

# **TOXICOLOGICAL REVIEW**

**OF**

# **ACROLEIN**

(CAS No. 107-02-8)

## **In Support of Summary Information on the Integrated Risk Information System (IRIS)**

February 2002

Notice

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U.S. Environmental Protection Agency  
Washington DC

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## FOREWORD

The purpose of this Toxicological Review is to provide scientific support and rationale for the hazard and dose-response assessment in IRIS pertaining to acute and chronic exposure to acrolein. It is not intended to be a comprehensive treatise on the chemical or toxicological nature of acrolein.

In section 6, EPA has characterized its overall confidence in the quantitative and qualitative aspects of hazard and dose response. Matters considered in this characterization include knowledge gaps, uncertainties, quality of data, and scientific controversies. This characterization is presented in an effort to make apparent the limitations of the assessment and to aid and guide the risk assessor in the ensuing steps of the risk assessment process.

For other general information about this assessment or other questions relating to IRIS, the reader is referred to EPA's Risk Information Hotline at 301-345-2870.

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Summaries of the external peer reviewers' comments and the disposition of their recommendations are in Appendix A.

## 1. INTRODUCTION

This document presents background and justification for the hazard and dose-response assessment summaries in 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 non-cancer dose-response assessments. The RfD is based on the assumption that thresholds exist for certain toxic effects such as cellular necrosis but may not exist for other toxic effects such as some carcinogenic responses. 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 non-cancer 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 (extrarespiratory or systemic effects). It is generally expressed in units of mg/m<sup>3</sup>.

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 low-dose extrapolation procedure and 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 µg/m<sup>3</sup> 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 identification and dose-response assessments for acrolein has followed the general guidelines for risk assessment as set forth by the National Research Council (1983). 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), *Proposed Guidelines for Carcinogen Risk Assessment (1996a)*, *Guidelines for Reproductive Toxicity Risk Assessment* (U.S. EPA, 1996b), and *Guidelines for Neurotoxicity Risk Assessment* (U.S. EPA, 1998a); *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, 1995b); Review Draft Guidelines for Carcinogen Risk Assessment (1999); *Science Council Policy Handbook: Peer Review* (U.S. EPA, 1998b,2000a); *Science Policy Council Handbook: Risk Characterization* (U.S. EPA, 2000b).

Literature search strategy employed for this compound were based on the CASRN and at least one common name. At a minimum, the following databases were searched: HSDB, CCRIS, GENETOX, EMIC, EMICBACK, DART, ETICBACK, TOXLINE, MEDLINE, AND MEDLINE backfiles. Any pertinent scientific information submitted by the public to the IRIS Submission Desk was also considered in the development of this document.

## 2. CHEMICAL AND PHYSICAL INFORMATION RELEVANT TO ASSESSMENTS

Acrolein is also known as acrylaldehyde, acrylic aldehyde, allyl aldehyde, ethylene aldehyde, 2-propenal, and prop-2-en-1-al (Izard and Libermann, 1978). Trade names include aqualin, aqualine, biocide, magnacide, magnacide B, and Slimicide (Ghilarducci and Tjeerdema, 1995). Some relevant physical and chemical properties are listed below.

CASRN: 107-02-8

Empirical formula:  $C_3H_4O$

Molecular weight: 56.06

Vapor pressure: 214 torr at 20°C

Vapor density: 1.94

Specific gravity: 0.8427 at 20°C

Boiling point: 52.7°C

Melting point: -86.9°C

Water solubility: 206 g/L at 20°C

Log octanol-water partition coefficient ( $K_{ow}$ ): 0.9

Conversion factor: 1 ppm = 2.3 mg/m<sup>3</sup>; 1 mg/m<sup>3</sup> = 0.44 ppm

At room temperature acrolein is a colorless to yellowish flammable liquid with a disagreeable, choking odor. It is extremely acrid and is irritating to mucous membranes (ACGIH, 1991). The odor threshold was stated to be 0.21 ppm (Leonardos, et al., 1969). The stability of acrolein in deionized water was established at 4 and 24 hr at concentrations of 0.5 and 6.0 mg/ml (Parent et al., 1993c). No evidence of decomposition was found at 0.5 mg/ml, but at 6 mg/ml losses of 0.5 and 3.9% were noted at 4 and 24 hr, respectively. At a temperature of 5 °C, there was a measured loss of 18% after 6 days and 27% after 3 days at 22°C (Lijinsky and Reuber, 1987). This suggests that acrolein in drinking water has the potential to be a source of exposure to humans. However, limited studies indicate that it has not often been detected in drinking or well water (Glaze et al., 1989; Staples et al., 1985).

The principal use of acrolein is as an intermediate in the synthesis of acrylic acid, which is used to make acrylates, and of DL-methionine, an essential amino acid used as an animal feed supplement. Other derivatives of acrolein are glutaraldehyde, pyridines, tetrahydrobenzaldehyde, allyl alcohol and glycerol, 1,4-butanediol and 1,4-butenediol, 1,3-propanediol, DL-glyceraldehyde, flavors and fragrances, polyurethane and polyester resins.

The most important direct use of acrolein is as a biocide: it is used as a herbicide and to control algae, aquatic weeds and mollusks in recirculating process water systems. It is further used to control the growth of microorganisms in liquid fuel, the growth of algae in oil fields and the formation of slime in paper manufacture. Acrolein has been used in leather tanning and as a tissue fixative in histological work (IARC, 1995).

Acrolein is released to the air as a result of manufacturing processes, through incomplete combustion of petroleum fuels, as a component of cigarette smoke, and as a photooxidation product of hydrocarbon pollutants (ATSDR, 1990). Combustion of fuels represents a major source of emissions of acrolein to the atmosphere. It is classified in air as a reactive chemical with a half-life on the order of 4 to 20 hours upon removal from the atmosphere by hydroxyl radicals (Grosjean, 1990). This range is in agreement with those calculated by others (Atkinson, 1985). Concentrations in indoor air may exceed outdoor levels (Environment Canada, 2000).

### **3. TOXICOKINETICS RELEVANT TO ASSESSMENTS**

#### **3.1. ABSORPTION AND DISTRIBUTION**

Studies relating to respiratory uptake of acrolein in dogs indicated that acrolein was retained primarily, but not exclusively, in the upper respiratory tract (URT). At inhaled concentrations of 400-600 mg/m<sup>3</sup>, 80-85% was retained at varying ventilation rates, indicating little distribution elsewhere (Egle, 1972). This is probably the result of its strong reactivity with tissues (Beauchamp et al., 1985). The fact that McNulty et al. (1984) saw no reduction in liver glutathione (GSH) following inhalation of acrolein by rats also suggests that acrolein does not reach the liver to any great extent via inhalation. The deposition efficiency of inhaled acrolein (nominal concentrations of 0, 2.1, 10.4, and 20.9 mg/m<sup>3</sup>; 0, 0.9, 4.5 and 9.1 ppm) in the upper respiratory tract of the anesthetized male F344 rat was examined by Morris (1996). During the nose-only exposure of the surgically-isolated URT, steady-state concentrations were not reached during 40 minutes of exposure, indicating limited uptake at these concentrations and durations.

Evidence for systemic absorption of acrolein from the gastrointestinal tract was reported by Draminski et al. (1983), who identified low levels of acrolein-derived conjugates in the urine of rats after ingestion of a single dose of 10 mg/kg body weight. This dose, however, resulted in 50% mortality and would be expected to cause severe gastrointestinal damage under these conditions. Damage to the stomach lining, especially endothelial cells (Patel and Block, 1993), may allow some absorption to occur. The likelihood of significant absorption from the gastrointestinal tract at lower concentrations is thus still uncertain.

While the possibility of some transport of acrolein or a metabolite of acrolein to systemic sites remains, the critical target sites, as noted in the toxicology section, are those at the point of contact, the respiratory system, the gastrointestinal tract, mucous membranes, and skin.

#### **3.2. METABOLISM AND EXCRETION**

Absorbed acrolein reacts directly with protein and non-protein sulfhydryl groups and with primary and secondary amines found in proteins and nucleic acids (Ghilarducci and Tjeerdema, 1995). In proteins, it preferentially attacks free SH groups of cysteine residues,  $\epsilon$ -amino groups of lysine residues and histidine residues (Esterbauer et al., 1991). Uchida et al. (1998a,b) has shown that, in vitro, acrolein binds to serum albumen and low-density lipoproteins. Acrolein's role as a lipid peroxidation byproduct and possible mediator in various human diseases has been recently reviewed by Uchida (1999). It is well-documented that the conjugation of the  $\beta$ -carbon of acrolein with sulfhydryl groups is rapid and essentially irreversible (Esterbauer et al., 1976), and leads to thiazolidine derivatives and a decrease in GSH stores without an increase in

oxidized GSH. This pathway results in an acrolein-GSH adduct which is then further metabolized by both high- and low-affinity forms of mitochondrial and cytosolic aldehyde and alcohol dehydrogenase (Mitchell and Peterson, 1989); one resultant product has been identified as 3-hydroxypropylmercapturic acid (Clapp et al., 1969; Kaye and Young, 1970). This product has been isolated from urine of rats after subcutaneous injection of acrolein (Kaye, 1973). The reduction of the acrolein-GSH adduct by alcohol dehydrogenase to 3-hydroxypropylmercapturic acid was postulated as a potentially important pathway (Mitchell and Peterson, 1989). There is increasing evidence that aldehydes such as acrolein are generated endogenously during the process of lipid peroxidation (Esterbauer et al., 1991); the rate constant for reaction of acrolein with cysteine at pH 7.4 was  $220 \text{ M}^{-1} \text{ sec}^{-1}$  compared to 121 with GSH. Among all  $\alpha$ ,  $\beta$ -unsaturated aldehydes, acrolein is the strongest electrophile, which accounts for its high reactivity with nucleophiles (Witz, 1989). Thiol adducts of acrolein are considerably more stable than adducts formed by all other  $\alpha$ ,  $\beta$ -unsaturated aldehydes (Esterbauer et al., 1991).

At neutral pH, acrolein reacts with guanosine and cytosine and adenine derivatives to yield several cyclic adducts (Chung et al., 1984; Sodum and Shapiro, 1988).

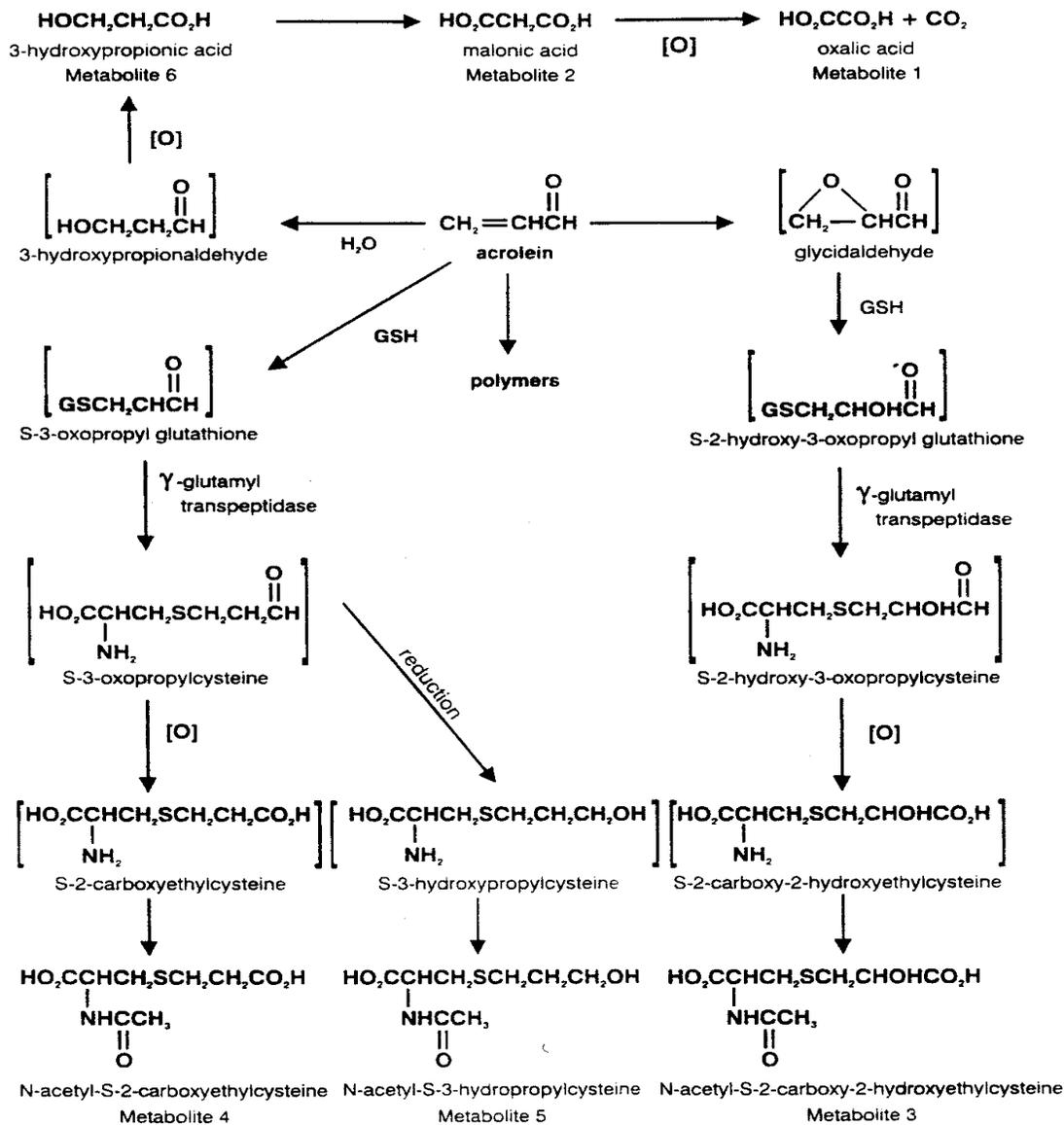
Elucidation of the major pathways of metabolism has been greatly enhanced by the studies of Parent and colleagues. Parent et al. (1998) synthesized and characterized the potential metabolites of acrolein in the feces and urine of rats administered acrolein either orally or intravenously. The pathways of metabolism proposed by Parent et al. (1998) are illustrated in figure 1. The main pathway appears to be an addition of GSH to the activated double bond, followed by processing to mercapturic acid derivatives, the three compounds at the bottom of the figure, which are then excreted in the urine after either oxidation or reduction of the aldehyde, with reduction predominating. Another pathway of metabolism is that of epoxidation of the double bond followed by attack of GSH on the epoxide. A third pathway involves addition of water to acrolein to form 3-hydroxypropionaldehyde, which is further oxidized to malonic acid and ultimately oxalic acid. Some of these compounds can be incorporated into normal metabolic pathways. For example, glycinaldehyde can be hydrated to glyceraldehyde (Patel et al., 1980).

None of the unconjugated metabolites resulting from the epoxidation of acrolein, such as those reported by Patel et al. (1980), were found in the excreta by Parent et al. (1998). A polar and a nonpolar fraction were extracted with a molecular weight range of 2,000-20,000 Da (Parent et al., 1998). They concluded that these compounds were either homopolymers of acrolein, or that the polyacrolein in this fraction was originally a copolymer with a natural polymer, either a protein or polysaccharide.

Marinello et al. (1984) incubated [ $^{14}\text{C}$ ]acrolein with purified P450 in the absence of NADPH and observed the binding of label. GSH inhibited the binding of label to hepatic microsomes by 90%. Binding to microsomes was substantially enhanced in the presence of NADPH. Addition of the P450 inhibitor, SKF-525A, in the presence of NADPH prevented binding of label.

Incubation of Wistar liver microsomes with 5 mM acrolein for 30 seconds resulted in a two-fold stimulation of GSH transferase and 0.1 mM for 30 minutes reduced GSH protection against lipid peroxidation (Haenen et al., 1988).

Figure 1. Proposed metabolism of acrolein in rats. The structures in brackets represent postulated intermediates (Parent et al., 1998). Reprinted with the permission of the Society of Toxicology.



## 4. HAZARD IDENTIFICATION

### 4.1. STUDIES IN HUMANS--EPIDEMIOLOGY, CASE REPORTS, CLINICAL CONTROLS

#### **Acute Exposures (<24 hours):**

The clinical study by Weber-Tschopp et al. (1977) provided the most comprehensive description of acute effects in humans. Three experiments were performed using male and female student volunteers: (1) a continuous exposure at constantly increasing acrolein concentrations, (2) short exposures to successively increasing concentrations, and (3) a one-hour exposure to a constant concentration.

In experiment (1) 31 males and 22 females were exposed to acrolein for 40 minutes in which acrolein was gradually increased to 0.6 ppm (1.4 mg/m<sup>3</sup>) during the first 35 minutes, then remained constant. Groups of unexposed students were used as controls. The subjects had to fill out a questionnaire for the first 5 minutes. After that, the eye blinking frequency of two subjects was measured and as well as the breathing frequency of a third subject during the entire exposure. Eye irritation was significantly higher (p<0.01) than controls beginning at 0.09 ppm and was increasing even at 0.6 ppm. Nasal irritation was significantly higher (p<0.01) than controls beginning at 0.26 ppm and was increasing even at 0.6 ppm.. Throat irritation increased significantly through 0.43 ppm. The eye blink frequency increased significantly beginning at 0.26 ppm (p<0.01).

In experiment (2) 17 males and 25 females were exposed, in groups of five, for 1½ minutes to successive concentrations of 0, 0.15, 0.3, 0.45, and 0.6 ppm (0, 0.3, 0.6, 0.9, and 1.2 mg/m<sup>3</sup>). After a minute of exposure, they were administered a questionnaire. Between each exposure they were allowed to recuperate in a clean room for 8 minutes. As in the first experiment, eye blink frequency and respiration rate were measured. The same controls as for the first experiment were used. Eye and nasal irritation was significantly higher (p<0.05) than controls beginning at 0.3 ppm and 0.06 ppm, respectively. Throat irritation was not evident.

In experiment (3) 21 males and 25 females were distributed into three groups and exposed for 60 minutes to a constant acrolein concentration of 0.3 ppm. As in the other two experiments, eye blink frequency and respiration rate were measured. As for controls, measurements of eye blink and breathing frequency, and subjective symptoms of irritation was assessed at the beginning of exposure. Each of the effects increased significantly (p<0.01) during the first 20-30 minutes of exposure compared to controls after which the irritation effects reached a plateau. Eye blink frequency reached a steady rate after 10 minutes of exposure. During exposure there was a decrease in the average respiration rate (16 individuals) after 40 minutes (p<0.01). Each individual that demonstrated an increase in eye blink frequency also reported a sharp increase in eye irritation. Throat irritation, not a significant response in the previous two experiments, was increased compared to controls after 10 minutes of exposure.

It was concluded by the investigators that the average threshold of sensation lies in the range of 0.09 (eye irritation) to 0.30 ppm (respiration rate, throat irritation) with nasal irritation at 0.15 ppm.. No adaptation to these effects were observed.

According to the review by Esterbauer et al. (1991), a level of 5.5 ppm (12.6 mg/m<sup>3</sup>) results in painful eye and nose irritation after 20 seconds and 22 ppm in air is immediately intolerable; in one case report exposure to 153 ppm (352 mg/m<sup>3</sup>) for 10 minutes was fatal

Sim and Pattle (1957) exposed volunteers (12 males/group) to 1.88 mg/m<sup>3</sup> and 2.80 mg/m<sup>3</sup> (0.8 and 1.2 ppm) acrolein for 10 and 5 minutes, respectively. Volunteers were exposed simultaneously in 100 m<sup>3</sup> exposure chambers with no restrictions on movement or smoking within the chamber. The vapor was described by the volunteers as “extremely irritating” to all exposed mucosal surfaces with lacrimation occurring within 20 and 5 seconds in the low and high exposures, respectively. Ten minutes of low dose exposure was described as “only just tolerable,” and exposure to the high exposure for more than 5 minutes “would have been extremely distressing.” The comments by the volunteers were subjective, and it does not appear that any other endpoints were monitored. The effects of acrolein were considerably more apparent than much higher concentrations of several other aldehydes.

In one of two case reports available a 27-month-old boy was exposed to acrolein from burning vegetable oil for one hour (Mahut et al., 1993). No exposure measurements were reported. Initial acute respiratory failure regressed in a few hours, but in the months following exposure diffuse bronchiectasis developed. In the other, a chemical worker was exposed to a sudden release of acrolein from a rupture in the workplace. The principal effect was chemical pneumonia and eye irritation, both of which resolved with treatment (Champeix et al., 1966).

Based on the available human data, levels as low as 0.09 ppm (0.02 mg/m<sup>3</sup>) for 5 minutes have been recorded as eliciting subjective complaints of eye irritation with increasing concentrations leading to more extensive eye, nose and respiratory symptoms.

### **Exposures >24 hours:**

No chronic studies of humans exposed to acrolein are available.

The only study relating to cancer was a case control study by Ott et al. (1989), in which individuals were classified as having been exposed to one of a large number of chemicals in the work environment. Odds ratios of 2.6 (2 cases) for non-Hodgkin’s lymphoma, 1.7 (1 case) for multiple myeloma, and 2.6 (3 cases) for nonlymphocytic leukemia were reported for workers exposed to acrolein. None of the lower confidence bounds for these 3 endpoints significantly exceeded one. Because of a lack of a statistically significant increase in the cancer endpoints and the likelihood of confounding by concomitant exposure to other chemicals in the workplace, the results must be considered to be equivocal.

## **4.2. ACUTE STUDIES IN ANIMALS—ORAL AND INHALATION**

### **4.2.1 Lethality Studies**

Ballantyne and coworkers (1989) examined the effects of 1- and 4-hour exposures to acrolein in male and female Sprague-Dawley rats (5/sex/exposure). Animals were exposed to 14, 22, 24, 31, and 81 ppm (32, 50, 55, 71, and 186 mg/m<sup>3</sup>)<sup>1</sup> acrolein for 1 hour or 4.8, 7.0, 9.1, and 12.1 ppm (11, 16, 20.8, and 27.7 mg/m<sup>3</sup>) for 4 hours.

One- and 4-hour LC<sub>50</sub> values of 65 and 25.8 mg/kg, respectively, were calculated for the combined sexes. Clinical signs of sensory irritation and toxicities were observed at all exposure concentrations. Lachrymation, perinasal and periocular wetness and encrustation, mouth and

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<sup>1</sup>Conversion to mg/m<sup>3</sup>: 1 ppm = 2.3 mg/m<sup>3</sup>.

audible breathing, decreased breathing rate, and hypoactivity were observed during exposure in all animals. Signs of respiratory distress and hypoactivity were observed for post-exposure days 1-6. Body weights of survivors decreased during the first week post-exposure but the weight was regained during the second week.

A necropsy of animals that died during the post-exposure period revealed perinasal and perioral encrustation, mottled discoloration of the lungs and liver, clear fluid in the trachea and thoracic cavity, gas-filled stomach and intestine, and opaque or cloudy corneas. Histology of the lungs revealed congestion and intra-alveolar hemorrhage, fibrin disposition in the smaller airways, and necrosis and exfoliation of the bronchiolar epithelium. Death was attributed to lung injury. Histopathology was not performed on surviving animals.

In another study examining acute exposure effects of acrolein in rats, Crane et al. (1986) exposed Sprague-Dawley rats to acrolein at concentrations ranging from 580-41,550 ppm (1,330-95,268 mg/m<sup>3</sup>) for exposure durations ranging from 2.8 to 36.5 minutes until animals were incapacitated. The time-to-incapacitation endpoint (i.e., when rats could no longer perform a coordinated act of walking in a rotating cage, and exhibiting stumbling, sliding, or tumbling) was recorded for each animal. Exposure was then continued until animals expired and the time of death was reported. After incapacitation, death occurred very quickly (in 1.9-19.7 minutes). Prior to death, animals exhibited clinical signs of respiratory distress, agitation, and convulsions. Ocular effects were not noted.

Mortality and clinical signs have also been reported in other species. Groups of 50, 20, and 5 mice, guinea pigs and rabbits, respectively, were exposed to 2,279 ppm (5,225 mg/m<sup>3</sup>) acrolein vapor for 13, 25, and 27 minutes, respectively, until the animals died (Salem and Cullumbine, 1960). In addition, the same species were exposed to 2,019 ppm (4,624 mg/m<sup>3</sup>) acrolein aerosol for 13, 24, and 26 minutes, respectively, until the animals died. Initial exposure to both types of acrolein exposure caused increased activity which was attributed to compound-related irritation. Respiration then slowed and animals convulsed just prior to death. Of the nine aldehydes tested, acrolein had the highest relative toxicity.

Beeley and coworkers (1986) examined the effects of acute acrolein exposure in female New Zealand rabbits. Animals (18/group) were exposed to 375 or 489 ppm (860 and 1,121 mg/m<sup>3</sup>) for 15 minutes. Animals were sacrificed at 3 days post-exposure, and lung and trachea were removed and examined for histopathological changes. Five animals in the 860 mg/m<sup>3</sup> exposure group and 8 animals in the 1,121 mg/m<sup>3</sup> exposure group died during the 3 day post-exposure period. The surviving animals exhibited edema, necrosis of the lung parenchyma, and damage to the bronchial linings of the large airways. Acute inflammatory reactions were found in conjunction with areas of necrosis.

To assess the potential of acrolein to impair escape, a signal avoidance task was developed in which a baboon's ability to escape from a chamber containing the noxious gas was monitored (Kaplan et al., 1986). Male juvenile baboons (1/group) were exposed to 12, 25, 95, 100, 250, 505, 1,025, or 2,780 ppm (28, 57, 218, 229, 573, 1,158, 2,350, and 6,374 mg/m<sup>3</sup>) for 5 minutes. After exposure, animals were allowed to exit the chamber by depressing a lever. Escape time, i.e., the time it took for the animal to select the correct lever and exit the chamber, was measured. Acrolein exposure did not inhibit escape time. However, irritant effects of the gas were noted at each concentration tested and which increased in severity with increasing concentration. Irritant effects manifested as blinking and closing of the eyes and rubbing the nose/eyes at lower concentrations to salivation, nasal discharge, violent shaking of the head, and nausea at higher concentrations. However, the exposures at which the more serious effects

occurred were not reported by the study authors. Animals exposed to 2,350 and 6,374 mg/m<sup>3</sup> acrolein expired after 24 and 1.5 hours, respectively. Severe pulmonary edema and hemorrhage were the significant histological changes observed in these two animals.

An acute oral LD<sub>50</sub> was found to be 29 mg/kg in Sprague-Dawley rats administered acrolein by gavage (Bioassay Systems Corp., 1981c); male rats were somewhat more sensitive with an LD<sub>50</sub> of 25 mg/kg compared to females with an LD<sub>50</sub> of 33 mg/kg. In contrast, LD<sub>50</sub>'s of 10.3 (males) and 11.8 mg/kg (females) were reported in another gavage study with acrolein with a stated purity of 97% (Microbiological Assoc., 1989). In male CD-1 mice the LD<sub>50</sub> was 14 mg/kg (Bioassay Systems Corp., 1981d). In female mice, the LD<sub>50</sub> was determined to be 18 mg/kg (Bioassay Systems Corp., 1981e). The acute dermal LD<sub>50</sub> in New Zealand white rabbits was 231 mg/kg with females somewhat more sensitive (223 mg/kg) than males (240 mg/kg) (Bioassay Systems Corp., 1981f).

#### 4.2.2 Sensory Irritation

Alterations in respiratory function have been used as an indicator of sensory irritation. Murphy et al. (1963) exposed male guinea pigs (n=10) to 0.6 ppm (1.4 mg/m<sup>3</sup>) acrolein for 2 hours. The study authors reported that expiratory flow resistance and tidal volume increased and respiratory rate decreased. These adverse responses were rapid and reached a maximum within 30 to 60 minutes. In a second experiment, male guinea pigs were exposed to 0.1, 0.2, 0.35, 0.6 or 1 ppm (0.2, 0.5, 0.8, 1.4, or 2.3 mg/m<sup>3</sup>) for 2 hours. Respiratory flow resistance during inspiration and expiration was significantly increased and respiratory rates decreased at levels of 0.35 to 1 ppm. The study authors also reported that several drugs (atropine, aminophylline, isoproterenol, and epinephrine) partially or completely reversed increased flow resistance. Statistically significant increases in respiratory resistance and tidal volume coupled with decreases in respiration rate and minute volume were observed in guinea pigs exposed to 17 ppm (40 mg/m<sup>3</sup>) acrolein for 60 minutes (Davis et al., 1967).

One measure of the potency of a sensory irritant is the exposure concentration at which respiratory rate is depressed by 50% (RD<sub>50</sub>). Table 1 shows RD<sub>50</sub>s for mice and rats. A comparison of rat and mouse values indicates that mice are more sensitive than rats to sensory irritation. Respiratory rate depression following acrolein exposure recovers rapidly, usually within 10 minutes (Cassee et al., 1996a; Nielsen et al. 1984; Steinhagen and Barrow, 1984). However, the recovery rate decreases as acrolein concentration increases. Cassee et al. (1996a) reported that 24 hours after exposure of Wistar rats to 1.7, 11.1 and 31.9 ppm (3.9, 25.4, and 73 mg/m<sup>3</sup>), breathing patterns were comparable to pre-exposure values, indicating that the effect was not persistent. The decrease in breathing frequency was maximal between 1 and 3 minutes of exposure with desensitization occurring only with the two lower concentrations. Kane and Alarie (1977) reported that four daily consecutive three-hour exposures to 0.5 and 1.7 ppm (1.1 and 3.9 mg/m<sup>3</sup>) acrolein caused further decreases in respiratory rate, which suggests that animals become sensitized to the irritant effect. However, when animals were exposed to 0.17 ppm (0.39 mg/m<sup>3</sup>) acrolein three hours/day for three days and then exposed to 0.44-11.2 ppm (1.0-26.7 mg/m<sup>3</sup>) acrolein for 10 minutes, there was a decrease in response compared to controls, i.e., the control RD<sub>50</sub> was 1.7 ppm compared to 3 ppm in pre-exposed animals.

**TABLE 1. RD<sub>50</sub>s for Rats and Mice**

Species	RD <sub>50</sub>	Reference
F-344 Rats (male)	13.7 mg/m <sup>3</sup> (6.0 ppm)	Babiuk et al., 1985
Wistar Rats (male)	10.5 mg/m <sup>3</sup> (4.6 ppm)	Bergers et al., 1996
Wistar Rats (male)	21.7 mg/m <sup>3</sup> (9.2 ppm)	Cassee et al., 1996b
Swiss Webster Mice (male)	3.9 mg/m <sup>3</sup> (1.7 ppm)	Kane and Alarie 1977
Ssc:CF-1 Mice (male)	6.6 mg/m <sup>3</sup> (2.9 ppm)	Nielsen et al., 1984
B6C3F1 Mice (male)	3.2 mg/m <sup>3</sup> (1.41 ppm)	Steinhagen and Barrow 1984
Swiss Webster Mice (male)	2.4 mg/m <sup>3</sup> (1.03 ppm)	Steinhagen and Barrow 1984

Davis and coworkers (1967) examined the respiratory irritant effect of acrolein in normal and tracheotomized guinea pigs. Groups of normal and tracheotomized guinea pigs were exposed to 17 ppm (39 mg/m<sup>3</sup>) acrolein for one hour. Normal animals exhibited clinical signs of sensory irritation, i.e., depressed respiratory rate as described by Murphy et al. (1963). However, tracheotomized animals did not exhibit respiratory rate depression. A similar finding was reported by Kane and Alarie (1977). Davis et al. (1967) theorized that tracheotomized animals lacked receptors for irritant responses that were present in the intact animal.

To further understand the mechanism through which acrolein elicits its irritant effect, Lee and coworkers (1992) examined the effect of capsaicin treatment of the cervical vagi followed by acrolein exposure in rats. Capsaicin treatment selectively blocked C-fiber afferent nerves and inhibited the respiratory rate depression normally observed during acrolein exposure. In addition, bilateral vagotomy also inhibited the respiratory rate depression. These results are consistent with a mode of action in which acrolein activates C-fiber afferent nerves.

Since acrolein exposure in the workplace is usually concurrent with other chemicals, particularly aldehydes, studies have been undertaken to examine the effects of acrolein exposure with pre-exposure and co-exposure to other chemicals. Babiuk et al. (1985) examined the effects of pre-exposure to 15 ppm (34 mg/m<sup>3</sup>) formaldehyde 6 hours/day, for nine days followed by exposure to acrolein for 10 minutes on the 10<sup>th</sup> day. The study authors reported that the RD<sub>50</sub> in pre-exposed animals increased to 29.6 ppm (68.1 mg/m<sup>3</sup>) compared to 6 ppm (13.8 mg/m<sup>3</sup>) in the controls. This would suggest that pre-exposure to lower concentrations of sensory irritants desensitizes animals to sensory irritation effects of acrolein. However, co-exposure to acrolein with other aldehyde sensory irritants, acetaldehyde and formaldehyde, resulted in a more pronounced decrease in respiratory rate in male Wistar rats than exposure to acrolein only (Cassee et al., 1996a). Groups of four rats were exposed to a mixture of the three at concentrations which were expected to result in a decrease in breathing frequency (DBF) between 10 and 35% for each. The observed DBF for the mixture was more pronounced than the DBF for each chemical separately, but was less than the sum of the DBFs for the single chemicals. Model prediction indicated that the combined effect was consistent with a competition for a common receptor, i.e., the trigeminal nerve.

The clinical signs and sensory irritation reported in the above mentioned animal studies indicate that the respiratory system is a principal target following acute exposure to acrolein. Further studies provide additional evidence. Kilburn and McKenzie (1993) exposed Syrian golden hamsters to 6 ppm (14 mg/m<sup>3</sup>) acrolein for four hours, which caused a > 50% exfoliation of ciliated cells in the bronchi. The cells were pale and swollen at 24 and 48 hours post-exposure. The basal lamina was indented or penetrated by proliferating basal cells. After 96 hours, there were areas of irregular epithelium with early stratification and hyperplasia. There was no recruitment of polymorphonuclear leukocytes (PMN) to the trachea or intrapulmonary airways; however, acrolein administered absorbed on carbon or simultaneous with carbon was chemotactic for PMN leukocytes. Formaldehyde behaved similarly to acrolein.

Acrolein has been reported to deplete the neuropeptides calcitonin-gene related peptide (CGRP) and substance P in the trachea of rats (Springall et al., 1990). Female Wistar rats exposed to 22, 81 or 249 ppm (51, 186, or 571 mg/m<sup>3</sup>) for 10 minutes exhibited a dose-dependent decrease in these two sensory neuropeptides. The study authors suggested that the neuropeptide decrease could be responsible for the observed vasodilation and bronchoconstriction that follows irritant exposure. Roemer and colleagues (1993) reported that respiratory tract cell proliferation in male Sprague Dawley rats occurred following an acute 6-hour exposure to 0.2 and 0.6 ppm (0.46 and 1.4 mg/m<sup>3</sup>) acrolein.

Bronchial hyperresponsiveness following acrolein exposure has also been reported. Leikauf and coworkers (1989a, 1991) exposed guinea pigs to 0.31-1.26 ppm (0.71-2.9 mg/m<sup>3</sup>) acrolein for two hours and determined bronchial responsiveness with an acetylcholine challenge up to 24 hours after exposure. The effective dose of acetylcholine sufficient to double specific resistance (ED<sub>200</sub>) was decreased at all post-exposure times. The authors interpreted these results as suggestive evidence that asthmatics may be pre-disposed to an asthmatic attack following acrolein exposure. In addition, thromboxane B<sub>2</sub>, the inactive form of the potent vasoconstrictor thromboxane A<sub>2</sub>, and prostaglandin F<sub>2α</sub> were increased immediately after exposure, and neutrophils were increased 24 hours after exposure. In a subsequent study, Leikauf and colleagues (1989b) reported that acrolein exposure resulted in an increase in leukotriene C<sub>4</sub> (LTC<sub>4</sub>) in bronchoalveolar lavage fluid in guinea pigs. It was also determined that hyperresponsiveness to acetylcholine following acrolein exposure could be abated if guinea pigs were pretreated with 5-lipoxygenase inhibitors and leukotriene receptor antagonists, which suggests that the sulfidopeptide leukotrienes play causal role in acrolein-induced bronchial hyperresponsiveness.

Acute exposure of Swiss-Webster mice to acrolein (0.3 or 0.6 µg/ml) decreased pulmonary compliance, pulmonary resistance, tidal volume and respiratory frequency (Watanabe and Aviado, 1974); pretreatment with a beta-adrenergic blocking agent indicated that lung effects were not mediated through adrenergic receptors. Similarly, chronic exposure (30 minutes, daily, 5 weeks) to a lower concentration reduced pulmonary compliance.

### 4.2.3 Other Effects

**Antibacterial defenses:** Several studies have assessed the effects of acrolein exposure on pulmonary antibacterial defenses. Jakab (1977) exposed Swiss CD-1 mice (18-24 animals/group) to 1- 2 ppm (2.3-4.6 mg/m<sup>3</sup>) acrolein for four or 24 hours following a 0.5- hour bacterial challenge to *Staphylococcus aureus* and *Proteus mirabilis*. After 24 hours of exposure, there was a statistically significant increase in the number of surviving bacteria (both *S. aureus*

and *P. mirabilis*) in exposed animals compared to controls. In a second study, Astry and Jakab (1983) exposed female Swiss mice (6/group) to 0.5, 3, 6.2, 7.5, or 9 ppm (1.1, 6.8, 14.2, 17.2, or 20.6 mg/m<sup>3</sup>) for eight hours following a 45-minute bacterial challenge to *S. aureus*. Exposure to 0, 1.1, 6.8, 14.2, 17.2, and 20.6 mg/m<sup>3</sup> resulted in survival of 3.2, 5.0, 12.8, 33.9, 35, and 40% of bacteria, respectively. The study authors reported significantly greater percent of surviving bacteria at exposures  $\geq$  6.8 mg/m<sup>3</sup>. Exposure to 0.09 ppm (0.21 mg/m<sup>3</sup>) acrolein for three hours following exposure to *Klebsiella pneumonia* had no effect on percent bacteria killed compared to controls in female CD1 mice (Aranyi et al., 1986). These studies suggest that acrolein exposure can inhibit pulmonary antibacterial defenses.

**Cardioinhibitory Effects:** Egle and Hudgins (1974) examined possible cardioinhibitory effects of acrolein exposure in male Wistar rats. Animals (6-11/group) were exposed to concentrations of acrolein ranging from 4 to 2,181 ppm (10-5,000 mg/m<sup>3</sup>) for 1 minute. Animals were assessed for changes in blood pressure and heart rate. The principal effects observed were significant increases in blood pressure and heart rate with exposure concentration, with statistically significant increases in heart rate occurring at exposures  $\geq$  50 mg/m<sup>3</sup>. However, exposure to 2,500 and 5,000 mg/m<sup>3</sup> acrolein generally caused a decrease in heart rate. Intravenous studies in Wistar rats with several aldehydes indicated that the relative pressor potency of acrolein was higher than that of formaldehyde, acetaldehyde and propionaldehyde.

**Biochemical Changes:** Biochemical changes have also been reported following inhalation exposure to acrolein. Alabert et al. (1971) found significant alterations in NAD/NADH ratios in liver, lung, and brain of rats exposed to high concentrations of acrolein. Murphy (1965) reported that liver alkaline phosphatase and tyrosine transaminase activities were increased 3.1- and 3.6-fold, respectively, in male Holtzman rats exposed to 8 ppm (18.3 mg/m<sup>3</sup>) acrolein for four hours; a dose-response relationship was also observed upon injection of acrolein. Cassee and coworkers (1996b) examined changes in the nasal epithelium of male Wistar rats exposed to 0, 0.25, 0.67, or 1.40 ppm (0, 0.45, 1.2, or 2.5 mg/m<sup>3</sup>) acrolein by nose only exposure for 6 hours. No effects in cell proliferation or treatment-related lesions were observed. Likewise, non-protein sulfhydryl levels were similar to controls. However, exposure to 1.2 or 2.5 mg/m<sup>3</sup> acrolein significantly decreased glutathione reductase activity in a dose-dependent manner.

**Glutathione and P450 levels:** When male rats were given a single i.p. dose of acrolein (89  $\mu$ moles/kg) and sacrificed at 30 min, 4 and 24 hr, hepatic GSH was decreased 51% only at the 4- hr period (Witz, 1989). Levels returned to normal by 24 hr. However, cytochrome P450 levels were 61-71% of controls at 24 hr. Walk and Hausmann (1989) found that acute inhalation exposure of rats to acrolein (0.7 to 4 ppm) resulted in a decrease in the total glutathione (GSH and GSSG) pool of nasal and olfactory epithelia and in trachea and lungs. These decreases were accompanied by complex changes in GSH enzyme activities. After a 4-hour exposure of rats to acrolein (1 to 15 ppm), a dose-dependent decrease in the total glutathione pool was observed in nasal olfactory and respiratory epithelia (Hausmann and Walk, 1989). Activities of GSH peroxidase, GSH reductase, and GSH transferase increased slightly in olfactory epithelium, but decreased in respiratory epithelium as exposures increased.

**Eye Irritation:** Eye lesions were reported in New Zealand white rabbits when acrolein was placed on the everted lower lids and examined for different time periods up to 7 days post-exposure (Bioassay Systems Corp, 1981a).

**Skin Irritation:** Acrolein was determined to be a skin irritant after 0.5 ml was placed on intact and abraded skin of six male New Zealand white rabbits with erythema and edema scored after 24 and 72 hours (Bioassay Systems Corp, 1981b)

### 4.3. PRECHRONIC AND CHRONIC STUDIES AND CANCER BIOASSAYS IN ANIMALS—ORAL AND INHALATION

#### 4.3.1. Non-Cancer Toxicity

Acrolein, like other aldehydes, is a known sensory irritant (Lyon et al., 1970; Cassee et al., 1996a,b) producing both nasal and eye irritation. Breathing frequency which is depressed upon initial exposure has been shown in Wistar rats to partially or fully recover during post-exposure. Sensory irritation and depressed breathing frequency is regarded as a defense mechanism to penetration to the lower respiratory tract. Acrolein was the most potent of 15 saturated and unsaturated aldehydes in sensory irritation potential as measured by the reflex decrease in respiratory rate in B6C3F1 and Swiss-Webster mice (Steinhagen and Barrow, 1984). The relationship of the RD<sub>50</sub> and other structure-activity properties of acrolein in relation to other sensory irritants have been documented by Alarie et al. (1998).

##### 4.3.1.1. Inhalation studies

Several studies have found that subacute exposure of guinea pigs, rats, and mice to acrolein causes pulmonary inflammation, decreases in respiratory rate, and nasal lesions, effects also seen upon acute exposure. The effects of inhaled acrolein on laboratory animals are shown in Table 2.

**RATS:** Male Wistar rats (5-6/group) were exposed 6 hr/day, for 3 consecutive days, in a nose-only exposure chamber to acrolein at concentrations of 0, 0.25, 0.67, or 1.40 ppm (0, 0.57, 1.5, or 3.2 mg/m<sup>3</sup>) (Cassee et al., 1996b). Variation in exposure concentration was 13%. Rats were examined for nasal lesions (6 levels of the nasal tract examined) immediately after the last exposure. **Histopathology:** After 6 hours of exposure, no treatment-related histopathological lesions were found. After three days, disarrangement, necrosis, thickening and desquamation of respiratory/transitional epithelium of a slight degree were reported in 4 of 5 animals exposed to 0.25 ppm and in 3/6 at 0.67 ppm. Slight changes were noted in 3/6 rats and moderate changes in the other 3 exposed to 0.67 ppm acrolein. Atrophy of the olfactory epithelium was not observed. **Proliferation:** Nasal cell proliferation indices were significantly increased by exposure to 0.25 and 0.67 ppm after the three-day exposure only. The increase was even more pronounced at 0.67 ppm. The concentrations of acrolein associated with the proliferation indices were considerably lower than those of formaldehyde and acetaldehyde. Cell proliferation data was expressed as the number of positive-stained cells per millimeter basement membrane. Effects at 1.4 ppm were not reported for pathologic changes or cell proliferation. **Enzymatic Changes:** Among biotransformation enzymes measured in homogenates of nasal tissue, glutathione S-transferase activity was significantly depressed in the 1.4 ppm exposure group (p<0.01) while formaldehyde dehydrogenase and aldehyde dehydrogenase activities were significantly increased (p<0.05). No changes were reported in the other dose groups, or for glutathione peroxidase

TABLE 2. Effects of Inhaled Acrolein on Laboratory Animals

Species	Exposure Duration	Concentration (ppm)	Principal Effects	Reference
<b>Rat</b>				
Wister, male	6 h/day 3 days	0, 0.25, 0.67, or 1.4	Nasal necrosis of respiratory epithelium and increased proliferation up to 0.67 ppm; 1.4 ppm group not evaluated.	Cassee et al. (1996b)
S-D, male	6 h/day 5 days/wk 3 wk	0, 0.1, 1.0, or 3.0	No effect on macrophage killing of inhaled <i>K. pneumonia</i> .	Sherwood et al. (1986)
S-D, male	6 h/day 5 days/wk 3 wk	0, 0.1, 1.0, or 3.0	Nasal exfoliation, erosion necrosis of respiratory epithelium and squamous metaplasia at 3 ppm; no effects on lungs or inlocal pulmonary antibody responsiveness to <i>L. monocytogenes</i> .	Leach et al. (1987)
F-344, male	6 h/day 5 days/wk 62 days (except for weekends)	0, 0.4, 1.4, or 4.0	<ol style="list-style-type: none"> <li>1. High mortality at 4 ppm.</li> <li>2. Increase in lung collagen at 1.4 and 4 ppm (<math>p &lt; 0.05</math>).</li> <li>3. Elastin content in 4 ppm group twice controls</li> <li>4. Bronchial necrosis and pulmonary edema at 4 ppm.</li> <li>5. Parenchymal restriction at 0.4 ppm and obstructive lesions at 4.0 ppm</li> <li>6. No cytogenic or sperm abnormalities</li> </ol>	Kutzman et al. (1981, 1985) Costa et al. (1986)
Dahl, female (selected for susceptibility or resistance to salt-induced hypertension)	6 h/day 5 days/wk for 61-63 days (excluding weekends)	0, 0.4, 1.4, or 4.0	<ol style="list-style-type: none"> <li>1. All susceptible 4 ppm rats died after 11 days and 60% of resistant rats survived to end of study.</li> <li>2. Lungs of susceptible rats had severe airway necrosis with edema and hemorrhage but only proliferative changes with resistant rats.</li> <li>3. No differences in histopath between rat groups at lower doses.</li> <li>4. No effect of exposure on blood-pressure changes.</li> </ol>	Kutzman et al. (1984, 1986)
SPF-OFA, male	Not explicitly stated, but up to 77 days	0, 0.55	<ol style="list-style-type: none"> <li>1. Decrease in alveolar macrophage.</li> <li>2. No effects on reproductive potential.</li> </ol>	Bouley et al. (1975, 1976)
S-D, male	7 h/day 3 consecutive days	1.7	<ol style="list-style-type: none"> <li>1. Olfactory degeneration in all exposed rats.</li> <li>2. Ulceration of respiratory epithelium in 4/10.</li> </ol>	Teredesai and Stinn (1989)
S-D, male	6 h/day 1 or 3 days	0, 0.2, and 0.6	Proliferative nasal and tracheal cells in epithelia at both concentrations.	Roemer et al. (1993)

TABLE 2 (cont'd). Effects of Inhaled Acrolein on Laboratory Animals

Species	Exposure Duration	Concentration (ppm)	Principal Effects	Reference
<b>Mouse</b>				
Swiss-Webster, male	6 h/day 5 days	1.7	1. Lesions of moderate severity in respiratory epithelium except for severe squamous metaplasia. 2. Lesions (ulceration and necrosis) of moderate severity in olfactory epithelium with squamous metaplasia mild. 3. Incomplete recovery after 72 hours.	Buckley et al. (1984)
Swiss, female	4 h/day 4 days	2.5	Coexposure to acrolein and carbon black increased pulmonary killing of <i>P. mirabilis</i> and impaired elimination of <i>L. monocytogenes</i> . Killing of <i>S. aureus</i> was suppressed on first postexposure day, but returned to normal on seventh day.	Jakab (1993)
CDI, female	3 h/day 5 consecutive days	0.1	Decreased ( $p < 0.01$ ) in percent killing of <i>S. zooepidemicus</i> and <i>K. pneumonia</i> .	Aranyi et al. (1986)
<b>Rat</b>				
White albino, male	6 h/day, one 5-day period or 6h/day for two 5-day periods	various	1. Lung lesions (but no mortality) in mice exposed for two 5-day periods (concentration unknown). 2. LC <sub>50</sub> of 66 ppm in group exposed for 6 h. 3. 91% mortality in mice exposed to 50 ppm for 5 days.	Philippin et al. (1970)
FVB/N, male	6 h/day 5 days/wk 3 wks	3.0	Acrolein-induced excessive macrophage accumulation was associated with mucus hypersecretion.	Borchers et al. (1999)
1. S-D rat, male and female 2. Beagle dogs, male 3. Princeton or Hartley guinea pigs, male and female 4. Squirrel monkeys, male	8 h/day 5 days/wk 6 wks	0.7 or 3.7	1. No concurred controls. 2. No nasal histopathology. 3. Excessive salivation and eye irritation in dogs and monkeys at 3.7 ppm. 4. Chronic lung inflammatory changes and occasional emphysema in all animals at 0.7 ppm.	Lyon et al. (1970)

**TABLE 2 (cont'd). Effects of Inhaled Acrolein on Laboratory Animals**

Species	Exposure Duration	Concentration (ppm)	Principal Effects	Reference
	24 h/day 90 consecutive days	0.22, 1.0, and 1.8	<ol style="list-style-type: none"> <li>Ocular and nasal discharges in dogs and monkeys at 1 ppm; severe at 1.8 ppm.</li> <li>Squamous metaplasia of trachea in all monkeys at 1.8 ppm.</li> <li>Two dogs at 1.8 ppm had confluent bronchopneumonia.</li> <li>Evidence of pulmonary inflammation (guinea pigs at 1 ppm) and fecal liver necrosis (rats and guinea pigs at 1 ppm).</li> <li>Nonspecific inflammatory changes in a variety of tissues in both rats and guinea pigs at 1.8 ppm.</li> </ol>	
Syrian golden hamsters, male and female Wister rats, male and female Dutch rabbits, male and female	6 h/day 5 days/wk 13 wks	0, 0.4, 1.4, and 4.9	<ol style="list-style-type: none"> <li>Mortality in rats at 4.9 ppm.</li> <li>Necrotizing rhinitis in rats at 4.9 ppm and squamous metaplasia at 1.4 ppm with neutrophilic infiltration.</li> <li>Lungs of hamsters unaffected. Severe nasal lesions in hamsters at 4.9 ppm; also tracheal hyperplasia in all female hamsters at 4.9 ppm.</li> <li>Nasal and tracheal lesions similar to rat and hamster in rabbits at 4.9 ppm; no nasal lesions at lower doses.</li> </ol>	Feron et al. (1978)
Guinea pigs	7.5 h/day 2 consecutive days	1.6	<ol style="list-style-type: none"> <li>Pulmonary inflammation.</li> <li>Prolonged increase in airway sensitivity to substance P.</li> </ol>	Turner et al. (1993)
Rabbits, New Zealand, female	15 min	375 and 489 ppm	<ol style="list-style-type: none"> <li>Mortality at both concentrations.</li> <li>Extensive lung damage at both concentrations.</li> </ol>	Beeley et al. (1986)

activity in any of the dose groups. Non-protein sulfhydryl (NPSH) depletion was not observed in this study. No biochemical effects were observed in olfactory tissue.

In a study designed to evaluate the effect of acrolein on bacterial defense systems, male Sprague-Dawley rats were exposed to 0.1, 1.0 or 3.0 ppm acrolein (0.23, 2.3 or 6.9 mg/m<sup>3</sup>) 6 hr/day, 5 days/week for 3 weeks (Sherwood et al., 1986). No change was noted in the clearance of <sup>35</sup>S-*Klebsiella pneumonia* at any of the concentrations. Alveolar macrophage lysozyme and 5'-nucleotidase of acrolein-exposed rats were significantly increased at all exposure concentrations [p<0.05 at the low and intermediate concentration, and p<0.01 at the high concentration], while alkaline phosphatase showed a non-statistically significant increase. Phagocytosis was significantly increased at the low and intermediate concentrations (p<0.01), but not at the 3.0 ppm. However, these changes had no apparent effect on macrophage killing of inhaled bacteria and were not indicative of extreme chemical toxicity.

Four groups of 40 male Sprague-Dawley rats were exposed by inhalation to target concentrations of 0, 0.1, 1.0, and 3.0 ppm (0.23, 2.3 or 6.9 mg/m<sup>3</sup>) acrolein 6 hr/day, 5 days/week for 3 weeks (Leach et al., 1987). Mean body weights were lower in the high-dose group, although differences were not statistically significant. There were no statistically significant effects of acrolein on immune responsiveness as measured by a hemolytic plaque assay performed on lung-associated lymph node cells. The ability of spleen- and lung-associated lymph nodes to respond to the T cell mitogen, PHA, and the B cell mitogen, STM, as well as resistance to infection by *L. monocytogenes* were not affected by acrolein exposure. Microscopic examination of the nasal turbinates of the high-dose group revealed acrolein-induced exfoliation, erosion and necrosis of the respiratory epithelium as well as squamous metaplasia. No effects were reported in the lungs of the high-dose group or at any location at the lower concentrations.

Kutzman et al. (1981, 1985) exposed male Fischer 344 rats (50/group) via inhalation to acrolein at 0, 0.4, 1.4, or 4.0 ppm (0, 0.9, 3.2 or 9.2 mg/m<sup>3</sup>) 6 hr/day, 5 days/week for 62 days of exposure (consecutive, except for weekends) to principally relate lung function with lung pathology. Of the 50 animals/group, 24 were assessed for pulmonary function, 8 for pathology only, 10 for cytology, and 8 for reproductive function. Eight females per group were exposed to assess reproductive potential in which weight gain and mortality also were followed. There was no histopathology for females. There were 10 control males and 8 control females. The duration-adjusted concentrations were 0, 0.16, 0.57, and 2.0 mg/m<sup>3</sup>. Cytological endpoints included sister chromatid exchanges (SCE) and cell proliferation kinetics. All examinations (including lung function) were measured 6 days after final exposure to reduce effect of acute exposure upon results. It should be noted that this recovery period undoubtedly allowed for compensatory changes. Sperm was examined for morphological abnormalities. Histopathology was performed on lung, peribronchial lymph node, nasal turbinates, brain, kidney, liver, spleen, testes, and heart (8 male rats from each dose group except only 3 from the 4.0 ppm group). Of the 24 animals/group examined for pulmonary function, the right lung was subsequently used for biochemical analyses and the left lung processed for pathological examination.

Mortality (32/57) was observed only in males at the highest concentration, with many displaying severe acute bronchopneumonia. Body weights were significantly lower in the high-dose males and females during the first 10 days after which they gained weight with females never achieving their starting weight throughout the study. Lung hydroxyproline per mg protein (as an index of lung collagen) was increased 113 and 137% above controls (p<0.05) in the 1.4 and 4.0 ppm groups, respectively. Lung elastin per mg protein did not change significantly in the two lower dose groups but was increased to 174% of control levels (p<0.05) in the group

exposed to 4.0 ppm. Histologically, the 4.0 ppm surviving animals demonstrated bronchiolar epithelial necrosis and sloughing, bronchiolar edema with macrophages, and focal pulmonary edema. Rats from the 0.4 and 1.4 ppm groups did not exhibit pulmonary lesions attributable to acrolein exposure. Changes in the non-respiratory organs appeared incidental. The severity of the pulmonary lesions was scored for the left lung with a concentration-related increase in severity noted. No adverse histopathology was noted in other tissues examined. The only finding in the nasal turbinates was an apparent dose-dependent increase in submucosal aggregates. In addition, no cytogenetic nor sperm abnormalities were observed nor was there any treatment-related effect on reproductive performance. In this latter aspect of the study, exposed male rats were mated with unexposed females for six days and also exposed females were mated with unexposed males.

Pulmonary function testing and the morphometric and compositional analyses in the male Fisher 344 rats from the Kutzman et al. studies was reported by Costa et al, 1986. Results indicated that at 0.4 ppm, parenchymal tissue density was significantly increased along with significantly increased maximal expiratory flow volume (MEFV), together inferring some degree of parenchymal restriction. Lung composition was similar to controls. The animals in the 1.4 ppm group did not differ functionally from controls. Parameters measured in the 4 ppm group, however, suggested obstructive lesions causing impaired ventilation in both the small and large airways. It was hypothesized that the functional effects from the restrictive lesions (0.4 ppm) and obstructive lesions at 4 ppm canceled in the 1.4 ppm group. Based on the restrictive changes, 0.4 ppm is considered a lowest-observed-adverse-effect level (LOAEL).

Female Dahl rats selected for either susceptibility (DS) or resistance (DR) to salt-induced hypertension were exposed to filtered air, 0.4, 1.4, or 4.0 ppm (0.9, 3.2 and 9.2 mg/m<sup>3</sup>) acrolein (Kutzman et al., 1984, 1986). Ten DS and 10 DR rats/group were exposed 6 hr/day, 5 days/week for 61-63 days (consecutive except for weekends). All of the DS rats exposed to 4.0 ppm acrolein died within the first 11 days of exposure, while 60% of the DR animals survived to the end of exposure. Neither-dose dependent blood pressure changes nor altered behavioral characteristics were evident following acrolein exposure. Measures of lung connective tissue, hydroxyproline and elastin, as well as several serum chemistry parameters, alkaline phosphatase, phosphorus, SGOT and SGPT were significantly increased (p<0.05) in the DR rats following exposure to 4.0 ppm acrolein. There was a marked difference in the pulmonary pathology observed in DS and DR rats exposed to 4.0 ppm acrolein. The lungs of the DS rats exhibited severe airway epithelial necrosis with edema and hemorrhage, while surviving DR rats primarily showed a proliferative change. These included collections of intraalveolar macrophages with foamy cytoplasm, terminal bronchiolar hyperplasia, squamous metaplasia of tracheal epithelium and terminal bronchial epithelium, as well as interstitial pneumonitis in 4 of 6 survivors. Alterations were more mild in the 0.4 and 1.4 ppm groups and generally did not involve metaplasia. Differences between the DS and DR groups at the 2 lower doses were minimal. Reasons for the difference in susceptibility of DS and DR rats to 4.0 ppm acrolein are unclear.

Bouley et al. (1975; 1976) exposed male SPF OFA rats (110/group) via inhalation to 0 and 0.55 ppm (1.3 mg/m<sup>3</sup>) acrolein. Daily length of exposure and duration of the exposure were not explicitly stated, although measurements were reported for exposures up to 77 days. Body weights decreased to slightly less than 80% of controls by 60 days of exposure. Signs of nasal irritation (sneezing) were consistently observed in exposed rats between the 7<sup>th</sup> and 21<sup>st</sup> day of exposure. Sneezing subsequently disappeared despite continuing exposure. Exposed rats also exhibited a significant decrease in the number of alveolar macrophages. No differences were

noted in liver weight after 22 days of exposure although liver/body weight ratios were decreased in exposed animals after day 15. Lung/body weight ratios were unchanged after day 15 or 32, but were significantly elevated ( $p < 0.002$ ) after 77 days. There was no effect on hepatic alcohol dehydrogenase after 15 days of exposure. Serum alkaline phosphatase was unchanged at days 15, 32 and 77. On the other hand, serum acid phosphatase was increased on day 15 ( $p < 0.001$ ), but not on days 32 and 77. An LD<sub>50</sub> inhaled dose of *Salmonella enteritidis* resulted in a higher death rate in treated animals than controls at 18 days, but not at 63 days. Results were negative in a reproduction study involving 21 females and 3 males. In this portion of the study, mating was started on the 4<sup>th</sup> day after exposure was initiated and female rats were sacrificed on the 26<sup>th</sup> day after exposure began. There were no significant differences between control and exposed animals in the number of pregnant animals or number and mean weight of foetuses. While a large number of animals were exposed in the study and numerous measurements were made, use of only one exposure concentration and lack of histopathology greatly limit the usefulness of this study.

In an abstract, Teredesai and Stinn (1989) reported that exposure of male Sprague-Dawley rats to 1.7 ppm (3.9 mg/m<sup>3</sup>) acrolein for 7 hours/day for three successive days caused ulceration of the respiratory epithelium in 4/10 rats and olfactory degeneration in all rats. Proliferative responses in nasal and tracheal epithelia of male Sprague-Dawley rats exposed were observed at levels of 0.2 and 0.6 ppm (0.5 and 1.4 mg/m<sup>3</sup>) acrolein for 6 hr/day on one or three successive days (Roemer et al., 1993); significant cell proliferative changes also noted with formaldehyde alone, but at 2 ppm and higher.

**MICE:** Male Swiss-Webster mice were exposed via inhalation 6 hr/day for 5 consecutive days to 1.7 ppm (3.9 mg/m<sup>3</sup>) acrolein, the estimated concentration resulting in a 50% decrease in respiration (RD<sub>50</sub>) (Buckley et al., 1984). Eight to ten animals were sacrificed for pathologic examination immediately post exposure and an approximately equal number were sacrificed 72 hours later. The nasal region was sectioned at 5 levels for examination. Changes were labeled as none, slight, minimal, moderate or severe. For respiratory epithelium, exfoliation, inflammation, erosion, ulceration and necrotic changes were considered to be moderate. Squamous metaplasia was considered severe. For olfactory epithelium, ulceration and necrosis were considered to be moderate while squamous metaplasia and serous exudate mild. No effects were reported in the lungs. Recovery after 72 hours was minimal to moderate, suggesting that the recovery period was insufficient for complete repair of lesions. During single exposures to acrolein, the reflex decrease in respiration was virtually eliminated by tracheal cannulation, providing additional evidence that the critical site for irritant effects of acrolein is the nasal region rather than the deep lung (Kane and Alarie, 1977).

The effect of coexposure to acrolein and carbon black upon lung defenses was evaluated by Jakab (1993). Female Swiss mice were exposed using a nose only inhalation chamber, 4 hr/day for 4 days to carbon black (10 mg/m<sup>3</sup>), acrolein (2.5 ppm; 5.7 mg/m<sup>3</sup>), or the two combined. Twenty-four animals per group were assayed for resistance to *Staphylococcus aureus* and *Proteus mirabilis* at 1, 4, and 7 days post-exposure. For *Listeria monocytogenes*, 30 animals/group were utilized, with 6 animals/group sacrificed at 3, 6, 10, and 13 days post exposure. For influenza virus, 30 animals/group were used and 6 animals/group were sacrificed at 3, 6, 8, and 11 days post exposure with an additional group of 6 lavaged for quantitative cell counts and determination of lung lavage albumin concentrations. *S. aureus* was used for the alveolar macrophage (AM) surveillance phagocytic system, *P. mirabilis* for the dual phagocytic

system composed of AMs and inflammatory polymorphonuclear leucocytes (PMNs), *Listeria monocytogenes* for the lymphokine-mediated arm of the acquired cellular immune response, and the influenza A virus for the cytotoxic T-cell mediated effector mechanism of cellular immunity.

Intrapulmonary killing of *S. aureus* was suppressed on the first day post-exposure to acrolein, with a return to normal by day 7. Coexposure enhanced pulmonary killing of *P. mirabilis*, which correlated with a significant increase in accessory phagocytic PMNs recovered from the lungs. Elimination of *L. monocytogenes* and influenza A virus from the lungs was impaired. Exposure to acrolein or carbon black alone had no effect upon lung defenses. Effects noted were likely due not only to the ability of carbon black to carry acrolein into the deep lung, but ingestion of particles by macrophages resulting in enhanced cellular penetration of acrolein. In an earlier study, Astry and Jakab (1983) found that an underlying viral pneumonia in mice compounded the pulmonary toxicity of 3 or 6 ppm (6.9 or 12.8 mg/m<sup>3</sup>) acrolein in that antibacterial (challenge with *S. aureus*) defense mechanisms were suppressed.

Aranyi et al. (1986) exposed female CD1 mice via inhalation to 0.10 ± 0.22 ppm (0.23 ± 0.50 mg/m<sup>3</sup>) acrolein, 3 hr/day for 5 consecutive days. To evaluate resistance to infection, the animals were simultaneously challenged with *Streptococcus zooepidemicus* (Group C) for measurements of mortality and <sup>35</sup>S-*Klebsiella pneumonia* (noncapsulated) for determination of *in vivo* bacteriocidal activity of alveolar macrophages. A non-significant increase in mortality from 6/140 among controls to 11/140 in exposed mice was recorded. The percent bacteria killed showed a small, but significant decrease from 84.3 to 76.6 (p < 0.01).

Philippin et al. (1970) examined the inhalation effects of acrolein in mice exposed for 6 hours and for two weeks (6 hours/day). Groups of white albino male mice were exposed (1) for 6 hours, (2) for two 5-day periods (not known if consecutive) at 6 hr/day, and (3) for one 5-day period. At the conclusion of the two 5-day exposures (47/mice/group), there was no mortality at 6, 15, and 25 ppm (2.6, 34, and 58 mg/m<sup>3</sup>); there was 91% mortality when 34 mice were exposed to 50 ppm (116 mg/m<sup>3</sup>) for 5 consecutive days. There was no mortality at the lowest concentration tested (31 ppm; 71 mg/m<sup>3</sup>) in the 6-hours-only exposure group. The acute LC<sub>50</sub> was determined to be 66 ppm (152 mg/m<sup>3</sup>). Primary lung lesions observed in the groups (15 mice each examined for histopathology) exposed for two 5-day periods and sacrificed 24 hours after the last exposure were atelectasis and inflammatory responses with edema.

Borchers et al. (1999) exposed FVB/N male mice to 3.0 ppm (6.9 mg/m<sup>3</sup>) acrolein for 6 hours/day, 5 days/week for three weeks and found a significant and persistent increase in macrophages in bronchoalveolar lavage fluid and evidence that acrolein-induced excessive macrophage accumulation is associated with mucus hypersecretion.

**MULTISPECIES:** Groups of 15 Sprague-Dawley rats, 7-8/sex; Princeton or Hartley guinea pigs, 7-8/sex; 2 male purebred beagle dogs; and 9 male squirrel monkeys (*Saimiri sciurea*) were exposed to acrolein, 8 hr/day, 5 days/week for 6 weeks at concentrations of either 0.7 or 3.7 ppm (1.6 or 8.4 mg/m<sup>3</sup>) (Lyon et al., 1970). According to the first author (Lyon, 2001), there were no concurrent controls in this study; control data (including histopathology) were obtained at a different time point. Histopathological examinations were stated to have been carried out on all dogs and monkeys and about one-half of the rats and guinea pigs. Nasal histopathology was not conducted. No deaths occurred and all animals appeared to be normal during exposure to 0.7 ppm acrolein. Lung sections from all animals exposed to 0.7 ppm showed chronic inflammatory changes and occasional emphysema. These changes were more prominent in dogs and monkeys. The inflammatory changes, consisting of interstitial infiltration of round

cells, were mild and ranged from focal to diffuse, and while some infiltrates were peribronchial in distribution, no alteration of the respiratory epithelium or of the peribronchial smooth muscle was noted. In the 3.7 ppm exposure groups, dogs and monkeys salivated excessively and blinked frequently during the first week, and during the next four weeks the dogs experienced continued eye irritation. Two monkeys died, but it is unclear if these deaths were related to exposure inasmuch as the condition of the monkeys upon arrival was not discussed. Nonspecific inflammatory changes were noted in sections of lung, liver, and kidney from all species. Focal calcification of renal tubular epithelium was noted in some of the rats and monkeys. Significant morphological changes, considered by the investigators to be related to acrolein exposure, consisted of squamous metaplasia and basal cell hyperplasia of the trachea of dogs and monkeys, and necrotizing bronchitis and bronchiolitis with squamous metaplasia of the lungs from 7 of the 9 monkeys, including two that died early in the study. The lung changes were commonly present in the bronchi rather than the bronchioles; the necrosis of the bronchial mucosa was associated with varying degrees of repair and regeneration of the epithelium. Bronchopneumonia was noted in dogs. There was no mention of any histopathological effects in control animals. Although data from control animals was under-reported, it appears that 0.7 ppm was associated with lung injury in all species evaluated.

Lyon et al. (1970), using identical group sizes, species (Sprague-Dawley rats, Hartley- or Princeton-derived guinea pigs, beagle dogs and squirrel monkeys) and strains as described above in the 6-week study, also carried out 90-day continuous inhalation exposures (24 hr/day) at concentrations of 0, 0.21, 0.23, 1.0, and 1.8 ppm (0, 0.5, 0.5, 2.3, and 4.1 mg/m<sup>3</sup>). The two lower exposures were combined as one experiment at 0.22 ppm. The same histopathological protocol was followed. All animals appeared normal during the 0.22 ppm exposure.

**Monkeys and dogs:** Two monkeys died as a result of apparent infections. Histopathology demonstrated inflammatory effects in the eyes of dogs and monkeys, although no detail regarding number of animals or degree of inflammation was given. Ocular and nasal discharges were reported in the dogs and monkeys exposed to 1.0 ppm, the latter keeping their eyes closed for extended periods. Morphological changes observed in tracheas of monkeys and lungs of dogs were considered related to exposure. At 1.0 ppm focal inflammatory reactions were reported in the lungs, liver, and kidneys of dogs. At 1.8 ppm the dogs and monkeys experienced severe irritation as evidenced by excessive salivation and ocular discharge. All monkeys in the 1.8 ppm group showed squamous metaplasia and 6/9 monkeys presented with basal cell hyperplasia of the trachea. The lungs from the two dogs at this concentration showed confluent bronchopneumonia. Lungs from 2/4 dogs in the 0.22 ppm group demonstrated moderate emphysema and focal splenic hemorrhage. The other two dogs showed hyperplasia of the thyroid. It is not clear if these observations from the 0.22 ppm group were treatment-related inasmuch as there was no discussion of the condition of the control dogs. The investigators, however, did consider the lung effects in dogs (at all exposure levels) to be treatment-related.

**Rats and guinea pigs:** While weight gain was significantly lower in rats exposed to 1.0 and 1.8 ppm ( $p < 0.005$ ), no statistically significant differences in weight gain were noted in the other three species. In the 1 ppm groups, guinea pigs showed various degrees of pulmonary inflammation and occasional focal liver necrosis while rats (3/9) had occasional pulmonary hemorrhage and focal liver necrosis. In the 1.8 ppm exposure groups nonspecific inflammatory changes were observed in sections of brain, heart, lung, liver, and kidney of all animals.

Given the similarities in lung effects across species seen during the repeated and continuous exposures as well as in other studies, 1.0 ppm can be considered a LOAEL.

Feron et al. (1978) exposed four equal groups, each consisting of 20 Syrian golden hamsters, 12 Wistar rats, and 4 Dutch rabbits (equal numbers of each sex) to 0, 0.4, 1.4, and 4.9 ppm (0, 0.9, 3.2, and 11.0 mg/m<sup>3</sup>) acrolein, 6 hr/day, 5 days/week for 13 weeks. Duration-adjusted values are 0, 0.16, 0.57, and 2.0 mg/m<sup>3</sup>. Histopathology was performed on all major organs/tissues, including three transverse sections of the nasal cavity.

**Rats:** Of the three species, rats seemed to be the most sensitive to the effects of acrolein. Mortality (6/24) occurred in the 4.9 ppm group and animals kept their eyes closed. No adverse clinical observations were reported for the other concentration groups. Hematological and serum enzyme levels were within the normal range. Body weight gain was significantly ( $p < 0.001$ ) depressed at 4.9 ppm and at 1.4 ppm ( $p < 0.05$ ). The decrease in weight gain appeared related to decreased food consumption. Of several rats that died, hemorrhage, perivascular and alveolar edema of the lung were seen. Focal broncho-pneumonia, bronchitis, hyper- and metaplasia of the bronchial and bronchiolar epithelium, increased numbers of mucous-producing cells in the bronchioles, macrophage accumulation, and focal interstitial pneumonitis were observed in surviving rats in the 4.9 ppm group. Incidence of nasal lesions was not reported. Only one male rat of the 0.4 ppm group showed any evidence of histopathological effects (metaplastic and inflammatory changes of slight severity) in the nasal tract. Squamous metaplasia and neutrophilic infiltration (moderate severity) of the nasal mucosa were observed in the 1.4 ppm group. In the 4.9 ppm group, necrotizing rhinitis was occasionally seen in the dorsomedial part of the nasomaxillary region, with normal epithelium being partly replaced by stratified squamous epithelium, and in some cases showing keratinization. Neutrophil infiltration was invariably observed, but substantial neutrophilic exudation was seen in only a few animals. The trachea of rats in the 4.9 ppm group was described as “severely damaged,” with nodules of granulation tissues protruding into the lumen.

**Hamsters:** There was one death among the hamsters, but it was not related to treatment. Body weight gain was depressed only in the 4.9 ppm group and there was no evidence of decreased food intake. Lungs were unaffected by exposure. Only minimal inflammatory changes were seen in the nasal cavity at 1.4 ppm; the nasal lesions observed in the 4.9 ppm group were similar (severe) to those seen in the rat. Hyperplasia and metaplasia in the trachea occurred in a few males and all females at 4.9 ppm. In females at 4.9 ppm, there were statistically significant increases in red blood cell packed cell volume, hemoglobin (Hb) content, and in numbers of lymphocytes accompanied by a decrease in the number of neutrophilic leucocytes. All serum enzyme activities were within normal ranges.

**Rabbits:** Body weight gain (males and females combined) was significantly depressed ( $< 0.05$ ) in only the 4.9 ppm group. Decreased weight gain appeared to be related to diminished food intake. No effects were detected in the nasal region in the low and mid dose groups. Nasal lesions in the 4.9 ppm group were similar to those in the rat, but less severe. Tracheal effects were seen only in the high dose group, primarily hyperplasia and metaplasia.

Based on the severity of respiratory tract lesions in the rat compared to diminished responses in the rabbit and hamster in the 4.9 ppm groups, the rat is considered the most sensitive species of the three with a minimal LOAEL for nasal lesions of 0.4 ppm. Although only 1/12 rats at this concentration demonstrated minimal metaplastic and inflammatory changes, these effects were consistent with the pathology demonstrated at the higher concentrations in which severity was increased.

**GUINEA PIGS:** Turner et al. (1993) found that exposure of guinea pigs for 7.5 hours per day for two consecutive days to 1.6 ppm (3.7 mg/m<sup>3</sup>) acrolein resulted in pulmonary inflammation and epithelial damage. Even after 28 days post-exposure there was a prolonged increase in airway sensitivity to aerosolized substance P, a sensitivity which may have been enhanced by an acrolein-induced reduction in neutral endopeptidase in bronchoalveolar lavage fluid.

**RABBITS:** In a study designed to evaluate the effects of corticosteroids on mortality and lung histopathology of female New Zealand rabbits exposed to acrolein, animals (18/group) were exposed to 375 and 489 ppm (862 and 1125 mg/m<sup>3</sup>) for 15 minutes (Beeley et al., 1986). Although treatment with methylprednisolone reduced mortality (no significant differences between the two groups), there was no evidence of an improvement in lung histopathology (hemorrhagic necrosis, edema).

#### 4.3.1.2. Oral administration

**RATS:** In a study that clearly shows acrolein-induced damage to cellular function, Arumugam et al. (1999b) exposed male Wistar rats, 5 animals/group, daily for 45 days to distilled water or acrolein (2.5 mg/kg bw) via intubation. Electron microscopic examination revealed a loss of mitochondrial lamellae in the treated livers. This was accompanied by a decrease in GSH as well as the activities of the citric acid cycle enzymes, isocitrate dehydrogenase,  $\alpha$ -ketoglutarate dehydrogenase, malate dehydrogenase, succinate dehydrogenase, NADH dehydrogenase and cytochrome-c-oxidase ( $p < 0.01$ ). According to the authors, glutathione peroxidase is the only defense available against the potential toxic effects of superoxide anion radicals generated by mitochondria under physiological and pathological conditions. The mitochondria do not contain catalase, the other enzyme responsible for clearance of H<sub>2</sub>O<sub>2</sub>. The reaction of GSH with acrolein decreases the availability of this substrate for formation of glutathione peroxidase, resulting in mitochondrial damage and diminished energy production. Limitations of the study for evaluation of chronic effects are the (1) use of a 45-day exposure period rather than a longer one, (2) use of only one dose level, and (3) lack of histopathology of the stomach which would have ascertained if intubation of acrolein damaged stomach lining.

In contrast, a summary of an unpublished study reported that administration of acrolein in water at 0.05, 0.5, and 5.0 mg/kg to rats daily, 5 days per week for 13 weeks did not demonstrate any significant toxic effects (Bioassay Systems Corp, 1981g); histopathology, hematology, and clinical chemistry were performed.

Parent et al. (1992c) administered acrolein in water daily via gavage to Sprague-Dawley rats, 70/sex/group, at dose levels of 0, 0.05, 0.5, and 2.5 mg/kg bw. Dosing volume was 10 ml/kg. Ten animals from each group were sacrificed after one year and the remainder after two years. An extensive array of tissues (including the stomach although it was not clear if both the glandular and forestomach were evaluated) was examined microscopically. Daily observations were made and various clinical, hematological and urinary parameters were measured after 3, 6, 12, and 18 months of treatment and immediately prior to termination. There were no significantly increased incidences of microscopic lesions in the treated rats, whether neoplastic or non-neoplastic. Food consumption and body weights were unaffected by treatment. With the exception of a statistically significant depression of phosphocreatin levels at all dose

levels and at most time intervals (except 12 months), clinical chemistry parameters, hematology and urinalysis measurements were unaffected by treatment. The significance of this depression is uncertain. The most definitive responses reported were treatment-related increases in early cumulative mortality. Data was provided in the form of survival curves. Among high-dose males, survival was significantly reduced after one year ( $p < 0.05$ ), and marginally reduced among mid-dose males ( $p$  value not reported). Among high-dose males, a trend test for survival during the first year indicated a highly significant ( $p = 0.003$ ) decrease; however, the statistical differences are nullified when the survival data for two years are included in the analysis. Survival among females during the first year corresponded closely to those obtained for males. A statistically significant decrease in survival ( $p < 0.05$ ) was reported in the high-dose group, while a decrease in survival in the mid-dose group was marginally significant ( $p$  value not reported). A highly significant trend towards reduced survival ( $p < 0.001$ ) in the high-dose group was also reported. Unlike responses in males, the significant associations between dosing and survival persisted in females through the end of the study. After two years, a statistically significant reduction in survival was noted based on four different statistical tests for the mid-dose group and in three of four statistical tests in the high-dose group ( $p$  values not reported). Although the differences in survival were statistically significant in females after two years, it should be noted that the differences were relatively small. No differences in survival were seen in the low-dose groups. The results in this study suggest that 0.5 mg/kg could be considered a frank-effect-level (FEL).

**MICE:** In a study (Parent et al., 1991b) designed to evaluate the potential carcinogenicity of acrolein, Swiss albino CD-1 mice (70/sex/group) were dosed via gavage (acrolein in distilled water and stabilized with hydroquinone) with 0, 0.5, or 2.0 mg/kg/day for 18 months. A separate group (75/sex) was similarly dosed at 4.5 mg/kg/day. All animals were sacrificed at 18 months. The primary effect was increased mortality only in high-dose males. There were no dose-related adverse histopathological or clinical findings.

**RATS and MICE:** In a 13-week daily gavage study of acrolein (in 0.5% methyl cellulose) in F344 rats and B6C3F1 mice conducted for the National Toxicology Program (NTP), 10 rats/sex/dose were administered 0.75, 1.25, 2.5, 5.0, and 10 mg acrolein/kg with 10 mice/sex/dose receiving 0, 1.25, 2.5, 5.0, 10 and 20 mg/kg. Dose volume was 5 ml/kg for rats and 10 ml/kg for mice. Treatment resulted in similar dose-related effects in both sexes of both species: hemorrhage and necrosis and other lesions of the forestomach and glandular stomach and secondary changes associated with acrolein-induced mortality in high-dose animals (NTP, 1995; Pathology Working Group Review, 1997). Abnormal breathing and nasal/eye discharge were among the clinical findings in high-dose rats, but there were no clinical signs of toxicity in mice. Nearly all high-dose animals died or were removed from study because of gastrointestinal toxicity. The no-observed-effect level (NOEL) for rats was 0.75 mg/kg, based on forestomach squamous epithelial hyperplasia in the 1.25 mg/kg group of females. There was no NOEL for the mouse. Pretreatment of male Charles river mice with high oral doses of L-ascorbic acid, L-cysteine, and an alpha adrenergic-blocking agent gave a high degree of protection against the lethality of orally-administered acrolein administered once after which animals were followed for 72 hours (Sprince et al., 1979).

**DOGS:** Six male and 6 female beagle dogs/group were administered acrolein (0.1% aqueous) in gelatin capsules at doses of 0, 0.1, 0.5, and 1.5 mg/kg/day, 7 days/week for 53 weeks (Parent et al., 1992a). At week 4, the high dose was increased to 2 mg/kg/day. Blood and biochemical measurements were made at pretest and at 3-month intervals. At termination, all dogs were subjected to full necropsy and histological examination. Body weights and food consumption were not significantly affected by treatment. A primary effect noted was a dose-dependent increase in the frequency of vomiting shortly after dosing. The frequency decreased with time indicating adaptation. Serum albumin, calcium and total protein levels were significantly depressed (p values not given) in high-dose animals throughout the study. Measurements for the other exposure groups were not listed. Some variability in red blood cell parameters and coagulation times were noted, but the significance of these effects was not obvious. It was reported that clinical signs, with the exception of vomiting, were evenly distributed among groups. At termination, gross necropsy indicated vascular congestion and mucosal reddening of the gastrointestinal tract of both males and females, but it is unclear if these effects were treatment-related. While the study was well designed and the methodology adequately reported, incomplete reporting of results limits its usefulness for quantitating risk. Another limitation is vomiting which undoubtedly reduced the dose received by the dogs.

Conclusions: Decreased survival was a feature in both the 13-week NTP study and Parent et al. two-year study with rats and mice. However, only the rat/mouse study performed for the NTP resulted in stomach lesions. The absence of stomach lesions in the Parent et al.(1991b; 1992c) studies involving rats and mice does not negate the findings of the NTP study. Reasons for the apparent dichotomy in results are unclear although it could relate to the strength of the daily dosing solution or strain and vehicle differences.

#### 4.3.1.3. Dermal administration

The toxicity of acrolein dissolved in water+ethanol in rabbits was evaluated by dermal application of 7, 21, and 63 mg acrolein/kg for three consecutive weeks, 5 days per week according to a FIFRA study design (Bioassay Systems Corp., 1982a). Observations included: slight to significant reduction in body weight, moderate to severe skin irritation, and histopathologic lesions in skin and lungs.

### 4.3.2. Cancer Assessment

#### 4.3.2.1. Inhalation Exposure

Feron and Kryusse (1977) exposed groups of 36 Syrian golden hamsters of both sexes to acrolein vapor at measured levels of 0 and 4.0 ppm (0 and 9.2 mg/m<sup>3</sup>), 7 hr/day, 5 days/week for 52 weeks. Six animals per group were sacrificed at 52 weeks and the remainder at 81 weeks. Overall mortality was 38% in exposed hamsters and 33% in controls. Histological changes were observed in the anterior half of the nasomaxillary turbinates, consisting of epithelial metaplasia, but not hyperplasia. In addition, exposure resulted in abnormal behavior and growth retardation. The only respiratory tract tumor observed was a small tracheal papilloma in an acrolein exposed female. The exposure period for this study was short for a cancer bioassay and sacrifice at 81 weeks may have been insufficient to allow for latency.

In a study by Le Bouffant et al. (1980), 20 female Sprague-Dawley rats/group were exposed to 8 ppm (18.3 mg/m<sup>3</sup>), 1 hr/day, 5 days/week for either 10 or 18 months. No tumors or

metaplasias were reported. Use of only one exposure concentration and less than lifetime exposure duration limit any inferences that can be drawn from this study.

#### 4.3.2.2. Oral administration

There have been three long-term cancer bioassays by the oral route: male F-344 rats via drinking water (Lijinsky and Reuber, 1987); CD-1 mice via drinking water (Parent et al., 1991b); and Sprague-Dawley rats via drinking water (Parent et al., 1992c).

Male Fischer 344 rats (20/group) were administered acrolein in the drinking water at concentrations providing average daily doses of 0, 1.9, 5.0, or 12.5 mg/day, 5 days/week for 104-124 weeks (Lijinsky and Reuber, 1987). High-dose animals stopped drinking the solution before the other groups. Drinking water solutions were prepared weekly and stored at unspecified refrigerator temperatures until dispensed. Each cage of four rats was given a measured amount (80 ml) of drinking water over the span of the study. The daily dose per kg bw could not be calculated from the data given. [Parent et al. (1992c), assuming that each of the four rats/cage drank the same amount of water, estimated a daily dose of 50 mg/kg bw at the highest concentration which exceeds the LD<sub>50</sub> for rats; this suggests that acrolein in solution was not as stable in solution as purported]. The maximum tolerated dose was not determined. Major organs and tissues were reported as being examined histopathologically (if there were any non-proliferative lesions they were not reported). One group of 20 females also received the highest dose on the same schedule as the males. Adrenocortical tumors (5/20) and hyperplastic nodules of the adrenal cortex (2/20) were found only in females in the high concentration group. The increased incidence of adrenocortical tumors was considered by the authors to be marginally significant as judged by the Fisher's exact test ( $p=0.091$ ) and significant for adrenocortical tumors plus hyperplastic nodules ( $p=0.022$ ). According to the authors, this type of tumor is rare in untreated female Fischer 344 rats; there was one reported in concurrent controls. The historical incidence is approximately 4.8% based on the findings of Solleveld et al. (1984) for untreated female F-344 rats allowed to die naturally. Significant increases in tumor incidence were not found in male rats. There was no treatment-related mortality. Lijinsky and Reuber (1987) also exposed rats to acrolein diethylacetal, acrolein oxime, and allyl alcohol, agents that can be expected to be hydrolyzed to acrolein in the stomach acids, with negative results. A reevaluation of the tumors in this study (reported by Parent et al., 1992) is described in Section 4.7.

Lijinsky and Reuber (1987) also exposed hamsters to acrolein, but it proved to be too toxic to carry out a cancer bioassay; a single one mg dose via gavage in corn oil killed all of the animals within a few hours; hamsters reportedly drank too little water to make the study feasible. Relevance of these study results in comparison to other studies has been discussed by Parent et al. (1992c) (see Section 4.7).

Four groups of 70-75 male and 70-75 female Swiss albino CD-1 mice, eight weeks of age, were administered 0, 0.5, 2.0, or 4.5 mg acrolein/kg bw via gavage in deionized water daily for 18 months, followed by sacrifice of survivors at the end of the treatment period (Parent et al., 1991b). Dosing levels were chosen based on a range-finding study demonstrating severe stomach lesions at higher dosing levels. Body weight gains were decreased and mortality increased in males, especially at the high dose. All mice killed at the end of treatment, as well as those found dead or moribund, were necropsied. Tissues from major organs were examined histologically. No treatment-related increase in tumor frequency was observed. The study was

near lifetime duration for mice and the maximum tolerated dose (MTD) appeared to be achieved. Thus, acrolein appears unlikely to be carcinogenic in mice by gavage.

Parent et al. (1992c) also gavaged 560 Sprague-Dawley rats about 6 weeks of age (70/sex/group) daily with 0, 0.05, 0.5 and 2.5 mg acrolein/kg in water (10 ml/kg). Ten rats/sex/group were sacrificed at 1 year for various clinical measurements. The remainder of the animals were treated for 102 weeks followed by sacrifice. Dosing solutions were prepared daily from stock solutions (prepared daily) and analyzed weekly by gas chromatography. Stability studies indicated losses at <10% after storage for 3 hr at room temperature. The only statistically significant changes noted in treated animals were consistent depression of creatine phosphokinase levels (significance unknown) and consistent increases in early cumulative mortalities in both males and females. There was no significantly increased incidence of either neoplastic or non-neoplastic microscopic lesions in treated rats. Analyses of survival took into account confirmed and possible accidental deaths (28 total). Decreased survival of high- and mid- dose males during the first year was highly and marginally significant, respectively; however, this trend did not persist into the second year. Unlike survival in male rats, decreased survival in females during the first year persisted until the end of the second year. Based upon results of this two-year exposure in which mortality indicated a maximum-tolerated dose (MTD) was achieved, it can be concluded that there was no evidence for carcinogenicity in an adequately designed and conducted study. While the doses/kg bw used in this study are most likely much lower than those used by Lijinsky and Reuber (1987), Parent and colleagues have raised concerns that the stability of the acrolein-in-water solutions used by Lijinsky and Reuber was actually less than reported (see section 4.7).

#### 4.3.2.3. Injection studies

The earliest reported study investigating the potential carcinogenicity of acrolein was reported by Steiner et al. (1943). Fifteen female partly inbred albino mice received subcutaneous injections (0.2 mg/kg) of acrolein weekly for 24 weeks. No sarcomas developed at the site of injection. The use of only one dose level and a small number of animals limits any conclusions.

#### 4.3.2.4. Initiation and promotion studies

Cohen et al. (1992) exposed 30 male Fischer 344 rats/group to acrolein, 2 mg/kg by i.p. injection twice weekly as part of a larger initiation/promotion study. All groups were sacrificed 53 weeks from the start of the study. No increases in tumor incidence were reported in groups exposed to acrolein alone for either 6 or 21 weeks (severe toxicity occurred during the 21 week study). Exposure to acrolein for 6 weeks followed by administration of uracil (3% by weight) for an additional 20 weeks resulted in the induction of 18 papillomas and one carcinoma, a significantly greater incidence ( $p < 0.05$ ) than following exposure to uracil alone (8/30). While it appears that acrolein may have some tumor initiating capability, it should be noted that the incidence of papillomas and nodular hyperplasias combined, was significantly greater in the uracil only group compared with the group initiated with acrolein ( $p < 0.05$ ).

A group of 15 "S" strain mice (sex and age unspecified) received 10 weekly skin applications of a 0.5% solution of acrolein in acetone at a total dose of 12.6 mg/animal (Salaman and Roe, 1956). Starting 25 days after the first application of acrolein, the mice received weekly skin applications of 0.17% croton oil for 18 weeks; for the second and third applications the concentration was reduced to 0.085%. When croton oil and acrolein were administered together, each compound was given alternately at three or four day intervals. Tumor incidence was

evaluated at the end of the croton oil treatment. Four skin papillomas were reported in 4 of 19 animals that received croton oil alone. A total of three papillomas were noted in 2 of 19 mice treated with acrolein and croton oil. The data suggest that acrolein lacks potential for initiation of skin tumors. However, small numbers limits any definitive conclusions.

Feron and Kryusse (1977) exposed groups of 30 male and 30 female Syrian golden hamsters, about 6 weeks of age, to 0 or 4 ppm (0 or 9.2 mg/m<sup>3</sup>) acrolein for 52 weeks, together with either weekly intratracheal installations of 0.175 or 0.35% benzo[a]pyrene (BP) in 0.9% saline, or subcutaneous injections of 0.0675% *N*-nitrosodiethylamine (DNA) in saline once every three weeks (total dose, 2 µL/animal). The experiment was terminated at 81 weeks, and all survivors were killed and autopsied. An increased incidence of papillomas, adenomas, adenocarcinomas and squamous-cell carcinomas of the respiratory tract were found in acrolein-exposed male and female hamsters treated with benzo[a]pyrene and *N*-nitrosodiethylamine. Exposure to acrolein vapor alone resulted in only one respiratory tumor (female).

Conclusions that can be drawn from the Feron and Kryusse (1977) inhalation and initiation/promotion studies are limited because of the use of only one dose level, although they did report toxic responses at concentrations of 9.2 mg/m<sup>3</sup>. Because respiratory tract tumors typically occur in hamsters administered BP or DNA, the evidence is insufficient to suggest that acrolein is a cofactor in carcinogenesis.

#### **4.4. REPRODUCTIVE/DEVELOPMENTAL STUDIES-ORAL AND INHALATION**

##### **In vivo:**

Kutzman et al. (1981) exposed female and male Fischer 344 rats (8/group) via inhalation to acrolein at 0, 0.4, 1.4, or 4.0 ppm (0, 0.9, 3.2 or 9.2 mg/m<sup>3</sup>) 6 hr/day, 5 days/week for 62 days. There were no treatment-related effects on reproductive performance. Exposed and control male rats were mated with unexposed females for six days and also exposed females were mated with unexposed and exposed males. Parameters evaluated were: corpora lutea, viable embryos, early and late deaths, and pre-implantation losses.

The only other inhalation-reproduction study reported to date was performed by Bouley et al. (1976). Three male and 21 SPF OFA rats were exposed continuously to acrolein at a measured concentration of 1.3 mg/m<sup>3</sup> then mated on the fourth day of exposure. Exposures were continued for an additional 22 days when the females were sacrificed. The exposure did not cover the entire period of spermatogenesis. No significant differences in the number of and mean weight of fetuses (no data given) were reported. While the results were negative, no conclusions can be drawn from this study.

Claussen et al. (1980) intravenously injected New Zealand white rabbits on day 9 of gestation with 3, 4.5, or 6 mg/kg acrolein. Embryo-lethal effects increased in a dose-dependent manner, but few malformations were noted. After direct injection into the rabbit embryos at doses of 10, 20 and 40 µL, resorptions and malformations increased in a dose-dependent manner. The highest dose by both routes showed that direct embryo injection of acrolein induced malformation at doses 50-60 times lower than those inducing embryo-lethal effects via iv injection.

Four groups of 30 male and 30 female Sprague-Dawley rats were gavaged daily with 70 doses of acrolein at levels of 0, 1, 3, or 6 mg/kg in a dosing volume of 5 ml/kg (Parent, 1992b). Rats within each dosing group (F<sub>0</sub> generation) were then assigned to a 21-day period of cohabitation. Dosing continued for females through cohabitation, gestation, and lactation.

A similar regime was carried out for F<sub>1</sub> generation offspring, resulting in F<sub>2</sub> generation pups. Mortality was significant (at 6 mg/kg) in both males and female of the F<sub>0</sub> and F<sub>1</sub> generations with the pattern continuing with F<sub>1</sub> mid-dose animals, most of the latter showing signs of respiratory distress and histopathological lesions in the lungs and stomach. Reproductive parameters (i.e., mating performance and fertility indices) were unaffected. Erosions of glandular mucosa and hyperplasia/hyperkeratosis of the forestomach were the most frequent stomach lesions observed. Significant depressions in body weight gains were noted in the high-dose groups and achieved statistical significance in the mid-dose animals on several occasions. No treatment-related gross or microscopic effects were observed in the reproductive tissues of any of the F<sub>0</sub> or F<sub>1</sub> animals. The data provide evidence that acrolein is not a selective reproductive toxicant but does produce toxicological effects at doses as low as 3 mg/kg/day.

Pregnant New Zealand white rabbits (20/sex/group) were dosed via gavage with 0, 0.1, 0.75, or 2.0 mg/kg/day for days 7 through 19 of presumed gestation and subjected to caesarean sectioning on day 29 (Parent et al., 1993). Three deaths were observed, but were considered a result of misdosing or aspiration. Transient effects on feed consumption and body weight gains were noted. Resorptions were elevated in the high-dose group, but the effect was not statistically significant. Fetal malformations were distributed evenly among the groups and were consistent with historical control data. Higher doses in a range-finding study (0, 0.5, 1.0, 2.0, 4.0 and 6.0 mg/kg/day) produced high incidences of maternal mortality (at 4 and 6 mg/kg), spontaneous abortion, resorption, clinical signs, gastric ulceration, and/or sloughing of the gastric mucosa. Thus, acrolein was not found to be a developmental toxicant or teratogen at maternally nontoxic doses.

#### **In vitro:**

Rat conceptuses were explanted from the uterus on day 10.5 of gestation, transferred to culture bottles and treated with acrolein at concentrations ranging from 100 to 250  $\mu$ M (Schmid et al., 1981). Slight, but statistically significant inhibition of growth was reported at 100 and 150  $\mu$ M. A concentrations of 200  $\mu$ M resulted in drastic inhibition of growth and differentiation and no gross structural defects, but 250  $\mu$ M completely arrested differentiation and growth. These findings indicate that acrolein is lethal to embryos in a narrow dose range, but has no teratogenic potential.

Slott and Hales (1986), however, reported 100% mortality in embryos cultured in a standard medium containing an acrolein concentration of 140  $\mu$ M, and teratogenic effects at 80-120  $\mu$ M. In a serum-free medium acrolein was 100% lethal to embryos at 20  $\mu$ M and was teratogenic in the range of 5-15  $\mu$ M. The EC<sub>50</sub> for malformations in the serum medium was 137  $\mu$ M, whereas that for embryo lethality was 115  $\mu$ M. In a further study, Slott and Hales (1987a) reported that acrolein induced 64 and 100% mortality at acrolein concentrations of 120 and 160  $\mu$ M, respectively. At concentrations of 80 and 120  $\mu$ M, 50 and 100% of the embryos were malformed, respectively. In addition, both concentrations of acrolein produced growth retardation manifested by significant decreases in the yolk sac diameter, crown-rump and head lengths, number of somites, and morphological score. Concurrent exposure to 100 or 500  $\mu$ M glutathione markedly protected embryos against all of these effects, but glutathione addition 2 hours after the beginning of acrolein exposure offered little protection. Because addition of GSH resulted in little change in concentration in the yolk sack or embryos, protection was believed to be primarily due to interaction between acrolein and GSH in the culture medium. The

embryotoxicity of acrolein, on the other hand, was significantly enhanced by addition of glutathione sulfoxamine (10 or 100  $\mu\text{M}$ ), an inhibitor of GSH synthesis (Slott and Hales, 1987b).

Stahlmann et al. (1985) tested acrolein in a mouse limb bud culture system. Concentrations of acrolein between 3 and 10 mg/ml (56 and 178  $\mu\text{M}$ ) induced a significant impairment of limb bud differentiation with explants from 12 day old mouse embryos. Scapula and paw skeleton were more affected than ulna and radius. With limbs from 11-day old embryos similar effects were reported at even lower concentrations. A contact time of 20-40 min was sufficient to induce abnormal development.

Mirkes et al. (1984) evaluated the role of acrolein in cyclophosphamide (CP) teratogenesis in a culture medium containing day 10 rat embryos. The dechloro derivative of cyclophosphamide (D-CP), which breaks down upon activation to acrolein and dechlorophosphamide mustard (D-PM), D-CP was teratogenic and resulted in decreases in growth parameters whereas D-PM did not. When embryos were exposed to acrolein alone (0.45 to 18  $\mu\text{M}$ ), all concentrations produced abnormal flexion in some embryos, but effects did not resemble those induced by D-CP. Complete lethality was produced at 8.9  $\mu\text{M}$ . This suggests that the high reactivity of acrolein limits its entry to sensitive sites, but when D-CP is transported into the cell, yielding acrolein, teratogenicity can result.

Using the embryo culture, Hales (1989) reported that while phosphoramidate mustard and acrolein are both teratogenic, they had differing effects on developing limbs, indicating different targets and/or mechanisms of action.

No evidence of acrolein-induced teratogenicity was found in chicken eggs treated on day 3 with acrolein at doses of 0.001-10  $\mu\text{mol}$  and examined on day 14 of incubation. The  $\text{LD}_{50}$  was estimated to be 0.05  $\mu\text{mol}$  (Kankaanpää et al., 1979). In a similarly designed study, however, Korhonen et al. (1983) reported malformation in chicks at doses of 0.05  $\mu\text{mol/egg}$ . Chhibber and Gilani (1986) also reported increases in malformations in chicks at doses as low as 0.001 mg/egg (0.02  $\mu\text{mol}$ ) when dosed at 48 hours of incubation and examined on day 13.

In summary, acrolein can induce teratogenic and embryotoxic effects if administered directly to the embryos or fetuses, but had no selective reproductive, developmental or teratogenic effects in two gavage studies. An inhalation study found no effect of acrolein on reproductive performance in exposed male and female rats. The high reactivity of acrolein may limit its ability to reach critical sites in the developing embryo.

## 4.5. OTHER STUDIES

This section focuses upon the toxicity of acrolein in a variety of tissues and cell cultures in in vitro experiments. In addition, the genotoxicity of acrolein and its conjugates in *Salmonella* is discussed.

### 4.5.1. In Vitro Toxicity

**Heart:** Perfusion of rat hearts with 0.01-0.03 mM acrolein led to cessation of beating within 15 minutes; no lesions were detected, but creatine kinase was reported to be inactivated (Sklar et al., 1991). Rat neonatal myocytes were unaffected by exposure to 0.01 mM acrolein, but stopped beating within 2 hours during exposure to 0.05 mM acrolein with accompanying cell lysis and release of lactic dehydrogenase (Toraason et al., 1989). While acrolein was shown to act as an inhibitor of mitochondrial electron transport, the effective concentrations for a 50%

inhibition (0.39-0.80 mM) are probably too great to invoke a direct action on electron transport as a primary mechanism for cardiotoxicity of acrolein (Biagini et al., 1990).

**Pulmonary Cells:** Patel and Block (1993) observed that acrolein exposure results in alterations in plasma membrane-dependent transport in cultured pulmonary endothelial cells, leading to decreased availability of precursor amino acids used in GSH and protein synthesis. Joseph et al. (1994) reported that acrolein at concentrations of 5-50  $\mu\text{M}$  resulted in disruption of actin cytoskeletal fibers in cultured pulmonary artery endothelial cells. The damage was postulated as possibly due to crosslinking of sulfhydryl groups.

Survival of human alveolar macrophages was significantly decreased following 24 hours exposure to acrolein concentrations of 25  $\mu\text{M}$  or greater (Li et al., 1997). Incubation of Type II alveolar macrophages cultured for 24 hours with acrolein led to a near-zero adenosine triphosphate (ATP) concentration at 50  $\mu\text{M}$  and a significant increase ( $p < 0.001$ ) in LDH at an acrolein concentration of 25  $\mu\text{M}$ . These effects were considerably muted when lung slices were used (Monteil et al., 1999).

Cytotoxicity of acrolein, as measured by the decrease in colony forming efficiency (CFE) of cultured human bronchial fibroblasts, was not observed at 1  $\mu\text{M}$  acrolein, but CFE decreased to less than 50% following 7-8 days incubation at 3  $\mu\text{M}$  (Krokan et al., 1985); intracellular thiol content was decreased and inhibited the DNA repair enzyme O<sup>6</sup>-methylguanine-DNA methyltransferase, but had no effect on activity of uracil-DNA glycosylase. Cell survival was significantly decreased at lower acrolein levels than those that reduced thiol levels. Grafström et al. (1988) and Grafström, (1990) also reported that less than 3  $\mu\text{M}$  acrolein was required to decrease-colony forming efficiency 50% in human bronchial epithelial cells. The 1-hour LD50 determined by trypan blue exclusion (a measure of cell permeability) was about 20  $\mu\text{M}$ . A small, but significant increase in single strand breaks and DNA protein crosslinks occurred at a concentration of 30  $\mu\text{M}$  acrolein. Fibroblasts (origin not stated) derived from patients with Xeroderma pigmentosum (XP) were more sensitive to the cytotoxic effects of acrolein than were cells from normal individuals (Curren et al., 1988).

Acrolein enhanced responsiveness of human bronchi sections to carbachol following 20 minutes exposure at 0.1  $\mu\text{M}$  (Marthan et al., 1996). After 60 minutes responsiveness was depressed indicating a toxic effect. The effects appeared to follow a time-concentration  $C \times T$  relationship, with a maximum response at a  $C \times T$  ( $\mu\text{M} \times \text{min}$ ) of slightly less than 10 and a depressed response at a  $C \times T$  of 60.

**Liver:** Acrolein induced a rapid dose-related depletion of GSH in rat hepatocyte cultures at concentrations of 25-500  $\mu\text{M}$  after 2 hr (Zitting and Heinonen, 1980); at 500  $\mu\text{M}$ , recovery did not occur and the integrity of cell membranes was impaired. Similarly, Silva and O'Brien (1989) reported that five minutes exposure to 25  $\mu\text{M}$  acrolein resulted in an approximately 25% decrease in viability of cultured rat hepatocytes, while 50  $\mu\text{M}$  resulted in a greater than 50% decrease (significance levels not given).

Several studies have evaluated the effects of inhibitors to aldehyde dehydrogenase (ALDH) and its effect on the toxicity of acrolein. ALDH metabolizes acrolein to the less-reactive acrylic acid. Silva and O'Brien (1989) showed that incubating rat hepatocytes with inhibitors of ALDH resulted in increased toxicity and greater depletion of GSH. Oxidation of acrolein by hepatic ALDH had been demonstrated to be a detoxification reaction (Rikans, 1987). Acrolein (0.4 mM) was lethal to cultured rat liver cells; inhibition of lipid peroxidation by disulfiram, an inhibitor of aldehyde dehydrogenase (ALDH), did not protect against lethality (Dogterom et al., 1988). A significant increase in lipid peroxidation ( $p < 0.01$ ) and a depletion of

GSH ( $p < 0.04$ ) also occurred within 5 minutes of exposure to 100  $\mu\text{M}$  acrolein (Watanabe et al., 1992). Acrolein was shown to irreversibly inhibit high affinity ALDH with a 91 and 33% reduction in mitochondrial and cytosolic ALDH activities, by incubating rat liver hepatocytes with 30  $\mu\text{M}$  acrolein (Mitchell and Petersen, 1988). N-acetylcysteine protects against acrolein-induced toxicity in isolated hepatocytes by presumably maintaining sulfhydryl levels (Dawson et al., 1984).

**Brain:** Recent evidence has established that increased lipid peroxidation is intimately involved in the pathogenesis of Alzheimer's disease and represents a marker of oxidative stress (Calingasan et al., 1999). Lovell et al. (2001) obtained evidence in brains (10) obtained from Alzheimer's patients at autopsy (8 age-matched controls) that acrolein is increased in brains of Alzheimer's patients; in hippocampal neuron cultures, acrolein was neurotoxic in a time- and concentration-dependent manner and disrupted calcium homeostasis.

Acrolein was found to be a potent inhibitor of ADP-induced mitochondria state 3 and calcium-induced respiration in whole brain mitochondria obtained from adult male Sprague-Dawley rats. It did not affect basal levels of state 3 respiration nor did it alter activity of complexes I-V nor alter mitochondrial calcium transporter activity or induce cytochrome c release (Picklo and Montine, 2001). Inhibition was prevented by GSH and N-acetylcysteine. These results were similar to those obtained using isolated rat hepatic mitochondria in which phosphate and glutamate transport were inhibited (Zollner, 1973). In isolated mitochondria from rat heart, acrolein did inhibit complex II-linked state 3 and uncoupled respiration (Biagini et al., 1990).

The concentration of acrolein required to induce a 50% change in sloughed cells, neurite formation, viability of sloughed cells, total cell number, and viability of harvested neuroblastoma cells, cultured for 24 hours, was reported to be 1, 7.6, 5.3, 580, and 30  $\mu\text{M}$ , respectively (Koerker et al., 1976). Neuronal survival was decreased to about 50% following 24 hours exposure to 600  $\mu\text{M}$  acrolein and less than 25% following 48 hour exposure (Smith et al., 1990b).

**Skin fibroblasts:** A one-hour exposure of Xeroderma pigmentosum cells to acrolein caused depletion of GSH and free protein thiols to a quantitative extent similar to that in normal skin fibroblasts without causing changes in the thiol redox state (Dypbukt et al., 1993).

**Tumor cells:** Acrolein was shown to be highly cytotoxic in two lung carcinoma cell lines and in a glioblastoma cell line (Rudra and Krokan, 1999); in one of the lung carcinoma cell lines, toxicity was partially reversed by vitamin E. Acrolein was shown to reduce AP-1 activation in human lung adenocarcinoma cells of the A549 cell line (Biswal, 2000). There was also an elevation in CYP2E1 and a >9-fold elevation in redox-related gene activity. In an earlier study of the A549 cell line (Horton et al., 1997), acrolein's ability to alter the proliferation of cells in vitro was dependent on cell density and total cell number.

**Transformed cells:** Four concentrations of acrolein failed to induce malignant transformation in C3H/10T<sub>1/2</sub> mouse embryo fibroblasts (Bioassay Systems Corp., 1982c).

**Immunotoxicity:** Topical administration of acrolein to the shaved skin of female guinea pigs (15) was shown not to result in positive skin reactions; positive controls were used (Susten and Breitenstein, 1990).

**Miscellaneous:** Survival of human umbilical artery cells was unchanged by exposure to 10  $\mu\text{M}$  acrolein, but reduced to 17 and 11% of controls by 50 and 100  $\mu\text{M}$  acrolein (Pino and Lyles, 1995). Inhibition of P450 in rat microsomal preparations by acrolein has been demonstrated by Gurtoo et al. (1981). Myeloperoxidase isolated from human neutrophils was

shown to convert L-threonine into acrolein (Anderson et al., 1997); activated neutrophils required the myeloperoxidase-H<sub>2</sub>O<sub>2</sub>-chloride system to produce acrolein in high yield. It was suggested that activated phagocytes have the potential to cause tissue damage at sites of inflammation.

**Toxicity of conjugates:** The toxicity of acrolein-thiol conjugates as well as acrolein mercapturates to human lung adenocarcinoma A549 cells was examined by Ramu et al. (1996). These conjugates were incubated with cells following a 2-hour exposure of cells treated with diethyl maleate (DEM) to deplete GSH. There was a dose-dependent inhibition of cell growth following treatment with acrolein, S-3-oxopropyl N-acetyl cysteine and its sulfoxide, with the sulfoxide also resulting in plasma membrane damage. A 24-hour (but not 2-hour) exposure of cells to S-3-oxopropylGSH also resulted in growth inhibition. Pretreatment with DEM increased the inhibition of cell growth seen with acrolein. Quantitative, pH, and rate considerations suggested that  $\beta$ -elimination of acrolein was not the sole mechanism of toxicity of S-3-oxopropyl N-acetyl cysteine and its sulfoxide. Acrolein itself has been demonstrated to inhibit NF- $\kappa$ B activation of A549 cells, consistent with formation of acrolein-NF- $\kappa$ B conjugates (Horton et al., 1999). NF- $\kappa$ B is a transcription factor controlling a number of genes, including those involved in proliferation and apoptosis.

Perry et al. (1995) evaluated the toxicity of several acrolein derivatives to A549 cells. No significant toxicity was observed with S-3-hydroxypropyl N-acetyl cysteine or S-3-oxopropylGSH. S-3-oxopropyl N-acetyl cysteine caused growth inhibition that was reversed by GSH and N-acetyl cysteine.

Eisenbrand et al. (1995) speculated that acrolein-GSH could potentially function as transport molecules for 2-alkenals, such as acrolein, if they reach tissues low in GSH and GST. These investigators found that, in the absence of GSH, acrolein-GSH conjugate decomposed slowly into aldehyde and GSH. The toxicological importance of GSH lies in its role as a substrate in detoxifying conjugation reactions catalyzed by glutathione S-transferase and as a substrate for GSH peroxidase which protects against membrane damage (Zitting and Heinonen, 1980). Incorporation of the GST isozyme P1-1 (Pi class) into Hep G2 cells was found to increase their resistance to acrolein toxicity suggesting that GSTs may play a role in cellular detoxication (Berhane et al., 1994). Comparison of the specific activities of GST A1-1 (alpha class), M1-1 (Mu class), and P1-1 indicated that the rate of reaction was in the order of A1-1 < M1-1 < P1-1.

#### **4.5.2. Intraperitoneal/Intragastric/Intravenous Toxicity**

Intraperitoneal administration of acrolein (0.5 to 6 mg/kg body weight) to male F344 rats was found to result in urinary bladder hyperplasia; hyperplasia was not observed upon intragastric administration at lethal levels (Sakata et al., 1989). The nephrotoxicity of a 1:1 acrolein-GSH adduct in the rat was examined by Horvath et al. (1992). Male Sprague-Dawley rats were given the adduct intravenously at 0.5 or 1 mmole/kg. In addition to gross and histologic changes in the kidney, glucosuria, proteinuria, and an elevation in serum urea nitrogen was observed. The nephrotoxicity was inhibited by acivicin, a  $\gamma$ -glutamyltranspeptidase inhibitor, indicating that metabolism through the first step in the renal mercapturic acid synthesis pathway is required.

### 4.5.3. Genotoxicity

#### 4.5.3.1. DNA adduct formation, sister chromatid exchange and DNA-protein crosslinks

Munsch et al. (1973) found that acrolein ( $2 \times 10^{-5}$  to  $8 \times 10^{-4}$ M) inhibited partially purified regenerating rat liver DNA polymerase, but not DNA polymerase I from *E. coli*. The site of action was at the -SH groups and was presumed to oxidize the -SH groups. In a confirmatory study, Munsch et al. (1974) observed that ( $^3$ H) acrolein was bound to regenerating rat liver DNA polymerase 10 to 20 times more than to *E. coli* DNA polymerase I, the latter which has no -SH groups at its active center. Acrolein has also been demonstrated to inhibit transcriptional activity of isolated liver nuclei from male Wistar rats and bacterial RNA polymerase (Moulé and Frayssinet, 1971).

Acrolein at concentrations of 5, 15, and 20  $\mu$ M, but not lower doses, induced significant increases in sister chromatid exchanges in cultured human lymphocytes (Wilmer et al., 1986). Acrolein was reported to induce formation of deoxyguanosine adducts at concentrations of 10  $\mu$ M, but not 4 or 7  $\mu$ M in *Salmonella* tester strain TA104 (Foiles et al., 1989). Lack of response at the lower doses suggests the presence of a saturable repair mechanism. Increasing adduct formation with dose was seen in tester strain TA100 at concentrations of 4 mM and greater. The responses are in agreement with increases in mutagenicity at about the same doses in these two tester strains. Using the same methodology, deoxyguanosine adduct formation increased progressively at concentrations ranging from 0.1 to 1 mM in Chinese hamster ovary cells (CHO) (Foiles et al., 1990). In studies by Chung et al. (1984), reaction of acrolein with deoxyguanosine or DNA under physiological conditions led to the formation of cyclic 1, $N^2$ -propanodeoxyguanosine and its adducts (Chung et al., 1984). Smith et al. (1990c) were able to determine the structure of an adduct formed from calf thymus DNA following acrolein exposure as 1, $N^6$ -propanodeoxyadenosine. Several putative adducts were observed in DNAs isolated from acrolein-treated human fibroblasts. One of these adducts was tentatively identified as the cyclic 1, $N^2$ -hydroxypropanodeoxyguanosine product, 3-(2'-deoxyriboyl)-5,6,7,8-tetrahydro-8-hydroxypyrimido[1,2-*a*]purine-10-one (Wilson et al., 1991). Chenna et al. (1992) reported that reaction of acrolein with thymidine resulted in one major product,  $N^3$ -(3''-oxopropyl)thymidine. Reaction of acrolein with 2-deoxyuridine under physiological conditions formed  $N^3$ -(3''-oxopropyl)-2'-deoxyuridine. This product was reduced to give  $N^3$ -(3''-hydroxypropyl)-2'-deoxyuridine (Chenna and Iden (1993).

Using a  $^{32}$ P-postlabeling method, Nath et al. (1996) found evidence of DNA-acrolein adducts (1, $N^2$ -propanodeoxyguanosine) in liver DNA of unexposed humans and untreated F344 rats, suggesting that they may be prevalent background lesions. Using the same technique, Nath and colleagues (Penn et al., 2001) found this adduct in aortic DNA of white leghorn cockerels exposed to 1 or 10 ppm acrolein for 6 hours. When cockerels were exposed to 1 ppm acrolein for 6 hr/day for 8 weeks to examine arteriosclerotic plaque formation potential, there was no effect of exposure on plaque development.

DNA-protein crosslinks were increased between calf thymus DNA and histone at acrolein concentrations of about 25  $\mu$ M, based upon graphic data, although numbers were not reported (Kuykendall and Bogdanffy, 1992). Costa et al. (1997) reported significant ( $p < 0.05$ ) increases in DNA-protein crosslinks in human Burkitt's lymphoma cells exposed 4 hours at concentrations greater than 150  $\mu$ M acrolein. It is uncertain if the differences in sensitivity reported are due to differences in methodology or cell type.

Inhalation exposure of male F344 rats to 2 ppm acrolein for 6 hours did not cause detectable DNA-protein cross-linking in the nasal respiratory mucosa whereas cross-linking was observed under in vitro conditions (Lam et al., 1985). It was hypothesized that acrolein reacted preferentially with sulhydryl-containing nucleophiles.

DNA single strand breaks were induced by acrolein in cultures of Namalva cells, a human lymphoblastic cell line poor in deactivating enzymes and low in GSH and in GST activity, at much lower concentrations than needed in primary rat hepatocytes (Eisenbrand et al., 1995). Acrolein caused a higher extent of DNA single-strand breaks (SSB) in XP cells (which normally have < 5% of excision repair capacity of normal cells) than normal cells (Dypbukt et al., 1993). Exposure to acrolein followed by incubation in fresh medium resulted in continued formation of DNA SSB in normal cells without further accumulation in XP cells.

#### 4.5.3.2. Mutagenic effects of acrolein in *Drosophila melanogaster*

Effects of acrolein on somatic mutations in *Drosophila* are shown in Table 3. Vogel and Nivard (1993) reported positive effects only with inhalation at toxic exposure levels, but not in feeding studies of larvae. Sierra et al. (1991), on the other hand, reported positive effects in feeding studies under similar conditions. Reasons for the difference in findings are uncertain although they could be due to the use of 48 hour cultures by Vogel and Nivard (1993) compared with 72 hour cultures by Sierra et al. (1991).

Effects of acrolein on sex-linked recessive lethals (SLRLs) in *Drosophila* are shown in Table 4. No effects of acrolein were reported for SLRL induction in feeding studies, but highly significant increases were noted in injection studies at high mM concentrations (Sierra et al., 1991). No effect on percent lethals, either by injection (200 ppm) or feeding (3,000 ppm) was observed in Canton-S wild-type males (Zimmering et al., 1985). Excision repair deficient *Drosophila* (*mus201*) mutations induced a greater incidence of SLRLs than repair efficient females. Based upon statistical analysis to evaluate hypermutability, it was concluded by the authors that acrolein induces lesions that are partially repaired by excision repair mechanisms. Since cyclic adducts can be repaired by excision mechanisms (Vogel 1989), and the only acrolein-derived lesions reported to date are cyclic adducts (Foiles et al., 1989; Smith et al., 1990b; Wilson et al., 1991), it appears that at least some of the SLRLs are derived from cyclic adducts. The response of *Drosophila* to acrolein with *mus308* mutation, which is thought to play a role in repair of cross-linking adducts, was no different than normal mice (Commendador et al., 1992; Barros et al., 1994a). These results thus provide additional support for the likelihood that cyclic adducts are the predominant forms induced by acrolein.

Barros et al. (1994b) tested the effects of metabolic modification upon induction of SLRLs. Diethyl maleate, a GSH-depleting agent, induced an increase in SLRLs in feeding studies with acrolein-exposed *D. melanogaster* (*Berlin K* and *Muller-5* strains). Phenobarbital, a cytochrome P450 inducing agent, eliminated response to acrolein via injection. Iproniazid and 1-phenylimidazole, potent inhibitors of cytochrome P450 oxidative enzymes of *Drosophila*, had no effect on SLRL induction by injection of acrolein. These results support the hypothesis that acrolein is a direct mutagen. Moreover, acrolein is deactivated by enzymatic activity induced by phenobarbital. The results also indicate that sensitivity to acrolein by the oral route is relatively low. This may be a function of its reactivity, with little reaching the reproductive organs by way of food.

**TABLE 3. Effects of acrolein on somatic mutations in *Drosophila melanogaster***

Sex	Conc (mM)	Dose Method	Endpoint	% spots	Average clone size	Reference
both	0	food	eye spots	4.2	2.9	Vogel and Nivard (1993)
both	10	food	eye spots	3.0 (-)	2.4	Vogel and Nivard (1993)
both	20	food	eye spots	4.1 (-)	3.8	Vogel and Nivard (1993)
both	80	food	eye spots	4.0 (-)	2.1	Vogel and Nivard (1993)
both	0	inhalation	eye spots	4.7	4.1	Vogel and Nivard (1993)
both	500 ppm	inhalation	eye spots	8.9 (+)	4.1	Vogel and Nivard (1993)
both	1,000 ppm	inhalation	eye spots	4.4	4.0	Vogel and Nivard (1993)
both	2,000 ppm	inhalation	eye spots	lethal	—	Vogel and Nivard (1993)
male	0	food	eye spots	1.4	5.0	Sierra et al. (1991)
male	5	food	eye spots	5.5 (+)	4.1	Sierra et al. (1991)
male	10	food	eye spots	3.8 (+)	4.7	Sierra et al. (1991)
male	20	food	eye spots	11.6 (+)	5.7	Sierra et al. (1991)
female	0	food	eye spots	3.3	7.0	Sierra et al. (1991)
female	5	food	eye spots	7.4 (+)	11.2	Sierra et al. (1991)
female	10	food	eye spots	7.4 (+)	4.4	Sierra et al. (1991)
female	20	food	eye spots	16.6 (+)	6.9	Sierra et al. (1991)
both	0	food	wing spots	17.5	1.3	Sierra et al. (1991)
both	5	food	wing spots	21.1 (-)	1.6	Sierra et al. (1991)
both	10	food	wing spots	29.3 (+)	1.8	Sierra et al. (1991)
both	20	food	wing spots	29.6 (+)	3.0	Sierra et al. (1991)

A statistical analysis was conducted according to Frei and Wurgler (1988): +, positive; -, inactive.

#### 4.5.3.3. Tests for gene mutation in mammalian cell cultures

Although acrolein has been shown to induce DNA adducts in a variety of cell types as well as mutagenesis in *Drosophila* and microorganisms under certain conditions, there is limited information regarding the ability of acrolein to induce mutations in normal mammalian cells. Acrolein was shown to be highly mutagenic to human fibroblast cells characterized by a deficiency in DNA repair (cells from xeroderma pigmentosum patients). While a positive dose response was observed between 0.2 and 0.8  $\mu\text{M}$  acrolein in the repair deficient cells, acrolein did not induce an increase in the mutant frequency of normal fibroblasts (Curren et al., 1988).

Acrolein was also mutagenic in V79 cells deficient in DNA repair (Smith et al., 1990a), but V79 cells with efficient repair mechanisms were not tested. *In vitro* chromosomal studies of acrolein have produced weakly positive findings in Chinese hamster ovary (CHO) cells (Au et al., 1980), and in cultured human lymphocytes (Wilmer et al., 1986). Chromosomal aberrations were not detected in CHO cells either in presence or absence of metabolic activation (Bioassay Systems Corp., 1982d) or in rat bone marrow cells (Bioassay Systems Corp., 1982e).

**TABLE 4. Effects of acrolein in the induction of sex-linked recessive lethal (SLRL) mutations in *D. melanogaster* exposed for 5 hr (feeding) and by injection.**

Concentration (mM)	Dose Method	Stage of Meiosis	Other Treatments	Percent Lethals	Reference
0.00 (broods pooled)	food	post	none	0.5	Sierra et al. (1991)
0.50	food	post	none	0.33	
1.00	food	post	none	0.16	
2.50	food	post	none	0.15	
5.00	food	post	none	0.16	
10.00	food	post	none	0.50	
5.00 (24-hr exposure)	food	post	none	0.19	
0.00	injection	post	none	0.17	
2.00	injection	post	none	0.31	
3.00	injection	post	none	0.39*	
3.00	injection	post	none	0.92***	
5.00	injection	post	none	0.61***	
5.00	injection	post	none	1.01***	
7.00	injection	post	none	0.60***	
7.00	injection	post	none	0.34	
0.00	injection	post	<i>mus201</i>	0.35	Barros et al. (1994a)
2.00	injection	post	<i>mus201</i>	0.82**	
3.00	injection	post	<i>mus201</i>	0.99***	
5.00	injection	post	<i>mus201</i>	1.06***	
0.00	injection	post	<i>mus308</i>	0.24	
1.00	injection	post	<i>mus308</i>	0.41*	
2.00	injection	post	<i>mus308</i>	0.25	
3.00	injection	post	<i>mus308</i>	1.41**	
5.00	injection	post	<i>mus308</i>	0.31	
0.00	injection	post	DEM	0.08	Barros et al. (1994b)
0.00	injection	pre	DEM	0.09	
3.00	injection	post	DEM	0.50*	
5.00	injection	post	DEM	0.46*	
5.00	injection	pre	DEM	0.14	
7.00	injection	post	DEM	0.20	
0.00	injection	post	PB	0.27	
0.00	injection	pre	PB	0.10	
3.00	injection	post	PB	0.30	
3.00	injection	pre	PB	0.03	

**TABLE 4 (cont'd). Effects of acrolein in the induction of sex-linked recessive lethal (SLRL) mutations in *D. melanogaster* exposed for 5 hr (feeding) and by injection.**

Concentration (mM)	Dose Method	Stage of Meiosis	Other Treatments Treatments	Percent Lethals	Reference
5.00	injection	post	PB	0.18	
5.00	injection	pre	PB	0.00	
0.00	injection	post	PHI + IPR	0.10	
0.00	injection	pre	PHI + IPR	0.12	
0.30	injection	post	PHI + IPR	0.18	
0.30	injection	pre	PHI + IPR	0.17	
0.50	injection	post	PHI + IPR	0.36*	
0.50	injection	pre	PHI + IPR	0.13	
0.70	injection	post	PHI + IPR	0.37*	
0.70	injection	pre	PHI + IPR	0.17	

*mus201* maternal excision repair deficiency.

*mus308* hypersensitive to crosslinking agents.

PB (phenobarbital): Induces xenobiotic metabolism.

DEM (diethylmaleate): Glutathione-depleting agent.

PHI (1-phenylimidazole): Inhibitor of cytochrome P450.

IPR (ipronazid): Inhibitor of cytochrome P450.

\* (p<0.05), \*\* (p<0.01), \*\*\* (p<0.001).

More recently Parent et al. (1991a) failed to detect mutagenic effects of acrolein using the sensitive Chinese hamster ovary hypoxanthine-guanine phosphoribosyl transferase (HGPRT) forward mutation assay system both with and without exogenous activation, even at toxic dose levels. These results confirmed earlier findings in which acrolein was found not to induce mutations at the HGPRT locus in CHO cells (Bioassay Systems Corp., 1982b). In order to determine the spectrum of mutations that acrolein may induce, a molecular analysis of mutations induced by acrolein in human fibroblast cells using *supF* shuttle vector plasmids was carried out by Kawanishi et al. (1998). The majority were base substitutions (76%) and the others were deletions and insertions (24%). Single base substitutions were most frequently found (46%), while multiple base substitutions accounted for 18% and tandem (two adjacent) base substitutions were 12%. Of the base substitutions, G:C to T:A transversions were 44% and G:C to A:T transitions were 24%.

#### 4.5.3.4. Tests for gene mutation in bacterial cells

Results are summarized in Table 5. In tests for frameshift mutagens without metabolic activation (TA98 and TA1538), TA98 gave some positive responses while TA1538 was negative. The only positive response for TA98 with S-9 activation was reported by Claxton (1985). With this exception, metabolic activation generally resulted in negative responses in all strains. Tests for base repair and point mutations (TA100, TA104 and TA1535) were positive in some tests with TA100, in most tests with TA104, but not with TA1535. TA104 has been reported to be more sensitive to carbonyl compounds (Marnett et al., 1985). Among strains

**TABLE 5. Tests for gene mutation in bacterial systems**

Species/Strain	Result <sup>a</sup>		Test Type	Reference
	+S9	-S9		
<i>Salmonella typhimurium</i> TA98	-	-	Reverse mutation	Basu and Marnett (1984)
	+	+		Claxton (1985)
	-	-		Haworth et al. (1983)
	-	-		Florin et al. (1980)
	-	+		Lijinsky and Andrews (1980)
	-	-		Loquet et al. (1981)
	-	±		Parent et al. (1996)
<i>Salmonella typhimurium</i> TA100	-	-	Basu and Marnett (1984)	
	toxi	+	Eder et al. (1993)	
	-	-	Florin et al. (1980)	
		+	Foiles et al. (1989)	
	±	-	Haworth et al. (1983)	
	-	-	Lijinsky and Andrews (1980)	
	-	-	Loquet et al. (1981)	
<i>Salmonella typhimurium</i> TA102	-	+	Lutz et al. (1982)	
	-	+	Parent et al. (1996)	
	-	-	Jung et al. (1992)	
<i>Salmonella typhimurium</i> TA104	-	-	Parent et al. (1996)	
	-	-	Watanabe et al. (1998)	
	0	+	Foiles et al. (1989)	
<i>Salmonella typhimurium</i> TA1535	-	+	Hoffman et al. (1989)	
	0	+	Marnett et al. (1985)	
	-	-	Parent et al. (1996)	
	-	-	Florin et al. (1980)	
	-	±	Hales (1982)	
<i>Salmonella typhimurium</i> TA1537	-	-	Haworth et al. (1983)	
	-	-	Lijinsky and Andrews (1980)	
	-	-	Loquet et al. (1981)	
	-	-	Parent et al. (1996)	
	-	-	Florin et al. (1980)	
<i>Salmonella typhimurium</i> TA1538	-	-	Haworth et al. (1983)	
	-	-	Lijinsky and Andrews (1980)	
	-	-	Parent et al. (1996)	
	-	-	Basu and Marnett (1984)	
<i>Salmonella typhimurium</i> TA1538	-	-	Lijinsky and Andrews (1980)	
	-	-	Parent et al. (1996)	
	-	-	Basu and Marnett (1984)	

**TABLE 5 (cont'd). Tests for gene mutation in bacterial systems**

Species/Strain	Result <sup>a</sup>		Test Type	Reference
	+S9	-S9		
<i>Salmonella typhimurium</i> <i>hisD3052/nopKM101</i>	-	-		Basu and Marnett (1984)
<i>Salmonella typhimurium</i> TA1535	0	-	SOS ( <i>umu</i> ) induction assay	Benamira and Marnett (1992)
<i>Escherichia coli</i> PQ37	0	+	SOS repair	Eder et al. (1993)
<i>Escherichia coli</i> HB101pUC13	0	+	DNA-histone crosslinks	Kuykendall and Bodanffy (1992)
<i>Escherichia coli</i> WP2 <i>uvrA</i>	-	±	Reverse mutation	Hemminki et al. (1980)
	±	-		Parent et al. (1996)
<i>Escherichia coli</i> WP2/pKM101	0	-		Watanabe et al. (1998)
<i>Escherichia coli</i> WP2	0	-		Watanabe et al. (1998)
<i>Escherichia coli</i> JTG10	0	+		Nunoshiha and Yamamoto (1999)
<i>Escherichia coli</i> AB1157	0	+		
<i>Escherichia coli</i> WP2	0	-		Aikawa and Miwa (1993)
<i>Escherichia coli</i> WP2( <i>uvrA</i> )A155	0	-		
<i>Escherichia coli</i> ZA159( <i>uvrB</i> )	0	-		

<sup>a</sup> + : ≥ 2 x background rate or statistically significant (P < 0.05); ± : equivocal; - : negative; 0 : not tested.

sensitive to crosslinking (TA102, TA2638, WP2 and *Escherichia coli* HB101), TA2638 and HB101 were positive in the only study reported for each strain, while TA102 and WP2 strains were negative. The *Escherichia coli* strains JTG10 and AR1157, which are lacking in GSH synthetase, are sensitive to induction of mutations as well as induction of cytotoxicity at very low concentrations.

It is clear from the studies reported that acrolein is highly reactive and cytotoxic. Acrolein has been shown to be mutagenic in some test systems within a narrow range of concentrations. Sensitivity to mutational effects is increased by GSH depleting agents and decreased by addition of metabolic activation, indicating that acrolein is a direct acting agent. While acrolein is capable of alkylating DNA and DNA bases (Maccubbin et al., 1990) and is known to inhibit purified DNA methylase activity from liver and bladder (Cox et al., 1988), it may never reach the target tissues of whole animals other than those at the site of insult. Even in the *in vitro* assays cited, acrolein is so reactive that special techniques must generally be employed to reduce cytotoxicity to induce positive effects. Parent et al. (1996) have suggested that the reactivity of acrolein precludes its reaching target cells at a sufficient concentration to initiate the carcinogenic process.

#### 4.5.4. Mechanistic Studies

A number of *in vitro* and *in vivo* studies demonstrated that acrolein has the potential to perturb the environments of human and laboratory animal cells in which GSH plays an important role, suppress host defense mechanisms, and elicit pro-inflammatory processes.

GSH depletion can lead to lipid peroxidation (Zitting and Heinonen, 1980). GSH protects cells by removing reactive metabolites such as electrophilic carbonium ions. Thus, GSH depletion deprives the cell of its natural defense against ubiquitous reactive metabolites and leaves the thiol groups in critical proteins vulnerable to attack by oxidation, crosslinking, and the formation of mixed disulfides or covalent adducts. For example, cellular constituents of the antioxidant defense system, including ascorbic acid,  $\alpha$ -tocopherol, GSH peroxidase, and catalase in rat lung were decreased following inhalation exposure to acrolein (Aramugam et al., 1999a). This led to enhanced lipid peroxidation, which produced extensive lung damage as indicated by elevated levels of the biochemical markers--angiotensin converting enzyme, LDH, protein, and lactate in the bronchioalveolar lavage.

The depletion of GSH by acrolein has clearly been established in cultured endothelial cells and rat hepatocytes (Patel and Block, 1993), in human bronchial epithelial cells (Grafström et al., 1988) and human and rat phagocytic cells (Witz et al., 1987). *In vivo* exposure resulted in GSH depletion in nasal respiratory mucosa (McNulty et al., 1984). This is likely due to the highly reactive nature of acrolein which reacts by virtue of its allylic function with GSH and similar compounds (Zitting and Heinonen, 1980). Meacher and Menzel (1999), using cultured adult rat type II alveolar cells, demonstrated with a fluorogenic reagent that the depletion of GSH by 1-5  $\mu\text{mol/L}$  of acrolein follows the nonenzymatic rate constant for the forward reaction. In addition, rates of GSH depletion by other alkenals and alkanals correlated with LD50 values for each compound leading the authors to conclude that structure-activity relationships are useful for predicting toxicity of aldehydes.

Adams and Klaidman (1992) reported that acrolein and its GSH adduct glutathionylpropionaldehyde can directly induce oxygen radical formation *in vitro*. The enzymes xanthine oxidase and ALDH were found to interact with this adduct to produce  $\text{O}_2^{\cdot-}$  and  $\text{HO}\cdot$ . Acrolein was also oxidized by xanthine oxidase to produce acroleinyl radical  $\text{O}_2^{\cdot-}$ .

It would appear that when a reactive chemical, such as acrolein, comes in contact with a cell, its first site of attack is the plasma membrane. Srivastava et al. (1992) have, in fact, reported that in *in vitro* studies, acrolein interaction at low concentration inhibited rat liver plasma membrane enzymes (i.e., ATPases) to varying degrees and attacked membrane surface proteins, suggesting at least a superficial change could lead to changes in ion transport and membrane potential. Pompella et al. (1991), on the other hand, determined that alkylation of macromolecules by acrolein is not a major factor in liver cell injury. Although acrolein was observed to rapidly bind to cytosolic soluble proteins and membrane-bound thiols *in vitro*, acrolein avoided membrane-bound thiols *in vivo*, even after GSH depletion. Gurtoo et al. (1981) have obtained convincing evidence that acrolein binds to cytochrome P450 resulting in its denaturation.

The role of acrolein in suppressing host defense mechanisms is also an area of increasing research interest. Using cultured human alveolar macrophages, Li et al. (1997) demonstrated that acrolein *in vitro* inhibited the release of the cytokines IL-1 $\beta$ , TNF- $\alpha$ , and IL-12, and induced apoptosis and necrosis in human alveolar macrophages. Subsequently, Li and Holian (1998)

provided preliminary information that inhibition of the transcription factor for many cytokine genes, NF- $\kappa$ B, may be responsible for the inhibition of cytokine release as well as acrolein induced apoptosis in alveolar macrophages. Most recently, Li et al. (1999) found that acrolein inhibited phosphorylation of the principal regulator of NF- $\kappa$ B. The activated form of NF- $\kappa$ B is of relevance to genes encoding cytokines involved in immune and proinflammatory responses, including viral genomes such as human immunodeficiency virus, type 1 (Müller et al., 1993). NF- $\kappa$ B also plays a central role in expression of adhesion molecules in human vascular endothelial cells (Collins et al., 1995). Inasmuch as acrolein in vitro acts as an inhibitor of NF- $\kappa$ B activity, immunomodulation by acrolein should be regarded as an area for further investigation, particularly at environmental levels.

Acrolein also has been demonstrated to inhibit in a dose-dependent manner the in vitro synthesis of prostaglandin E<sub>2</sub> in rat resting and zymosan-stimulated alveolar macrophages (Grundfest et al., 1982). This resulted in a relative increase in release of thromboxane B<sub>2</sub>, the inactive form of the potent vasoconstrictor, thromboxane A<sub>2</sub>. GSH protected the macrophages from acrolein-induced changes in arachidonic acid metabolism. Acrolein was found to increase bronchial reactivity to intravenously administered acetylcholine in guinea pigs with a maximum at 2-4 hours postexposure. Upon bronchoalveolar lavage, thromboxane B<sub>2</sub> and prostaglandin F<sub>2 $\alpha$</sub>  was shown to be increased immediately after exposure followed by an influx of neutrophils 24 hours later (Leikauf et al., 1990). Prostaglandin F<sub>2 $\alpha$</sub>  has been demonstrated to increase bronchial reactivity in asthmatics (Mathe et al., 1973). Evidence suggests that acrolein-induced bronchial hyperresponsiveness may be the result of damage to epithelial cells (Costa et al., 1986).

Using freshly isolated rat tracheal smooth muscle myocytes, Hyvelin et al. (2000) found that acrolein modulates the Ca<sup>++</sup> signaling pathway by increasing production of inositol triphosphate and does not directly affect the muscarinic cholinergic receptor or inositol triphosphate receptor sensitivity. This extends previous work (Ben-Jebria et al., 1993, 1994) in which it was reported that acrolein exposure increased the reactivity of human bronchial and rat tracheal rings to muscarinic agents in a dose-dependent manner.

The mode of action whereby acrolein produces nasal irritation in Fischer 344 rats has been investigated by Morris et al. (1999). At 20 ppm for 50 min, acrolein induced vasodilation and plasma protein extravasation into nasal tissues which were inhibited by capsaicin. Vasodilation, but not protein extravasation, was also elevated over controls at 2, 5, and 10 ppm (4.6, 11.4, and 22.9 mg/m<sup>3</sup>). Inhibition by capsaicin was regarded as evidence of C-fiber involvement. While there was evidence of tachykinin release, substance P and neurokinin were not thought to be involved. On the other hand, exposure of female Wistar rats to 22, 81, and 249 ppm (50.4, 185.5, and 570.2 mg/m<sup>3</sup>) acrolein for 10 minutes resulted in a significant decrease in nerves of the trachea immunoreactive for substance P, less so for calcitonin gene-related peptide, with the effect spreading further down the respiratory tract with increasing dose (Springall et al., 1990). There appeared to be no evidence of nerve damage.

It is clear that GSH plays a major role in acrolein toxicity. The depletion of GSH and the formation of acrolein GSH adducts resulting in an increase in reactive oxygen species is undoubtedly a major factor in the induction of toxic and mutagenic effects. Although membrane binding, inhibition of regulatory proteins, and modulation of cytokine release at the gene transcription level have been demonstrated, their importance at low levels of exposure is still uncertain.

## **4.6. SYNTHESIS AND EVALUATION OF MAJOR NON-CANCER EFFECTS AND MODE OF ACTION—ORAL AND INHALATION**

### **4.6.1. Oral administration**

No human studies are available regarding exposure by the oral route. Several animal studies are available.

An NTP (1995) 13-week gavage study in Fischer 344 rats (up to 10 mg acrolein/kg) and B6C3F1 mice (up to 20 mg/kg) has been made available in the form of the Chairperson's Report of the Pathology Working Group. Principal effects were forestomach and glandular stomach lesions in both species with the NOAEL at 0.75 mg/kg (rats); mortality only occurred in high-dose animals; there was no NOAEL for the mouse. However, 0.5 mg/kg was an FEL in the gavage study in the Sprague-Dawley rat (Parent et al., 1992c). Mortality over a two year span in females was reported as statistically significant in the high-dose group and marginally significant in the mid-dose group.

The cause is not readily apparent inasmuch as there was an absence of histopathological lesions. Differences in the strains of the test animals, in concentration of the daily dosing solution, study length, or vehicles used are possible factors in the inconsistent results of the NTP and Parent studies. In an 18-month chronic gavage study in CD-1 mice (Parent et al., 1991b), there were no dose-related adverse histopathological or clinical findings. The primary effect was increased mortality only in high-dose males.

Arumugam et al. (1999b) reported oxidative damage to hepatocyte mitochondria and decreasing activities of the citric acid cycle enzymes in Wistar rats intubated with 2.5 mg/kg bw/day acrolein, daily for 45 days. While this dose level is a clear-cut adverse effect level for a subacute exposure, the use of a 45-day exposure period and only one dose level limits the usefulness of this study for quantitatively assessing risk from lifetime exposure.

In the beagle dog 53-week study (Parent et al., 1992a), 2 mg/kg/day was found to be an adverse effect level inasmuch as significant decreases in serum protein, albumin and calcium were observed. Because of inadequate reporting it is uncertain if adverse effects occurred at the lower doses. It is also likely that actual exposure levels were lower than those reported because vomiting, which increased in a dose-related manner, can be expected to have eliminated some of the acrolein.

### **4.6.2. Inhalation Exposure**

#### **Acute Exposures:**

In the few clinical studies that have examined the effects of low-level acrolein exposure, it is clear that levels considerably lower than 1 ppm elicit subjective complaints of eye and nasal irritation and a decrease in the respiratory rate. Such effects should be considered adverse inasmuch as longer-term studies in laboratory animals at higher concentrations have demonstrated more severe nasal lesions as well as pronounced adverse effects on lung function leading to lethality.

Acrolein was reported by male and female volunteers as causing eye irritation at 0.09 ppm (0.2 mg/m<sup>3</sup>) for 5 minutes, nasal irritation at 0.15 ppm (0.3 mg/m<sup>3</sup>) for 15 minutes, and a decrease in respiratory rate at 0.6 ppm (1.4 mg/m<sup>3</sup>) for 35 minutes (Weber-Tschopp et al., 1977).

In an inhalation study by Sim and Pattle (1957) male volunteers reported 0.8 ppm (1.9 mg/m<sup>3</sup>) for 10 minutes acrolein as extremely irritating.

Signs of respiratory distress and irritation were noted in rats exposed to as low as 4.8 ppm (11 mg/m<sup>3</sup>) for one hour (Ballantyne et al., 1989). These clinical indicators were not observed when rats were exposed to levels of 0.25 to 1.4 ppm for 6 hours or 6 hr/day for three days (Cassee et al., 1996b). Nor were there any acrolein-induced histopathological nasal lesions after 6 hours of exposure. Other exposure studies of laboratory animals involved much higher concentrations with expected results of lethality associated with respiratory distress.

The weight-of-evidence suggests it would be prudent to regard 0.09 ppm (0.2 mg/m<sup>3</sup>) as a human reference level of concern for acute exposures of < 1 hour. The limited human data as well as data from animal studies at higher concentrations and longer durations suggest that clinical symptoms of distress (and histopathological lesions in the case of laboratory animals) become more pronounced as exposure increases. It becomes more problematic to identify reference levels of concern for durations beyond 1 hour based on the human data available.

In order to protect humans against acrolein-induced symptoms at longer exposure durations, it may be desirable to employ a default intraspecies uncertainty factor of 10 to the 0.09 ppm value, in the same manner as has been done to derive the RfC shown in Section 5.1.4. The resultant value of 0.009 ppm (0.02 mg/m<sup>3</sup>) can be considered as protective against adverse lung effects and nasal/eye irritation .

#### **Long-term exposures:**

No chronic studies involving human or laboratory animal exposure are available. Two studies with exposures of 90 days and 4 with exposures of 60 days or more have been reported. Feron et al. (1978) exposed groups of Syrian golden hamsters, Wistar rats, and Dutch rabbits 30 hours/week for 90 days to 0, 0.4, 1.4, and 4.9 ppm (0.9, 3.2, and 9.2 mg/m<sup>3</sup>) acrolein. At the highest dose, mortality occurred in rats, while ocular and nasal irritation, growth depression and histopathologic changes in the respiratory tract were seen in all three species. At the intermediate dose, squamous metaplasia and neutrophilic infiltration of the nasal mucosa was seen in the rat, whereas in hamsters, minimal inflammatory changes were seen in the nasal cavity. No effects were detected in the nasal region in the mid and low-dose rabbits. Slight inflammatory effects were reported in the nasal mucosa of the rat, the most sensitive region at the lowest dose. The LOAEL for rats, the most sensitive species, is therefore 0.4 ppm for slight inflammatory changes of the nasal mucosa. The NOAEL for hamsters was determined to be 0.4 ppm and the LOAEL, 1.4 ppm, based on inflammatory changes in the nasal cavity. The NOAEL for rabbits was determined to be 1.4 ppm and the LOAEL 4.9 ppm.

Lyon et al. (1970) exposed rats, guinea pigs, dogs, and monkeys 24 hours/day for 90 days to 0, 0.22, 1.0 and 1.8 ppm (0, 0.5, 2.3, and 4.1 mg/m<sup>3</sup>) acrolein. One ppm may be a LOAEL with inflammation reported in several organs of one or more of the species. The principal deficiency in this study was the lack of use of concurrent control groups. In addition, it was not clear which changes were definitively related to exposure.

Acrolein is highly reactive and can induce toxicity in a variety of ways. Increases in reactive oxygen species resulting from reaction with and depletion of glutathione is considered the primary mechanism of toxicity (Zitting and Heinonen, 1980; Aramugam et al., 1999a), although reaction with cell membrane proteins and inhibition of regulatory proteins may also play a role.

As a result of acrolein's high degree of reactivity during inhalation, deposition occurs primarily in the nasal mucosa with accompanying pathological effects. As concentrations increase, penetration deeper into the respiratory system occurs with accompanying toxic effects. Effects were occasionally reported for other organs such as the liver (Lyon et al., 1970), but only at concentrations higher than those affecting the respiratory system and cause-effect is uncertain given acrolein's high reactivity. The nasal mucosa, therefore, is considered to be the critical target site in the most sensitive species (rat) with a LOAEL of 0.4 ppm (Feron et al., 1978). A NOAEL has not been established.

#### **4.7. WEIGHT-OF-EVIDENCE EVALUATION AND CANCER CHARACTERIZATION—SYNTHESIS OF HUMAN, ANIMAL, AND OTHER SUPPORTING EVIDENCE, CONCLUSIONS ABOUT HUMAN CARCINOGENICITY, AND LIKELY MODE OF ACTION**

Applying the criteria outlined in the Guidelines for Carcinogen Risk Assessment (U.S. EPA, 1986) for evaluating the overall weight-of-evidence for carcinogenicity to humans, acrolein is most appropriately designated a Group D-Inadequate Evidence for Carcinogenicity. Using the Draft Guidelines for Carcinogen Risk Assessment (U.S. EPA, 1999), the data are inadequate for an assessment of the human carcinogenic potential for the following reasons.

1. The two inhalation bioassays in laboratory animals are inadequate to make a determination because of protocol limitations.
2. Two gavage bioassays failed to show an acrolein-induced tumor response in two species of laboratory animals.
3. The findings of suggestive evidence of an extra-thoracic tumorigenic response in a drinking water study in female rats was not supported by a re-analysis of the data by an independently-convened pathology working group. In addition, there is information that suggests that administered levels of acrolein in this study were other than those reported.
4. Because of the high reactivity of acrolein, it is unlikely that inhalation of acrolein, the most important route of exposure, it would be distributed systemically in concentrations that would be expected to cause adverse effects.
5. Although positive mutagenic results in bacterial systems have occasionally been reported, these invariably occur at high concentrations, near the lethal dose.
6. There are no adequate human data.

#### **Human Studies:**

Ott et al. (1989) reported a series of nested case-control studies in relation to various work areas, specific chemicals, and chemical activity groups. An odds ratio of 2.6 for nonlymphocytic leukemia was found for workers who had exposure to acrolein during employment. The small number of cases (3) and the likelihood of exposure to other chemicals, however, precludes drawing a conclusion from this study. Thus, this study presents inadequate evidence for acrolein-induced leukemia.

### Laboratory Animal Studies:

Two cancer bioassays failed to show an increase in tumor incidence when rats (Parent et al., 1992c) and mice (Parent et al., 1991b) were administered acrolein by gavage. In both studies the Maximum Tolerated Dose was demonstrated by a significant increase in mortality.

Although administration (Lijinsky and Reuber, 1987) of acrolein in drinking water to female F344 rats resulted in an elevation of adrenocortical tumors (only in females) over 104-124 weeks (total dose=115 mmoles), the increase was only significant when the tumors were combined with hyperplastic nodules. However, the incidence of the tumors appeared to exceed the historical control range for female F344 rats reported by Goodman et al. (1979) and Solleveld et al. (1984).

Because of the difference in findings between the Parent et al. (1992c) and Lijinsky and Reuber, 1987) studies, an independent pathology working group (PWG) was convened to reevaluate the cortical tumors reported by Lijinsky and Reuber. According to the PWG (cited in Parent et al., 1992c), the “slightly elevated incidence of pheochromocytomas in the treated females were (*sic*) “well within limits for historical controls and were of no biological significance.” “It is the opinion of the PWG that there is no evidence of any carcinogenic effect of acrolein on the adrenal glands of female rats in this study.” However, the original slides for the high-dose females were not available for re-examination and only one of the original control slides was available. Thus, the PWG was unable to clarify the findings for cortical adenomas, the basis for the “positive” findings in the Lijinsky and Reuber study. An additional confounder is the lack of a carcinogenic response when rats were exposed to acrolein diethylacetal, acrolein oxime, and allyl alcohol, agents that can be expected to be hydrolyzed to acrolein in the stomach acids. Furthermore, Parent et al. (1992c), assuming that each of the four rats/cage in the Lijinsky and Reuber study drank the same amount of water, estimated a daily dose of 50 mg/kg bw at the highest concentration which exceeds the LD<sub>50</sub> for rats; this suggests that acrolein in solution was not as stable in solution as purported.

Evidence that acrolein may have some tumor-promoting activity was shown in the study by Cohen et al. (1992). Intraperitoneal injection of acrolein, 2 mg/kg bw for either 6 or 21 weeks into male Fischer 344 rats did not induce cancer, but 6 weeks treatment with acrolein, followed by uracil induced urinary bladder papillomas in 18 of 30 rats compared with 8 of 30 administered uracil alone. However, when the incidence of nodular hyperplasia (considered to be precursors to papillomas) was combined with the incidence of papillomas, significance (<0.05) was achieved with the the uracil-only group, but not with acrolein.

Based upon the (1) negative findings in two of three studies testing acrolein as a complete carcinogen, (2) questionable findings of the Lijinsky and Reuber study, and (3) significance of the results of the intraperitoneal study by Cohen et al. (1992), the animal carcinogenicity gavage data must be considered inadequate.

Two inhalation studies, one in hamsters (Feron and Kryusse, 1977) and one in rats (Le Bouffant et al., 1980) did not result in any carcinogenic response. However, the insufficient length and use of only one concentration in each study precluded any definitive conclusions about the carcinogenic potential of acrolein after inhalation.

### **Genotoxicity Studies:**

Mutagenicity and genotoxicity studies provide little support to the possibility that acrolein may be carcinogenic. Acrolein is capable of inducing sister chromatid exchange in cultured human lymphocytes, DNA crosslinking with protein, and mutations under certain conditions. *In vitro* assays with bacterial or mammalian cells either deficient in DNA repair or treated to deplete GSH were quite sensitive to the mutagenic and toxic effects of acrolein. While mutagenic activity has occasionally been shown, positive results generally occurred only in a narrow, near lethal, dose range. Moreover, because of its highly reactive nature and known irritant effects, acrolein is unlikely to reach systemic sites in mammalian species except at high dose levels.

### **Mode of Action:**

There is no evidence for uptake of acrolein in significant amounts. Acrolein and its GSH adduct does, however, directly induce oxygen radical formation *in vitro* (Adams and Klaidman, 1992) that could induce DNA damage. Extensive lung damage due to lipid peroxidation after inhalation exposure of rats to 1 or 2 ppm acrolein was demonstrated by Arumugam et al. (1999a); also antioxidant levels were significantly decreased. The high reactivity of acrolein, its lack of uptake, and the absence of adequate inhalation bioassay data preclude the identification of a mode of action leading to a potential carcinogenic response.

### **Conclusions:**

The available laboratory animal data are inadequate for an assessment of human carcinogenic potential. Based upon the negative findings in two gavage studies and a skin tumor initiation-promotion study, inadequate studies via inhalation, findings of uncertain significance from an intraperitoneal injection study, and questionable results in a drinking water study, the available animal carcinogenicity data are not sufficiently robust to categorize acrolein as not having carcinogenic potential, but rather must be considered to be inadequate. Although acrolein has been shown to be capable of inducing sister chromatid exchange, DNA crosslinking and mutations under certain conditions, its highly reactive nature and the lack of tumor induction at portals of entry make it unlikely that acrolein reaches systemic sites at biologically-significant exposure levels. Thus, using the Draft Guidelines for Carcinogen Risk Assessment (U.S. EPA, 1999), the potential carcinogenicity of acrolein by either the oral or inhalation routes of exposure cannot be determined because the existing data are inadequate for an assessment of human carcinogenic potential.

## **4.8. SUSCEPTIBLE POPULATIONS**

### **4.8.1. Possible Childhood Susceptibility**

No information is available regarding early life susceptibility to acrolein.

### **4.8.2. Possible Sex Differences**

No sex-related differences in toxicological responses to acrolein were reported in dogs exposed for up to 53 weeks orally to 2 mg/kg/day acrolein (Parent et al., 1992a). Sex-related differences were also absent in rats, guinea pigs, monkeys and dogs exposed 40 hours/week for

6 weeks, or continuously for 90 days to concentrations up to 3.7 ppm (8.5 mg/m<sup>3</sup>) acrolein (Lyon et al., 1970). Feron et al. (1978) reported no sex differences in responses among rats, hamsters and rabbits exposed to acrolein 30 hours/week for 13 weeks. Kutzman et al. (1985) reported that 32 of 57 male Fischer 344 rats exposed 30 hours/week to 4.9 ppm (9.2 mg/m<sup>3</sup>) acrolein died compared with none of 8 exposed females. While LD<sub>50</sub> values were nearly identical for male and female Sprague-Dawley rats in the Ballantyne et al. (1989) study, Parent et al. (1992c) found that female Sprague-Dawley rats intubated daily with 2.5 mg/kg acrolein had a statistically significant (p < 0.001) decrease in survival over a two-year period compared to male rats, which showed some evidence of dose-related mortality during the first year of treatment, but not the second. On the other hand, the Parent et al. (1991b) study in mice showed increased mortality in high-dose males only.

### **4.8.3. Other Susceptible Populations**

As noted in section 4.5 depletion of GSH increases sensitivity to cytotoxic effects and the induction of mutations. Also, male Wistar rats intubated with acrolein for 45 days evidenced decreased GSH levels leading to mitochondrial damage in liver. Individuals with metabolic defects, such as decreased ability to synthesize GSH, could be expected to be more sensitive to the toxicity of acrolein. Differences in cytochrome P450 activity may affect sensitivity in humans, although this possibility has not been tested.

## **5. DOSE-RESPONSE ASSESSMENTS**

### **5.1. ORAL REFERENCE DOSE (RfD)**

#### **5.1.1. Choice of Principal Study and Critical Effect—With Rationale and Justification**

There are no chronic human studies suitable for dose-response assessment. Long-term studies with Sprague-Dawley rats and CD-1 mice (Parent et al., 1991b; 1992c), dogs (Parent et al., 1992a), and Fisher-344 rats and B6C3F<sub>1</sub> mice (NTP, 1997) indicate that mortality and stomach lesions are the predominant effects. The two-year gavage study with the Sprague-Dawley rat (Parent et al., 1992c) is considered to be the most suitable for developing an RfD. This study was a lifetime study and used an adequate number of animals (70/sex/group), whereas only 10/sex were used in the 13-week NTP study. Statistically significant mortality at doses as low as 0.5 mg/kg/day was observed in female Sprague-Dawley rats over the two-year span of the study whereas mortality was only seen in high dose animals (10 mg/kg/day) of the NTP study. The dog study (6 animals/sex/group) was deemed unsuitable as a principal study because vomiting following administration of acrolein resulted in uncertain amounts of retained dose. Thus, the Parent et al. (1992c) study in rats was selected as the principal study with 0.5 mg/kg/day as the FEL and 0.05 mg/kg/day as the NOAEL, based on mortality as the critical effect.

### 5.1.2. Supporting Studies

Parent et al. (1992a) administered acrolein (0.1% aqueous) in gelatin capsules at doses of 0, 0.1, 0.5, and 1.5 mg/kg/day, 7 days/week for 53 weeks to 6 beagle dogs/group. After four weeks the high dose was increased to 2.0 mg/kg/day. At termination all dogs were subjected to full necropsy and histological examination. Body weights and food consumption were not significantly affected by treatment. The most commonly reported effect was a dose-dependent increase in the frequency of vomiting. The incidence, however, decreased greatly with duration of treatment. Observed treatment-related lesions on gross necropsy included vascular congestion and mucosal reddening of the gastrointestinal tract. The results of this study are difficult to evaluate. Although there were some alterations in blood parameters, they were unsupported by pathology evaluation. Some of the clinical parameters may have been changed as a result of vomiting. Moreover, adaptation appears to occur, as noted by the decreased vomiting with duration of exposure. Lack of changes in food consumption and body weight also suggest that any effects noted were mild.

Arumugam et al. (1999b) exposed male Wistar rats, 5 animals/group, daily to acrolein via intubation (2.5 mg/kg bw) for 45 days. This study clearly shows damage to mitochondria, through loss of mitochondrial lamellae, a decrease in the availability of GSH, a substrate for glutathione peroxidase, and a decrease in activities of citric acid cycle enzymes, resulting in decreased energy production in liver cells. The study was well designed and showed definitive effects. While the duration of the study was less than subchronic in duration and included only a single dose level it provides support and a plausible explanation for the mortality increases reported in the Parent et al. (1992c) study. It also indicates that at least a portion of an oral dose is absorbed and reaches the liver. Incidence of mortality, if any, was not reported in this study.

The NTP (1995) 13-week gavage study also reported significant mortality in rats and mice, but at much higher levels than in the Parent et al. (1992c) study. Principal effects were stomach lesions at doses as low as 0.75 mg/kg/day in mice.

### 5.1.3. Methods of Analysis

The method employed for development of an RfD was determination of an NOAEL for mortality in the Parent et al. (1992c) study and the application of uncertainty factors. A benchmark dose approach was unsuitable for RfD development because the data were presented graphically, with statistical evaluation at one and two-year time points but no numerical values.

### 5.1.4. RfD Derivation—Including Application of Uncertainty Factors (UFs) and Modifying Factors (MFs)

The dose level of 0.05 mg/kg/day is determined to be the NOAEL for mortality (Parent et al., 1992c). The adjusted NOAEL is modified by an uncertainty factor of 10 for interspecies adjustment, and a 10 for susceptible human subpopulations. Based on gavage studies in rats and rabbits, it does not appear that acrolein causes reproductive or teratogenic effects. Application of the total uncertainty factor of 100 to the NOAEL of 0.05 mg/kg/day gives the reference dose of  $5 \times 10^{-4}$  mg/kg/day.

## **5.2. INHALATION REFERENCE CONCENTRATION (RfC)**

### **5.2.1. Choice of Principal Study and Critical Effect**

Chronic inhalation studies designed to evaluate the toxicological effects of acrolein are unavailable. Previously on IRIS, the Kutzman et al. (1981, 1985) study was cited as co-principal along with the Feron et al. (1978) study. A reexamination concluded that the Kutzman et al. study suffered limitations that precluded its choice as a co-principal study. Discussion of these limitations is provided in Section 5.2.2.

The most suitable study for development of an RfC is Feron et al. (1978). In this study, 10 Syrian golden hamsters/sex/dose, 6 Wistar rats/sex/dose, and 2 Dutch rabbits/sex/dose were exposed 6 hours/day, 5 days/week for 13 weeks to 0, 0.4, 1.4, or 4.9 ppm (0.9, 3.2, or 11.0 mg/m<sup>3</sup>) acrolein. Hematological parameters were unaffected by acrolein in rats and rabbits. However, female hamsters in the high-dose group exhibited statistically significant increases in red cell counts, packed cell volume, hemoglobin content, and number of lymphocytes accompanied by a decrease in neutrophils. Body weight gain was significantly inhibited at the high dose in rats and rabbits and less so at the intermediate dose, but food consumption appeared to be decreased in these groups as well. At the intermediate dose, only male and female rats showed significantly retarded weight gain ( $p < 0.05$ ). Three male and three female rats died during exposure at the highest dose ( $p$  value not reported). No other deaths considered to be treatment-related were reported in any of the species or dose groups. Histopathological changes were graded as minimal, moderate or marked.

Minimal histopathologic changes were found in the nasal cavity of rats exposed to 0.4 ppm, and none were reported in other species at this concentration. The severity of nasal lesions was dose-related in all three species, most clearly so in the rat. The 1.4 ppm level induced moderate histopathology in the nasal cavity of rats and minimal histopathology in the nasal cavity of hamsters. In the 4.9 ppm groups of all three species, slightly to markedly increased lesions was reported in the nasal cavity and trachea; moderate to marked effects were seen in the bronchi and lungs of rats and rabbits (but not hamsters). Based upon the dose-related severity of lesions, the rat is clearly the most sensitive species, with hamsters and rabbits intermediate in sensitivity.

Although the Feron et al. study was adequately designed, the incidence of nasal lesions for treated groups was not reported. Grading of histopathology allowed determination of NOAELs, LOAELs and FELs for the three species, determination of the critical target site, and a comparison of sensitivity among the three species tested. Other limitations of this study, apart from the lack of incidence data for nasal lesions, include an exposure duration of 3 months rather than lifetime, histopathological examination of only three sections of the nasal cavity, lack of characterization of the type of nasal lesions by sex, and only 6 rats/sex exposed.

### **5.2.2. Supporting studies**

The Kutzman et al. (1981, 1985) study is supportive in sense that acrolein exposure was associated with nasal effects. However, only male F344 rats were examined and the changes in the nasal region consisted of only minimal evidence of submucosal lymphoid aggregates at 0.4 ppm (0.9 mg/m<sup>3</sup>). Although the degree of involvement increased to moderate at higher

concentrations, more extensive damage to the nasal epithelium was not observed. The principal limitation was that histopathology was not performed until the sixth day post-exposure.

The results of the three-day inhalation study in male Wistar rats by Cassee et al. (1996b), in addition to supporting the findings by Feron and colleagues, suggest that levels lower than 0.4 ppm (the LOAEL in the Feron et al. study) causes adverse nasal effects. This may be the result of extended sectioning (six sections) of the nasal tract compared to only three in the Feron et al. study.

The Lyon et al. (1970) continuous 90-day inhalation study (dogs, guinea pigs, rats and monkeys), on the other hand, did not examine the nasal tract by light microscopy.

### 5.2.3. Methods of Analysis

The nasal cavity has been shown by Feron et al. (1978) to be the most sensitive target site for the pathological effects of acrolein. Use of a benchmark dose approach is not possible because nasal pathology incidence data were not provided. The approach used is, therefore, determination of an LOAEL with application of uncertainty factors.

### 5.2.4. RfC Derivation

The endpoint for development of an RfC is nasal histopathology (necrotizing rhinitis, keratinization, squamous metaplasia); nasal lesions appeared to be dose-related in severity (describes as slight to severely affected) in all three species (rat, rabbit, and hamster) of the Feron et al. (1978) study. The identified LOAEL is 0.4 ppm (0.92 mg/m<sup>3</sup>) which was associated with minimal nasal histopathology in the Wistar rat (1/6), the most sensitive of the three species each exposed to acrolein 6 hr/day, 5 days/week for 13 weeks. The LOAEL was adjusted to continuous exposure by 0.9 mg/m<sup>3</sup> x 6/24 hours/day x 5/7 days/week to equal 0.16 mg/m<sup>3</sup>. A Regional Gas Dose Ratio (RGDR) was derived using standardized minute volumes (0.23 and 0.17 m<sup>3</sup>/day) for male and female rats, respectively and 20 m<sup>3</sup>/day for humans (U.S. EPA, 1988) and surface areas of 11.6 sq. m (rat) and 177 sq. m (human) for the extrathoracic region (EPA, 1994) as follows:

$$\begin{aligned} \text{RGDR} &= \frac{\text{Minute volume (rat)} / \text{surface area (rat)}}{\text{Minute volume (human)} / \text{surface area (human)}} \\ &= \frac{0.23/11.6}{20/177} \\ &= 0.18 \end{aligned}$$

Applying the RDGR of 0.18 to the adjusted LOAEL of 0.16 mg/m<sup>3</sup> yields an equivalent human concentration (HEC) of 0.03 mg/m<sup>3</sup>. This latter value is identical to the value previously reported on the IRIS database.

- A default uncertainty factor (UF) of 10 is applied for adjustment from subchronic to chronic duration because there are insufficient inhalation data to preclude an increase in severity (or incidence) with an increase in exposure duration from subchronic to chronic.

- An uncertainty factor of three (10<sup>1/2</sup>) is adopted for use of the LOAEL of 0.4 ppm in lieu of a NOAEL. The three-day study in the male Wistar rat by Cassee et al. (1996b) found nasal

effects in the respiratory/transitional epithelium (six sections of nasal tract evaluated) at 0.25 ppm, suggesting a likelihood of effects below the 0.4 ppm level noted by Feron and colleagues.

- A default UF of 10 was applied for intraspecies variability in sensitivity. In the Kutzman et al. 62-day study (1981, 1985) in the male F344 rat, nasal lesions of the nature observed by Feron and colleagues were not observed, suggesting rat strain-dependent responses. Because the Sprague-Dawley has not been evaluated, an UF of 10 is deemed appropriate.

- A three ( $10^{1/2}$ ) was selected for interspecies variability in sensitivity based on application of a dosimetric adjustment explicit in the RfC methodology. Since the rat was shown to be more sensitive to nasal pathology than either hamsters or rabbits in the Feron et al. (1978) study, increased confidence that the rat is the most sensitive animal species obviates the need for the default UF of 10.

- No additional uncertainty factor for the lack of adequate inhalation reproductive and developmental studies was adopted because oral studies indicated that acrolein is unlikely to be a reproductive or teratogenic agent.

Application of a total uncertainty factor of 1,000 to the HEC of  $0.03 \text{ mg/m}^3$  yields an RfC of  $3 \times 10^{-5} \text{ mg/m}^3$ . This value is the same as the value previously listed on IRIS as the RfC.

### **5.3. CANCER RISK ASSESSMENT**

A dose-response assessment for carcinogenicity is precluded inasmuch as there is inadequate evidence to establish a link between exposure to acrolein and cancer.

## **6. MAJOR CONCLUSIONS IN THE CHARACTERIZATION OF HAZARD AND DOSE RESPONSE**

### **6.1. HUMAN HAZARD POTENTIAL**

Acrolein is a colorless to yellowish flammable liquid at room temperature with a disagreeable, choking odor. It is extremely acrid and irritating to mucous membranes. Acrolein and its derivatives are used as an intermediate in the synthesis of acrylic acid for making acrylates and DL-methionine, an essential amino acid. It is used as a herbicide and to control algae aquatic weeds and molluscs in recirculating process systems, growth of microorganisms in liquid fuel, growth of algae in oil fields, and the formation of slime in paper manufacture. It is also used in leather tanning and as a tissue fixative in histological work.

Acrolein is released to the air as a result of manufacturing processes, through incomplete combustion of petroleum fuels, as a component of cigarette smoke, and as a photooxidation product of hydrocarbon pollutants (ATSDR, 1990). Combustion of fuels represents a major source of emissions of acrolein to the atmosphere.

Inhaled acrolein is retained primarily in the upper respiratory tract (Egle, 1972) because of its high solubility and reactivity. No direct evaluations of uptake via oral administration have been reported. However, Draminski et al. (1983) identified a low level of acrolein derived conjugates in the urine of rats following oral dosing, while Arumugam et al. (1999) reported toxicological effects in the liver of rats exposed by daily intubation (acrolein in water) for 45 days, indicating at least some uptake by this route; however, the stomach was not examined by

light microscopy. A main pathway of metabolism for acrolein is the addition of GSH to the activated double bond followed by processing to mercapturic acid. Another pathway is that of epoxidation of the double bond followed by attack of glutathione on the epoxide. A third pathway involves addition of water to acrolein to form 3-hydroxypropionaldehyde, which can be further metabolized and ultimately incorporated into normal metabolic pathways (Parent et al., 1998).

Chronic human inhalation studies designed to evaluate toxicological effects of acrolein are unavailable. However, acute studies (Weber-Tschopp et al., 1977; Esterbauer et al., 1991) have documented that acrolein can cause pronounced eye and nasal irritation. Studies in laboratory animals indicate that principal target sites are the nasal membranes (Feron et al., 1978) and the lung (Lyon et al., 1970; Kutzman et al., 1981, 1985). When acrolein was administered to laboratory animals by gavage, the principal sites affected were the stomach (Parent et al., 1992a; NTP, 1995) and liver (Arumugam et al., 1999).

At present the carcinogenicity of acrolein cannot be determined by the inhalation route because of a lack of human data and adequate chronic bioassays in laboratory animals. Two chronic oral bioassays, one with rats (Parent et al., 1992c) and one with mice, were negative. Marginally positive effects were reported in another chronic oral study (Lijinsky and Reuber, 1987), but these results were rendered questionable following reevaluation of the tissues at a later date by a pathology work group. A weak tumor initiating effect was reported in an intraperitoneal injection study (Cohen et al., 1992), but results were negative in a skin tumor initiation study (Salaman and Roe, 1956).

Because of acrolein's reactivity, toxicity can be induced by more than one mode of action. A major mode of action, however, has been shown to be related to depletion of GSH. Reaction of acrolein with GSH deprives the cell of its natural defense against reactive oxygen species (Arumugam et al., 1999). Moreover, the acrolein GSH adduct has been shown *in vitro* to directly induce oxygen radical formation (Adams and Klaidman, 1992).

Using EPA's (U.S.EPA, 1999) draft cancer risk assessment guidelines, the weight-of-evidence for evaluation of the cancer hazard indicates that the data are inadequate for classifying the human carcinogenic potential of acrolein. Under EPA's (1986) cancer risk assessment guidelines, acrolein is most appropriately classified as Group D - Inadequate Evidence for Human Carcinogenicity.

## 6.2. DOSE RESPONSE

Data are inadequate to develop a cancer potency estimate for acrolein by either the oral or inhalation route.

A quantitative estimate of noncancer risk by both the oral and inhalation routes of exposure are based upon animal experiments since no adequate human data are available. An RfD of  $5 \times 10^{-4}$  mg/kg/day was derived based upon a NOAEL (with increased mortality as the critical effect) of 0.05 mg/kg/day in Sprague-Dawley rats (Parent et al., 1992c), modified by a 10-fold interspecies uncertainty factor and a 10-fold uncertainty factor for intraspecies variability in sensitivity.

Confidence in the study is high; several supporting studies involving other species also indicated that mortality increases sharply with elevated dose. Two studies (Parent et al., 1992b; Parent et al., 1993) provide evidence that reproductive and developmental effects are not critical

endpoints, supporting high confidence in the data base. The overall confidence in this RfD assessment is high; a variety of studies across different durations of exposure and in several different laboratory animal species has been consistent in demonstrating that in the absence of mortality there are no clear indications of adverse effects.

An RfC of  $2 \times 10^{-5}$  mg/m<sup>3</sup> was derived based upon a 13-week inhalation study with rats, hamsters and rabbits (Feron et al., 1978). The critical endpoint was a LOAEL for lesions of slight severity in the nasal epithelium of rats following 13 weeks exposure to 0.4 ppm (0.9 mg/m<sup>3</sup>) acrolein. Severity of the lesions increased with exposure concentration. This concentration (0.4 ppm) was a NOAEL for hamsters and rabbits in the same study although at the 4.9 ppm level severity of nasal lesions across the three species was similar.

The RfC was derived by duration adjusting the LOAEL of 0.9 mg/m<sup>3</sup> for 30 hour/week exposure to 0.16 mg/m<sup>3</sup> for continuous exposure. Applying an RGDR of 0.15 (EPA, 1994) to convert dose/unit surface area of the extrathoracic region in the rat to that in humans, results in an equivalent human concentration for continuous exposure of 0.02 mg/m<sup>3</sup>. A three-fold UF was applied for use of a LOAEL and three for interspecies, the latter being decreased from ten because of the use of a dosimetric adjustment, and also because neither hamsters and rabbits exhibited nasal effects at the study concentration of 0.4 ppm. Support for the point of departure is provided by NOAELs for 2 of the 3 species in the same study. The UF for adjustment from subchronic to chronic exposure was ten. Finally, an UF of ten was applied for intraspecies variability in sensitivity. Adjustment of the LOAEL of  $2 \times 10^{-2}$  mg/m<sup>3</sup> by a total uncertainty factor of 1,000 yields an RfC of  $2 \times 10^{-5}$  mg/m<sup>3</sup>.

Confidence in the study is medium. Although the RfC was based on an adequately-designed subchronic study in three species, for which a wide range of endpoints were measured, incidence data were not provided and only a small number of rats were exposed. These limitations coupled with the observation that levels lower (Cassee et al., 1996b) than the study LOAEL of 0.4 ppm are consistent with a medium confidence in the RfC. The primary limitation in the database is the lack of a chronic inhalation study and the attendant uncertainty relating to severity of nasal lesions as exposure duration increases.

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