A BRIEF REVIEW OF INHALATION TOXICOLOGY AND THE DEVELOPMENT OF A RESEARCH PROPOSAL TO DEMONSTRATE THE RELEVANCE OF AN ESTABLISHED MOUSE BIOASSAY TO BIODEFENSE OBJECTIVES

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Submitted to the Graduate Faculty of

The Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Master of Public Health

University of Pittsburgh

2006

UNIVERSITY OF PITTSBURGH

GRADUATE SCHOOL OF PUBLIC HEALTH

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Abstract

In a program announcement in 2005, The Office of Biodefense Research, the National Heart, Lung and Blood Institute, and the National Institutes of Health expressed concern about an issue of significant public health importance: the "US population's potential exposure to aerosolized, inhaled harmful chemicals possibly liberated as part of bioterrorism attacks against assembled groups of the civilian populace". The primary stated objective of the program was to "encourage research about how the upper respiratory tract and lungs respond to acute exposure to highly toxic chemicals and subsequent inhalation, so that preventive strategies can be improved, antidotes devised to lessen initial irritation of mucosal surfaces, mucosal absorption minimized, and acute lung injury causing pulmonary edema counteracted".

In the context of this objective, a review of the basic and applied science of inhalation toxicology was undertaken and a research proposal developed to demonstrate the relevance of an established mouse bioassay, documented to identify and quantify the effects of inhaled agents at all three regions of the respiratory tract, by evaluating the inhalation toxicity of methyl isocyanate, an agent known to affect all three regions of the respiratory tract. Human observational methyl isocyanate exposure data from the Bhopal industrial accident are available,

as are experimental animal exposure data, offering the opportunity to further establish the reproducibility and validity of the bioassay. In addition, the mouse biosassay detects effects of inhaled agents at concentrations below those at which histopathological changes occur, enabling the rapid screening of administered treatments and antidotes for effectiveness. This capacity is of fundamental importance in the development of future therapeutic agents.

With experience gained in the practical management of the experimental apparatus with the methyl isocyanate proposal, a study using the mouse bioassay to reproduce unpublished data investigating therapeutics against the inhalation toxicity of ricin would be proposed at a future date. Ricin is of outmost importance for biodefense since it is extremely potent and readily available. Furthermore, no antidote or treatment exists against this agent and the unpublished data on possible treatment should be pursued.

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1.0 INTRODUCTION

Inhalation exposure to various toxic agents is a not uncommon event that presents to hospital emergency rooms throughout industrialized countries, most often as a consequence of a workplace exposure due to an unplanned chemical release or a smoke inhalation secondary to a residential fire (Rabinowitz and Siegel 2002; Miller and Chang 2003). However, when chemical disasters, fires, or laboratory release of biologic agents expose large numbers of a population, acute damage to the respiratory tract as a result of inhalation of toxic agents can become commonplace (Rabinowitz and Siegel 2002). In fact, in the past 30 years significant numbers of people have been affected by exposures occurring under such circumstances, and disturbingly some of the most recent mass exposure events have been due to terrorism attacks.

The deadliness of weaponized anthrax was clearly seen in 1979 when at least 68 people (unofficial estimates range from 200 to 2000) were reported to have died as a result of an accidental release of weapons-grade anthrax spores by a research facility in Sverdlovsk (Miller and Chang 2003). As a result of the 1984 Union Carbide methyl isocyanate release in Bhopal, the government of the state of Madhya Pradesh has reported that more than 200,000 persons were exposed, more than 6000 died, and an estimated 50,000 continue to suffer from long-term health effects (Dhara, Dhara et al. 2002). In 1995, the Aun Shinrikyo terrorist organization released the nerve agent Sarin on an underground train in Tokyo, with a resultant 12 deaths and 1038 organophosphate poisonings. In the aftermath of the attack, the acute medical care system

was overwhelmed by 4460 'worried well' presenting to Tokyo hospitals (Miller and Chang 2003; Schecter and Fry 2005). Subsequent to the 2001 World Trade Center terrorist attack in New York City, acute inhalation injury was the most common reason for survivors to present for medical care. It was also the most common condition in survivors with injuries requiring hospitalization (CDC 2002).

In a program announcement in 2005, The Office of Biodefense Research, the National Heart, Lung and Blood Institute, and the National Institutes of Health expressed concern about the "US population's potential exposure to aerosolized, inhaled harmful chemicals possibly liberated as part of bioterrorism attacks against assembled groups of the civilian populace" (DHHS and NIH 2005). The primary stated objective of the program is to "encourage research about how the upper respiratory tract and lungs respond to acute exposure to highly toxic chemicals and subsequent inhalation, so that preventive strategies can be improved, antidotes devised to lessen initial irritation of mucosal surfaces, mucosal absorption minimized, and acute lung injury causing pulmonary edema counteracted" (DHHS and NIH 2005). In the context of this objective, and with the goal of developing a research proposal, the applicability of a mouse bioassay for sensory irritation (Alarie 1966; ASTM 1984) will be explored, including an overview of the basic anatomy, physiology and the physicochemical properties relevant to toxic inhaled agents, a brief review of other experimental inhalational toxicology methods, and with emphasis on modifications of the bioassay.

2.0 RELEVANT ANATOMY, PHYSIOLOGY AND INHALED TOXIN PHYSICOCHEMICAL PROPERTIES

Toxic exposures can produce a broad range of clinical effects. The ultimate manifest injury is dependent upon factors related to the toxin or toxin mixture, the route, intensity, frequency and duration of exposure and a multiplicity of host factors present in the exposed organism (Casarett, Klaassen et al. 1996). In inhalation toxicology, the dose of an aerosol toxin arriving at its target is dependent upon concentration and duration of exposure, particle size and breathing pattern during the exposure (Pauluhn 2003). Inhaled toxins vary substantially in their physical and chemical properties, and these properties as well as the anatomy and physiology of the host organism impinge upon the observed toxic clinical effect.

2.1 RELEVANT RESPIRATORY TRACT ANATOMY AND PHYSIOLOGY

Conceptually, the respiratory tract and can be separated into three compartments based upon anatomical features, particle deposition and clearance of inhaled agents (Pauluhn 2003). Traditionally these three divisions have been named the nasopharyngeal, tracheobronchial, and pulmonary regions (Figure 1). The nasopharyngeal compartment begins at the entrance of the anterior nares and continues to the level of the larynx, including both the nasopharynx and oropharynx. In this region, water soluble toxins are absorbed and relatively rapidly transported

into the circulation. Nonsoluble particulates are cleared by mucociliary transport and ultimately expectorated or swallowed; in the latter case the inhaled particulate may ultimately be absorbed by the gastrointestinal route. The time required for the mucociliary clearance in this region can be as much as 1 to 2 days. The tracheobronchial division of the respiratory tract begins at the larynx and follows the trachea and conducting airways down to the level of the terminal bronchioles. Similarly to the nasopharyngeal region, here water soluble toxins have access to the systemic circulation relatively rapidly and particulates are eliminated from the region by mucociliary transport (Pauluhn 2003).

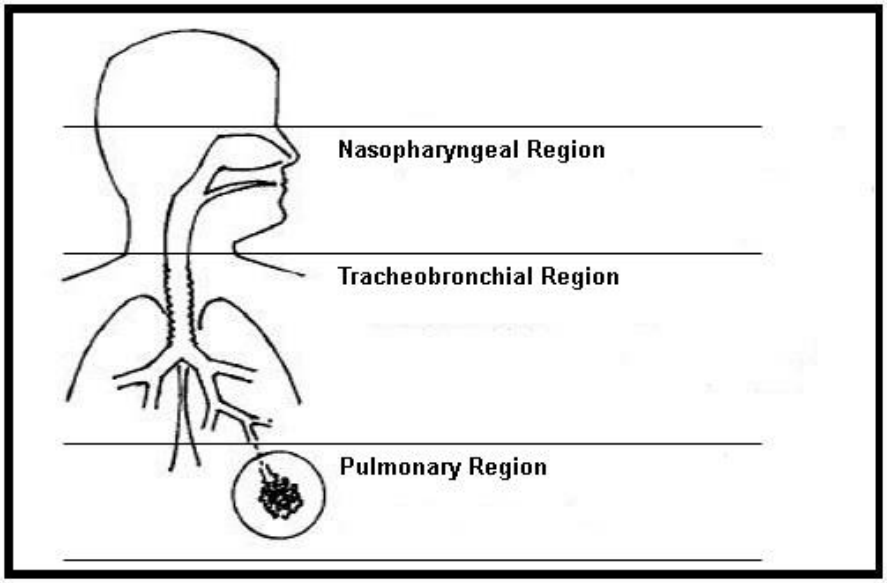

Figure 1. The Major Divisions of the Respiratory Tract (Johnson and Vincent 2003)
Adapted from Johnson, D. L. and J. H. Vincent (2003). Reproduced by permission of AIHA Press

The pulmonary region of the respiratory tract includes the alveoli and is the functional gas exchange site for the lung. The alveolar wall consists of a thin barrier of type I and type II pneumocytes. Type I cells cover approximately 90% of the alveolar wall surface, and therefore make up the vast majority of the gas transfer surface area of the lung: the large surface area to

mass ratio characteristic of type I pneumocytes has been used to explain why type I cells are injured in preference over the type II pneuomcytes in many toxic exposures (Pauluhn 2003). Figure 2 is a photomicrograph displaying the histologic features of the alveolus, with specific reference to type I pneumocytes, type II pneumocytes and interstitial cells.

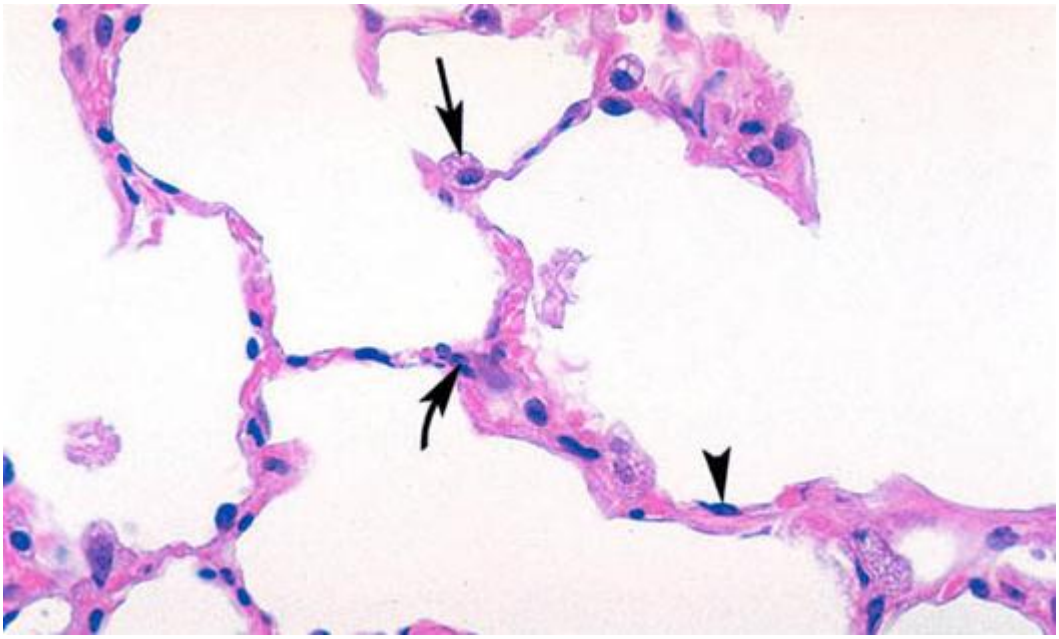

Figure 2. Alveolar Histology (Wilson, Kestenbaum et al. 2006)

Type II pneumocytes (arrow) are located in the walls of the alveoli and secrete surfactant material to reduce the surface tension along the alveoli. The flattened nucleus of a type I pneumocyte, the major cell type lining the alveoli, is located by the arrowhead. Interstitial cells (curved arrow) synthesize and secrete the connective tissue components of the pulmonary interalveolar septum (Wilson, Kestenbaum et al. 2006). From Wilson, F. J., M. G. Kestenbaum, et al. (2006), Histology Image Review: A complete illustrated review course in basic histology, Copyright ©2006 by The McGraw-Hill Companies, Inc. Reproduced by permission of The McGraw-Hill Companies.

In the pulmonary region, clearance of inert insoluble particulates is biphasic, with the first phase likely related to ingestion by macrophages and having a half-life in the order of 2 to 6 weeks, and

the second phase being related to dissolution with a half-life of months to years (Schlesinger 1995).

After inhalation of a toxic agent, reflex physiologic changes may occur in the individual to protect against further inhalation and increased exposure. Deposition of the offending agent in the nasopharyngeal region can result in stimulation of the trigeminal nerve endings and a sensation of burning in the nasopharnyx, oropharynx and eyes. Rhinorrhea, profuse tearing, coughing and sneezing may accompany this symptom. In addition, respiratory distress may occur as a result of inflammation of the glottis, excessive secretions and laryngospasm (Greenfield, Brown et al. 2002). In the case of mice and other small rodents, sensory irritation of the nasopharyngeal region rapidly results in a decrease in respiratory rate secondary to a characteristic delay in the expiratory phase of respiration (Alarie 1966; ASTM 1984). If not accounted for, this reflex decrease in respiratory rate, and therefore minute ventilation, can result in substantial reductions in the inhaled toxin dose (Pauluhn 2003) and could be a source of error in rodent bioassays**.**

When toxin deposition is in the tracheobronchial region, many agents will cause injury to the epithelial lining of the conducting airways and result in symptoms of cough and sputum production. Physiologic response in this region of the respiratory tract also includes bronchoconstriction, either on the basis of direct stimulation of parasympathetic nerve endings or as a result of local inflammation leading to release of chemical mediators that exacerbate bronchoconstriction (Greenfield, Brown et al. 2002).

In the case of toxins that penetrate into the lower respiratory tract, acute inhalation injury may result in damage to airway epithelium, subepithelial mucosa, alveolar lining cells, vascular tissue, and supporting structures. Diffuse inflammation of the pulmonary parenchyma can occur

and atelectasis may result from destruction of the pulmonary surfactant layer. The release of inflammatory mediators and increased permeability of the pulmonary capillaries may result in alveolar filling with protein-rich exudate producing pulmonary edema (Rabinowitz and Siegel 2002). Activated neutrophils appear to play a dominant role in the development of acute lung injury, and experimental animal models where neutrophils have been eliminated prior to hemorrhagic or endotoxin insult have shown reductions in indices of lung injury including degree of capillary permeability (Abraham 2003). The most common presentation of acute injury to the pulmonary parenchyma is that of self-limited shortness of breath, cough, and hypoxemia related to pneumonitis (Rabinowitz and Siegel 2002). Delayed-onset progression from pneumonitis to pulmonary edema and acute respiratory distress syndrome is also seen after exposure to pulmonary irritants such as phosgene, and overwhelming exposure to agents such as chlorine may result in immediate destruction of alveolar epithelial cells, as well as capillary endothelium, and cause death secondary to acute respiratory failure (Greenfield, Brown et al. 2002).

2.2 RELEVANT PHYSICOCHEMICAL PROPERTIES OF INHALED TOXICANTS

The physical form of an inhaled agent or mixture has a direct effect on the site of action in the host respiratory tract. Industrial hygiene literature defines a gas as a formless fluid that expands to fill its enclosure. Aerosols are liquid or solid particles suspended in air that are sufficiently small that they remain dispersed for a period of time, and include fumes, dust, smoke and mists (Table 1). Fumes are defined as minute solid particles generated by condensation from the gaseous state, generally after evaporation from melted substances. Dusts are described as solid

particles that are capable of temporary suspension in air or other gases. Smoke is described as a mixture of dry and liquid particles, generated by incomplete combustion of an organic material, combined with and suspended in the gases from combustion. Mists are a dispersion of suspended liquid particles, formed when a finely divided liquid is suspended in the atmosphere (Johnson and Vincent 2003). Even the shape if the particulate may determine toxicity, as demonstrated by the importance of fiber length in the potency of the different asbestos species (Schwartz 2005).

Table 1. Definitions of Various Types of Aerosols (Johnson and Vincent 2003)

Aerosol Type	Definition
Fumes	Minute solid particles generated by condensation from the gaseous state, generally after evaporation from melted substances
Dust	Solid particles that are capable of temporary suspension in air or other gases
Smoke	A mixture of dry and liquid particles, generated by incomplete combustion of an organic material, combined with and suspended in the gases from combustion
Mists	A dispersion of suspended liquid particles, formed when a finely divided liquid is suspended in the atmosphere

The total retained dose of an inhaled toxicant can be described by the following equation (Alarie 2004):

$$\textbf{Retained Dose} = \alpha \times (VT \times f) \times C \times t$$

where,

α = deposition fraction

VT = tidal volume

f = respiratory frequency

C = concentration

t = duration of exposure

In this equation, the deposition fraction (α) is the probability that an inhaled particle of a given size will be retained and not exhaled. However, total dose is not the only factor of importance, and the ultimate toxic response to an inhaled particulate is dependent on the amount, pattern of distribution and residence time of the toxin in the specific regions of respiratory tract. The regional deposition of toxin within the respiratory tract not only dictates the primary site of contact, but also determines the mechanism of toxin clearance and redistribution and has been reported to be more frequently a determinant of ultimate toxic effect than the total quantity of the toxin retained within the respiratory tract (Schlesinger 1995).

2.2.1 Relevant particle physical characteristics

The total deposition and the regional deposition pattern of particles within the respiratory tract are related to physical mechanisms that are dependent on the factors of physical particle characteristics, air flow patterns and respiratory tract anatomy (Schlesinger 1995). In the case of physical particle characteristics, these factors can also be further subdivided to include particle size, shape, density, electrostatic charge and hygroscopicity (Alarie 2004).

2.2.2 Determinants of regional particle deposition

There are five significant physical mechanisms which describe the processes by which deposition of an airborne particle occurs in the respiratory tract. These mechanisms include impaction (inertial), sedimentation or settling (gravitational), diffusion (Brownian movement), electrostatic precipitation (attraction) and interception (Figure 3) (Schlesinger 1995).

The first two mechanisms are of primary importance for non-fiber particles >0.5 μm in diameter. Impaction refers to inertial deposition and occurs when a particle's inertia is such that

it cannot follow a sudden change in direction of air flow and impacts onto an airway surface. Factors increasing the probability of impaction include increased velocity of the air stream, respiratory rate, particle size and particle density (Schlesinger 1995). Sedimentation or settling describes the mechanism by which a particle contacts the respiratory tract lining after reaching terminal settling velocity and occurs within a relatively quiescent air stream (primarily small bronchi and bronchioles). The likelihood of a particle depositing in the respiratory tract by this gravitational mechanism is increased by particle size, density, and residence time within the airway. It is decreased with increasing respiratory rate (Schlesinger 1995).

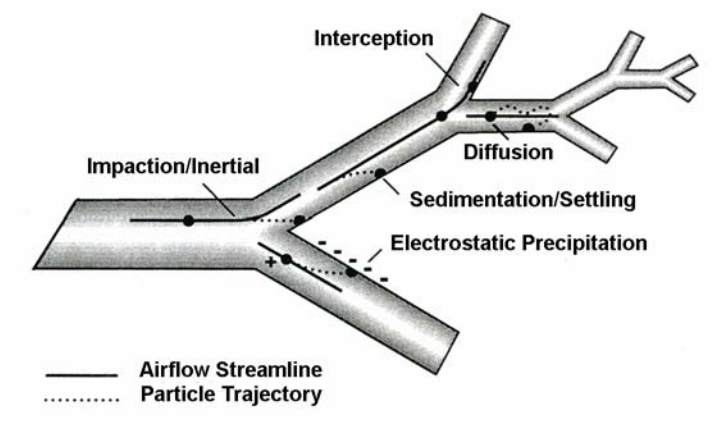

Figure 3. Schematic Diagram of Particle Deposition Mechanisms (Schlesinger 1995)
Adapted from Schlesinger, R. B. (1995), Copyright ©1995 From Concepts in inhalation toxicology by R. O. McClellan and R. F. Henderson. Reproduced by permission of Routledge/Taylor & Francis Group, LLC

Diffusion or Brownian movement is the dominant mechanism of deposition for particles <0.5 μm in diameter (and especially for particles <0.1 μm in diameter). These particles of submicron size are subject to random motion as a result of inertial impact with surrounding air molecules, and ultimately this random movement may result in contact with a respiratory tract surface. This process is independent of particle density and is dominant in areas with minimal or

no mass air flow such as the terminal bronchioles and alveoli (Schlesinger 1995). Regardless of this mechanism, extremely small particles can deposit to a significant degree in the upper respiratory tract, likely related to turbulence of the air stream (Schlesinger 1995).

Electrostatic precipitation or deposition is generally a minor factor in overall particle deposition within the respiratory tract; however, it can be of importance in the laboratory environment, especially with freshly generated particles having polymer or protein components that readily hold a charge (Schlesinger 1995; Alarie 2004). Finally, the interception mechanism is significant for fiber particulates with length to diameter ratio >3:1 (Schlesinger 1995).

As discussed for impaction and sedimentation mechanisms, the behavior of the particle in an air stream is a complex interaction related to a number of factors which include particle density and particle resistance (a function of the size shape of the particle). In predicting the behavior of a particle in an air stream, and ultimately the probability of deposition within a specific region of the respiratory tract, the mass median aerodynamic equivalent diameter (MMAD) of the particle is used. The MMAD of a particle is an experimentally determined measurement that takes into account both density and the complex issue of particle resistance: a particle is assigned a MMAD of 1 μm if it impacts (on an impactor previously calibrated with spherical particles of unit density) where a 1 μm spherical particle of unit density impacts (Alarie 2004).

For a given animal species, the MMAD of an inhaled particle predicts the deposition fraction and also predicts the site of deposition of the particle within the respiratory tract of that species. For human subjects, particles of aerodynamic diameter greater than 10 μm are mainly trapped in the nasopharynx or larynx and do not penetrate further into the conducting airways or deep lung tissues. Particles with aerodynamic diameter range 3-10 μm deposit in the conducting

airways and those in the range of 0.5-3 μm are deposited in the alveoli. Particulate size is of particular importance for dusts and mists, where size distribution may range from <1 to 50 μm (Miller and Chang 2003). These numbers are generally descriptive of the regional distribution of inhaled particles in human subjects, and Table 2 also provides similar information. However, the reality of the situation is that there are no abrupt cut-off values and graphical representations of the data are available that better describe the actual situation (Figure 4) (Alarie 2004).

Table 2. Particle Deposition within Regions of the Respiratory Tract (Alarie 2004)

Region	**Importance of Mechanism of Deposition**			**Primary Particle Size Deposited**
	Impaction	Sedimentation	Diffusion	
Nasopharyngeal	+++	+	+	5-30 μm
Tracheal	+	+	+	1-5 μm
Bronchial	+++	++	+	1-5 μm
Alveolar	+	+++	++++	<1 μm

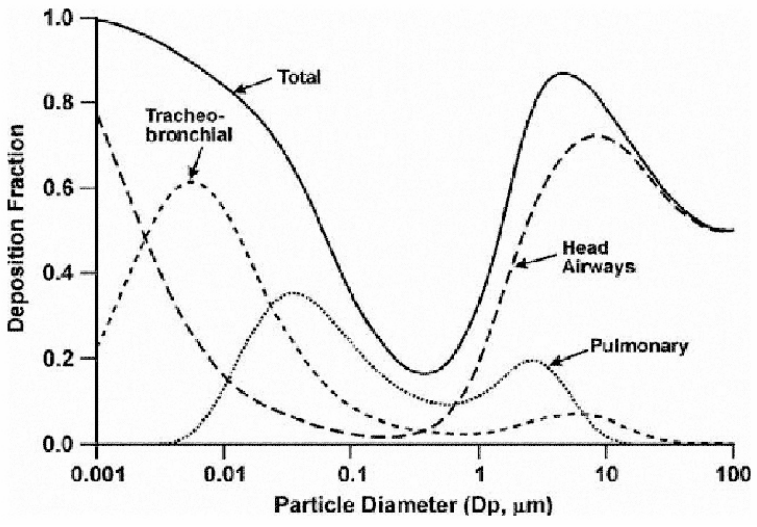

Figure 4. Fractional Regional Deposition of Inhaled Particles in the Human (Snipes 1994)
From Snipes (1994) Reproduced by permission of Medical Physics Publishing

A given particulate may or may not be in and of itself toxic to the respiratory tract, however, gases, mists or liquid aerosols can also be adsorbed onto otherwise innocuous particulates. Therefore, in the case of complex mixtures, this may affect the ultimate distribution of the toxic agent or mixture when carried into the respiratory tract. Finally, hygroscopicity may also be a factor in determining regional particle deposition since a small particle may increase significantly in size after entering the high humidity environment of the respiratory tract depending on chemical composition (Alarie 2004).

2.2.3 Air flow patterns and respiratory tract anatomy

As discussed previously, respiratory rate has an effect on the probability that a particle may deposit via a particular mechanism within the respiratory tract. Likewise, tidal volume, respiratory rate, and mouth versus nose breathing are factors that affect total deposition and regional deposition of aerosols by impacting air flow patterns (Alarie 2004). From the perspective of experimental design of aerosol exposures in laboratory animals, it is important to realize that there are substantial differences between the respiratory tract anatomy and breathing patterns of animals and human subjects, and therefore, the total deposition, regional respiratory tract distribution and clearance for particles of a given size may differ substantially. However, there are a number of generalizations that can be made concerning this complex situation (Alarie 2004):

1. The primary determinant of total dose is minute ventilation, and thus smaller animals will receive a proportionately higher dose due to higher minute ventilation to body weight ratios.
2. The smaller the animal to greater the likelihood of nasal deposition.

3. As particle size increases >3 μm MMAD, there is increasing likelihood that regional respiratory tract distribution will differ from that of human subjects, and although the total dose may be proportionate, the biologic effect may be substantially different.
4. Generating an aerosol with particle size of 1-3 μm MMAD will result in a situation where human subjects as well as mice and small laboratory animals obtain the same rate of lung deposition for a set concentration of toxin in the exposure atmosphere.

2.2.4 Gas phase toxicants

In the case of gas phase toxicants, the site of deposition within the respiratory tract is primarily determined by considering the factors of water solubility and degree of reactivity. In the case of highly water soluble or highly reactive gases, the retained dose equation for particulates may also be used, where 100% of gas is assumed to dissolve or react and the deposition fraction term (α) is assumed to be 1 (Alarie 2004):

$$\textbf{Retained Dose} = \alpha \times (VT \times f) \times C \times t$$

where,

α = deposition fraction

VT = tidal volume

f = respiratory frequency

C = concentration

t = duration of exposure

As mentioned, the water solubility of a gas plays a dominant role in determining the site of deposition and ultimately the location of a gas or vapor inhalation injury (Miller and Chang 2003). Henry's Law dictates that for a given temperature, when equilibrium is achieved between a mixture of gas and liquid phases, the partial pressure of a compound in the gas (air) phase is equal to the partial pressure of the compound dissolved in the liquid phase. At equilibrium then, the concentration of the compound in the air phase is proportional to the concentration in the liquid phase and the concentrations of the compound in the two phases are related by the Henry's Law constant:

$$\textbf{Henry's Law Constant} = C_{liquid} / C_{gas}$$

Therefore, the concentration of the compound in the liquid phase is equal to the concentration of the compound in the gas phase multiplied by the Henry' Law constant (Rabinowitz and Siegel 2002; Jayjock 2003). This Henry's Law constant is also referred to as a solubility, partition or distribution coefficient.

Following this principle, highly water soluble gases are 'scrubbed' from the inhaled air by the upper respiratory tract via preferential distribution into the aqueous and mucus liquid phase overlying the mucus membranes of the upper respiratory tract according to the Henry's Law constant. This results in decreased levels of exposure in the lower respiratory tract. However, with exposures of sufficient intensity and duration, the scrubbing effect of the upper respiratory tract can be overwhelmed and the lower respiratory tract irritation may still occur with these agents. If also reactive, as well as being water soluble, as in the case of ammonia or SO_2, these dissolved agents can cause acute irritation of the nose, larynx, nasopharynx, and eyes.

This peripheral sensory irritation causes unpleasant symptoms and may act as a biologic warning and encourage the exposed individual to leave the area, ultimately reducing exposure and the risk of greater toxic effects. There is also evidence for a number of compounds such as SO_2 that water solubility alone is not sufficient to describe the degree of removal by the upper respiratory tract. Chemical reactions between these inhaled toxicants with components of the nasopharyngeal lining (protein in the case of SO_2) function to trap these compounds in the upper respiratory tract (Alarie 2004).

Low water solubility gases do not dissolve immediately in the upper respiratory tract aqueous film and pass through the upper airway, penetrating into the conducting airways and deeper to the alveoli (Rabinowitz and Siegel 2002). If these agents are also reactive, such as in the case of phosgene or ozone, they exert their toxic effect in the conducting airways, bronchioles and alveoli. Because these agents do not necessarily cause unpleasant upper airway symptoms, the exposed individual may remain in the exposure area for a longer period of time, ultimately increasing exposure to the agent and the risk of toxicity (Schwartz 2005).

Similarly, noxious gases with intermediate solubility, such as chlorine, can display their toxic effects throughout the whole of the respiratory tract, ranging from rhinitis and tracheobronchitis through to pneumonitis and pulmonary edema (Rabinowitz and Siegel 2002).

Completing the continuum, extremely insoluble and non-reactive gases such as inhalational anesthetics or CO will pass through the respiratory tract in its entirety, cross the alveolar wall and continue on into the systemic circulation, exerting toxic or pharmacologic effects at remote sites within the body. In the case of these compounds the retained dose equation presented previously does not apply, and physiologically based pharmacokinetic models are used to describe gas uptake, distribution and metabolism. Since there is always

sufficient time to reach equilibrium between the alveolar air and alveolar capillaries, the partial pressure of the inhaled gas in the exiting alveolar capillary will always be equal to the partial pressure of the inhaled gas in the alveolar air. Once equilibrium is established between the inhaled air, the alveolar air and capillary blood, the concentration of the inhaled gas in capillary blood (arterial blood) is determined solely by the concentration in the inhaled air and is independent of the duration of exposure: no further quantity of the inhaled gas will be taken up into blood regardless of the length of exposure, except if the inhaled chemical is biotransformed, then further uptake will take place to the extent that biotransformation occurs (Alarie 2004).

3.0 REFLEX REACTIONS RELEVANT TO THE DEVELOPMENT OF THE BIOASSAY FOR EVALUATION OF SENSORY AND PULMONARY IRRITATION

3.1 THE INNERVATION OF THE RESPIRATORY TRACT

The entire respiratory tract, from external nares to the alveoli, is abundantly supplied with sensory nerves and morphologically distinct receptors (Figure 5) (Alarie 1973). Within the mucous membranes of the nasopharynx and oropharynx there are peripheral neurologic structures identifiable on light or electron microscopy that respond to certain airborne chemical stimuli to give the characteristic perceived sensations of smell or taste for that compound. However, there are also additional sensory nerve endings of trigeminal nerve origin (unmyelinated C-fiber type) in the cornea and nasal mucosa that terminate just a few micrometers below the mucosal surface. These afferent trigeminal nerve endings histologically resemble primitive nerve endings of the fetal skin prior to development of definitive receptor organs and terminate not only in relation to adjacent epithelial cells but also in relation to the intercellular fluid. As such they are subject to easy stimulation by any airborne xenobiotics that contact the overlying mucosal or epithelial surface (Alarie 1973).

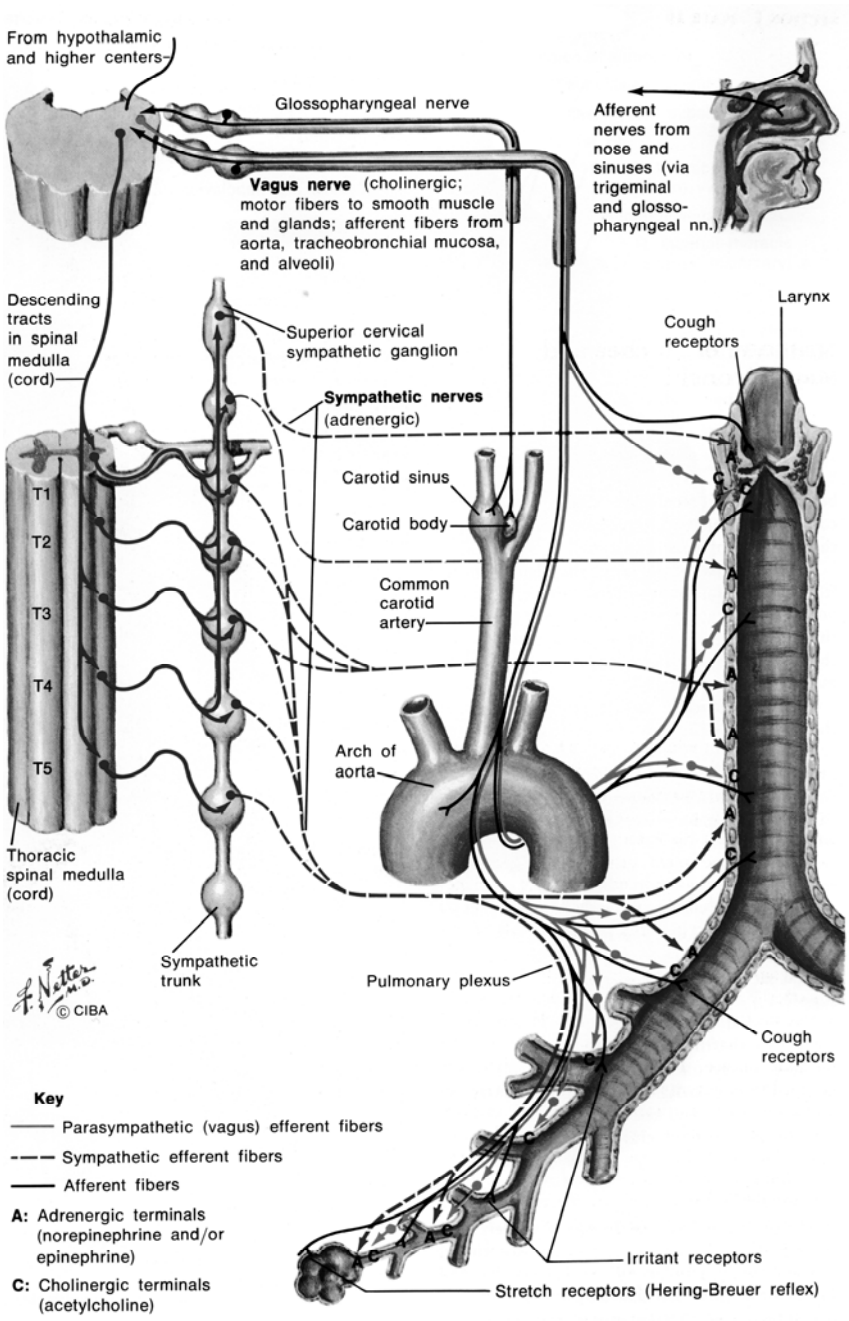

Figure 5. The Innervation of the Human Respiratory Tract (Netter, Divertie et al. 1979)
Adapted from Netter, Divertie et al. (1979)

The airways and pulmonary parenchyma are innervated by both myelinated and unmyelinated afferent fibers of vagal origin that contribute to the reflex control of ventilation by modulating ventilatory patterns (Leff and Schumacker 1993). The receptors innervated by

myelinated fibers are usually grouped into slowly adapting receptors and rapidly adapting receptors on the basis of whether ongoing stimulation of the receptor results in transient or prolonged afferent nerve discharge. Slowly adapting receptors, also termed stretch receptors, are responsible for the abbreviation of inspiration produced by vagal afferent discharge and are also responsible for the Hering-Breuer reflexes. The Hering-Breuer inflation reflex is the prolongation of expiration produced by steady lung inflation; increased lung volume triggers these receptors and vagal afferents stimulate 'off-switch' medullary neurons resulting in a termination of inspiration (Leff and Schumacker 1993). The Hering-Breuer deflation reflex is the abbreviation of expiration resulting from the significant deflation of the lung (Ganong 2005).

Rapidly adapting receptors, also termed irritant receptors, are known to be stimulated by chemicals such as histamine. Stimulation of irritant receptors located in the larynx results in coughing, stimulation of the bronchi and bronchioles results in bronchoconstriction and mucous secretion, whereas stimulation of irritant receptors in the pulmonary parenchyma may produce increased respiratory rate (Ganong 2005). Hering-Breuer reflexes are relatively inactive during quiet, resting respiration in humans, as demonstrated by the response of patients with heart-lung transplants in whom Hering-Breuer reflexes are absent (surgical transection of vagal afferent fibers), yet their pattern of resting respiration is normal (Ganong 2005). The unmyelinated vagal afferent fibers (C-fiber type) terminate in specialized receptors in the airways that facilitate the ventilatory responses resulting from inhalation of irritating substances such as chemicals, noxious gases and cold air (Leff and Schumacker 1993). Within the lung parenchyma similar receptors linked to unmyelinated C-fiber type vagal afferents have been termed juxta-alveolar, juxta-capillary or type J receptors. Type J receptors are responsive to chemical or mechanical stimuli in the interstitium of the lung, and behave as interstitial stretch receptors to mediate the

tachypnea seen in response to interstitial lung edema or lung parenchymal inflammation (Alarie 1973; Leff and Schumacker 1993). In addition to being stimulated by hyperinflation and airborne chemicals, type J receptors also respond to intravenous administration of xenobiotics such as capsaicin, and result in the pulmonary chemoreflex consisting of tachypnea, bradycardia and hypotension (Ganong 2005).

3.2 THE PERIPHERAL SENSORY IRRITANT EFFECT

A number of published reviews express concern over the somewhat imprecise manner in which the word *irritation* is used in some toxicological texts. When properly applied, irritation may be used to describe either pharmacologic or non-destructive pathologic processes. In the case of a pharmacologic process, it is used to describe the interaction of a xenobiotic with sensory nerve receptors in the skin, mucous membranes or smooth muscle. In appropriate use in the case of a pathologic process, the term may be used to describe effects such as tissue inflammation. In both cases described above, the biologic effects are clearly reversible following the removal of the toxicant or removal from exposure. However, it is incorrect to use the word irritation to describe irreversible or destructive respiratory tract tissue effects, such as those seen resulting from massive exposures to sulfur dioxide, ammonia, or methyl isocyanate (Alarie, Schaper et al. 2000; Ballantyne 2006).

The peripheral sensory irritant effect is essentially a pharmacologic process where a xenobiotic acts locally at a skin or mucosal barrier by stimulating sensory nerve receptors to produce local sensations (discomfort, itching, burning, or pain) in addition to reflex responses, some of which are local and some of which are systemic (Ballantyne 2006). Consistent with the

definition of irritation discussed previously, many xenobiotics that initially result in a peripheral sensory irritant pharmacologic effect at a lower applied concentration may also result in an inflammatory pathologic effect at higher concentrations. A number of terms have been used over the years to describe the action of sensory irritants on nerve endings within the skin, cornea and respiratory tract mucous membranes, and it was originally thought that peripheral sensory irritant effects were attributable to a 'common chemical sense' independent of touch, temperature and deformation stimuli. This was later shown not to be the case, and at least in the skin, most of the same receptors that propagate peripheral sensory irritant effects also respond to noxious stimuli of mechanical or thermal origin (Green 2000; Ballantyne 2006). As such, the terms peripheral sensory irritation or peripheral chemosensory irritation have come into more frequent use in the literature (Ballantyne 2006).

The peripheral sensory irritation effect in the respiratory tract is experienced as discomfort or pain when stimulation occurs at the level of the nasal mucosa, nasopharynx, oropharynx, throat, larynx and conducting airways, and can be associated with reflex coughing, sneezing, bronchospasm and increases in respiratory tract secretions (Ballantyne 2006). Discussed in more detail later, there may be an associated increase or decrease in respiratory rate contingent upon the regional level of the respiratory tract being affected. The effects of sensory irritation in the case of the lower respiratory tract also depend on whether the stimulation is occurring at the level of the larynx, main bronchi and conducting airways or whether the stimulation is occurring more peripherally in the pulmonary parenchyma and alveoli. Stimulation of receptors located in the conducting airways results in reports of painful sensations. However, deeper within the respiratory tract and at the level of the pulmonary parenchyma, stimulation of irritant receptors does not result in complaints of pain and instead

unpleasant sensations of shortness of breath and discomfort are typically reported. In addition to these complaints, when lung irritant receptors are directly stimulated there can be an associated reflex increase in respiratory rate, bronchoconstriction, increase in mucus production and pulmonary congestion (Alarie 1973).

The stimulation of nerve endings in the respiratory tract by peripheral chemosensory irritant compounds results in a broad variety of reflex reactions. Taken as a whole, the biological importance of peripheral chemosensory irritant reactions are clear: these reactions are protective of the host organism and either function as a biological warning in the case of unpleasant local sensory responses to encourage the affected individual to seek an uncontaminated area or function physiologically by altering ventilatory patterns in order to minimize or prevent exposure to potentially noxious xenobiotics.

3.3 REFLEX REACTIONS OCCURRING WITH SENSORY IRRITATION OF THE UPPER RESPIRATORY TRACT

The study of reflex reactions to airborne chemicals began well over a century ago, with initial work being done in 1870 by Kratschmer, who described the responses to various irritants on the nasal mucosa and larynx. Kratschmer's protocol for laryngeal exposures involved exposing only this portion of the respiratory tract and selectively preventing exposure of either the nasal mucosa or the lower respiratory tract of the subject. Unfortunately, 50 years of controversy followed Kratschmer's original work when numerous researchers generated conflicting results, likely due to the "poor control over the concentration of the sensory irritants used, (although) various anesthetics and species differences may also have contributed" (Alarie 1973). However,

in a four-year period between 1925 and 1929, both Magne and Allen working independently confirmed the earlier results of Kratschmer, and arguably more significantly, Allen was able to identify that the same reflex reactions that occurred in humans also occurred in rabbits, dogs and cats (Alarie 1973).

In both 1966 and 1973, Alarie reviewed and summarized earlier data obtained by other researchers reporting the modifications of ventilatory pattern in experimental animals exposed to irritant xenobiotics. The following general conclusions were reached concerning reflex reactions resulting when irritant compounds were delivered only to the upper airway (animals were tracheally cannulated to protect the lower respiratory tract from irritant compound exposure) (Alarie 1966; Alarie 1973):

1. Decrease in respiratory rate secondary to a lengthened expiratory pause
2. Decrease in heart rate
3. Increase in systolic arterial blood pressure
4. Bronchoconstriction-bronchodilation
5. Decrease in pulmonary blood flow
6. Closure of the glottis
7. Closure of the nares and increase in nasal airflow resistance
8. Reflex reactions were of rapid onset and resolution

Of these reactions, the first three are of particular interest in the context of the development of the bioassay for evaluation of sensory and pulmonary irritation.

3.3.1 Decrease in respiratory rate secondary to lengthened expiratory pause

When peripheral chemosensory irritation of the trigeminal nerve endings in the nasopharynx occurs, there is an immediate inhibition of respiratory movements where the duration of the expiratory phase increases proportionately with the concentration of the irritant xenobiotic. This characteristic pattern of inhibition of respiration with peripheral chemosensory irritation of the nasal mucosa has been consistently seen across many species (man, dogs, rabbits, cats, mice, rats, guinea pigs, hamsters, and ducks) and with a broad range of chemical irritants. With research prior to 1950, experimental techniques were such that exposures were typically of short duration and high exposure concentration. The data obtained from these exposures clearly demonstrated the nature of this reflex reaction, however, these experiments were insufficient to determine whether sustained exposure would result in a sustained response and whether a concentration-response relationship could be determined (Alarie 1973).

3.3.2 Decrease in heart rate

The decrease in heart rate with peripheral chemosensory irritation of the upper respiratory tract has been reported to occur only when the respiratory rate is significantly depressed and as such, is not usually seen at low concentrations of irritant xenobiotic. Transection of the vagal nerve will abolish this reflex (Alarie 1973).

3.3.3 Increase in systolic arterial blood pressure

The increase in systolic arterial blood pressure seen with sensory irritation of the upper respiratory tract occurs despite the concurrent decrease in heart rate described above, and as in

the case of the decrease in heart rate, is not usually seen without sufficiently high irritant xenobiotic concentrations and associated depression of respiratory rate. Transection of the splanchnic nerves and sympathetic blockade both eliminate this reflex (Alarie 1973).

3.3.4 Bronchoconstriction-bronchodilation

Because of the complex nature of vagal nerve afferent innervation, the efferent nerve involved, interspecies differences and issues of experimental design, Alarie reported in 1973 that one might expect bronchoconstriction, bronchodilation or no effect with trigeminal nerve stimulation by sensory irritants. In fact, for the references evaluating the peripheral sensory irritation effect, this was the case at the time of the review article publication. However, a definitive role for stimulation of trigeminal nerve endings in the generation of reflex bronchoconstriction was established by research carried out evaluating the effects of cold applied to the face in man, cats and rats (Alarie 1973).

3.4 REFLEX REACTIONS OCCURRING WITH SENSORY IRRITATION OF THE LOWER RESPIRATORY TRACT

In 1925, Magne et al. reviewed the experimental conclusions of previous investigators and also performed more in-depth experiments assessing the effects of irritant xenobiotics on the upper respiratory tract, the lower respiratory tract and on both regions concurrently (Alarie 1966). These researchers were the first to identify that although an irritant stimulus to the upper respiratory tract resulted in a decrease in respiratory rate, the opposite effect occurred when the irritant stimulus was delivered to the lower respiratory tract. Additional cardiovascular reflexes

also occurred with sensory irritation of the lower respiratory tract, although they were not as intense as those occurring when the upper respiratory tract was stimulated (Alarie 1973). The following general conclusions were reached concerning reflex reactions resulting when irritant compounds were delivered only to the lower airway (irritants were delivered via tracheostomy to protect the upper respiratory tract from exposure) (Alarie 1966; Alarie 1973):

1. Increase in respiratory rate
2. Decrease in heart rate
3. Decrease in blood pressure
4. Reflex reactions were of more gradual onset and resolution

As mentioned, these reflex reactions are of lesser intensity than those seen with peripheral chemosensory irritation of the upper respiratory tract. This is particularly true in the case of the decrease in heart rate and decrease in blood pressure, where despite a high concentration of irritant these reflexes continue not to be pronounced and may in fact be completely absent (Alarie 1973).

3.4.1 Increase in respiratory rate

In most cases, an associated decrease in tidal volume is also seen with the increase in respiratory rate seen during the stimulation of irritant receptors in the pulmonary parenchyma. The overall effect of this reflex reaction would be to limit contact of the irritant compound with the alveoli, while increasing the deposition of the compound in the conducting airways. This reaction is once again consistent with the physiologic goal of altering ventilatory patterns in order to minimize or prevent exposure to potentially noxious xenobiotics. Table 3 (Alarie 1973) summarizes much of the preceding sections and tabulates the reflex reactions of respiratory rate,

blood pressure and heart rate dependent on site of stimulus and subject to modifying factors such as nerve block or peripheral nerve transection.

Table 3. Reactions to Stimulation by Sensory Irritants Observed under Various Conditions (Alarie 1973)

Reflex Effect	**Conditions of Exposure**							
	A	Same as A with addition of:	Same as A with addition of:	Same as A with addition of:	Same as A with addition of:	Same as A with addition of:	Same as A with addition of:	
	Unanesthetized and breathing via nose	Transection of superior laryngeal nerves	Transection of trigeminal nerves or local anesthesia with cocaine	Transection of olfactory nerves or removal of olfactory bulb	Transection of the splanchnic nerves or sympathetic blockade	Transection of vagus nerve or parasympathetic blockade	Transection of vagus and splanchnic nerves (or sympathetic blockade)	Breathing via tracheal cannula
Respiratory Rate	↓	↓	No effect	↓	↓	↓	↓	↑
Systolic Arterial Blood Pressure	↑	↑	No effect	↑	No effect	↑	No effect	No effect/↓
Heart Rate	↓	↓	No effect	↓	↓	No effect	No effect	No effect/↓

In contrast to the respiratory rate increase in response to pulmonary irritation that is seen in most animals (Table 3), the mouse develops a decrease in respiratory rate with pulmonary irritation secondary to the development of a pause between the end of expiration and the initiation of the next inspiration. In a manner analogous to the respiratory rate depression seen with peripheral chemosensory irritation of the upper respiratory tract where the duration of the expiratory phase increases proportionately with the concentration of the irritant xenobiotic, with pulmonary irritation in the mouse progressive respiratory rate depression is seen as the duration of the pause between the end of expiration and the initiation of the next inspiration increases with irritant exposure concentration (Ballantyne 2006).

3.5 REFLEX REACTIONS OCCURRING WITH ADMINISTRATION OF IRRITANTS TO THE UPPER AND LOWER RESPIRATORY TRACTS SIMULTANEOUSLY

In a 1966 review, Alarie noted that earlier investigators had reported that when respiratory tract irritants were administered to intact animals breathing normally through their noses (administration of the irritants simultaneously to both the upper and lower airways), at times they obtained a classic upper airway reflex response, at times a classic lower airway reflex response, and at times a combination of both responses, dependent upon the compound and the concentration of the compound administered. On the basis of this finding, the authors had classified the experimentally administered agents on the basis of the reflex reactions occurring following their administration, and identified acrolein as an upper airway irritant, phosgene as a lower airway irritant and chlorine as a total airway irritant (Alarie 1966).

For any compound, there is an expected difference between the concentration causing an *in vivo* peripheral chemosensory irritation response via the stimulation of nasal mucosa trigeminal afferent receptors and the concentration that would be required to stimulate pulmonary irritant receptors (Ballantyne 2006). It is generally true that for most substances, in an intact conscious animal, the reflex reaction to nasal trigeminal afferent stimulation will predominate (Ballantyne 2006). Akin to the therapeutic index measure utilized in pharmacology (the ratio of toxic dose to effective dose) (Mycek, Harvey et al. 2000), the ratio of the exposure concentration resulting in a 50% depression in respiratory rate in tracheal-cannulated mice (pulmonary irritation) to the concentration resulting in a 50% depression in respiratory rate in intact mice (nasal irritation) has been utilized in studies with the goal of quantifying the margin

of safety that the sensory irritant response offers against possible pulmonary parenchymal injury from the inhaled agent (Alarie 1981; Ballantyne 2006).

4.0 METHODS FOR ASSESSING SENSORY AND PULMONARY IRRITATION

Methods for assessing sensory irritant potential range from biological approaches, including the controlled exposure of human volunteer subjects and the use of *in vitro* and *in vivo* biological models, through non-animal methods including quantitative structure activity relationships and chemical models.

4.1 HUMAN EXPOSURE DATA

Although animal studies may allow the detection of a chemical compound's ability to cause sensory and pulmonary irritation, human response may in fact vary from the response seen in animal models. In addition, these models do not allow for an assessment of the degree of discomfort or pain that may be experienced in an exposed human subject. In this context, well-designed studies utilizing human volunteer subjects can allow assessment of subjective responses to peripheral chemosensory irritation, measurement of physiologic variables and reflex responses (respiratory rate, heart rate, blood pressure), and determination of threshold limits (Ballantyne 2006). There are obviously ethical concerns in the design of such studies that put limits on the utilization of this approach. Regardless of the situation, studies involving human cutaneous exposure (Green 2000), corneal and conjunctival irritation, and respiratory tract exposure have been undertaken (Ballantyne 2006).

The case of voluntary human exposure to pulmonary irritants is obviously a different matter, and clearly not possible for ethical reasons (Alarie, Schaper et al. 2000). However, it is possible to correlate observed human health outcomes and symptoms to extrapolated human pulmonary irritant exposure conditions from reported occupational and accidental exposures (Alarie, Schaper et al. 2000). This same approach is also clearly applicable to peripheral chemosensory irritant exposures. In the case of accidental exposures where no exposure atmosphere monitoring data exists, such as the Bhopal methyl isocyanate release, useful information may still be obtained despite an inability to obtain dose-response relationships (Dhara, Dhara et al. 2002).

4.2 QUANTITATIVE STRUCTURE ACTIVITY RELATIONSHIP ANALYSIS

Quantitative structure activity relationships do have a role in the assessment of sensory and pulmonary irritation; however, this approach requires an experienced toxicologist or chemist to evaluate the irritant potential of a new compound in the context of existing chemicals because of the similarities in chemical structure or function. As such, for the approach to be effective a well-established database of toxicologic effect needs to exist for the comparison or reference chemical category. Quantitative structure activity relationship computer programs are available for some human health end points, and the Environmental Protection Agency provides working categories for chemical compounds with similar physicochemical, structural and toxicological properties (Dodd and Brock 2006). Potency of nonreactive volatile organics as sensory irritants (expressed as RD_{50}) has been robustly correlated with physicochemical properties of vapor pressure and gas-liquid partition coefficients (Alarie, Nielsen et al. 1995). In addition, the

potency of chemically reactive -CH_2-halogen compounds as sensory irritants has been correlated with lipophilicity (octanol-water partition coefficient) and a descriptor variable for chemical reactivity (Alarie, Nielsen et al. 1998).

Taken in the context of *a priori* assumptions, quantitative structure activity relationship algorithms can be valuable. However, although substantial numbers of chemical categories are documented as capable of causing peripheral chemosensory irritation effects and relationships have been determined that correlate to relative potency, the 'gold standard' to which these quantitative structure activity relationships are often held is based on the experimentally determined reflex respiratory rate depression previously discussed (Ballantyne 2006).

4.3 CHEMICAL MODELS

Various artificial membranes have been developed in attempt to simulate biological membranes, including monolayers and bilayers of lethicin. A recent review reports that the use of such a simulated membranes has not yielded reliable predictions of peripheral chemosensory irritation potential (Ballantyne 2006).

4.4 *IN VITRO* MODELS

Nonspecific methods such as the isolated intestinal segment and frog flexor reflex involve the progressive exposure of segments of small intestine or frog hind-limb preparation respectively to increasing concentrations of the test compound in solution. Several neurophysiological methods also exist where peripheral nerves known to carry afferents that transport sensory irritant induced

action potentials from skin, mucosa or cornea are used to detect the peripheral chemosensory irritant potential various compounds. These include a ciliary nerve preparation using an excised cat eye, and a number of rat models in which recordings from nasopalatine, ethmoidal, sphenoidal and laryngeal nerves are made subsequent to nasal and laryngeal mucosal irritation respectively. However, these methods are generally less sensitive than *in vivo* models, and are usually evaluated on the basis of their performance in comparison to *in vivo* models like the guinea pig blepharospasm test and various inhalation methods (such as experimentally determined reflex respiratory rate depression) discussed below (Ballantyne 2006).

4.5 *IN VIVO* MODELS

In vivo models for the assessment of peripheral chemosensory irritation may include topical application of peripheral chemosensory irritant to the cornea or skin as well as any of a number of methods that deliver the toxic compound via the inhalation route. Blepharospasm in conscious animals as a response to the topical or aerosol application of an irritant compound to the eye is a method commonly used to assess peripheral chemosensory irritation of the cornea. In general, the guinea pig is the species of choice for this method, and the technique is reported to be reproducible, free from tachyphylaxis, and able to differentiate the peripheral chemosensory irritant potential of various compounds that are of similar chemical structure. Because of the similarity of the guinea pig dose-response relationship with human response, the method has been utilized in the pharmaceutical industry in assessing the tolerability of ophthalmic drug preparations (Ballantyne 2006). A wide array of inhalation exposure systems have been designed and utilized to expose both human and animal subjects to a broad range of

agents over the past 50 years (Phalen 1996) and these will be briefly reviewed in the subsequent section.

4.6 METHODS OF INHALATION EXPOSURE

A 1996 review suggests a useful classification of inhalation exposure systems is based on the region of the test subject's body that is in direct contact with the exposure atmosphere (Table 4) (Phalen 1996). Chamber or whole-body exposure systems provide an exposure environment in which the test subjects are unrestrained and either move about freely or within cages. The systems can provide efficient exposure of large numbers of subjects, are useful for chronic exposures where daily exposure duration may as much as 24 hours, and because the subjects are unrestrained, are thought to be relatively non-stressful. Disadvantages of such systems include the exposure of not only the entire body surface of the subjects, but also exposure of the food and water, resulting in possible oral and dermal exposure to the test compound (Phalen 1996).

Chamber exposure methods also generally limit the ability of the investigator to accurately quantify physiologic parameters. As such, they restrict the investigator's ability to evaluate real-time physiologic response to test compounds as well as to administered prophylactic or therapeutic agents. The result is that time-consuming histopathologic examination of tissue may be required to evaluate toxic, therapeutic or prophylactic effects when physiologic responses, such as reflex decrease in respiratory rate, may identify these effects earlier and with greater sensitivity (Alarie 1966). From an experimental design perspective, what is not measured cannot be accounted for, and unrestrained chamber rodent bioassays have been criticized on the basis that the reflex decrease in respiratory rate, and therefore decrease in

minute ventilation, can result in substantial reductions in the inhaled toxin dose and can be a significant source of error (Pauluhn 2003).

Table 4. Major Advantages and Disadvantages Associated with Various Methods of Inhalation Exposure (Phalen 1996)

Method	Advantages	Disadvantages
Chamber	Large number of subjects	Dermal, eye, and oral exposure in addition to inhalation
	Suitable for chronic studies	Large amounts of test material required
	Minimal restraint	Expensive
	Can house animals in chambers	Excreta can interact with pollutants
	Labor efficient	
Head-only	Good for repeated exposure	Can be stressful
	Minimal skin contamination	Pollutant losses can be large
	Efficient dose delivery	Neck seal problems
	Better control of dose	Labor intensive
Nose/mouth-only	No skin contamination	Can be stressful
	Can be used for repeated exposures	Needs good face seal
	Uses much less material	Labor intensive
	Exposures can be pulsed	Technically difficult
	Personnel and facility contamination minimized	
Lung-only	Precision of dose	Anesthesia or tracheostomy required
	Uses less material (efficient)	Bypasses nose (could be an advantage)
		Artifacts in deposition in response
		Technically difficult

Additional exposure methodologies exist that are designed to expose only selected regions of the respiratory tract to the test atmosphere, and typically the lung parenchyma is the region of choice in these methods. Anesthesia of the test subjects is required in the case of these systems for the surgical implantation of a tracheostomy tube or installation of liquids into the lower respiratory tract. As such, the systems tend to be non-physiologic in their delivery of test agents and are often criticized on the basis of creating trauma locally within the airway (Phalen 1996). In addition, test animals need to be sacrificed following exposures with these methods, and therefore the animals cannot be repeatedly challenged for any length of time that the

experimental design dictates (Schaper 1984). However, there are experimental designs under which these approaches may offer important advantages, such as those utilized in the early investigation of sensory irritation of the upper respiratory tract where animals were tracheally cannulated to protect the lower respiratory tract from irritant compound exposure (Alarie 1966; Alarie 1973).

Head-only, nose-only and mouth-only exposure systems offer the advantage of preventing a substantial amount of the dermal exposure; in the case of nose-only exposures, non-respiratory pathways of uptake can be totally eliminated (Phalen 1996). Because of the decreased volume of exposure atmosphere required in comparison to the whole-body exposure chamber, these systems require lesser quantities of test compound and allow exposure concentrations to be adjusted more rapidly. Disadvantages of these systems include difficulty maintaining a comfortable, effective seal around the neck of the subject in the case of head-only exposures, and the technical requirement of a precisely fitted mask or tapered tube in the case of nose-only exposures (Phalen 1996). In addition, the use of exposure helmets, collars, masks or tapered tubes may produce stressful restraint of the test subjects, as well as possibly inflicting thermal stress by increasing temperature, relative humidity and decreasing the test subject's ability to maintain thermal equilibrium (e.g. the tail is known to be important for thermal regulation in the rodent) (Cheng and Moss 1995). Regardless of these disadvantages, the control of the delivery of study material can be quite precise and these systems are felt to offer a significant overall advantage, especially in the case of nose-only methods (Phalen 1996). The bioassay for sensory irritation (Alarie 1966; ASTM 1984) utilizes one of these methodological approaches, and will be reviewed in greater detail in the following chapter.

5.0 THE BIOASSAY FOR THE EVALUATION OF SENSORY AND PULMONARY IRRITATION

In 1966, Alarie provided the first description of a mouse bioassay that had utility in detecting the sensory-irritating properties of airborne chemicals that also permitted qualitiative extrapolation to human responses (Alarie 1966). In that review, Alarie noted that earlier investigators had been able to classify experimentally administered agents on the basis of the reflex reactions occurring following the administration of the compounds to intact animals breathing normally through their noses. It was concluded that one of the physiologic parameters recorded by the earlier investigators might allow classification of agents administered via total airway exposure, and respiratory rate was chosen as the test criterion on the basis of the ease with which it could be measured. At that time an experimental technique using body plethysmography with mice was chosen (Alarie 1966).

5.1 PLETHYSMOGRAPHY

The two important techniques for the assessment of respiratory function are spirometry and plethysmography. Although spirometry is used with clinical success in the evaluation of respiratory parameters in human subjects, the degree of cooperation required to obtain reproducible results precludes its use in animals (Schaper 1984). For this reason,

plethysmography has been, and remains to be, the method of choice in animal experimental studies. There are two basic principles that govern the use of plethysmography as an approach to measuring respiratory function in laboratory animals (Gad 2006):

1. The animal (which may or may not be anesthetized and which may or may not be restrained) is placed in a chamber(s) with pressure transducers and/or pneumotachographs
2. As a result of inspiration and expiration, variations in pressure within the chamber(s) or flow in and out of the chamber(s) through the pneumotachograph(s) make it possible to obtain respiratory parameters for the animal

There are three main types of body plethysmographs used experimentally (Figure 6): volume displacement ("flow box"), constant volume ("pressure box"), and barometric ("Fenn box", named for an early user, W. O. Fenn) (Mauderly 1995; Gad 2006).

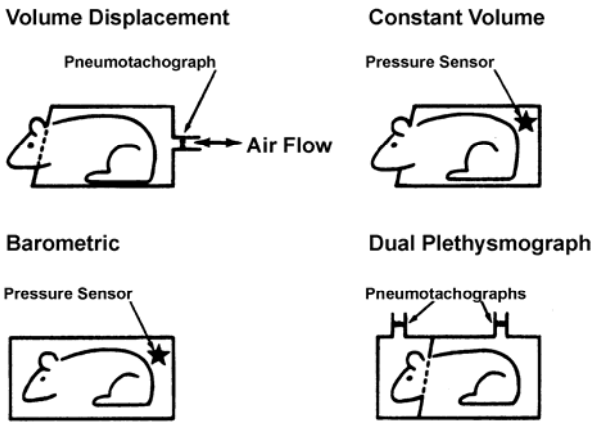

Figure 6. Types of Plethysmographs (Mauderly 1995)

Adapted from Mauderly (1995), Copyright ©1995 From Concepts in inhalation toxicology by R. O. McClellan and R. F. Henderson. Reproduced by permission of Routledge/Taylor & Francis Group, LLC

In the first two types of plethysmographs, the test subject breathes the exposure atmosphere outside the plethysmograph, either via an airway or by having the head and neck extend through an opening in the plethysmograph wall (Mauderly 1995). The constant volume body plethysmograph is a sealed constant volume container and a relatively airtight seal between the neck of the subject and the container maintains the constant volume status required by the method. While inside the plethysmograph, inhalation of test atmosphere (from outside the plethysmograph) by the animal causes an increase in lung volume, resulting in chest expansion, and therefore causing an increase in pressure within the plethysmograph according to Boyle's Law ($P_1V_1 = P_2V_2$). Similarly, a fall in pressure within the plethysmograph occurs when the animal exhales. Sensitive pressure transducers detect the change in plethysmograph pressure, and this pressure change can be related to a change in lung volume through the application of a calibration factor (Schaper 1984; Gad 2006). For the constant volume body plethysmograph there is an additional experimental condition imposed through the application of Boyle's Law, and for results to be valid maintenance of constant temperature within the body plethysmograph chamber during the test is required.

The volume displacement body plethysmograph is a constant volume container with the addition of a pneumotachograph port in its wall that measures flow rate through the port and integrates the flow rate in order to calculate a volume change. The pneumotachograph records equivalent flow out of the plethysmograph when the subject inhales and records equivalent flow into the plethysmograph when the subject exhales.

In the barometric plethysmograph, the test subject breathes the exposure atmosphere inside the plethysmograph. Within this closed container, respiratory volumes are reflected as small pressure changes due to the heating and humidification of the inhaled exposure atmosphere

by the test subject (Mauderly 1995). Although differential pressure transducers are most commonly used for all three types of plethysmographs, microphones have been used in the barometric variety (Ellakkani, Alarie et al. 1984; Mauderly 1995).

The dual plethysmograph is an important variation on a theme based on the first two types of plethysmographs that warrants mention (Figure 6). In this application, the head of the test subject is contained in one plethysmograph and the body of the subject is contained in another plethysmograph. This arrangement allows the investigator to measure differences in flow or volume at the mouth versus the thorax due to airway restriction, and thus evaluate airway resistance, conductance and other parameters while avoiding the requirement for an invasive transpulmonary catheter recording of transpulmonary pressure (Mauderly 1995).

5.2 CHARACTERISTIC SENSORY IRRITANT BREATHING PATTERN CHANGES

When trigeminal peripheral chemosensory irritation receptors are stimulated in the nasal mucosa, there is a reflex closure of the glottis at the end of inspiration causing in a delay in the initiation of expiration. This unique pattern is characterized by a lengthening of the time of expiration due to a pathognomonic pause occurring after inspiration, resulting in a decrease in respiratory rate (Figure 7). The duration of the pause is proportional to the intensity (or logarithm of the exposure concentration) of the airborne chemical stimulus to which the mouse is exposed (Alarie 1973). Depending on the chemical, this decrease in respiratory rate reaches a maximum and plateaus while the exposure continues, or reaches a maximum response and is then followed by a weakening of the response over the remainder of the exposure. Regardless of the fashion in which it occurs, this maximum response level can be used in the development of a concentration-

response relationship for the particular chemical exposure (analogous to a dose-response relationship) (Schaper 1993). From this concentration-response relationship, a determination of the concentration at which the pre-exposure respiratory rate is depressed by 50% can be made, and in a 1966 paper Alarie termed this concentration the RD_{50} (Alarie 1966). Of greater importance is the fact that in that 1966 study, 52 chemicals were tested both in mice and in human volunteers and it was concluded that "every compound tested in humans and reported as irritating or nonirritating at a particular concentration was also classified accordingly when tested in mice at similar concentrations" (Alarie 1966). In addition, the same author later reported in 1973 that the concentration of a chemical resulting in an RD_{50} response in male Swiss-Webster mice was the same concentration of that chemical which would result in human subjects reporting intolerable irritation of the eyes, nose or throat (Alarie 1973).

5.3 CHARACTERISTIC PULMONARY IRRITANT BREATHING PATTERN CHANGES

Stimulation of unmyelinated vagal afferent fibers (C-fiber type) via type J receptors can result in a change in respiratory pattern characterized by two distinct phases:

1. The first phase occurs with low exposure concentration and is identified by rapid shallow breathing with a corresponding decrease in tidal volume and decrease in both the duration of inspiration and duration of expiration. There is no associated change in the tidal volume waveform such as that described during sensory irritation (Figure 7). This change is not very prominent in mice, slightly more prominent in rats, and is prominent in guinea pigs, dogs, rabbits and humans (Alarie, Schaper et al. 2000). In

addition, the first phase pulmonary irritation effect can be augmented and intensified by the addition of 10% CO_2 to the inhalation exposure chamber atmosphere, and this effect is especially prominent in guinea pigs (Matijak-Schaper, Wong et al. 1983; Schaper 1984).

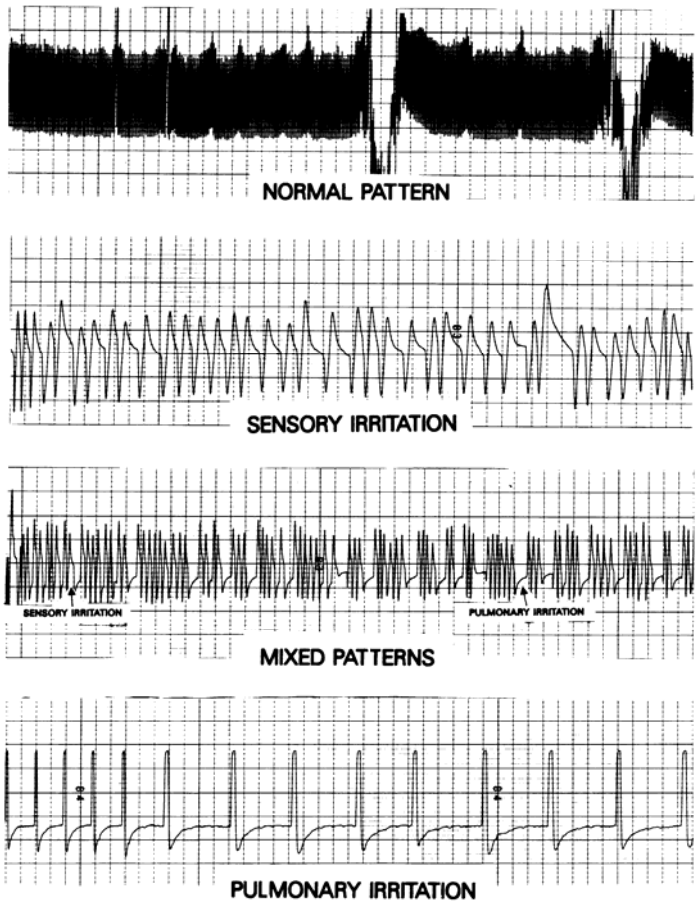

Figure 7. Respiratory Patterns in Mice (Ferguson, Schaper et al. 1986)

First tracing: obtained in normal mice and mice fitted with a tracheal cannula. A slightly reduced respiratory frequency is usually observed in the latter. **Second tracing**: characteristic pattern obtained with sensory irritants, showing a pause during expiration. Taken from one animal exposed to methyl isocyanate. **Third tracing**: characteristic pattern of sensory irritation but with some indication of pulmonary irritation. Taken from one animal exposed to methyl isocyanate. **Fourth tracing**: characteristic pattern of pulmonary irritation, showing a pause between the end of expiration and the beginning of inspiration. Taken from one animal fitted with a tracheal cannula during exposure to methyl isocyanate (Ferguson, Schaper et al. 1986). Reprinted from Toxicol Appl Pharmacol **82**(2), Ferguson, J. S., M. Schaper, et al., "Sensory and pulmonary irritation with exposure to methyl isocyanate," p. 329-35, Copyright ©1986, with permission from Elsevier

2. The second phase of the pulmonary irritant response appears subsequent to the first, and is identified by the development of a pause of increasing duration of the end of active expiration, resulting in what appears to be a pause between breaths on a tidal volume tracing (Figure 7). In contrast to the first phase response, the second phase response is much more prominent in mice than in the other species listed above (Alarie, Schaper et al. 2000).

As such, the second phase (development of a pause between breaths) is the physiologic response that indicates an airborne chemical's tendency to cause pulmonary irritation in mice, whereas the first phase (development of rapid shallow breathing) is the appropriate response to recognize in order to identify pulmonary irritation in the other species. In mice, as the pause between breaths increases in duration as a result of increasing intensity of pulmonary irritation (increasing exposure concentration), there is an associated decrease in respiratory rate or frequency of breaths per minute. The potency of a chemical as a pulmonary irritant in mice can then be defined by the concentration of the chemical required to decrease the respiratory rate by 50%. This measure is termed the $RD_{50}P$ and is analogous to the RD_{50} value used in the determination of the potency of the sensory irritant (Alarie, Schaper et al. 2000).

5.4 SELECTION OF ANIMAL SPECIES AND EXPERIMENTAL DESIGN

In 1966, Alarie developed the bioassay using mice as a tool to screen multiple chemicals for suitability as potential tear gas agents by rapidly identifying sensory irritant agents while ruling out those agents that also had the capacity to cause pulmonary irritation and alveolar injury

(Alarie 1966; Alarie, Schaper et al. 2000). Various laboratory animals were tested with chemicals known to cause sensory irritation and pulmonary irritation, as well as chemicals known not to cause sensory irritation and not to cause pulmonary irritation. At that time, the mouse bioassay was chosen because of its ability to discern between these two effects (Alarie 1966; Alarie, Schaper et al. 2000).

A review of animal bioassays in 2000 recommended that for the purpose of studying pulmonary irritation, it is best to use either guinea pigs for their first phase pulmonary irritant response or mice for their second phase effect. Rats were described as a poor choice to study pulmonary irritation using this bioassay because their first phase effect is more pronounced than in mice while their second phase effect is less pronounced than in mice, with the result that neither effect is particularly prominent. Rats and guinea pigs were also described as poor choices to study sensory irritation in the same review because of their decreased sensitivity in comparison to mice (Alarie, Schaper et al. 2000). An earlier literature review performed in the development of a database for sensory irritants found that rats were generally less sensitive to sensory irritants than mice, however, at that time the dataset was described as too limited to allow any firm conclusions to be drawn regarding "the appropriateness of using rats to evaluate sensory irritants" (Schaper 1993). In addition, it has also been documented that substantial intra-species variation in sensitivity also occurs, and a factor of 10 has been reported between the most sensitive and the least sensitive mouse strains (Alarie, Kane et al. 1981; Ballantyne 2006). The 1984 American Society for Testing and Materials (ASTM) standard method describing the bioassay was clearly developed with an understanding of this marked difference in sensitivity between different strains and sexes of mice: in the section on test animals within the description

of the standard test method it is stated that it is imperative that only male Swiss-Webster mice be used as the test animals for the bioassay (ASTM 1984).

5.5 TECHNICAL DESCRIPTION OF THE SENSORY AND PULMONARY IRRITATION BIOASSAY

A detailed technical description of the mouse bioassay is available in the 1966 publication in which Alarie first presented a description of the method (Alarie 1966), as well as being accessible in the American Society for Testing and Materials (ASTM) 1984 standard (ASTM 1984) where it is presented with some additional refinements. A brief technical summary to provide an overview of the method is provided in this section.

The objective of the sensory and pulmonary irritation bioassay method as first described in 1966 (Alarie 1966) and then established as an ASTM standard in 1984 (ASTM 1984) is to continuously monitor respiration in unanesthetized male Swiss-Webster mice during exposure to a set concentration of agent in order to identify the characteristic change in breathing pattern, and when this breathing pattern reaches a maximum or a plateau, to record the corresponding decrease in respiratory frequency (Alarie, Schaper et al. 2000). This is accomplished by placing the mouse in a body plethysmograph, with the head of the animal outside the body plethysmograph chamber and a (relatively) air-tight seal at the level of the neck of the animal. A pressure transducer is attached to the body plethysmograph chamber, and in the case of a sealed controlled temperature environment, the change in tidal volume will result in a proportional change in pressure recorded by the transducer. As such, when the animal modifies its respiratory pattern in response to a sensory or pulmonary irritant, the characteristic change in the breathing

pattern is reflected in the tidal volume (pressure transducer) waveform (Alarie, Schaper et al. 2000). The duration of exposure allowed by the method quite broad, and although the duration of exposure for sensory irritants is usually set at 30 minutes, the recommended duration for pulmonary irritants is three hours owing to the slower development of the characteristic change in respiratory pattern (Alarie, Schaper et al. 2000). The sensory and pulmonary irritation bioassay has also been used with much shorter or repeated exposures, depending on the specific requirement of the investigator (Alarie, Schaper et al. 2000).

The sensory and pulmonary irritation bioassay as described in the ASTM standard test method requires the exposure of four animals simultaneously and provides detailed equipment specifications as well as precise guidelines for preparation of the exposure chamber and the test agent sample, calibration of the equipment, operating procedure, and interpretation and reporting of results.

5.6 VALIDATION AND CALIBRATION OF THE BIOASSAY

5.6.1 Validation and calibration of the sensory irritation bioassay

The term validation refers to the correct prediction of negative and positive findings between the *qualitative* response obtained with a bioassay and *qualitative* response obtained in humans (Alarie, Schaper et al. 2000). The qualitative response in the test species need not be the same as a qualitative response in humans for a bioassay to be valid, but the response observed in the bioassay does need to predict response of interest in humans (Alarie, Schaper et al. 2000). For the Swiss-Webster male mouse sensory irritation bioassay it has been shown that for a broad range of chemicals as well as mixtures, a perfect rank order correlation exists between the

respiratory rate depression seen in mice and the subjective reports of sensory irritation in exposed humans (ASTM 1984).

The Swiss-Webster male mouse bioassay has also been described as having been calibrated in that the results of the mouse bioassay have been shown to be capable of being extrapolated to human response in a *quantitative* manner. In studies performed in the 1970s and 1980s, it was shown that human subjects would report slight sensory irritation at a chemical concentration equal to $0.1\times RD_{50}$, and that they would report minimal or no effect that a concentration equal to $0.01\times RD_{50}$. Alarie later published work demonstrating the ability of the bioassay to predict acceptable levels of exposure and also to function as a basis of establishing acceptable levels of exposure through the correlation between the American Conference of Governmental Industrial Hygienists (ACGIH) Threshold Limit Value (TLV) for 40 known sensory irritants and $0.03\times RD_{50}$ (the geometric mean of $0.1\times RD_{50}$ and $0.01\times RD_{50}$) (Schaper 1993). Some additional proposed protocols for the establishment of occupational exposure guidelines utilizing the RD_{50} include simply recommending that the TLV be established somewhere between $0.1\times RD_{50}$, and $0.01\times RD_{50}$, and establishing the short-term exposure limit for an agent at $0.1\times RD_{50}$ (Ballantyne 2006).

Schaper also produced and published a database in 1993 encompassing 295 chemicals and mixtures that had been tested either using the ASTM mouse bioassay or variations of the bioassay, establishing the ASTM mouse bioassay as one of the most extensively validated and calibrated toxicologic methods for airborne chemicals. In the review, correlation between the RD_{50} and the ACGIH TLV was strongest for the male Swiss-Webster mouse ($R^2 = 0.90$), although other mouse strains also showed high degrees of correlation (Schaper 1993).

5.6.2 Validation and calibration of the pulmonary irritation bioassay

In the case of the pulmonary irritation bioassay, ethical considerations clearly dictate that no experimental validation or calibration is possible. However, a review of available literature in 2000 indicated that the first phase and second phase pulmonary irritant reflex responses have been remarkably consistent across all species tested with agents known to be pulmonary irritants in humans, although as discussed the relative intensity of each phase may vary significantly between species (Alarie, Schaper et al. 2000). The mouse bioassay has shown itself to reliably identify both sensory and pulmonary irritation and the 1984 ASTM standard formally recognizes this capacity by approving the mouse bioassay to recognize and quantitate both the sensory and the second phase pulmonary irritation effects. Although protocols have been proposed that utilize the $RD_{50}P$ to estimate safety factors for pulmonary irritants, in terms of calibration no formal relationship exists for the pulmonary irritation bioassay $RD_{50}P$ in the way that it has been established for the sensory irritation bioassay RD_{50} (Alarie, Schaper et al. 2000).

The Swiss-Webster mouse sensory irritation bioassay has been a robust method used by many investigators, and as discussed has been established as an American Society for Testing and Materials (ASTM) standard method since 1984 (ASTM 1984). Although the mouse bioassay allows for the assessment of both sensory and second phase pulmonary irritation effects by generating a tidal volume (pressure transducer) waveform, it does not have the capacity to measure air flow velocity and therefore does not allow the investigator to detect air flow limitation. Per the American Society for Testing and Materials protocol, the method has been reevaluated and re-approved every 5 years since its acceptance as an ASTM standard (Alarie, Schaper et al. 2000).

5.7 ADAPTATIONS OF THE SENSORY AND PULMONARY IRRITATION BIOASSAY

5.7.1 Mixtures

Both the sensory and pulmonary irritation bioassay method as first described in 1966 (Alarie 1966) and the ASTM standard method established in 1984 (ASTM 1984) are capable of evaluating complex mixtures without the investigator knowing the detailed specifics of the mixture composition. Notably, the ASTM standard method has been used in conjunction with mixture generating systems to evaluate complex mixtures including photochemical smog, fly ash, air fresheners, fragrance products, organic dusts and emissions from *Stachybotrys chartarum*, as well as combustion gases, thermal decomposition products from plastics, aerosols of metalworking fluids (Alarie, Schaper et al. 2000), and asphyxiants (Alarie 2002).

5.7.2 10% CO_2 challenge and related modifications to the ASTM method

In 1982, Wong and Alarie developed an experimental model utilizing a single unrestrained guinea pig placed in a whole body plethysmograph that is actually open to the atmosphere, but because of the inlet-outlet system and airflow flow used, actually behaves like a sealed barometric (whole body) plethysmograph (Wong and Alarie 1982). The inlet and outlet through which the exposure atmosphere continually flows is designed and constructed such that the fast pressure wave created by respiration is not lost to the outside and is able to be measured by a pressure transducer or microphone (Alarie, Schaper et al. 2000). The method assesses the pulmonary 'performance' of unrestrained and un-anesthetized guinea pigs by measuring the increase in tidal volume, respiratory frequency and minute ventilation induced by exposure to an

exposure atmosphere containing 10% CO_2. One of the primary findings of study was that the CO_2 response was shown to be highly reproducible, especially for individual animals. Sulfuric acid mist is known to increase airway flow resistance and decrease lung compliance, and exposures to sulfuric acid mist were shown to attenuate the CO_2 response in a concentration-dependent manner. In addition, the recovery from acute lung injury induced by exposure to the sulfuric acid mist was evident from a daily CO_2 challenge (Wong and Alarie 1982). Although it is clearly better suited to less active animals such as guinea pigs, the 10% CO_2 challenge method offers the advantage of being a noninvasive procedure and therefore has excellent applicability for chronic studies of airway responsiveness because of the capability of performing repeated evaluations on the same animals (Alarie, Schaper et al. 2000).

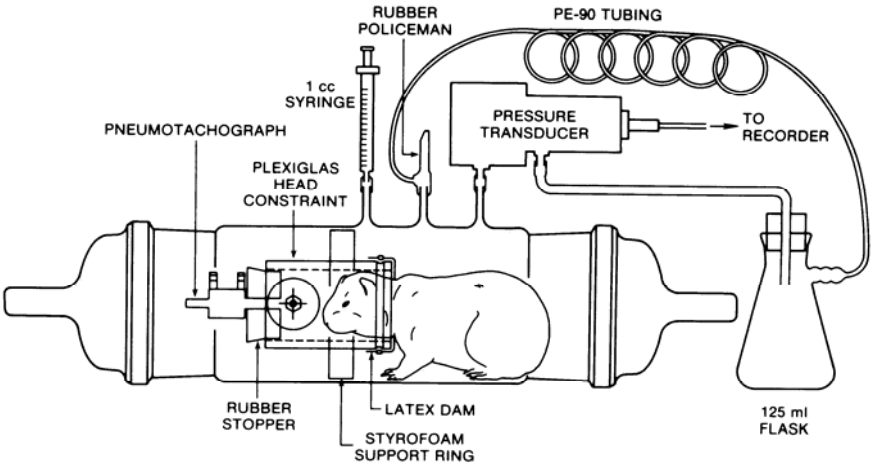

Figure 8. Exposure System for CO_2 Challenge with Head Chamber (Matijak-Schaper, Wong et al. 1983) Reprinted from Toxicol Appl Pharmacol **69**(3), Matijak-Schaper, M., K. L. Wong, et al., "A method to rapidly evaluate the acute pulmonary effects of aerosols in unanesthetized guinea pigs," p. 451-60, Copyright ©1983, with permission from Elsevier

A modification of the CO_2 challenge method was subsequently developed in 1983 where in addition to the guinea pig being placed in a barometric plethysmograph (Figure 8) in order to

measure tidal volume and breathing frequency, a head chamber with a pneumotachograph was added to the apparatus to enable the measurement of inspiratory and expiratory flow (Matijak-Schaper, Wong et al. 1983; Ferguson 1988). This development allowed the calculation of flow-volume and plethysmograph pressure-volume loops which subsequently enabled distinctions to be made between airflow limitation and first phase pulmonary irritation responses (Schaper, Thompson et al. 1985).

5.7.3 Additional modifications to the ASTM method

In 1993, Vijayaraghavan et al. presented a modification of the ASTM bioassay that enabled the recognition and quantification of the effects of airborne chemicals at any of the three levels of the respiratory tract. The first modification was made using a dual-plethysmograph method (Figure 9) and tested for respiratory responses to a known sensory irritant, airway constrictor, vagal nerve stimulant and a pulmonary irritant. The approach clearly identified and was able to quantify characteristic changes in flow and flow-volume measurements indicative of upper respiratory tract sensory irritation, airflow limitation, and first or second phase pulmonary irritation (Vijayaraghavan, Schaper et al. 1993). This adaptation of the ASTM method starts with the standard mouse bioassay with the modification that tidal volume is measured by integrating the output of a pneumotachograph attached to the body plethysmograph instead of using calibrated pressure transducer recordings (Alarie, Schaper et al. 2000).

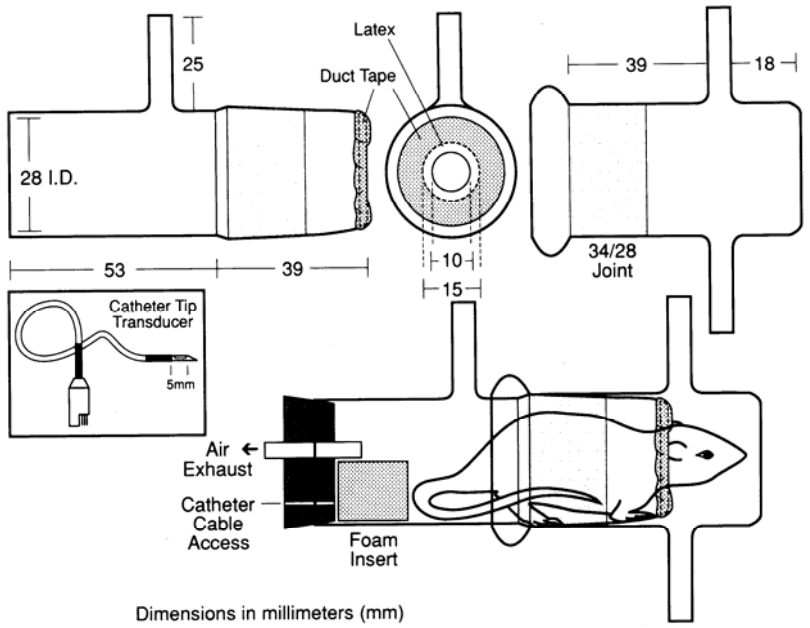

Figure 9. Head Chamber/Body Plethysmograph for Mice (Vijayaraghavan, Schaper et al. 1993)
Both the head chamber and body plethysmograph have inlet and outlet ports to attach a pneumotachograph on the inlet side and a pump for air exhaust on the outlet side (Vijayaraghavan, Schaper et al. 1993). Reprinted from Arch Toxicol **67**(7), Vijayaraghavan, R., M. Schaper, et al., "Characteristic modifications of the breathing pattern of mice to evaluate the effects of airborne chemicals on the respiratory tract," p. 478-90, Fig. 2, Copyright ©1993. With kind permission of Springer Science and Business Media

This study also included the use of trans-thoracic pressure measurements to document the validity of the substitution of a flow-volume curve measurement as a surrogate for invasive trans-thoracic pressure measurements in the preliminary identification of airflow limitation effects (Figure 9) (Vijayaraghavan, Schaper et al. 1993). Subsequent studies improved upon the 1993 modifications by developing rule-based computer programs to aid in the recognition of the types of effect on the respiratory tract by classifying each breath automatically, rather than by visual examination of the flow-time and volume-time curves (Figure 10) (Vijayaraghavan, Schaper et al. 1994; Boylstein, Luo et al. 1996). A review of animal bioassays in 2000 identified this system the best method to evaluate all possible effects, i.e. sensory irritation, airflow

limitation and pulmonary irritation, although restraint of the animal at the neck is required (Alarie, Schaper et al. 2000).

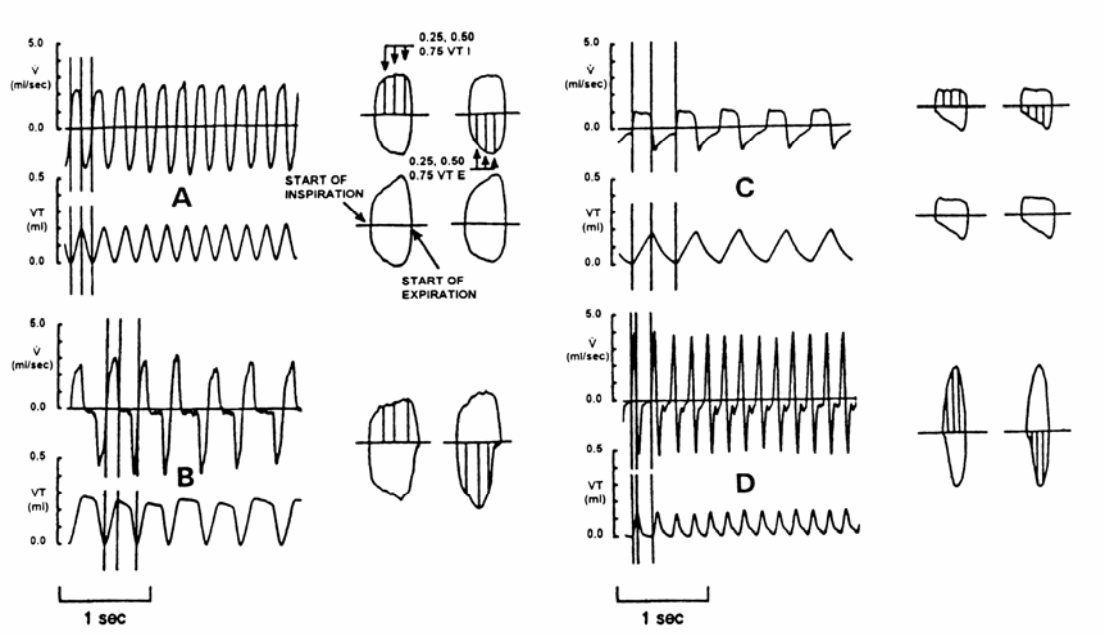

Figure 10. Plots of Digitized Flow and Integrated Tidal Volume (Vijayaraghavan, Schaper et al. 1993) The horizontal line drawn at zero flow (V) separates inspiration (upward) from expiration (downward). At the right are flow-volume loops. **A**: Control mouse. **B**: During exposure to a sensory irritant. **C**: During exposure to a airway obstructor. **D**: During exposure to a vagal stimulant (pulmonary irritation response) (Vijayaraghavan, Schaper et al. 1993). Reprinted from Arch Toxicol **67**(7), Vijayaraghavan, R., M. Schaper, et al., "Characteristic modifications of the breathing pattern of mice to evaluate the effects of airborne chemicals on the respiratory tract," p. 478-90, Fig. 4, Copyright ©1993. With kind permission of Springer Science and Business Media

5.8 CRITICISMS OF THE SENSORY AND PULMONARY IRRITATION BIOASSAY

Many occupational exposure limits are based on irritancy (Ballantyne 2006) and the calculated $0.03 \times RD_{50}$ value is generally excellent starting point to assign as a preliminary Threshold Limit

Value (TLV): a TLV may need to be decreased to prevent toxic effects not related to peripheral chemosensory irritation, but it will never be higher than the $0.03 \times RD_{50}$ level (Alarie, Schaper et al. 2000). In approaching this concern, the ASTM 1984 standard clearly states that the method "will predict values are too high for compounds of low reactivity that are metabolically activated, and also for pulmonary irritants" (ASTM 1984). These clearly stated caveats concerning the application of the bioassay notwithstanding, some authors have expressed concern about the suitability of this method for estimating occupational exposure limits. In 1992, Bos published a critique of the use of the ASTM method for assessing occupational health risk and expressed a number of concerns including that inflammatory and other pathologic changes were seen at levels below the RD_{50}, and that the $0.03 \times RD_{50}$ threshold was established empirically and not on the basis of scientific evidence of correlation between $0.03 \times RD_{50}$ and systemic toxicity (Bos 1992; Ballantyne 2006). With this in mind, Bos recommended a number of specific procedures be followed with respect to the use of the sensory irritation bioassay (Bos 1992; Ballantyne 2006):

1. In view of interspecies variation, 2 species should be used with the results for the most sensitive species being chosen to establish the RD_{50}
2. Time-response and log concentration-response curves should be obtained
3. Exposure should be continued until a plateau is obtained
4. There should be verification that no pulmonary irritation is occurring
5. For every material tested, it should be determined whether the response is due to irritation or toxicity, and that the toxicity is not occurring below the concentration causing sensory irritation

In addition, the author also stated that, on principle, occupational exposure limits should be based on a no adverse effect level. These recommendations, and the criticism underlying them, present more of a challenge to the manner in which results of the method are applied by than to the validity and degree of calibration of the ASTM method itself.

Finally, there have also been reports that the method results in hemorrhagic pituitary gland lesions in the test animals in studies involving multiple exposures (Kaempfe and Dudek 1994). Subsequent studies with Swiss-Webster mice given single sham and dust exposures performed to address this concern did show hemorrhagic pituitary lesions within both the sham and the test material groups and the pituitary lesions were felt to be secondary to increased pressure in the blood supply to the pituitary gland due to the neck restraint (Werley, Burleigh-Flayer et al. 1996). However, given the usual short duration of sensory irritation studies that employ the ASTM bioassay (a total of seven days including post-exposure observation), and the fact that disturbances in the function of the pituitary gland as a result of the pituitary lesions would not be expected to alter normal physiology in the short term (hemorrhages were not severe enough to cause a mass effect and brain or brainstem compression), the authors concluded that the lesion would have little effect on the usefulness of the procedure as a screening test. It was also concluded that effects on the pituitary gland produced by some inhalational agents could potentially be obscured by the pituitary lesions secondary to neck restraint and therefore, it was "suggested that similar studies be conducted using concurrent control groups of mice" (Werley, Burleigh-Flayer et al. 1996; Ballantyne 2006).

6.0 APPLICABILITY OF THE BIOASSAY TO DEPARTMENT OF HEALTH AND HUMAN SERVICES AND NATIONAL INSTITUTES OF HEALTH OBJECTIVES

In a 1966 monograph, Alarie published the first description of an animal bioassay for the detection of sensory irritation from airborne chemicals that also permitted qualitative extrapolation to human responses (Alarie 1966). Reflex reactions resulting from respiratory tract exposure to irritant xenobiotics are the foundation upon which this bioassay was based and these have been reviewed in previous chapters. In the original 1966 publication, the stated criteria for the development of the method were as follows (Alarie 1966):

1. Ability to identify that a substance delivered as an aerosol or vapor is irritating to the upper respiratory tract.
2. Sufficient simplicity of the method such that it allowed screening of a large number of compounds.
3. Ability to develop minimum effective concentration and concentration-response curves for comparison purposes.
4. Reproducibility of results.

These criteria continue to be guidelines for the assessment of experimental approaches relevant to the Department of Health and Human Services and National Institutes of Health objective in 2005 of encouraging "research about how the upper respiratory tract and lungs respond to acute exposure to highly toxic chemicals and subsequent inhalation, so that preventive strategies can be improved, antidotes devised to lessen initial irritation of mucosal surfaces, mucosal absorption

minimized, and acute lung injury causing pulmonary edema counteracted" (DHHS and NIH 2005). This is especially true in the context of the stated goal of development of preventive strategies and antidotes, since the same qualities of an experimental method that allow the development concentration-response curves are essential to allow the following of an endpoint response to a treatment or an antidote. Similarly, the features of a method that enable detection of irritant effects before tissue pathological changes are observed should also allow rapid effective screening of possible treatments for efficacy, rather than initially relying on time consuming histopathological tissue examination to assess outcomes.

The text of the 1984 ASTM Standard Test Method for Estimating Sensory Irritancy of Airborne Chemicals, in describing the significance and use of the test method, comments that the method was sufficiently simple to permit the testing of large numbers of materials, possessed the qualities of good reproducibility, and was capable of generating concentration-response curves for the purposes of compound comparison (ASTM 1984). Per the American Society for Testing and Materials protocol, the method has been reevaluated and re-approved every 5 years since its acceptance as an ASTM standard (Alarie, Schaper et al. 2000). The establishment of the method as an ASTM standard does not imply that there aren't caveats to the use of the method, and a number of criticisms of the method have been tabled concerning the use of the RD_{50} in setting occupational exposure limits, but as a rule these concerns are associated with the application of the results obtained using the method, and are not criticisms of the methodological approach itself.

Modifications to the method in 1993 extended its capacities to allow for screening of effects of airborne chemicals at all three levels of the respiratory tract (including peripheral chemosensory irritation, airflow limitation due to either bronchoconstriction or inflammatory

reaction along the conducting airways, and first and second phase of pulmonary irritation), based on the recognition of a characteristic modification of the normal breathing pattern of un-anesthetized mice. The publication that reported this advancement also reported the experimental system to be easy to construct as well as to operate (Vijayaraghavan, Schaper et al. 1993). Subsequent studies utilizing the method also reported the system to be simple and easily implemented, and also improved upon reproducibility by developing rule-based computer programs to aid in the recognition of the types of effect on the respiratory tract by classifying each breath automatically, rather than by visual examination of the flow-time and volume-time curves (Vijayaraghavan, Schaper et al. 1994; Boylstein, Luo et al. 1996). The application of rule-based computer programs to data obtained with the modified method also enabled well-defined criteria to be applied in an objective way to easily classify the observed effect, and also provided rapid quantitation of the effect (Vijayaraghavan, Schaper et al. 1994; Boylstein, Anderson et al. 1995).

6.1 POTENTIAL BIOLOGICAL AND CHEMICAL TERRORISM AGENTS

In April 2000, the Centers for Disease Control and Prevention of the US Department of Health and Human Services published a strategic plan for preparedness and response to biological and chemical terrorism. Potential biological and chemical terrorism agents are numerous, and in the process of formulating a plan to best protect the public, attention was focused upon agents known to be highly contagious and that could be readily dispersed or could be engineered for widespread dispersion as a small-particle aerosol and therefore, have the greatest impact on US health and security (CDC 2000). This resulted in the development of a list of critical biological

agents as well as chemical agents that bore the features of easy dissemination or transmission in the case of biological agents, and in the case of chemical agents, were known to already be used as weaponry or were felt to be of significant availability to terrorists. The additional qualities of likelihood of high mortality or morbidity, potential to generate panic and social disruption and the requirement for special action for public health preparedness were also included as criteria in the selection of the high priority chemical and biological agents.

The highest priority, Category A, critical biological agents included smallpox, anthrax, plague, botulism, tularemia and the hemorrhagic fever viruses. The categories of high priority chemical agents included nerve agents (organophosphates) such as sarin, GF and VX, blood agents including hydrogen cyanide, blister agents such as nitrogen and sulfur mustard, pulmonary agents such as phosgene, chlorine and vinyl chloride, and a selection of other agents including pesticides and poisonous, flammable or corrosive industrial chemicals (CDC 2000).

6.2 POTENTIAL TERRORISM AGENTS PREVIOUSLY ASSESSED USING THE BIOASSAY

As discussed, the range of toxins that have been assessed using the bioassays presented in the previous chapter is extremely broad, and includes pure compounds, mixtures, organic dusts, emissions from *Stachybotrys chartarum*, as well as thermal decomposition products, smoke (Alarie, Schaper et al. 2000) and asphyxiants (Alarie 2002). In the context of the Department of Health and Human Services and National Institutes of Health concern in 2005 surrounding the "US population's potential exposure to aerosolized, inhaled harmful chemicals possibly liberated as part of bioterrorism attacks against assembled groups of the civilian populace" (DHHS and

NIH 2005), there are many published studies investigating exposure to potential terrorism agents employing commonly used laboratory animal species that have utilized the bioassays described above as well as similar bioassays. In the following sections there are a few specific examples of high priority chemical or biological agents where the bioassay has contributed significantly to the understanding of the toxicity due to the substance.

6.2.1 Methyl Isocyanate

Although methyl isocyanate is not on the Centers for Disease Control and Prevention of the US Department of Health and Human Services published list of high priority chemical agents, it was the causative agent in the world's worst industrial disaster (Dhara and Dhara 2002) and warrants consideration from the point of view of the large numbers of human civilian population exposed, injuries caused, and deaths that occurred.

A 1987 study evaluating methyl isocyanate inhalation toxicity utilized the standard ASTM method with Swiss-Webster mice as the experimental animals to estimate potency of methyl isocyanate as a sensory and pulmonary irritant and also used a guinea pig model employing a 10% CO_2 challenge to follow and assess the recovery after a single high level exposure (Alarie, Ferguson et al. 1987). The investigation revealed that methyl isocyanate was a highly potent sensory and pulmonary irritant in mice and demonstrated that the primary pulmonary effect of methyl isocyanate in guinea pigs was that of airways obstruction. It was also noted in the report that the human responses to methyl isocyanate exposure indicated by the flow-volume curves obtained in survivors of the Bhopal disaster displayed a good deal similarity with the guinea pig responses seen in this study, increasing confidence in the flow-volume

analysis method in guinea pigs to predict pulmonary toxicity in humans (Alarie, Ferguson et al. 1987).

6.2.2 Phosgene

Phosgene, or carbonyl chloride, is a highly reactive gas of historical interest, a high-production industrial chemical intermediate, and a current public health concern as one of the high priority chemical pulmonary agents listed by the Centers for Disease Control and Prevention of the US Department of Health and Human Services. It was the most lethal gas used in World War I, and up to 80% of the deaths caused by poison gas during that war have been attributed to phosgene exposure (Borak and Diller 2001). Phosgene-induced lung injury has been investigated extensively in animal models (Greenfield, Brown et al. 2002) and the pulmonary toxicity produced by the agent has also been utilized as a model for non-cardiogenic, or permeability-type pulmonary edema such as seen in acute respiratory distress syndrome (Borak and Diller 2001). Because of low water solubility, phosgene passes through the upper respiratory tract without being 'scrubbed', and reaches the lower respiratory tract to cause extensive parenchymal damage.

Many techniques have been used to evaluate the mechanisms of toxicity and the progression of acute lung injury after exposure to phosgene. The experimental animal models that have been used often involve whole-body exposure inhalation techniques and have focused on dealing with the question of whether the concentration or the concentration × exposure time ($C \times t$) product for phosgene exposure determines the biologic end point (known as Haber's Law) (Sciuto 2006). Especially for phosgene, nose-only exposure is generally considered preferable to whole-body exposure because of more rapid attainment of steady-state exposure concentration.

In a recent study, the concentration × exposure time ($C \times t$) relationship of phosgene was examined in rats using a directed-flow nose-only exposure design and exposure durations of concern for accidental exposures (10 to 240 minutes) followed by a post-exposure period of 2 weeks (Pauluhn 2006). The exposure atmosphere was sampled and analyzed from the vicinity of the animal breathing zone, and individual animal respiratory patterns during exposure were analyzed using a plethysmograph fitted with differential pressure transducers and a pneumotachograph, similar to approach used in the mouse bioassay described in the previous chapter. Initial and concentration-dependent increases in apnea time, with associated decrease minute volume were observed on exposure to phosgene, similar to the changes observed in rats exposed to other respirable irritant aerosols, but these changes leveled off after exposure exceeded 10 to 15 minutes. The authors concluded that the observed response appeared to follow Haber's Law, although departures may occur with high-level, short-term exposures as a result of changes in breathing pattern and ventilation changes specific to the rodent (Pauluhn 2006).

6.2.3 Mustard gas

Sulfur mustard is a blister agent, one of categories of high priority chemical agents selected by the Centers for Disease Control and Prevention of the US Department of Health and Human Services when the agency published a strategic plan for preparedness and response to biological and chemical terrorism in 2000. Mustard gas is a potentially effective terrorist or chemical warfare agent and it is widely available with over a dozen countries maintaining known stockpiles. The chemical is also relatively easy and inexpensive to manufacture. An alkyating agent with short and long-term effects on the skin, eyes, and respiratory system, when absorbed

in large amounts, it can also produce a wide range of systemic toxicities, including hematologic, immunologic, and digestive disorders (Greenfield, Brown et al. 2002).

In 1997, an experimental model using a modified ASTM approach described by Vijayaraghavan in 1994 was used for exposing mice to varying concentrations of sulfur mustard vapor (Vijayaraghavan 1997). The respiration of the exposed mice within the body plethysmograph was monitored using a rule-based computer program (capable of identifying the breathing pattern as sensory irritation, airflow limitation, or pulmonary irritation) for the one-hour exposure, and respiration was also followed for modifications in the breathing pattern until seven days post-exposure. Sulfur mustard induced sensory irritation during exposure, and the characteristic concentration dependent decrease in respiratory rate was observed. The exposed animals' respiratory rate decreased over the following days, dependent upon the exposure concentration, and the breathing pattern at that time was characteristic of airflow limitation. During the experiment there was no evidence of pulmonary irritation identified through the modification of breathing patterns. These findings correlated with pulmonary complications and complaints observed in sulfur mustard exposed victims: humans exposed to sulfur mustard display asthma-like symptoms where the primary affected regions of the respiratory tract are the laryngeal mucosa and tracheal mucosa, not the pulmonary region (Vijayaraghavan 1997).

6.2.4 Ricin

Ricin is a protein cytotoxin derived from the castor bean and is one of the most potent toxic compounds known with an estimated lethal human dose as low as 1 μg/kg (Wannemacher and Anderson 2006). Throughout the world approximately 1,000,000 tons of castor beans are mechanically processed on an annual basis in the production of castor oil and residual waste

mash is approximately 5% ricin by weight (Greenfield, Brown et al. 2002). The toxin is widely available and can also be purified in an inexpensive, low-tech manner, justifying its placement on the Centers for Disease Control and Prevention of the US Department of Health and Human Services published list of high priority biological agents. The agent is stable under ambient conditions, and is effective by either the oral or inhalation route (Greenfield, Brown et al. 2002). In the case of ricin, where the target organ may be determined by the site of initial deposition of the toxin, variations in the toxin distribution can potentially have a substantial impact on pathogenesis. Therefore, methods and equipment must be carefully evaluated when interpreting research data. This would include both the methods and equipment used to generate and deliver a ricin aerosol, as well as the mode of administration, be it a whole-body exposure chamber, nose-only, head-only or closed-loop exposure system (Wannemacher and Anderson 2006).

In terms of the efficacy of therapeutic agents against inhaled ricin, a recent review reported that approximately 100 compounds have been screened utilizing an *in vitro* cell method, and though a few were protective in the *in vitro* model, none were efficacious in preventing or treating ricin toxicity in a mouse model (Wannemacher and Anderson 2006). A recent review in clinical medical literature also reported that although there is some hope that a primary prevention approach may be effective with the development of a vaccine against ricin, there is no currently available antidote to the toxin and that there are no specific treatment protocols in existence for ricin exposure: treatment is symptomatic and supportive only (Audi, Belson et al. 2005).

However, there are unpublished data included in a 1990 University of Pittsburgh PhD thesis suggesting that dibutyryl cyclic adenosine monophosphate might be effective as an antidote to decrease or prevent pulmonary effects post-ricin exposure (Rosato 1990). This study

assessed the pulmonary effects of inhaled ricin in mice utilizing the ASTM standard method with the objectives of investigating the mechanisms of pulmonary toxicity from ricin following inhalation exposure and determining if the increase in respiratory frequency in mice (a reflex reaction due to pulmonary irritation) could be blocked utilizing either a galactose containing sugar or dibutyryl cyclic adenosine monophosphate (dBcAMP), an agent that increases intracellular levels of cyclic adenosine monophosphate. This monograph reported that although the increase in respiratory rate seen in mice following exposure to 0.3 mg/m^3 ricin was unaffected by the galactose containing sugar, it was completely blocked by i.p. injections with 0.72 mg/kg dBcAMP in saline administered 30 minutes post-exposure and then daily for the next 5 days. One sub-group of mice exposed to 0.3 mg/m^3 ricin and given the dBcAMP was followed to day 29 with no significant increases in respiratory rate noted (Figure 11).

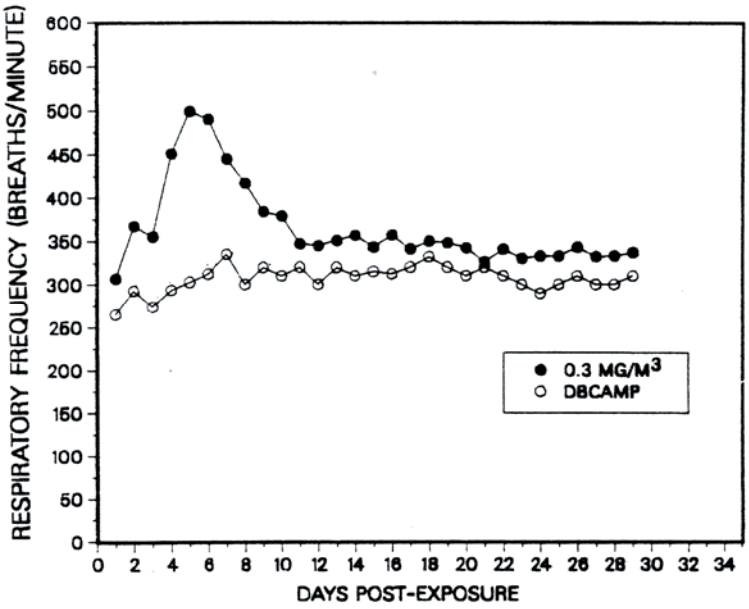

Figure 11. Respiratory Response of Mice Exposed to Ricin Followed by dBcAMP Compared to Ricin Exposure without dBcAMP (Rosato 1990)

Histology in the ricin-exposed dBcAMP-treated mice showed slight hyperinflation with very little hemorrhagic areas (day 5), whereas in comparison, mice exposed to 0.3mg/m3 ricin without receiving dBcAMP injections showed large areas of hemorrhage and frothy fluid in the trachea (day 3) (Rosato 1990).

In this particular instance, the qualities of the bioassay that allow detection of irritant effects before tissue pathological changes are observed has also allowed the investigator to rapidly discern between the effectiveness of two trial antidotes to ricin, well before histopathological tissue examination confirmed the finding.

7.0 RESEARCH PROPOSAL UTILIZING THE BIOASSAY TO ASSESS THE RESPIRATORY TRACT EFFECTS OF METHYL ISOCYANATE

7.1 BACKGROUND

The world's worst industrial disaster occurred in Bhopal, India on December 3, 1984, when water inadvertently entered into a storage tank containing approximately 40,900 kg of methyl isocyanate. The exothermic reaction that ensued resulted in the atmospheric release of an estimated 24,545 kg of methyl isocyanate and 12,800 kg of reaction products, spreading as a cloud over an area of approximately 40 km^2. The Indian council of Medical Research has estimated that 100,000 people lived within a 1 km radius of the Bhopal Union Carbide pesticide plant at the time of the accident (Mehta, Mehta et al. 1990). As a result of the release, the government of the state of Madhya Pradesh has reported that more than 200,000 persons were exposed to methyl isocyanate, more than 6000 died, and an estimated 50,000 continue to suffer from long-term health effects (Dhara, Dhara et al. 2002). Before the Bhopal incident, neither deaths nor cases related to toxicity from methyl isocyanate had been recorded (Mehta, Mehta et al. 1990), and only one published report existed in the literature on the toxicity of inhalation exposure to methyl isocyanate, although some limited data had been published indicating significant acute toxicity as well as dermal and corneal irritation (Alarie, Ferguson et al. 1987).

Since that time, a significant body of research has developed around human toxicity related to methyl isocyanate exposure, and review papers have been published that summarize

toxic effects on the human ocular, respiratory, reproductive, hematologic and immune systems, and also report genotoxicity and pediatric toxicity data (Mehta, Mehta et al. 1990; Dhara and Dhara 2002). Many of the reviewed human studies reportedly had methodological problems (retrospective design without suitable controls, bias in history taking, etc.), but well-designed animal studies exist that substantiate the development of acute lung injury and persistent airways obstruction after methyl isocyanate exposure (Mehta, Mehta et al. 1990).

As discussed in the previous chapter, a 1987 study evaluated methyl isocyanate inhalation toxicity utilizing the standard ASTM method with Swiss-Webster mice exposed to methyl isocyanate at 0.5 to 7.6 ppm for 90 minutes and revealed that methyl isocyanate was a highly potent sensory and pulmonary irritant (Alarie, Ferguson et al. 1987). The same investigation also included a guinea pig model employing a 10% CO_2 challenge to follow (for 35 days) and assess the recovery after a single high level exposure at 37 ppm for 3 hours and demonstrated that the primary pulmonary effect of methyl isocyanate in guinea pigs was that of airways obstruction. It was also noted in the report that the human responses to methyl isocyanate exposure indicated by the flow-volume curves obtained in survivors of the Bhopal disaster displayed a good deal of similarity with the guinea pig responses seen in this study, increasing confidence in the flow-volume analysis method in guinea pigs to predict pulmonary toxicity in humans. The investigation also revealed "the high potency of methyl isocyanate as a sensory and pulmonary irritant in mice and as a severe airway obstructor in guinea pigs; therefore, methyl isocyanate would be classified as a respiratory irritant", with potential to affect all 3 regions of the respiratory tract (Alarie, Ferguson et al. 1987). Other studies also confirmed the highly irritating nature of methyl isocyanate as a sensory and pulmonary irritant (Nemery, Dinsdale et al. 1987), and the development of fibrotic and occluded airways in a number of

commonly used animal species (Boorman, Brown et al. 1987; Boorman, Uraih et al. 1987; Nemery, Dinsdale et al. 1987; Srivastava, Vijayaraghavan et al. 1987).

7.2 RATIONALE FOR THE SELECTION OF AGENT AND METHOD

Evidence presented in previous chapters supports the conclusion that the 1994 modification of the mouse bioassay for sensory irritation (Vijayaraghavan, Schaper et al. 1994) is an ideal method to evaluate the effects of an inhaled agent on all three regions of the respiratory tract through its documented ability to identify and quantitate sensory irritation, airflow limitation and pulmonary irritation (restraint of the animal at the neck notwithstanding).

Methyl isocyanate is not on the Centers for Disease Control and Prevention of the US Department of Health and Human Services published list of high priority chemical agents, however, it is a good choice as an experimental agent for a number of reasons. As presented previously, experimental animal data exist indicating that methyl isocyanate is a respiratory irritant with potential to affect all three regions of the respiratory tract, and although the Department of Health and Human Services and National Institutes of Health stated program objectives in 2005 (DHHS and NIH 2005) focus on sensory irritation and pulmonary edema, airway obstruction is part of the continuum of clinically relevant human endpoints. In addition, methyl isocyanate is a good choice as an experimental agent because there are corroborating human observational exposure data available from the Bhopal incident. Finally, the combination of agent and method offers many opportunities to demonstrate outcomes for administered treatments and antidotes.

7.3 MATERIALS AND METHODS

An appropriate pilot study would reproduce some of the data of Ferguson et al. (Ferguson, Schaper et al. 1986), and also that of Boorman et al. (Boorman, Uraih et al. 1987) by exposing groups of four mice to methyl isocyanate at concentrations ranging from 0.3 to 30 ppm for 90 minutes, and then following the exposed groups of mice for 90 days to assess for evidence of recovery (as indicated by respiratory parameters) and correlate with histopathological findings.

The proposed method to evaluate the effects of inhalation exposure to methyl isocyanate on all 3 levels of the respiratory tract has been well described by Vijayaraghavan et al. (Vijayaraghavan, Schaper et al. 1994) and is firmly based on the sensory irritation bioassay presented by Alaire in 1966 (Alarie 1966) and now established as an ASTM standard method (ASTM 1984).

7.3.1 Animals

Pathogen-free male Swiss-Webster mice weighing between 22 and 28 g are to be used for all experiments. They should be held at least one week before being used for experiments to ensure healthy status, housed 4 per cage with food and water *ad libitum*, and maintained on a 12 hour light/dark cycle.

For exposure of mice via tracheal cannula (determination of pulmonary irritation exclusively), the animals are first anesthetized with i.m. sodium pentobarbital 50 mg/kg body weight and following an incision in the neck, the trachea is cut and a polyethylene tube is inserted and secured. Skin sutures are made to hold the cannula firmly in place and the region

around the sutures infiltrated with local anesthetic prior to recovery from general anesthesia. The animals are allowed to recover from anesthesia before exposure.

An application will be submitted to the Institutional Animal Care and Use Committee of the University of Pittsburgh for approval of the protocol prior to the initiation of any laboratory work.

7.3.2 Chemicals

Methyl isocyanate will be used as the exposure chemical.

7.3.3 Exposure apparatus

The exposure chamber consists of a 2.5 L glass cylinder with a baffle at the entrance to which 4 glass body plethysmographs are attached through ground glass joints as previously described (Vijayaraghavan, Schaper et al. 1994). A collar is made at the ground glass end of each body plethysmograph with an 8 mm (ID) hole in dental latex rubber and 10 mm (ID) hole in adhesive duct tape. These values can be increased when using larger mice. With the tubes removed from the chamber, the mouse is gently guided into the collar and the seal at the neck inspected. The mouse is then held in position using a foam insert in front of the cork sealing the end of the body plethysmograph.

7.3.4 Airflow measurements

Measurements will be made according to a method previously described (Vijayaraghavan, Schaper et al. 1993). Briefly, a calibrated pneumotachograph and a differential pressure

transducer are attached to the top part of each body plethysmograph. Continuous airflow of 170 ml per minute is maintained into each plethysmograph and pneumotachograph by a pump and critical orifice, and attached to the port at the rear of each plethysmograph (Vijayaraghavan, Schaper et al. 1994).

7.3.5 Computer programs for data collection and breath classification

The data acquisition, analysis, and printing program procedure to be used is available through Notocord Systems SAS (www.notocord.com) and is based on the rule-based algorithms developed and presented by Vijayaraghavan et al. in 1994 (Vijayaraghavan, Schaper et al. 1994). A detailed description of the criteria for breath classification is available in Vijayaraghavan et al. in 1994. Briefly, a breath is classified as normal, sensory irritation, pulmonary irritation, airflow limitation or combinations of these based upon three variables: the duration of braking during stage I of expiration (induced by sensory irritants), the duration of the pause before inspiration (induced by pulmonary irritants), and the decrease in expiratory airflow at the midpoint of tidal volume (a measure of airflow limitation) (Vijayaraghavan, Schaper et al. 1994).

7.3.6 Generation of methyl isocyanate exposure atmospheres

The desired exposure concentrations of methyl isocyanate in the range of 0 (control) and 0.3 to 30 ppm are to be obtained by method previously described by Ferguson et al. (Ferguson, Schaper et al. 1986). Briefly, methyl isocyanate vapor is obtained via the evaporation of methyl isocyanate (10 ml) held in a 500 ml bottle maintained at 0°C in an ice bath. The evaporating bottle is sealed with a rubber septum and covered with Teflon film. One 16-gauge hypodermic needle is passed through the septum from the inside and attached to a polyethylene tube, 160 mm

in length, for delivery of methyl isocyanate vapor to the exposure chamber. One 18-gauge hypodermic needle (50 mm in length) penetrates the septum from the outside for constant delivery of dry air to the evaporating bottle. This needle is connected to a microflowmeter equipped with a micrometer valve to regulate air delivery. To obtain various concentrations of methyl isocyanate, methyl isocyanate vapor from the evaporating bottle is mixed in an exposure chamber with no body plethysmographs attached. After allowing for steady state to establish, analysis for methyl isocyanate exposure concentration is carried in using a calibrated gas chromatograph, and when target levels are attained, the methyl isocyanate delivery tube from the evaporating bottle is changed over to a duplicate exposure chamber with body plethysmographs attached and operated at the same airflow as the calibrating exposure chamber (Ferguson, Schaper et al. 1986).

7.3.7 Exposure conditions and measurement of response

Duplicating the exposure conditions of Ferguson et al. (Ferguson, Schaper et al. 1986), a mouse is placed in one of the four body plethysmographs that are attached to the glass exposure chamber so that the head of the animal protrudes into the chamber. After introduction of a group of four mice (normal or fitted with a tracheal cannula) into the body plethysmographs, a 10 to 15 minute adaptation period is observed and subsequently a 10 minute measurement is made to obtain a baseline record. Each exposure is then to be conducted for a period of 90 minutes, followed by a 30 minute recovery period during which the respiratory patterns of the individual animals and group of animals is recorded. As previously described, recordings of respiratory parameters are obtained via a calibrated pneumotachograph and a differential pressure transducer attached to each body plethysmograph.

7.3.8 Statistical analysis

The data are to be evaluated using the log of the exposure concentrations versus the measured variables to obtain concentration-response relationships. For each data set, linear least squares regression analysis will be performed and a test for linearity will also be performed using individual values (n=4) at each exposure concentration used according to the method of Draper and Smith (Draper and Smith 1998). From the linear least squares analysis slope, intercept and correlation coefficient will also be obtained, and the slope coefficient will be tested via analysis of variance for significant difference from zero (Draper and Smith 1998). For the purposes of analysis, level of significance is to be set at $p < 0.05$.

8.0 SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

In a program announcement in 2005, The Office of Biodefense Research, the National Heart, Lung and Blood Institute, and the National Institutes of Health expressed concern about the "US population's potential exposure to aerosolized, inhaled harmful chemicals possibly liberated as part of bioterrorism attacks against assembled groups of the civilian populace" (DHHS and NIH 2005). The primary stated objective of the program is to "encourage research about how the upper respiratory tract and lungs respond to acute exposure to highly toxic chemicals and subsequent inhalation, so that preventive strategies can be improved, antidotes devised to lessen initial irritation of mucosal surfaces, mucosal absorption minimized, and acute lung injury causing pulmonary edema counteracted" (DHHS and NIH 2005).

In the context of this objective, and with the goal of developing a research proposal, the applicability of a mouse bioassay for sensory irritation (Alarie 1966; ASTM 1984) has been explored, including an overview of the basic anatomy, physiology and the physicochemical properties relevant to toxic inhaled agents, a brief review of other experimental inhalational toxicology methods, and with emphasis on modifications of the bioassay.

8.1 CONCLUSIONS AND RECOMMENDATIONS

8.1.1 Methyl isocyanate research proposal

An adaptation of the original mouse sensory irritation bioassay (Alarie 1966; ASTM 1984; Vijayaraghavan, Schaper et al. 1994) has been shown to be capable of evaluating the effects of inhaled agents at all three levels of the respiratory tract through its documented ability to identify and quantitate sensory irritation, airflow limitation and pulmonary irritation. The method also detects effects at concentrations below those at which histopathological changes occur and therefore, offers opportunities for rapid screening for effectiveness of administered treatments and antidotes.

Methyl isocyanate is a good choice as an experimental agent in a research project to demonstrate the capabilities of the modified mouse bioassay. Experimental animal data exist indicating that methyl isocyanate is a respiratory irritant with potential to affect all 3 regions of the respiratory tract, and corroborating human observational exposure data are available from the Bhopal industrial accident.

Therefore, a research project utilizing the modified mouse bioassay with methyl isocyanate as the experimental agent is proposed.

8.1.2 Potential future role of the model in the evaluation of the pulmonary effects of inhaled ricin

As presented in a previous chapter, Rosato assessed the pulmonary effects of inhaled ricin in a Ph.D. thesis completed at the University of Pittsburgh in 1990. This document also reported that the reflex increase in respiratory rate seen in mice following exposure to 0.3 mg/m^3 ricin was completely blocked by post-exposure i.p. injections with 0.72 mg/kg dBcAMP in saline. Histology in the ricin-exposed dBcAMP-treated mice was also reported to have shown slight hyperinflation with very little hemorrhagic areas (day 5) whereas in comparison, mice exposed to 0.3 mg/m3 ricin without receiving dBcAMP injections showed large areas of hemorrhage and frothy fluid in the trachea (day 3).

A recent review reported that approximately 100 compounds have been screened utilizing an *in vitro* cell method with a few showing some effect, however, none were efficacious in preventing or treating ricin toxicity in a mouse model (Wannemacher and Anderson 2006). In addition, a recent review in the *Journal of the American Medical Association* reported that although there is some hope that a primary prevention approach may be effective with the development of a vaccine against ricin, there is no currently available antidote to the toxin and that there are no specific treatment protocols in existence for ricin exposure: treatment is symptomatic and supportive only (Audi, Belson et al. 2005).

With this evidence, and experience gained in the practical management of the experimental apparatus with the methyl isocyanate proposal, a study using the modified mouse bioassay to reproduce Rosato's data would logically be proposed at a future date.

8.1.3 Potential future role of the model in developing countermeasures against aerosolized bioterrorism agents

A recent report from the Committee on Animal Models for Testing Interventions against Aerosolized Bioterrorism Agents recognized that the National Institute of Allergy and Infectious Diseases Strategic Plan for Biodefense Research (2002) gives highest priority to the development of countermeasures against aerosolized bioterrorism agents. Since it is not ethically appropriate to deliberately expose human subjects, the "development of countermeasures relies on the ability of the scientific community to adequately test effectiveness of countermeasures in animal models" (DHHS and NIH 2006).

The characteristics of the modified mouse bioassay that make it capable of assessing effects of inhaled agents at all three levels of the respiratory tract and also detecting effects at exposure levels below those at which histopathologic changes occur also give it a potential future role as an animal model for testing interventions against aerosolized bioterrorism agents. In support of this thought, the mouse bioassay has already been used to assess organic dusts and emissions from *Stachybotrys chartarum* (Alarie, Schaper et al. 2000). In addition, and as presented, the mouse bioassay was used by Rosato at the University of Pittsburgh in 1990 to assess not only the pulmonary effects of inhaled ricin, but also the effects of two proposed antidotes to the toxin (Rosato 1990).

APPENDIX A

KEYWORDS

The following keywords were commonly seen in the literature retrieved from MEDLINE and OLDMEDLINE databases using PubMed and Ovid search engines and cited in this thesis:

Experimental Technique
Drug Evaluation, Preclinical/instrumentation
Respiratory organs Pathophysiology.
Lungs Pathophysiology
Inhalation exposure
Pulmonary toxicology
Animal models
Atmosphere exposure chambers
Administration, inhalation

Pathophysiology of Pulmonary Injury
Pulmonary Alveoli/*physiology
Pulmonary Edema/*etiology/physiopathology/therapy
Respiratory Distress Syndrome, Adult/*diagnosis/drug therapy/*therapy
*Pulmonary Edema/etiology/pathology/therapy
Respiratory Tract Diseases/*chemically induced/diagnosis/therapy
Respiratory Distress Syndrome, Adult/complications/physiopathology
Respiratory Distress Syndrome, Adult/*etiology/metabolism
Respiratory Tract Diseases/chemically induced/diagnosis/therapy
Respiratory Distress Syndrome, Adult/blood/chemically induced/*enzymology

Phosgene
Pulmonary Edema/*chemically induced/*prevention & control
Phosgene/*poisoning
Lung/drug effects/metabolism
Administration, Inhalation
Lung/drug effects/physiopathology
Lung Diseases/chemically induced/physiopathology/*prevention & control

Pulmonary Edema/chemically induced/metabolism/*prevention & control
Pulmonary Edema/chemically induced/*drug therapy/metabolism
Chemical Warfare Agents/*toxicity
Pulmonary Edema/*chemically induced/*physiopathology/therapy
Respiratory Distress Syndrome, Adult/*chemically induced/pathology/physiopathology
Respiratory Distress Syndrome, Adult/chemically induced/*immunology/pathology
Pulmonary Edema/*chemically induced/physiopathology

Mustard Gas
Respiration Disorders/*chemically induced/metabolism/pathology
Pulmonary Fibrosis/chemically induced/pathology
Bronchial Diseases/chemically induced/*complications/diagnosis
Chemical Warfare Agents/adverse effects
Mustard Gas/*poisoning
Chemical Warfare Agents/*poisoning

Ricin
Ricin/*immunology/*toxicity
Pulmonary Edema/drug therapy/mortality/prevention & control
Chemical Warfare Agents/pharmacokinetics/*toxicity
Pulmonary Edema/chemically induced
Chemical Warfare Agents/chemistry/metabolism/*toxicity
Ricin/chemistry/metabolism/*toxicity

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