REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188
Public reporting burden for this collection of information is e reviewing the collection of information. Send comments reg Information Operations and Reports, 1215 Jefferson Davis Hig	stimated to average 1 hour per response, including the time f arding this burden estimate or any other assoct of this collec hway, Suite 1204, Arlington, VA 22202-4302, and to the Ol	for reviewing instructions, searching existing d tion of information, including suggestions for ffice of Management and Budget, Paperwork Re	ata sources, gathering and maintaining the data needed, and completing and reducing this burden, to Washington Headquarters Services, Directorate for sduction Project (0704-0188), Washington, DC 20503.
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AFIT/CIA, BLDG 125			
2950 P STREET			98-104
WPAFB OH 45433			
11. SUPPLEMENTARY NOTES			
12a. DISTRIBUTION AVAILABILITY STATE Unlimited distribution In Accordance With AFI 35-20	MENT 95/AFIT Sup 1		12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 words)			
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14. SUBJECT TERMS			15. NUMBER OF PAGES
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17. SECURITY CLASSIFICATION OF REPORT	1B. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATI OF ABSTRACT	ION 20. LIMITATION OF ABSTRACT
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## AN ACUTE, TIME-WEIGHTED EXPOSURE OF HEXAMETHYLENE DIISOCYANATE (HDI) AND THE HDI–ETHANOL URETHANE MONOMER, HEXAMETHYLENE BIS(ETHYL CARBAMATE) (HDC)

by

#### **RAY ANTHONY SMITH, B.S., M.S.**

#### THESIS

Presented to the Faculty of the Graduate School of the University of Texas at Austin in Partial Fulfillment of the Requirements for the Degree of

#### MASTER OF SCIENCE IN PHARMACY

The University of Texas at Austin

August 1998

# AN ACUTE, TIME-WEIGHTED EXPOSURE OF HEXAMETHYLENE DIISOCYANATE (HDI) AND THE HDI-ETHANOL URETHANE MONOMER, HEXAMETHYLENE BIS(ETHYL CARBAMATE) (HDC)

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#### ACKNOWLEDGEMENTS

I would like to thank the members of my thesis committee for their time, guidance, and support. I would especially like to thank Dr Joseph D. Wander and the Air Force Research Laboratory, Airborne and Environmental Technology Division for programming and awarding research funds to support my work.

I would also like to thank some people at the University of Texas for their help with my work. This includes Dr James P. Kehrer, Dr John D. Richburg, Ms Christine Cinege, Ms Cindy Ehrig, and Mr Mark McPhee of the College of Pharmacy Division of Pharmacology/Toxicology, and Dr Noel Horton formerly of the College of Pharmacy Division of Pharmacology/Toxicology.

This effort is dedicated to Samuel Smith, my father, who passed on the eighteenth of September, 1996, at the age of 63. Dad, your example and wisdom have not been taken in vain as I have tried to do as you advised...follow my dreams. I miss you dearly and I will ALWAYS love you.

July, 1998

## AN ACUTE, TIME-WEIGHTED EXPOSURE OF HEXAMETHYLENE DIISOCYANATE (HDI) AND THE HDI–ETHANOL URETHANE MONOMER, HEXAMETHYLENE BIS(ETHYL CARBAMATE) (HDC)

by

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#### The University of Texas at Austin, 1998

#### **SUPERVISOR:** Alan B. Combs

Hexamethylene diisocyanate (HDI) is a compound that is condensed with a diol or triol to form polyurethanes, which form the structural matrix of some industrial paints. HDI is readily used in high-performance paints such as those used to protect aircraft, and is easily released (mechanically) during industrial painting operations. Like other isocyanates, HDI is believed to cause occupational asthma via a type-I hypersensitive reaction in the lung epithelium.

Since no satisfactory correlation has been established between the maximum allowable time-weighted average based on an eight-hour standard and the actual concentration of HDI present in most aircraft paint formulations, the immunologic effects of polyurethanes formed from isocyanates, if any, are less severe than those caused by HDI. This study consisted of a time-weighted, in vivo, subchronic, whole-body inhalation study on adult male Sprague-Dawley rats. Since the effects of a HDC were less than HDI, this study provided a better understanding of some of the toxicological impacts of HDI versus its proposed regulatory limits.

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#### **CHAPTER I – INTRODUCTION**

#### **1.1 Occupational Health**

The health and wellness of personnel working in industrial facilities should not be compromised by exposure to chemicals used in the workplace. Whereas new chemicals are constantly introduced into the workplace, the toxicological fates of the chemicals following exposure may not be fully understood by persons using them. Thus, conservative regulatory limits are established for exposure, until the research and legislative communities can attain a better understanding of a chemical's toxicity.

The Occupational Safety and Health Administration (OSHA) and The American Conference of Government Industrial Hygienists (ACGIH) are two government agencies that possess the responsibility and authority to tremendously influence occupational health issues. OSHA, which was founded by the enactment of the Occupational Safety and Health Act of 1970, is chartered to establish reasonable control measures to limit toxic exposure to those chemicals used in the workplace (LaGrega *et al.*, 1994). Additionally, ACGIH determines toxicological limits to chemicals by various routes of exposure emphasizing those found in the workplace. The charter

of each organization outlines specific duties and responsibilities that assist in maintaining a healthy and safe workplace for all employees.

Other bodies of law also regulate many chemicals used at industrial facilities. For instance, the Toxic Substances Control Act (TSCA) and the Resource Conservation Recovery Act (RCRA), each enacted in 1976, regulate the production, release, and disposal of toxic and hazardous substances. The Clean Air Act as amended in 1990, the Clean Water Act as amended in 1987, and the Safe Drinking Water Act as amended in 1986, which govern air, surface water, and drinking water, respectively, impact the methods of operation in and out of the workplace (LaGrega *et al.*, 1994). These bodies of law, along with standards and limits established by OSHA and the ACGIH, form a regulatory framework, whereby the health and wellness of personnel are safely maintained.

#### **1.2 Occupational Asthma**

Occupational asthma (OA) was first recognized by Hippocrates (*circa* 460 B.C.) when he noticed respiratory symptoms in some farmers, metal workers, tailors, and fishermen (Bernstein, 1996). Since then, the frequency and severity of OA cases have risen drastically, and the impact of OA in the workplace continues to challenge the industrial community.

Although modern industry currently uses industrial hygiene controls to minimize human exposure to chemicals that cause OA, the toxicological effects of acute and chronic exposures to agents producing OA symptoms warrant further scientific investigation. Additionally, while sufficient worker protection is both desired and essential, excessive use of protective equipment increases the cost to perform the work operation and degrades the overall comfort and efficiency of the worker.

OA is defined as "a disease characterized by airflow limitation and/or airway hyperresponsiveness due to causes and conditions attributable to a particular occupational environment and not to stimuli encountered outside the workplace" (Bernstein, 1996). Thus far, two distinct categories of OA have been identified: 1) OA with a defined latency period (the time following exposure during which sensitization occurs prior to the onset of clinical symptoms), and, 2) OA without a defined latency period [*i.e.*, irritantinduced asthma, reactive airways dysfunction syndrome] (Bernstein, 1996).

Most experts in the field further describe OA to occur via an immune response. In some cases, foreign airborne substances, or antigens, trigger a hypersensitive-immune response in the pulmonary epithelium. Both lowmolecular-weight (LMW) and high-molecular-weight (HMW) antigens have been known to cause OA (Baur *et al.*, 1996). LMW antigens, such as the

diisocyanates, are characterized by molecular weights less than 1000 kDa. They contain reactive chemical groups (-NCO) and react as haptens after binding to certain endogenous, cellular proteins (*e.g.*, albumin). The hapten complex initiates the ensuing immune response. On the other hand, HMW antigens act as complete antigens without the need to bind with a cellular protein to initiate an immune response (Baur *et al.*, 1996; Bernstein, 1996). The capability of either type of antigen is subject to the exposed individual's immunologic sensitivity, as well as the concentration and frequency of exposure(s).

OA is identified by a few distinct alterations in basal physiology. Symptoms of diisocyanate-induced OA in humans include a decreased FEV<sub>1</sub> (forced expiratory volume in the first second after exhalation) for about 30 to 60 minutes, followed by a LAR (late asthmatic response) lasting 4 to 12 hours after exposure in most cases (Baur, 1995; Bernstein, 1996). Moreover, the additive effects of the early and late responses can cause death via suffocation if the bronchial inflammation is not controlled and corrected shortly following exposure.

#### **1.3 Aircraft Coatings and HDI**

Industrial paints are complex materials that often contain a variety of organic constituents with low vapor pressures. One such constituent used in durable paints is hexamethylene diisocyanate (HDI). When used in a paint formulation, HDI condenses with a diol or triol to form polyurethane polymers, which form the structural matrix that provides the protective properties of the paint (Karol, 1984). The preparation of diethyl hexamethylene bis(ethyl carbamate) [HDC], the monomeric urethane used in this study, is depicted in Figure 1 as a representative urethane-forming reaction.



**Figure 1 – Reaction forming the HDI-Ethanol Urethane Monomer, Hexamethylene bis(ethyl carbamate)** – This diagram, which assumes complete reaction in excess ethanol, illustrates the preparation of the urethane monomer, Hexamethylene bis(ethyl carbamate) (Loudon, 1984). Substitution of a diol [e.g., 1,2-ethanediol] for ethanol would give a linear polyurethane, and inclusion of a triol [e.g., glycerol] would produce a branched polymer. HDI is commonly used in high-performance paints such as protective topcoats for aircraft. It has moderate volatility, but it is easily released (mechanically) into the atmosphere during industrial painting operations. Via both acute and chronic exposures to toxic aerosol components released during painting operations, the health of the personnel may be hampered due to accidental or systematic exposures to HDI, or to its urethane derivatives.

Federal law requires that personal protective equipment (PPE) must be worn when paints containing isocyanates are used at both indoor and outdoor industrial facilities. Examples of proper PPE include the following: gloves, half-face and full-face respirators, full-face respirators accompanied by full-body suits that protect the skin from dermal exposure (such as those suits produced by Tyvek<sup>®</sup> Inc.), and supplied-air suits that consist of stronger full-body suits equipped with self-contained breathing apparatuses. OSHA is chartered with implementing the use and wear of PPE, and establishes its criteria for protection on the current understanding of the nature of the chemical in question and the possible route(s) and rate of exposure(s). Using all known factors and inputs, the ACGIH sets a numerical value for permissible exposure, commonly known as the threshold limit value (TLV). For example, the ACGIH has set 0.005 ppm (5

ppb) as the TLV for HDI in air. The TLV concentration, which is based on an eight-hour standard as proposed by the ACGIH, is expressed as a timeweighted average (TWA). The TWA in this case is an exposure concentration averaged over a specified period of time, which is usually eight hours, based on the duration of the usual workday.

At Kelly AFB, Texas, as an example, aircraft such as the Lockheed C-141 are repaired and painted on a scheduled basis. Two planes, the C-141 and the McDonnell Douglas C-17, which are serviced by Kelly AFB, are shown in Figures 2 and 3, respectively. The isocyanate-containing paints used on the planes as topcoats are usually applied via electrostatic and high-volume, low-pressure (HVLP) compressed air paint spray guns. While the majority of the droplets produced from the paints actually impact the surface of the aircraft part, many of the droplets remains airborne as paint overspray (Ihry, 1997). It is the unreacted isocyanates in the paint overspray that cause the chronic reaction(s) in exposed workers.



Figures 2 and 3 – Photographs of the Lockheed C-141 and the McDonnell Douglas C-17 (photographs were obtained from website for the US Air Force Air Mobility Command, May 1998.) The paints used on these planes, depending on the color, contain different concentrations of isocyanates. Isocyanates are added to the paint formulation to provide structural hardness. Current literature contains an abundance of documentation concerning the toxicology associated with the inhalation of isocyanates, including HDI. Like other isocyanates, HDI is known to cause occupational asthma. For this reason, it is regulated as one of the 189 Hazardous Air Pollutants (HAPs) listed under the Clean Air Act Amendments.

Additionally, a controversy exists regarding the aspect of sensitization and HDI. Sensitization is a term defined as immunological familiarity with a given antigen. Some members of the occupational health community believe that prior exposure to isocyanates such as HDI is required for the initiation of an immune response. Others state that isocyanates can trigger an immune response without prior sensitization (Bernstein, 1996; Redlich, 1996).

A significant amount of uncertainty remains regarding the toxicological differences between bioavailable HDI and HDI bound within the urethane subunits that comprise most polyurethanes. The aims of this study were: 1) to determine if the toxicological potency of HDI is drastically different for HDI than for a derived ethyl urethane monomer, hexamethylene bis(ethyl carbamate) (HDC), and, 2) to further investigate the aspect of immune sensitization caused by exposure to HDI via inhalation. In addition to information gathered from prior toxicologic and immunologic studies

conducted on HDI, this research provides experimental verification that forming condensation products from HDI tremendously decreases the potency of HDI to induce OA. This suggests that the traces of free HDI in sprayed droplets of topcoats cause grossly less-profound physiological effects that do cured-paint dusts containing derived urethanes or ureas bound within a polymeric chain. Adult, male Sprague–Dawley rats were used as the toxicological model to test this hypothesis. A whole-body inhalation chamber was used to measure and then compare the resultant respiratory toxicological effects of HDI and the derived urethane HDC.

#### CHAPTER II – MORPHOLOGY, PHYSIOLOGY, AND TOXICOLOGY

To complete a valid extrapolation, the baseline conditions from which any toxicological conclusions will be drawn must be established. These conditions are based on the morphologic, physiologic, and toxicologic similarities and differences between the human and the rat.

#### 2.1 Respiratory Systems

The respiratory systems of humans and rats are similar in many ways. Both animals are mammals and, thus, obligate aerobes. Mammalian lungs have high surface areas within the alveolar walls for the purpose of gas exchange. Additionally, the respiratory systems of both humans and rats are innervated by the autonomic (sympathetic and parasympathetic) nervous system (Rang *et al.*, 1995). This is important because both of these portions of the autonomic nervous system affect breathing rate.

While the similarities between rats and humans are numerous, there are some differences that must be considered for an inhalation study. Perhaps the most significant difference between humans and rats with regard to inhalation toxicological assessments are anatomical. Rats are

obligate nasal breathers, whereas, humans have the ability to breathe from both the nose and the mouth. This difference can be an important distinction when deposition of an aerosol is considered. Additionally, the patterns in which breathing airways bifurcate within the lung vary between humans and rats. The human lung is dichotomous while the rat lung is monopodial (McClellan et al., 1995). As shown in Figure 4 (below), humans have a nearly symmetrical branching system, whereas, rats and other simple rodents effectively do not have respiratory bronchioles (McClellan et al., 1995). The symmetry exhibited by higher mammalian (e.g., humans) lungs and the asymmetry shown by lower mammalian (e.g., rats) lungs result in distinct variances in length-to-diameter ratios. The length-todiameter ratios within the bronchioles decrease at a much lower rate with decreasing diameter in higher mammals than in lower mammals. Such differences must be considered when a study such as this is planned. Notwithstanding the differences outlined above, the rat is a good model for this study because of cost, immunologic, and other similarities to humans, and their temperament.



### Figure 4 - Casts of Mammalian Respiratory Systems – taken from

**McClellan** *et al.*, **1995**, **page 137**. a) cast of a human lung showing bifurcation into bronchioles. The branches usually make about a 60° angle with the parent airway. b) Cast of a rat lung where the lack of a bifurcating flow system is evident. Lower mammals also have fewer divisions of respiratory bronchioles. In fact, respiratory bronchioles are virtually absent in laboratory rats and mice.

Many other characteristics between rats and humans must also be considered. For instance, rat lung tissue contains far more xenobioticactivating enzymes (*e.g.*, cytochromes P-450) than do humans. Also, breathing rate per unit of surface area within the lung also varies between species. In other words, rats have higher turnover of and shorter contact time with the air they breathe during a given time interval. For instance, although their breathing rate is significantly higher than humans, rats will inhale a much lower volume of air within a one-minute time span. Tables 1, 2, and 3 outline some additional similarities and differences among rats, humans, and other animals.

Characteristic	Rat	Rhesus Monkey	Human
Turbinate Complexity	Double Scroll	Simple Scroll	Simple Scroll
Volume (cm <sup>3</sup> )	0.4	8	16
Surface Area (cm <sup>2</sup> )	10	62	181

Table 1 - Interspecies Comparison of Nasal Cavity; InterspeciesComparison of Nasal Characteristics – taken from Dorman et al., 1997.This table lists the differences in nasal cavity characteristics betweenhumans and some inhalation test animals. Through evolutionaryprocesses, humans have developed respiratory systems that are moreprotective with aerosols and particulates. Rats also have a higher nasalsurface area / body weight ratio than humans. Thus, models using ratsmust consider these factors prior to any sort of extrapolation.

Parameter	Rat	Squirrel Monday	Human
Length of Trachea	3.2	2.5	11.0
Internal Diameter	3	3	20
Goblet Cells per cm	8	150	200
Glands	+/-	+	+++

Table 2 - Species Differences in Tracheal Anatomical Data – takenfrom Dorman et al., 1997. This table details intraspecies differences intracheal parameters. Humans have larger trachea, which alters flowconditions in the airways. Rats will have more turbulent flow due to thedecreased diameter. Glands are another adaptation present in the humanlung. The glands serve to secrete mucous for lubrication.

Parameter	Mouse	Rat	Human
Respiratory Bronchioles	Absent	Present	Present
Pseudostratified, ciliated epithelium	Trachea	Trachea; main and lobar bronchi	Trachea; main and lobar, segmental and subsegmental bronchi
Simple Columnar Ciliated Epithelium with Clara Cells	Bronchi; main, lobular and segmental	Bronchi; main, lobular and segmental	Peripheral and Terminal Bronchioles
Simple Cuboidal Epithelium, Mainly Clara Cells with Occasional Ciliated Cells.	Subsegmental bronchi; bronchioles and terminal bronchioles	Terminal Bronchioles	Respiratory Bronchioles

Table 3 - Histology of Airways Showing Different Cell Types – taken from Dorman *et al.*, 1997. This table fully explains the anatomical differences between humans and some test animals. The variances in parameters are of critical importance for airflow and metabolism. Humans have a more complex network of air vessels which impacts aerosol flow and contaminant deposition. Additionally, clara cells are non-ciliated cells in the lung epithelium with high concentrations of P-450. Research has shown that the P-450 concentrations in rats are much higher than those found in the human. These differences must be accounted for when extrapolating data.

#### 2.2 Type I Hypersensitivity

The terms hypersensitivity and allergy are often used interchangeably by different members of the scientific and medical communities. Most immunologists use *hypersensitivity* synonymously with *allergy*. However, some toxicologists regard hypersensitivity reactions as "the exaggerated response of a certain minority of the population at the lower end of any dose–response curve" (Burrell *et al.*, 1992). Neither of these definitions is applicable to immunotoxicology, or toxicity to the immune system by xenobiotics. For the purpose of this paper, the immunological definition of hypersensitivity will be used.

The actual conditions of the exposure dictate the type of hypersensitivity that is initiated. Variations with the ensuing reaction include the antigen (foreign substance that causes the immune response), the class(es) of immunoglobulin that is generated, the tissue or organ in which the hypersensitivity reaction occurs, and the other immunological mediators present as a result of the stimulation. This paper focuses on hypersensitive reactions within the lung to HDI. Additional tests will be conducted regarding the impact of antigen sensitization in exposed animals. Furthermore, a comparison will be made between the diisocyanate-induced and suspected HDC-induced asthmatic responses in the lung. A wide array of materials causes hypersensitive reactions in the lung. Reactions of this sort are triggered by the immune system, whereby antigens, such as some pollens and insect venoms, stimulate a hypersensitive reaction. (Some scientists and clinicians use the term *allergen* in place of *antigen*.) Hypersensitivities of this sort are also called immediate-type hypersensitivities because of the rapid tissue response following stimulation by the antigen (Burrell *et al.*, 1992). Initiation times are usually between 2 and 30 minutes following exposure (Kuby, 1997).

Type I reactions are primarily indicated by production of IgE-class immunoglobulins by plasma cells (Sandford *et al.*, 1996). IgE is also the most-abundant immunoglobulin class found in serum following this type of exposure, comprising around 70–75 percent of total immunoglobulins on average. As shown in Figure 5 (below), HDI binds to a cellular protein in the lung epithelium and that conjugate acts as the antigen. The HDI– protein conjugate activates B cells and commits them to become IgE-secreting plasma cells.



Figure 5 - Type I Hypersensitivity Mechanism – adapted from Kuby, 1997, page 415. The generally accepted mechanism for a Type I

hypersensitivity reaction is shown above. Here, the allergen, or antigen, binds to a B cell, and initiates the production of IgE antibodies. The IgE antibodies then sensitize mast cells in the epithelium. The next time the same allergen is introduced to the tissue, it triggers a degranulation of the mast cell and the release of pharmacological mediators of inflammation.

The elevated levels of allergen-specific IgE antibodies bind to select  $F_c$  receptors on mast cells.  $F_c$  receptors are cell-surface receptors that bind to only certain classes of immunoglobulins (Kuby, 1997). Binding of the antibodies sensitizes mast cells and blood basophils. (Mast cells are essentially basophils located within tissue epithelium such as the lining of the lung.) This sensitization usually occurs after the first exposure to the antigen. Upon the second exposure, the antigen may bind *directly* to the sensitized mast cells, causing rapid degranulation, and a release of pharmacologically active mediators (*e.g.*, histamine, leukotrienes, and prostaglandins) from the mast cells (Kuby, 1997). The occupational health community continues to debate the necessity for sensitization as a step in isocyanate-mediated inflammation (Cartier *et al.*, 1989; Pauluhn, 1997; Ruffilli and Bonini, 1997; Sandford *et al.*, 1996).

Mast cells form the critical cellular link in immediate-type (type I) hypersensitive reactions. Mast cells, whose precursors arise from pluripotent hematopoietic stem cells, are granulated inflammatory cells that are resident within most organs with connective tissues and epithelial tissues. Mast cells were originally named by the German physician Paul Ehrlich, who used the term *mastzellen*, meaning "fattened [or well-fed] cells" because of the large cytoplasmic granules found within their cytosol (McNeil, 1996). Each mast cell releases 3–6 pg of histamine per degranulation event (Witteman *et al.*, 1996). Mast cells can degranulate numerous times within their life spans, and near-constant histamine release may occur with constant dose of an antigen (McNeil, 1996).

Following mast cell degranulation, the elevated levels of pharmacologically active mediators cause a cascade of physiological events such as smooth muscle contraction, vasodilation, gastric acid secretion, and increased vascular permeability (Kuby, 1997; McNeil, 1996). Collectively, these events produce inflamed tissues. Under high, uncontrolled, chronic, and acute exposures, the inflammation within the lung can be so severe that breathing impairment causes the patient to suffocate.

While most occupational health scientists agree that diisocyanateinduced OA is triggered through the immune system, differing opinions exist as to whether IgE is the only mediator of the inflammatory response. This controversy is most likely attributed to the fact that no specific IgE antibodies are produced (Lummus, 1996). Thus, the presence of other antigens (*e.g.*, pollens) capable of triggering an elevation in IgE adds complexity to the studies and must be considered with *in vivo* analyses.

#### 2.3 Genetics of Asthma

While most immunologists agree that a major hereditary component exists to the etiology of asthma, the actual nature and process by which the genetic information is passed has not yet been resolved (Boushey and Fahy, 1995; Ruffilli *et al.*, 1997; Sandford *et al.*, 1996). OA, like clinical asthma, is a complex disorder with multivariate genetic links. Like hypertension, type-II diabetes, and arthritis, neither OA nor clinical asthma follows classical Mendelian inheritance patterns that can be linked to a single gene (Sandford *et al.*, 1996). While evidence supporting multi-gene factors as well as environmental factors are accepted as possible reasons for this phenomenon, no conclusive data have been reported (Boushey and Fahy, 1995; Sandford *et al.*, 1996).

The word *asthma* is originally derived from the Greek word for panting. Since that time, research has revealed more biomedical links to asthma and has changed the understanding of its pathophysiology. Most scientists now believe that allergic inflammation usually occurs when genetically susceptible individuals are exposed to aerosolized allergens, or *aeroallergens*. In addition to the effects of smoking, atmospheric pollutants, household allergens, and respiratory infections that cause

bronchoconstriction further complicate the understanding of the true cause(s) of asthma (Boushey and Fahy, 1995; Sandford *et al.*, 1996).

#### 2.4 Metabolism

After xenobiotics are introduced into metabolically active tissues in the body, metabolism usually occurs in two distinct phases, Phase I and Phase II. The reactions occurring in these phases are collectively called *metabolic pathways*. The chemical structure and dosage of the xenobiotic dictate the nature of the dominant metabolic pathway.

The biochemical result of Phase I is to make the xenobiotic more polar, which usually increases its solubility in water. Phase-II reactions generally promote transfer across hepatic, renal, and/or intestinal membranes and, thus, encourage elimination of the xenobiotic (in its metabolized form) in urine, feces, sweat, or exhaled air. Phase-I reactions are usually oxidative, reductive, or hydrolytic. Phase-II reactions usually involve formation of conjugates [*e.g.*, glucuronides, glutathionates, sulfonates] (Klaassen, 1996).

The literature describing the metabolism of HDI ingested via inhalation is not conclusive. Two primary mechanisms, however, have been widely accepted. One mechanism suggests a covalent attachment of HDI to macromolecular cellular components or other components in serum. The attachment of HDI triggers the aforementioned immune response and mast cell degranulation. A more widely accepted metabolic pathway of HDI includes hydrolysis of the isocyanate group with the formation of the related amine, hexamethylene diamine (HDA). Hydrolysis reactions of this type are common with diisocyanates in the presence of water (Baur *et al.*, 1996; Skarping *et al.*, 1996), but stronger nucleophiles [*e.g.*, amino, thio, or oxy groups, as in protein subunits] react much faster. While details concerning the metabolic pathway involved with HDI exposure are not contained in the literature, a traceable metabolite is produced and eliminated in urine. Like many other products of metabolite derivative produced after HDI exposure. HDA can be found in urine hydrolysates. The literature revealed no information regarding the physiological and toxicological effects of HDC.
# CHAPTER III – EXPERIMENTAL METHODS

### 3.1 Experimental Design

A whole-body inhalation chamber was procured from In-Tox Products, Inc., Albuquerque, New Mexico. The chamber uses a flowcontrol system that aids airflow and ensures even mixing via connections to bench-top air attachments and to a vacuum pump. The chamber also has adjustable pressure gauges to regulate flow and contaminant mixing. A sixjet collision nebulizer, purchased from BGI, Inc., of Waltham, Massachusetts, was used to create the aerosol. A process-flow diagram, and photographs of the nebulizer and inhalation chamber system are shown in Figures 6, 7 and 8, respectively.

To maintain uniformity, the nebulizer was operated at 30 psi for each of the studies. At 30 psi, the nebulizer aerosolized the liquid at a rate of 20 mL/hour. For the test groups, 0.1%, 1.0%, and 10% solutions of both HDI and of HDC were made in dimethyl sulfoxide (DMSO) as the solvent. Although water was used to determine the actual nebulizer aerosol rate, both solutions of HDI and of HDC in DMSO have similar viscosities. Additionally, in accordance with vendor instructions, the pressure within the inhalation chamber was kept between 0.5–1.0 inches (negative) of H<sub>2</sub>O.



Figure 6 – Inhalation Chamber System, Process-Flow Diagram (PFD).

The PFD shown above represents the inhalation exposure apparatus purchased from In-Tox Products, Inc. The control panel of the chamber allows for real-time control of the flow conditions within the chamber. By varying the make-up and dilution airflow, the concentration of aerosolized material entering the chamber can be controlled and, thus, the concentration of airborne xenobiotics delivered to the animals is also controlled.



# **Figure 7 – Nebulizer Used With Inhalation Chamber (photogtaph taken by author, April 1998).** The nebulizer is one of the most critical components of an inhalation chamber system. This nebulizer, purchased from BGI, Inc., Waltham, Massachusetts, uses six jets of highly focused air to generate the aerosol that is finally delivered to the animals in the chamber. For the sake of continuity, the pressure gauge was maintained at 30 psig for all of the experiments. Thus, the nebulization rate was assumed to remain constant.



**Figure 8 – Inhalation Chamber System (photograph taken by author, March 1998).** The photograph shown above shows the complete inhalation chamber system. From left to right, the gauges read: 1) Dump Leg, 2) Make-Up Air, 3) Chamber Exhaust, and 4) Dilution Air. In-line flowmeters are shown above on either side of the gauge that measures the pressure within the chamber. For the safety of those in the lab, the chamber pressure was maintained between 0.5–1.0 inches (negative).

Eight groups (*n*=8) of 8-to-10-week old Sprague–Dawley rats were used during the experiment:: control group (exposed to pure DMSO), HDI in DMSO at three exposure concentrations (0.1%, 1.0%, and 10%), and HDC in DMSO at the same three concentrations. Additionally, one group of animals was sensitized to an HDI-protein conjugate and exposed to the 1.0% solution of HDI. Table 4 keys the group identification numbers to the exposure conditions. The animals were exposed for three hours per day, on Mondays, Wednesdays, and Fridays, or Tuesdays, Thursdays, and Saturdays during a two-week period. Since the inhalation chamber held no more than four animal cages with two animals per cage, one group was exposed during the morning, and another was exposed during the afternoon. For instance, if Group 1 were exposed on Monday morning, Wednesday afternoon, and Friday morning during the first week, they would have been exposed on Monday afternoon, Wednesday morning, and Friday afternoon during the second week. Additionally, Groups 1-4 were exposed during a two-week period preceding Groups 5-8. To average diurnal effects [because rats are nocturnal], exposure times were alternated between the morning and afternoon for each animal group. Blood was drawn after the third dosing day of each week.

Group Number	Xenobiotic Administered	Concentration in DMSO (%)	8-hr TWA exposure/TLV	Sensitized Before Exposure?
1	HDI	0.1	10	No
2	HDC	0.1	10	No
3	HDI	1.0	100	No
4	HDC	1.0	100	No
5	HDI	1.0	100	Yes
6	HDC	10.0	1000	No
7	Control	0.0	0	No
8	HDI	10	1000	No

**Table 4 – Experimental Exposure Conditions.** Each exposure group contained eight male Sprague–Dawley rats. The rats were 8-to-10-weeks old at first exposure and all were purchased from the Animal Resource Center at the University of Texas at Austin.

Animals in Group 5 were sensitized by subcutaneous injection of ratserum albumin (RSA)–HDI conjugate 16 days before exposure to HDI in the chamber to allow for the production of antibodies. The RSA–HDI conjugate solution was prepared from a method adapted from methods used in similar assays (Cartier *et al.*, 1989; Waserman *et al.*, 1992). For this study, purified RSA, purchased from Chemicon, Inc., and HDI were each added in 1.5 mLvolumes and incubated for 30 minutes at room temperature. The mixture was then added to 27 mL of PBS to give a 1:10 dilution of the RSA–HDI conjugate solution. When sensitized, each animal was dosed subcutaneously with a 10 mL/kg body weight convention.

# 3.2 Gaseous Analysis

Another critical aspect of an inhalation toxicology study is the measurement and control of the toxic material (gaseous or particulate) being administered to the test subjects. Since the fate and transport of the aerosol within the chamber is determined by the principles of fluid mechanics and physics, a strong understanding of those principles is imperative for the development of an acceptable exposure/response curve. This is especially crucial in a whole-body inhalation study. As a result, a reasonable and applicable method to determine the quantity of HDI in the chamber during the study was required.

The method of analysis adapted from Bagon *et al.*, (1984), collects the airborne HDI for 10 minutes into a solution of 1-(2methoxyphenyl)piperazine [2-MOPP] in toluene contained within a midget impinger. (A photograph of the complete air-sampling system is shown in Figure 9.) Under these conditions, HDI reacts quantitatively with 2-MOPP to form a bis(urea), 1,6-bis{4-[1-(2-

methoxyphenyl)piperazino]carbonylamino}hexane [HDU], which is conveniently separated by HPLC (high-performance liquid chromatography) and quantified by UV spectroscopy.

The Bagon *et al.* method required the use of a few solutions that were prepared in the laboratory. The mobile phase used with the HPLC was prepared by dissolving 5.0 g of anhydrous sodium acetate in 400 mL of distilled H<sub>2</sub>O. Six-hundred mL of acetonitrile (GR) were then added, and the pH was adjusted to 6.0 with glacial acetic acid. A solution of 2-MOPP in toluene (the *absorber solution*) was prepared by dissolving 100 mg of 2-MOPP in a beaker with 200 mL of toluene (GR).



**Figure 9 – Photograph of air-sampling system.** This shows the air pump, flowmeter, and midget impinger that were used to sample air within the chamber for the gaseous analyses. In accordance with the assay, 10 mL of diluted (1/10 in toluene) absorber solution was placed in the midget impinger. The flowmeter was set for 1.0 Lpm. Then, the pump was run for 10 minutes and the solution within the impinger was analyzed.

To develop a standard curve for HPLC analysis of HDI, 1 mL of the absorber solution was diluted with 9 mL toluene. Aliquots (10mL) were placed in six glass (60x15 mm) Petri dishes. Known concentrations of HDI were then added. (The volumes for the standard solutions were 100, 80, 60, 40, 20, and 0  $\mu$ L, respectively.) After evaporation of the toluene, 1 mL acetonitrile containing 0.5% acetic anhydride was added to dissolve the residue. These solutions were used in development of the standard curve.

The HPLC used for these analyses was a Shimadzu Model LC-10AS liquid chromatograph equipped with a Shimadzu Model SPD-10A ultraviolet (UV) spectrophotometer. In a manner similar to the method described by Bagon *et al.*, the HPLC was used in isocratic mode and the flow rate of the mobile phase was set at 1.0 mL/min. The UV detector was also set at 293 nm in accordance with the method. A Whatman Partisil (model 5, ODS-3,  $C_{18}$ , 4.6x100 mm) column was used for these analyses.

Although steady-state conditions were assumed, limitations apply to flow conditions within the chamber. In reality, the rates of administration of HDI or (HDC) and the cumulative breathing rates of all animals within the chamber were not identical. Because of the additive effect of fluctuations in breathing rates of the animals, efforts were made to minimize the stress and anxiety of the animals prior to, during, and following the dosing periods. Using the method outlined by Bagon *et al.*, an airborne-concentration standard curve was developed based on variances in loading rates into the chamber. In theory, the nebulizer follows zero-order drug administration, and the chamber follows first-order elimination. While a constant flowrate was maintained through the nebulizer airstream, concentration ranges within the chamber were tested for the HDI and HDC solutions at 15-minute intervals until satisfactory concentrations were attained. True steady-state conditions with real-time monitoring and negative feedback systems could not be attained with this inhalation chamber system.

# 3.3 Preparation and Attempted Derivatization of HDC

HDC was prepared with 100 g of HDI and 500 mL of pure ethanol as a solvent. The solution was stirred overnight in an Erlenmeyer flask sealed with a rubber stopper. Once the reaction was completed, the hardened HDC cake was collected and ground with a mortar and pestle. The powdered HDC was then transferred, without purification, into a sealed, glass jar. Proton (<sup>1</sup>Hnmr) and carbon (<sup>13</sup>Cnmr) nuclear magnetic resonance spectroscopy, and infrared (IR) spectroscopy confirmed the identity of the product. The spectra are shown in Figures 10, 11, and 12, respectively.



**Figure 10** – **Nmr Spectrum (**<sup>1</sup>**Hnmr) run on HDC.** <sup>1</sup>H-nmr data (250 MHz, CDCl<sub>3</sub>):  $\delta$ 1.20, 6H, t, CH<sub>3</sub>(CH<sub>2</sub>O);  $\delta$ 1.31, 4H, m, -C(3,4)H<sub>2</sub>-;  $\delta$ 1.46, 4H, m, -C(2,5)H<sub>2</sub>-;  $\delta$ 3.14, 4H, apparent td, -C(1,6)H<sub>2</sub>-;  $\delta$ 4.08, 4H, q, -OCH<sub>2</sub>(CH<sub>3</sub>);  $\delta$ 4.65, 2H, br s, -NH-.



**Figure 11 – Nmr Spectrum (<sup>13</sup>C-nmr) run on HDC.** <sup>13</sup>C-nmr data (62.9 MHz, CDCl<sub>3</sub>):  $\delta$ 14.63, -(OCH<sub>2</sub>)CH<sub>3</sub>; 26.23, C-3,4; 29.91, C-2,5; 40.70, C-1,6; 60.62, -OCH<sub>2</sub>-; 156.69, -NC(=O)O-.



**Figure 12 – IR Spectrum Performed on HDC**. The IR spectrum shown above was run as a potassium bromide (KBr) pellet. Characteristic bands are seen at 3335 cm<sup>-1</sup> (CON-H);  $2900\pm100$ cm<sup>-1</sup> aliphatic C-H; 1690 and 1592 cm<sup>-1</sup> (amide). Completeness of conversion of HDI is verified by the absence of the intense –N=C=O band near 2300cm<sup>-1</sup>.

The Bagon *et al.* assay was adjusted slightly for use with HDC. Since HDC was not expected to react with 2-MOPP at room temperature, the HDC standards were heated to 80°C for one hour prior to the evaporation step in an attempt to form the bis(urea) HDU. HPLC analysis of the resulting sample indicated that no detectable amount of HDU had formed.

# 3.4 Biochemical Assays and Tissue Analyses

Pulmonary responses to injury originate from a variety of chemicals and materials. Aside from differences with the origin of the agents that may cause tissue injury, *i.e.*, drugs, foods, plant alkaloids, industrial chemicals, etc., there are a few typical response mechanisms involved in mediating the ensuing inflammation and consequential fibrosis. Biomarkers of inflammation such as immunoglobulins, cytokines, interleukins, and other mediators involved in pulmonary injury are linked to many inflammationmediated pulmonary responses. Elevated levels of those biomarkers may be measured in some biochemical fluids.

# 3.4.1 IgE Analyses

Since isocyanates are known to cause Type I hypersensitivities, elevations in immunoglobulins (Ig), or antibodies, are expected to be found in serum. IgE is the most probable antibody to be measured since it is the antibody produced in greatest quantities following a Type I hypersensitivity reaction. Levels of IgE were monitored in both sensitized and unsensitized animals.

The following assay describes the analytical protocol for the determination of non-specific IgE production in rat serum following exposure to HDI. To accomplish the assay, blood was drawn from each rat and the serum for the IgE analyses was separated following centrifugation.

The assay required primary goat anti-rat IgE antibodies and fluorescein-conjugated secondary anti-goat IgE antibodies. Both antibodies were obtained from Chemicon International, Inc. The primary antibodies, which were packaged as antisera, required a 1:100 dilution with phosphatebuffered saline (PBS) at pH 7.2. The secondary antibodies, which were lyophilized, also required 1:100 dilution with PBS. The secondary antibodies had an absorption peak at 492 nm and emission peak at 520 nm. (PBS is prepared by dissolving 8.0 g of sodium chloride, 0.2 g of potassium chloride, 1.44 g of sodium biphosphate (dibasic), and 0.24 g of

potassium biphosphate in 800 mL of distilled  $H_2O$ . The volume and pH were adjusted to 1L and 7.2, respectively.)

At the end of each week (three-day dosing period), approximately 500  $\mu$ L of blood was drawn from each rat by puncture of the retroorbital sinus with a heparinized microcapillary tube. The rats were fasted the night prior to blood sampling. The blood was collected in 2-mL heparinized microcentrifuge tubes.

To test for IgE, 96-well polystyrene plates were coated with 100 µL of the primary antibody solution and stored overnight at room temperature. In the morning, the blood samples were centrifuged at 2000 rpm for 3 minutes. The serum, or purified rat-IgE antibody for the standard curve, was added to the appropriate well. The plates were incubated with agitation for 3 hours at 37°C. The plates were then washed twice with a PBS–0.5% Tween-20 solution. Tween-20 functions as a surfactant to remove any excess lipids present in the serum.

Next, 150 µL from the secondary antibody preparation was added to each well, and the plates were incubated with agitation for 45 minutes at 37°C. Following a third wash (two times), the samples were run in the Cytofluor analyzer. Positive controls and standard curves were made from purified rat-IgE antibodies also purchased from Chemicon, Inc.

# 3.4.2 TNF $\alpha$ Analysis

Cell-to-cell communication in lung tissue, as well as in tissues in other organs, is primarily conducted by a class of biochemical messengers called cytokines. Cytokines, as a group, are mediators of cellular growth and proliferation. Many pharmacologists and toxicologists agree that cytokines are biochemical messengers that regulate cellular activity in close proximity to their effector cells. Cytokines are analogous to hormones, which serve as biochemical messengers that are located away from their effector cells. Research has shown that they serve as biochemical regulators of tissue repair from damage caused by a variety of toxins. Their levels have been shown to greatly increase following pulmonary inflammation and fibrosis resulting from tissue injury (McClellan et al., 1996). Since the injured cells are expected to die via necrosis (inflammation-mediated cell death) from HDI exposure, any cytokines or other mediators of inflammation normally found in the cytosol in a living cell can be found in extracellular fluids.

Tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), first identified as a cytokine in 1975, is a primary initiator of lung responses to cellular injury. Levels of TNF $\alpha$  increase tremendously following damage to highly sensitive cells in the lung such as alveolar macrophages and monocytes (McClellan *et al.*, 1996).

Recent research has shown TNF $\alpha$  to have greater activity in the lung than other cytokines such as Interleukin-1 (IL-1). For this reason, TNF $\alpha$  levels were measured in bronchoalveolar lavage (BAL) fluid.

Levels of TNF $\alpha$  following HDI exposure were monitored as follows: all rat specimens were euthanized, lungs were removed, and the BAL fluid was then collected using 5 mL of PBS solution. (Elevated levels of TNF $\alpha$ are expected in the extracellular necrotic fluid produced from this type of injury (*i.e.*, BAL fluid). According to a TNF $\alpha$  enzyme-linked immunosorbent assay (ELISA) kit produced by Biosource International, Inc. (Cat No. KRC3012), levels of TNF $\alpha$  can be monitored following ELISA analysis.) Levels of TNF $\alpha$  from the test groups were compared with the control group.

The principle of the ELISA method is to form a solid-phase "sandwich" using the antibodies of concern. Ninety-six well microtiter plates included within the TNF $\alpha$  kit were precoated with an antibody specific for rat TNF $\alpha$ . Once the experimental samples and controls are placed in each well, another biotinylated antibody is then added and incubated. The rat TNF $\alpha$  antigen binds to the capture antibody on one site and to the solution phase on another site during this incubation. The plates were then washed.

Following the wash, a solution containing streptavidin peroxidase is added to each well. This enzyme binds to the biotinylated antibody and competes for a binding site on the four-member sandwich. After a second incubation and washing, an active, color-producing substrate solution is added to each well. A plate reader measures intensity of the color, which is directly proportional to the concentration of rat TNF $\alpha$ . Concentrations from the experimental samples are then compared to the rat TNF $\alpha$  standards included with the analytical kit. Figure 13 shows a diagram of a well in the ELISA plate used for these analyses.

Essential reagents and standard solutions were reconstituted in accordance with the kit instructions. First, 10 mL of streptavidin–HRP 100x concentrate was mixed with 100 mL of streptavidin–HRP diluent (as specified to fill 10 rows of eight wells.) The wash buffer concentrate from the kit was also diluted in a 1:24 ratio with deionized water. This solution became the working wash buffer. Both solutions were refrigerated for a maximum of 14 days until use.



Figure 13 – Enzyme-Linked Immunosorbent Assay (ELISA) Diagram, adapted from BIOSOURCE Cytoscreen<sup>TM</sup> Immunoassay Kit Protocol Booklet, 1997. The following diagram is a graphic depiction of a well from an ELISA kit protocol specific for TNF $\alpha$  analysis in rat. Ninety-six well plates, precoated with the rat TNF $\alpha$  antibody, are coated with the test specimen (standard or sample), biotinylated antibody, and the streptavidinconjugate enzyme. To make standard solutions, the lyophilized rat TNFα standard was reconstituted with 1.80 mL of the standard diluent buffer solution for 10 minutes. (Both the buffer and the lyophilized standard were included with the kit.) This formed a 5000 pg/mL solution. Next, 0.100 mL of the reconstituted standard was added to a tube containing 0.400 mL of the standard diluent buffer. This formed a 1000 pg/mL solution. Six microcentrifuge tubes were then labeled as follows: 500, 250, 125, 62.5, 31.2, and 15.6 pg/mL. For each tube, 0.250 mL of standard diluent buffer and 0.250 mL of the preceding standard were combined to form serial dilutions in the aforementioned concentrations. A seventh microcentrifuge tube contained only 0.250 mL of the standard diluent buffer. The standards were also used within 1 hour of reconstitution. All excess was discarded appropriately.

Prior to execution of the assay, all reagents were allowed to come to room temperature and were gently mixed. Since there were 64 animals planned for use in this study, 10 eight-well strips were required for assay completion. Extra strips were rebagged and refrigerated for subsequent tests.

During the assay, 50  $\mu$ L of standard diluent buffer was added to the zero wells, but wells reserved for a chromogen (colored reagent used in the

procedure) blank were left empty. Fifty microliters of standards, samples, and controls were also added to each of the appropriate wells. Next, 50  $\mu$ L of biotinylated anti-TNF $\alpha$  solution was added to each well except the chromogen blank wells. The wells were then lightly tapped, covered, and incubated at room temperature for 90 minutes. Following incubation, the plate was washed four times. One-hundred microliters of streptavidin–HRP working solution was added to each well except the chromogen blank wells. The plate was then covered and incubated at room temperature for 45 minutes. The plate was again washed four times following the second incubation.

Next, 100 µL of stabilized chromogen was added to each well. (This made the liquid in the wells turn blue.) The plate was then incubated for 30 minutes in the dark. One-hundred microliters of stop solution was then added to each well, which made the fluid in the wells turn yellow. Absorbance was then read at 450 nm against a chromogen blank well (which contains only stabilized chromogen and stop solution). All readings were completed within 2 hours of the addition of the stop solution.

The asthmatic response is actually a long process usually divided into two phases. The release of histamine from mast cells and subsequent release of some leukotrienes, cytokines, and prostaglandins signify the first phase, commonly known as the early phase. Increases in levels of leukotrienes and prostaglandins lead to such events such as vasoconstriction and secretion of mucus. All of this occurs within a matter of minutes (Kuby, 1997). The second phase, also known as the late phase, is much longer in duration. During the late phase, other cytokines (including TNF $\alpha$ ) and some leukotrienes are released which lead to the recruitment of inflammatory cells. The late phase, unlike the early phase, can last for hours (Kuby, 1997). All of this is depicted graphically in Figure 14.



Figure 14 – Diagram of Early and Late-Phase Asthmatic Response (taken from Kuby, 1997). This cross-sectional diagram illustrates the release of intracellular and extracellular mediators released following the asthmatic response. The release of  $TNF\alpha$  is shown near the top of the diagram.

# 3.4.3 Histopathology

The third series of analyses performed on the test subjects was intended to visually examine HDI-induced and HDC-induced damage in the lung epithelium. The tissue was expected to show obvious signs of damage via inflammation and scarring from repeated exposure. Expert histopathological analyses were provided by the Histology Laboratory [HLab] (Dr Irma Conti) of the M.D. Anderson Cancer Research Center (Smithville, Texas). Tissue specimens were delivered to HLab staff, who prepared all of the slides.

Traditional staining procedures were employed. Hematoxylin and eosin staining is a conventional tissue-staining technique used historically as a means to aid the visual analysis of tissue(s) under a light microscope. Color slides and black-and-white photographs were taken of tissue samples from the inner lung and the trachea.

Following euthanasia, the complete lung was removed from each animal, packaged in a storing case, marked appropriately, and preserved in formalin (formaldehyde) for histological analysis. The lungs were shipped to HLab, stained with hematoxylin and eosin (H & E), made into slides, transported back to the UT College of Pharmacy, and then photographed.

The H & E staining assay is a multi-step process that allows different tissues to be viewed under a light microscope. The assay shown below is the procedure employed by the HLab. The assay begins with a 15-to-20-minute incubation procedure at 60°C. This helps the tissue adhere to the slide. The next steps include three baths in xylene to remove paraffin from the tissue. These baths are followed by three, five-minute baths in 100% ethanol, followed by two, five-minute baths in 95% ethanol. A two-minute water bath follows the ethanol baths. The ethanol and water baths are intended to rehydrate the tissue prior to staining.

The first stain, Gill 2 hematoxylin, is applied for four minutes and is followed by a one-minute wash with water. Next, HCI (1/24 in water) is added for 15 seconds to lighten the color of the stained nuclei. A 30second wash is then performed followed by a one-minute bluing step. Hydration of the tissue is then adjusted with a two-minute wash of 95% ethanol prior to the eosin-staining segment.

The Y eosin-staining segment of the assay is added to stain the cytoplasm. Y eosin is added for a period of two minutes. Dehydration steps with ethanol (95% ethanol for three minutes; and 100% ethanol for five minutes repeated three times) follow the eosin staining-step. Two final washes in xylene precede mounting of the tissue to the cover slip. The

tissue is mounted to the cover slip with a permanent-mounting medium and is dried for 12 hours.

The benefit of hematoxylin as a stain is that it easily penetrates into the nuclei and other cellular components of stained cells. Since it is dark blue in color, it contrasts strong with eosin, a red dye. The two stains together provide an excellent means to view cellular organelles under a microscope.

# CHAPTER IV – RESULTS AND DISCUSSION

A significant portion of the principles governing the dosimetry employed with a whole-body inhalation study stem from traditional fluid mechanics. The manner in which air moves through the nebulizer, and through the tubing in the chamber assembly under laminar conditions dramatically affects the likelihood that the animals within the chamber will inhale the nebulized xenobiotics, HDI and HDC, in the expected concentrations during the entire exposure period. To eliminate some of the uncertainty with the flow of the fluid, preliminary experiments were conducted to predict the points at which air flows optimally through the nebulizer, as well as when a steady-state concentration is achieved within the chamber. Initial estimates of significant exposure parameters were made from measurements of the volume of the inhalation chamber, and the total flowrate through the chamber. These measured factors, for example, provided reasonable estimates of other parameters of concern (i.e., number of air exchanges per hour, volume of dissolved xenobiotic required for a given exposure period, time needed to evacuate the chamber following an exposure period, etc.).

The experiments used to determine at which time a measurable concentration of HDI was present in the chamber were performed using the modified assay developed by Bagon *et al.* Those experiments revealed that a 15-to-20-minute "warm-up" period was required for the concentration in the chamber to approach a measurable level. The data obtained from these tests are shown in Figure 15 with a standard curve. Attempts to derivatize HDC by heating the standards were not successful. Additionally, approximately 50% of the nebulized HDI was lost due to unavoidable physical processes such as the formation of droplets on the walls of the chamber tubing.



**Figure 15 – Warm-up Period Measurements.** The graphs shown above were derived from HPLC data using the modified assay developed by Bagon *et al.* By starting the sampling pump in 15-minute intervals after initiating flow through the nebulizer, measurements were drawn of when a known concentration of HDI could be measured. A 15-minute "warm-up" period was used for all experiments.

The animals were fed, watered, and placed in clean cages in accordance with the rules and regulations of the Institutional Animal Care and Use Committee (IACUC). The animals were monitored for illness, or breathing difficulties, as well as behavioral changes that would indicate some unforeseen source of stress. As mentioned previously, an effort was also made to compensate for the nocturnal nature of rats. By alternating exposure times between morning and afternoon for each group during the experiment, any possible detrimental effects on the animals were minimized.

Three toxicological endpoints were planned for this study: 1) measurement of IgE in blood serum, 2) measurement TNF $\alpha$  levels in BAL fluid, and 3) examination of histopathological slides made from lung and tracheal samples. Measurement of IgE is undoubtedly the best indicator of the asthmatic response following exposure to a given allergen. The experiments, as planned, were expected to have provided a reasonable probability for IgE to have been produced in response to HDI, and presumably, to HDC. Some of the uncertainty with the immunologic and/or toxicologic response(s) of HDI and HDC, which are two materials that have diametrically different physical properties, was removed by the use of DMSO as vehicle. DMSO was chosen for several reasons: 1) it is an

acceptable solvent for both xenobiotics, 2) it does not initiate an immunologic response itself (Klaassen, 1996; Kuby, 1997), 3) it is not volatile at room temperature, 4) it does not excessively irritate or inflame the lung epithelium (Klaassen, 1996), and 5) its viscosity is low enough that it is easily nebulized.

Some unfavorable observations made during the experiments could have been related to the exposure regimen used in the study. For instance, two animals assigned to Group 5 (sensitized subcutaneously to HDI– albumin conjugate and then to be exposed to the 1.0% HDI solution) died within 24 and 48 hours, respectively, following injection of the conjugate. Rigor mortis had already set in by the time the deaths were identified. Although no autopsy was done on the 24-hour death because the carcass was discarded prematurely, an autopsy performed on the 48-hour carcass revealed some lime-green discoloration on the fascia adjacent to the abdominal muscles. This may have been due to septic injection. Additionally, there was no discoloration beneath the abdominal muscles. As a result, these deaths may have been caused by an extreme anaphylactic reaction with the conjugate, or a large blood vessel or section of the intestine may have been punctured during the injection process. Originally, the experimental duration for each test group was scheduled to be three weeks. However, the bench-top vacuum pump used to evacuate the chamber malfunctioned at the end of the second week and could not be repaired or used after it broke. This eliminated three of the nine exposure periods planned for the experiments. After a five-day search for an alternative means to move air through the chamber, the decision was made to euthanize the animals, obtain final blood samples, obtain BAL fluid samples, and remove the lungs for histopathological staining. For any immunologic study, exposure times to the allergens as well as the mean time between exposures significantly impact the production of immunoglobulins.

The experiments conducted to measure IgE levels in serum were not successful. After several consultations with many people (*i.e.*, outside faculty, technical service personnel from vendors, other graduate students, etc.) experienced with immunologic assays, the IgE assay could not be completed successfully. For example, changes to buffers, antibody concentrations, manufacturers of 96-well plates, incubation times and temperatures, and sensitivities set on the Cytofluor analyzer were attempted to complete a successful assay.

The assay used to measure TNF $\alpha$ , however, was successful. Samples of BAL fluid were obtained immediately following euthanasia, and were stored on ice. Once all of the required samples were obtained, the assay was run. For each experimental group, four animals were used for TNF $\alpha$  measurement, and the other four were used as samples for histology. Figure 16 is a graph showing the average levels of TNF $\alpha$  in BAL fluid.

According to the ELISA kit vendor, some uncertainty still remains about the lifetime of TNF $\alpha$  following cell death in biological fluids other than serum. Current toxicological literature does not contain an abundance of information addressing this issue. Although levels of TNF $\alpha$  are expected to correlate directly with increased levels of HDI and HDC, the average levels measured in the BAL fluid did not exhibit a significant correlation with exposure concentration in this experiment. This is most likely due to the fact that a five-day period was allowed to transpire prior to euthanasia after the air pump failed. Any inflammation and associated increased levels of TNF $\alpha$  presumably subsided as the tissue healed.



Group Number	Xenobiotic Administered	Concentration in DMSO (%)	8-hr TWA exposure/TLV	Sensitized Before Exposure?
1	HDI	0.1	10	No
2	HDC	0.1	10	No
3	HDI	1.0	100	No
4	HDC	1.0	100	No
5	HDI	1.0	100	Yes
6	HDC	10.0	1000	No
7	Control	0.0	0	No
8	HDI	10	1000	No

**Figure 16 – Levels of TNF** $\alpha$  in **BAL Fluid with Standard Curve.** The graph shown above illustrates the variations in TNF $\alpha$  levels in BAL fluid five days after euthanasia. Since the plate reader used for the measurement reads in total light absorbance at 450nm, a standard curve was run to translate absorbance into terms of concentration. The key is the same key provided in Table 1.
The histologic analyses encountered only minor difficulties. Following euthanasia, the lungs were removed from the carcass, and immediately placed in tissue cases provided by the Histology Laboratory at the M.D. Anderson Cancer Research Center. The lungs were then placed in formalin to fix the tissue. According to the technicians at M.D. Anderson, the cases they provided were too small for the lung samples. As a result, the tissues were slightly compressed, which may have caused some dimunition of alveoli before the tissue samples were stained.

Permanent slides were made of the alveolar tissue as well as the trachea from individuals within each test group. One sign of significant damage to the alveolar tissue is the absorption of hematoxylin and eosin into the nuclei and cytoplasm of damaged cells. Additionally, a sign of damage for tracheal tissue is absorption of both stains into the tracheal epithelium. Photographs from each test group are shown in Figures 17–24.



**Figure 17 – Histological Slides of Group 1 – Group Exposed to HDI at 10x's TLV for HDI.** The two cross-sectional photographs above are of the alveolar lining, above, and the trachea, below. At this exposure concentration, the cells in the epithelium did not absorb high levels of either dye.



**Figure 18 – Histological Slides of Group 2 - Group Exposed to HDC at 10x's TLV for HDI.** The two cross-sectional photographs above are of the alveolar lining, above, and the trachea, below. At this exposure concentration, the cells in the epithelium did not absorb high levels of either dye.



Figure 19 – Histological Slides of Group 3 – Group Exposed to HDI at 100x's TLV for HDI. The two cross-sectional photographs above are of the alveolar lining, above, and the trachea, below. At this exposure concentration, the cells in the epithelium did not absorb high levels of either dye.



**Figure 20 – Histological Slides of Group 4 - Group Exposed to HDC at 100x's TLV for HDI.** The two cross-sectional photographs above are of the alveolar lining, above, and the trachea, below. At this exposure concentration, the cells in the epithelium did not absorb high levels of either dye.



**Figure 21 – Histological Slides of Group 5 – Group Sensitized to HDI and exposed to HDI at 100x's TLV for HDI.** The two cross-sectional photographs above are of the alveolar lining, above, and the trachea, below. At this exposure concentration, the cells in the epithelium did not absorb high levels of either dye.



**Figure 22 – Histological Slides of Group 6 – Group Exposed to HDC at 1000x's TLV for HDI.** The two cross-sectional photographs above are of the alveolar lining, above, and the trachea, below. At this exposure concentration, the cells in the epithelium did not absorb high levels of either dye.





**Figure 23 – Histological Slides of Group 7 – Control Group, Group Exposed to pure DMSO.** The two cross-sectional photographs above are of the alveolar lining, above, and the trachea, below. At this exposure concentration, the cells in the epithelium did not absorb high levels of either dye.



**Figure 24 – Histological Slides of Group 8 – Group Exposed to HDI at 1000x's TLV for HDI.** The two cross-sectional photographs above are of the alveolar lining, above, and the trachea, below. HDI at this exposure severely ruptured the tracheal epithelium as is indicated by the increased absorbance of the tissue dyes. The alveolar lining, however, was not damaged apparently because its exposure was limited by the ability of the aerosol to pass through the bronchial tubes.

As shown in Figures 17–24, only the tissue sections from Group 8 exhibited significant damage by HDI or HDC five days after the last exposure. In those photographs, the epithelial tissue readily absorbed the stains and the structural damage to the tracheal epithelium is evidenced by the high absorbance of the dyes in the epithelial cells. This suggests that high- concentration, acute exposures of HDI causes severe inflammatorymediated responses in the tracheal epithelium, and that the structural limitations of the rat respiratory system inhibit the ability of the aerosol to flow into the deep lung. Thus, the epithelium shows little or no damage.

One other possible marker of bodily injury is body weight. In some cases, the immunological and toxicological effects induced from xenobiotics have a detrimental effect on metabolism. As a result, changes in body weight may exhibit a direct correlation with the physiological stress induced from HDI and HDC. The graphs for changes in body weight are shown in Figures 25 and 26. However, no significant conclusions are supported by these data.

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# Effect of Treatment on Changes in Body Weight

## **CHAPTER V – CONCLUSION**

Since the true immunologic fate of HDI is not yet confirmed, the occupational health community must take measures to eliminate or reduce exposures to HDI and other isocyanates in the workplace when possible. Proper wear of PPE is the only realistic method that can reduce occupational exposures.

The information obtained from this study demonstrates that the metabolic activation cause by unreacted HDI in the lung epithelium is far more severe than was shown by a condensation product of HDI, HDC. In fact, the inability for HDC to form a conjugate with 2-MOPP (a fairly aggressive nucleophile) at 80°C (more forcing than the conditions under which HDI form the condensation product for HPLC analysis) suggests that isocyanates condensed into a urethane form will not react readily with any ordinary nucleophile at normal body temperature. In other words, a urethane formed from HDI will not decompose under normal physiological conditions (Matsuda, 1989) and, thus, is not likely to elicit the same physiological responses as HDI.

The gross differences in physicochemical properties of HDI and HDC presented a challenge to the design of this study. Dissolution in DMSO

solved the mechanical problems of uniform delivery, but substituted the possibility of quantitative effects owing to both the solvency and the physiological properties of DMSO. Accordingly, the results support only the qualitative conclusions stated in this thesis.

Quantification of IgE is the best measure of a physiological response to HDI. Complications with assay development made analyses of IgE impossible. However, measurement of TNF $\alpha$  was accomplished. Although the levels measured five days following the last exposure to HDI and HDC did not show direct correlations to exposure concentrations, higher levels of TNF $\alpha$  should be found in tests conducted on the groups exposed to HDI without a time delay prior to euthanasia. This premise follows the expectation that a higher degree of inflammation is expected with HDI than would be found with an equivalent concentration of HDC (or any other exogenous condensation product). Since TNF $\alpha$  is produced during the late-phase asthmatic response, we would expect to see increases in its concentration within 12 hours following exposure (Kuby, 1997).

The only group in this study where measurable physiological responses were found is Group 8. Members of Group 8 tucked their noses into their fur during the exposure sessions for longer periods of time than members of the other groups. Members of Group 8 were also sneezing following their release from the chamber, and their noses were pinkish-red in color. Visible fluids were also present on their noses. Finally, although mechanical problems terminated the exposure series prematurely, the histopathology results demonstrate conspicuous differences exist between the toxicological effects of an acute exposure of a high concentration of HDI via inhalation and of the equivalent exposure of inhaled HDC.

## CHAPTER VI – FUTURE RESEARCH

This project was intended to improve the current understanding of HDI-induced and HDC-induced occupational asthma and to evaluate the premise that the physiological responses to unreacted HDI and to HDI condensed in urethane subunits are identical. Before stronger conclusions can be drawn comparing the effects of HDI and HDC (or other exogenous condensation products, all of which are structurally analogous to the antigenic HDI–protein conjugates) via inhalation, more quantitative research must be conducted on HDC. Although specific statements about its toxicological effects would be speculative, this study points out the need for more research to be conducted comparing unreacted isocyanates with representative condensation products.

To begin this effort, immunologic experiments with similar exposure durations should be performed with HDI and HDC, but for longer periods of time. Medical literature suggests that the asthmatic response exhibited by painters exposed to HDI is usually not developed until the painters have been exposed for several weeks, or even months (Vandenplas *et al.*, 1993; Bignon *et al.*, 1994). A subchronic, or even a chronic study should provide the immunologic framework within which immunoglobulins would be produced against the antigen. This, in turn, would sufficient time for immunoglobulin production, mast cell sensitization, and the strong presence on asthmatic symptoms in the test subjects.

Additionally, since the toxicological literature does not contain significant information about HDC, further research should be conducted to understand its metabolic and/or immunologic fate in the body. Only continued experiments will provide this information.

Another possible experiment involves the use a multi-urethane compound. For instance, a diol-based compound produced with HDI that contains two or three urethane subunits could be generated, administered to rats or some other animal model, and its toxicological impacts compared to those of HDI. The toxicological effects of the multi-urethane compound would be expected to be less severe than HDI alone as was suggested by this study. This follow-on experiment would present additional questions addressing differences in the manner in which isocyanates bound within externally generated urethane structures and unreacted isocyanates behave within the body. Finally, more tests should be conducted on the biuret diisocyanate prepared from HDI and widely used in modern polyurethane paint formulations.

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## VITA

Ray Anthony Smith was born on February 17, 1971, at Chanute Air Force Base (AFB), Illinois. After a 26-year Air Force career, Msgt Samuel Smith (deceased) and his wife, Rosa Lee, moved their family to Arlington, Texas. Ray, along with his older siblings, Gregory and Belinda, attended and graduated from Sam Houston High School in Arlington. Ray was a member of the Class of 1989.

Following high school, Ray was appointed to the United States Air Force Academy in Colorado Springs, Colorado. As a member of the Class of 1993, Ray graduated with a Bachelor of Science degree in Biology, was commissioned as an Air Force 2<sup>nd</sup> Lieutenant, and became a Bioenvironmental Engineer in the Air Force. In October of 1993, while assigned to Brooks AFB, Texas, for the Bioenvironmental Engineering Officer Course, Ray took, and passed the Fundamentals of Engineering Test (formerly known as the EIT).

In January 1994, Ray was reassigned to the Armstrong Laboratory Environics Directorate, Tyndall AFB, Florida. During his 2½-year term as a project officer, Ray worked as an Air Force technical liaison for the research and development of environmental technologies addressing Air Force problems. Some of the areas of research included application and removal of aircraft coatings, firefighter wastewater treatment and purification, and electroplating bath purification. In the summer of 1994, he was accepted as a graduate student (seeking a Master's degree) in the Department of Civil Engineering at The Florida State University College of Engineering. The program, with a focus in Environmental Engineering, was completed in the summer of 1997.

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In August 1996, the Air Force Academy sent Ray to the University of Texas at Austin College of Pharmacy as a graduate student (seeking a Master's degree) in the Department of Pharmacology/Toxicology. The focus of his research program is inhalation toxicology.

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