is initially placed in the mode of action for AN (**Section 4.3**), confidence is increased by the large body of epidemiology data which do not support the carcinogenic results for AN reported in animal studies. Accordingly, based upon a nonlinear assessment for AN-induced brain tumors in the rat, inhalation exposures to AN below 0.1 mg/m^3 are not expected to pose an appreciable risk to human populations.

Figure 5-1. Comparison of Neurological Endpoints in Rats Following Oral Exposure to AN (Gagniere *et al.*, 1998)

(a) Post-Exposure



(b) Post-Recovery

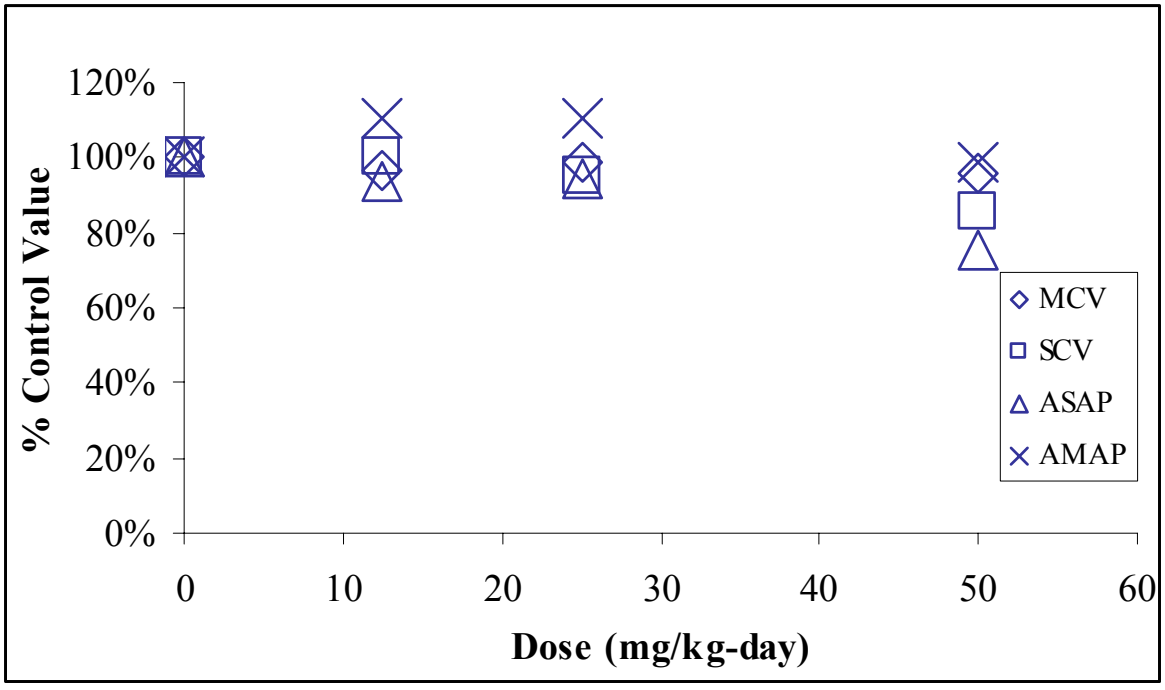


Figure 5-2. Dose-Response Relationship for Neurological Effects in Rats Using Internal Dose Measures

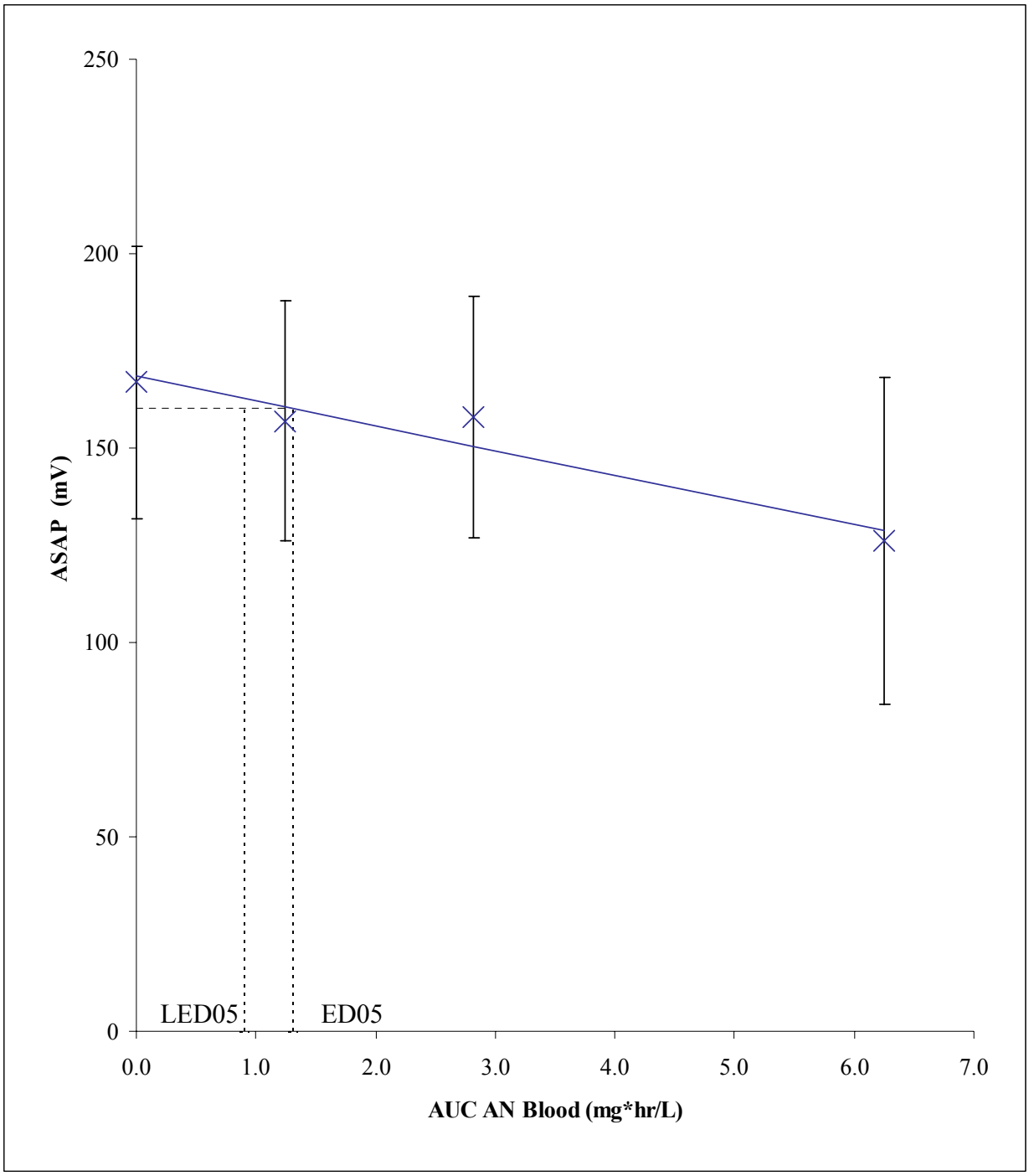
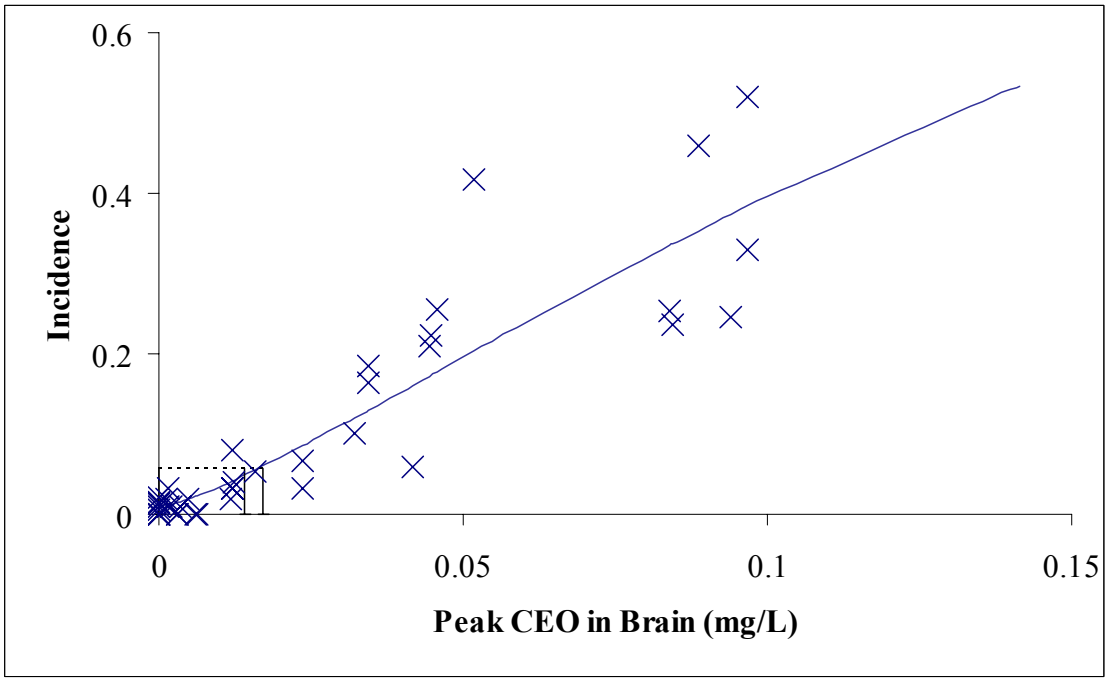
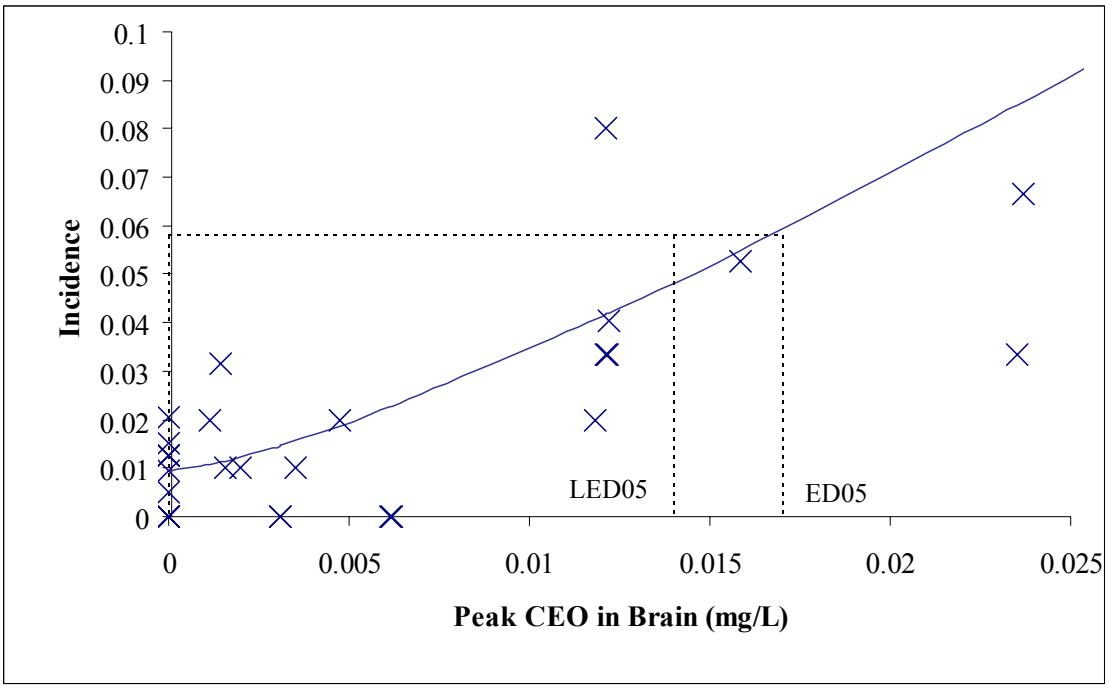


Figure 5-3. Oral and Inhalation Dose-Response Data for Brain Tumors in Rats Using Pooled Data Expressed in Terms of Internal Dose

(a)



(b)



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