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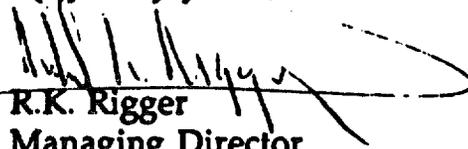
On May 3, 1995, the III submitted Bickis' Ph.D. thesis abstract. We now have a full copy of the thesis, and are forwarding this to EPA:

Ugis Bickis: Investigations of dermally induced airway hyperreactivity to toluene diisocyanate in guinea pigs. (Queen's University, Kingston, Canada, November, 1994).

We have been orally informed that an 80:20 mixture of TDI isomers (2,4-TDI:2,6-TDI, CAS # 26471-62-5), was used.

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Very truly yours,


R.K. Rigger
Managing Director

**Investigation of Dermal Induced
Airway Hyperreactivity to Toluene Diisocyanate
in Guinea Pigs**

by

Ugis Ilgvars Bickis

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**Investigation of Dermally Induced
Airway Hyperreactivity to Toluene Diisocyanate
in Guinea Pigs**

by

Ugis Ilgvars Bickis

A thesis submitted to the Department of Pharmacology and Toxicology
in conformity with the requirements for the degree of
Philosophiae Doctor (Ph.D.)

Queen's University at Kingston
Ontario, Canada
November, 1994

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ABSTRACT

Ugis Bickis: *Investigation of dermally induced airway hyperreactivity to toluene diisocyanate in guinea pigs*. PhD thesis. Department of Pharmacology and Toxicology, Queen's University, Kingston, Canada. November, 1994.

Hyperreactivity to environmental factors, with asthma as one manifestation, is becoming increasingly prevalent. An understanding of etiological mechanisms is of interest from both preventive and therapeutic standpoints. Isocyanates are responsible for more cases of occupational asthma than any other industrial chemical; toluene diisocyanate (TDI) has been most commonly involved. Isocyanate sensitization remains enigmatic: degree of chronic airborne exposure, genetic allergic predisposition ("atopy"), blood level of TDI-specific antibodies, and nonspecific bronchial hyperreactivity (methacholine sensitivity) do not appear to be directly related to the development of airway obstruction in response to airborne TDI challenge.

This thesis posits that worker skin contact with TDI may be a critical event in the development of the hypersensitive state, culminating for some in bronchospasm upon subsequent TDI inhalation.

By means of a guinea pig (gp) model involving sensitization/humoral immunity determination, *in vivo* plethysmographic challenge and *in vitro* tissue bath studies, four hypotheses were tested:

1. persistent immune response ("sensitization") to TDI can be induced by a single dermal exposure to dilute TDI;
2. the 'early' response to TDI challenge, while associated with sensitization, is not directly related to the level of TDI-specific antibody; response to methacholine inhalation challenge is independent of sensitization status;
3. *in vivo* sensitivity to airborne challenge correlates with *in vitro* airway tissue responsiveness to TDI;
4. *in vitro*, airways from sensitized animals can be differentiated pharmacologically from control airways.

Immune response was assessed by ELISA: within two weeks of the application of 12 mg of TDI, as a 5 % solution in acetone to the animals' skin, all developed a high level of IgG directed against TDI; 63-71 weeks post-sensitization the mean IgG effect in sensitized animals was six times that of the control animals. No immune response was observed after TDI inhalation.

Airway hyperreactivity *in vivo* was assessed in awake, unrestrained animals situated in flow-through whole-body plethysmographs. Animals were challenged (as groups of 3 sensitized and 1 control gp) with both methacholine and TDI in step-wise dose increments; the challenge atmosphere was actively generated for 60 seconds and the animals' respiratory pattern was monitored for 5 or 10 minutes (depending on the specific protocol). Methacholine challenges were conducted on two consecutive days, and two or more weeks prior to the TDI challenges, which were also conducted on two

consecutive days. A third methacholine challenge was conducted immediately following the second TDI challenge. During the methacholine challenges, animals were removed from their plethysmograph chamber if they progressed to severe bronchospasm, from which they then recovered spontaneously within seconds; endpoint was defined as the methacholine concentration at which the animal's ΔP (plethysmograph pressure amplitude) doubled from the pre-exposure ΔP . The respiratory response to TDI was quite different, and the animals did not progress to the paroxysmal breathing characteristic of methacholine endpoint. Ultimately, endpoint was defined in terms of a characteristic pattern of flattened waveforms associated with a retardation of the exhalation phase. With both challenge agents, when comparing by animal the consecutive day concentrations required to achieve endpoint, there was a significant correlation; also, there was no difference between the group mean concentrations required on the two days. Following the TDI inhalation challenge, the animals' sensitivity to methacholine challenge had decreased (by a factor > 2). There was no difference between control and sensitized animals in their methacholine sensitivity; the TDI 'sensitivity' of sensitized animals was seven times greater than that of control animals, when assessed almost a year after a single dermal sensitizing event; only 2 of 14 were not airway-hypersensitive (i.e. at least $1.96 \text{ sd} > \text{mean control sensitivity}$) to TDI. There was no association between TDI sensitivity and TDI-IgG level in sensitized animals.

Tracheal ring responsiveness was evaluated in conventional tissue baths, over a year following the single, dermal sensitization. Tissues from sensitized animals contracted when TDI was added to the baths; control tissues relaxed. A 'confounding' control animal (with baseline levels of TDI-IgG, yet in several respects more characteristic of sensitized animal response *in vivo* and *in vitro*) is discussed. Atropine, diphenhydramine and indomethacin did not inhibit the TDI response. Removal of the tracheal epithelium had no effect on this response, nor that to methacholine; conversely it increased the maximal tension (g_{max}) produced in response to histamine (in both control and sensitized tissues), and in sensitized tissues it decreased the degree of relaxation produced by isoproterenol. Epithelium removal also rendered tissues from control gps more sensitive to histamine than the corresponding intact sensitized tissue; otherwise, control and sensitized tissues showed no difference in g_{max} or EC_{50} to histamine or methacholine on their own. However, when methacholine response was assessed after the addition of TDI to the baths, the g_{max} demonstrated a change in opposite directions, for control and sensitized gps. The extent of TDI-induced contraction *in vitro* correlated, by animal, with its *in vivo* TDI sensitivity ($p < 0.01$) and, TDI-IgG level at the time of death ($p = 0.02$).

In summary, a single dermal contact with a small quantity of isocyanate in dilute form was sufficient to cause a specific airway hyperreactivity that could be determined *in vivo* and *in vitro* a year later. Occupational exposure limits do not currently recognize the dermal route as a potential for airway sensitization to isocyanates; the TLV should be assigned a "skin" notation, and appropriate warnings issued to all users.

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This program of studies certainly provided ample opportunity for my edification, in a multitude of ways; I would like to thank all who have supported me in this effort.

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I dedicate this work to all those who experience adverse health effects as a result of contact with their surroundings.

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LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate); C.A.S. RN30931-67-0
ACGIH	American Conference of Governmental Industrial Hygienists
ACh	Acetylcholine
AChE	Acetylcholinesterase
ATP	Adenosine triphosphate
AU	Absorbance units
BAL	Bronchoalveolar lavage
BPM	Breaths per minute
BSA	Bovine serum albumin
BSM	Bronchial smooth muscle
cAMP	Cyclic adenosine monophosphate
CAS	Chemical Abstracts Service
CGRP	Calcitonin Gene Related Peptide
DE	Delayed exhalation
DMSO	Dimethyl sulfoxide
DRO	Deionized reverse-osmosis
EC ₅₀	Effective concentration, 50%
EDRF	Endothelium Derived Relaxing Factor
EFS	Electric Field Stimulation
ELISA	Enzyme linked immuno sorbent assay
EpDCF	Epithelium Derived Contracting Factor
EpDRF	Epithelium Derived Relaxing Factor
ER	Early response
ET	Endothelin

FEV ₁	Forced expiratory volume, 1 second
GSA	Guinea pig serum albumin
H	Histamine
HDI	Hexamethylene diisocyanate
HMDI	Dicyclohexylmethane-4,4'-diisocyanate
HRPO	Horseradish peroxidase
HSA	Human Serum Albumin
IPE	Isoproterenol
L-NMMA	N-monomethyl-L-arginine
LR	Late response
M	Muscarinic
MCh	Methacholine; Acetyl- β -methylcholine
MCS	Multiple chemical sensitivity
MDI	Methylene bisphenyldiisocyanate; diphenylmethane diisocyanate
MKH	Modified Krebs-Henseleit
MOL	Ministry of Labour (Ontario)
NANC	Non-adrenergic non-cholinergic
NCO	-N=C=O functional group
NEP	Neutral endopeptidase
NKA	Neurokinin A
NO	Nitric oxide
NS	Not significant
NSBH	Nonspecific bronchial hyperreactivity
OA	Ovalbumin

p	-log probability
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate buffered saline
PCA	Passive cutaneous anaphylaxis
PC ₂₀₀	Provocation concentration 200 %
PEFR	Peak expiratory flow rate
PGF _{2α}	Prostaglandin F _{2α}
ppb	parts per billion
ppm	parts per million
PVC	Polyvinyl chloride
RADS	Reactive airway dysfunction syndrome
RAST	Radio allerge sorbent testing
R _{Aw}	Airway resistance
RD ₅₀	Respiratory rate decrease, 50 %
R _L	Pulmonary resistance
RIA	Radio Immuno Assay
RUDS	Reactive upper airway dysfunction syndrome
SBS	Sick building syndrome
sd	standard deviation
SD ₅₀	Skin sensitization dose, 50 %
SDS	Sodium dodecyl sulfate
SP	Substance P
STEL	Short term exposure limit
TDA	Toluene diamine

TDI	Toluene diisocyanate (unless otherwise specified, an 80:20 mixture of the 2,4- and 2,6- isomers)
TLV	Threshold Limit Value
TMA	Trimellitic anhydride
TMI	Tolyl monoisocyanate
TNBS	2,4,6-trinitrobenzenesulfonic acid; picryl sulfonic acid
TSM	Tracheal Smooth Muscle
WHMIS	Workplace Hazardous Materials Information System
ϵ	Extinction coefficient

1. GENERAL INTRODUCTION

1.1 Review of the literature

1.1.1 Asthma; environmental / occupational etiological factors

"Since the Cure of the Asthma is observed by all Physicians, who have attempted the Eradicating of that Chronical Distemper, to be very difficult, and frequently unsuccessful; I may thence infer, That either the true Nature of the Disease is not thoroughly understood by them, or they have not yet found the Medicines by which the Cure may be effected". (1726) ¹

"In all of medicine asthmatic patients may well be the most difficult and complex for physicians to understand and treat. Current knowledge... does not allow simple unifying concepts about its nature...". (1991) ²

"Asthma is more easily described than defined" ³. Such truisms notwithstanding, asthma is best defined clinically as episodic, reversible airway obstruction ⁴, which can itself be objectively determined in a number of ways. Most commonly this involves a measure of volumetric airflow rate, for example Forced Expiratory Volume in one second (FEV₁), or Peak Expiratory Flow Rate (PEFR). There is currently considerable debate in the literature regarding which of the three contributors to the obstruction (bronchoconstriction, inflammation / mucosal thickening, and mucus secretion) is the most significant. In fact, there is likely to be considerable variability in this respect, depending on the individual patient and on the time course of attack and phase of disease. It is likely that asthma involves multiple interactions; there is not necessarily any consistent sequential time course, and in fully developed disease the three constitute a self-perpetuating cycle. For example, Clifford ⁵ has characterized asthma generally as airway hyperreactivity with rapid variations in airway diameter which occur mainly because of smooth muscle contraction, but which can nevertheless be easily reversed by β_2 -agonists; it is only in *status asthmaticus* that spontaneous reversal of bronchoconstriction disappears, and β -agonist reversal diminishes due to the mucosal edema and mucus plugging. Perhaps it will prove most productive not to speak of "asthma" as if it were one disease entity, but rather the "asthmas", which share certain symptomatology, but which can differ significantly in etiology and pathology.

Asthma is the most common chronic condition encountered in the general practice of medicine ⁶. It is currently thought to affect about 5% of the adult population, and there are some indications that it is becoming more common and more severe ⁷. Approximately a million Canadians are currently classified as asthmatics; in several recent Canadian studies, it has been shown that the prevalence is increasing. For example, among Manitoban male children younger than four years old, there was a 41% increase in the prevalence of asthma between 1983 and 1988; overall, the increase was 31% ⁸. Perhaps more disconcerting societally is the fact that the death rate is also increasing. Among Ontario females in the 15 - 24 years of age bracket, the age standardized mortality rate due to asthma doubled between 1979 and 1985 ⁹. According to references reviewed by Svenson ¹⁰ increasing illness and death due to asthma have been reported in the United States, England, New Zealand, Australia and Hong Kong over the last 20 years. In the U.S. the age adjusted death rate for asthma increased by 46% from 1980 through 1989 ¹¹. The fact that the substantial increases are occurring among young people, and despite modern pharmacotherapy (some ^{12,13} would say because of it), is particularly troubling. This renders an understanding of the etiology, and an elucidation of the (pharmacological) mechanism(s), a more compelling pursuit, both in an effort to prevent new occurrences and to promote the development of efficacious therapies that do not have associated with them potentially life-threatening adverse effects.

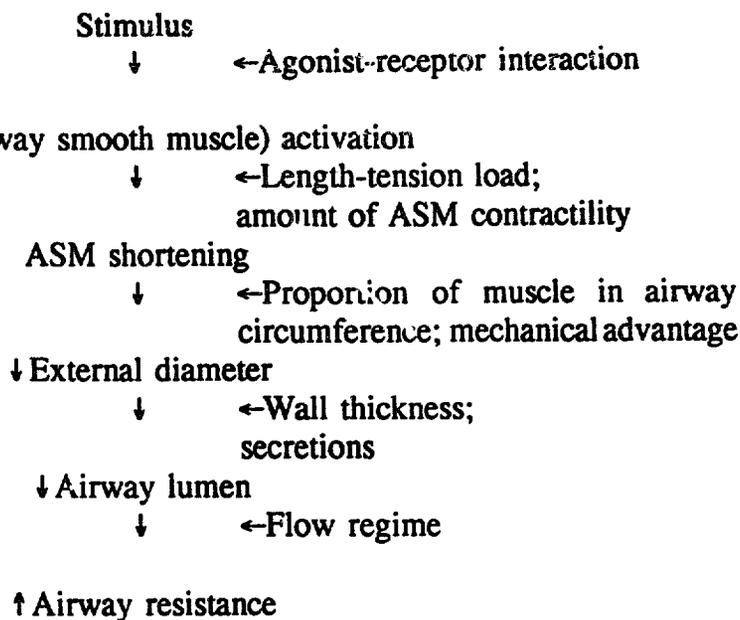
Over the past few decades (and with the advent of the β -adrenergic agonist inhaler) the pharmacological focus has been on the treatment of the bronchoconstriction, as this is the most acute, (externally) obvious and reversible manifestation of asthma. It has been suggested that the (over)-use of these inhalers has contributed to the increased mortality rate ^{14,15}, for example self-administered use of the inhaler in cases where more aggressive therapy is indicated ¹⁶. However, there are more elegant (mechanistic) suggestions as to how the β -inhalers are contributing to mortality in asthmatics. These range from selectively increased responsiveness to different airway spasmogens ¹⁷, and β -adrenoceptor tachyphylaxis ¹⁸, through to an actual promotion of the pathology due to the agonist's inhibition of mast cell degranulation, resulting in an increased antigen load on

5

the lower airways, which in turn leads to increased inflammation and formation of repair tissue^{13,19}. The epidemiologic findings have been presented concomitantly with a change in focus toward inflammation as the underlying pathology. Accordingly, there has been a shift in therapeutic emphasis towards the treatment of the inflammatory process.

However, there are ongoing struggles to establish whether it is the bronchoconstriction or the inflammation that is a more important factor to control chronically in asthma. Reed²⁰ states that airway hyperresponsiveness is now viewed as being secondary to inflammation and describes the disease in those terms, as "chronic desquamating eosinophilic bronchitis". Nevertheless, authorities such as Barnes²¹ regard edema as "contributory", but of minor significance relative to the role of airway smooth muscle contraction (except, perhaps, in terms of the effect that airway submucosal thickening has on airway responsiveness to spasmogens). Similarly, there is considerable controversy over whether the airway epithelium (which is commonly damaged in established asthmatics) has a (pharmacologically) functional role or is merely a passive diffusion barrier. Recently, Bramley *et al*²² have suggested mechanical reasons for the excessive bronchoconstriction occurring in asthmatics: they found reduced tissue elastance (which would normally limit smooth muscle shortening) in the airway preparation from their (one) asthmatic patient. These are not just academic exercises: their outcome will have a very real effect on the quality of life enjoyed by many thousands of asthmatics. The U.S. 1990 economic burden due to asthma was estimated as \$6.2 billion; expenditures for asthma medication alone currently approach one billion dollars annually²³. This is likely to increase with the shift towards more expensive therapies such as cromolyn and corticosteroids in metered dose inhalers. Therefore, there are socio-economic considerations relating to drug efficacy, as well.

The illustrative sequence adapted from Moreno²⁴ summarizes several of these etiological considerations:



Airway resistance varies with the fourth power of the lumen diameter²; accordingly, a patent airway is critical for unobstructed flow. To paraphrase Pezzini *et al*²⁵ regardless of whether caused by mucus plugging, edema or bronchoconstriction, the increase in airway resistance results in a low airflow which is particularly evident during expiration, as the pressure surrounding the airways is positive at this point, therefore promoting the closing of the airways. This factor will become particularly significant later, in relation to the plethysmograph endpoint developed in this study for the *in vivo* challenges with toluene diisocyanate.

A multitude of host (patient) and environmental etiological factors have been identified, and more have been postulated, for asthma. It is unclear to what extent the two are important in the development of the disease. It has been suggested that common environmental agents such as sulphur dioxide (in air and/or food)²⁶, ground-level ozone²⁷ and diesel exhaust/air pollution²⁸, or general indoor air pollution²⁹ may be responsible for the increasing prevalence. In certain cases, specific (but widespread) local factors appear to be implicated. For example, the introduction of the *Prosopis* tree to Kuwait has been offered as a (partial) rationale for the increase in asthma there from virtual nonexistence, to a prevalence of 10%²⁶. Community outbreaks of asthma in Barcelona³⁰, Cartagena³¹ and Valencia³² have been associated with the unloading of soya beans in

the respective city harbours.

Similarly, we are making progress in elucidating the innate characteristics of the asthmatic individual. The term "atopic" (meaning genetically predisposed to develop IgE-type allergies) has long been used in association with the asthmatic patient. Quite recently, some allelic determinants have been identified that are associated with the development of asthma in isocyanate-exposed individuals, as well as others that appear to convey protection from the development of the disease ³³. Meggs ³⁴ points out that asthma was (in the past) dichotomized as "extrinsic" (triggered by proteinaceous aeroallergens) and "intrinsic" (of unknown cause). He suggests that asthmas should now be classified as **neurogenic** or **immunogenic**. As indicated below, the distinction only applies to the "catalysts" for the common outcome (bronchial inflammation):

ASTHMAS:	Immunogenic	Neurogenic
Triggered by:	Protein aeroallergens	Volatile organics
Interacting with:	IgE antibody	Irritant receptors
Located on:	Mast cells	Sensory nerve C-fibres
Releasing:	Histamine, leukotrienes, prostaglandins, chemotactic factors	Neuropeptides: substance P, neurokinin A, CGRP
Stimulating:	Sensory nerve C-fibres	Mast cell degranulation
Producing:	Bronchial inflammation	Bronchial inflammation
Manifesting as:	Asthma	Asthma

In spite of Meggs' valiant effort to consolidate much of the literature, and to rationalize a commonality to Reactive Airways Dysfunction Syndrome (RADS), Reactive Upper Airways Dysfunction Syndrome (RUADS), Multiple Chemical Sensitivity (MCS) and Sick Building Syndrome (SBS), this model is not entirely consistent with all of the literature. Various recent papers, while reinforcing the "cross-over" between the classic neural and immune concepts, suggest that there may be greater inherent differences than assumed

by Meggs, or alternatively, that the neural system is more critical in the manifestation of an "immune" response. Larsen *et al*³⁵, for example, present data to indicate that repeated airway exposure of mice to IgE-stimulating allergen (ovalbumin) leads to changes in airway neural control and to increased airway responsiveness due to increases in the amount of acetylcholine (ACh) released in response to stimulation. This increased release is associated with the loss of function of a muscarinic (M₂) autoreceptor in the sensitized mice. Jacoby and Fryer³⁶ implicated the same mechanism in the case of infection by (neuraminidase-containing) influenza and parainfluenza viruses, which lead to increased bronchial responsiveness (in part) due to increased acetylcholine release. With sensitized guinea pigs, Whicker *et al*³⁷ showed that allergen challenge increases the carbachol sensitivity of peripheral airway tissue six-fold, but not by altering receptor characteristics or by impairment of relaxation mechanisms. Classic neurotransmitters / autocooids are variously involved in the modulation of airway response to antigen challenge. For example, Bertrand *et al*³⁸ demonstrated that indomethacin (an inhibitor of the cyclooxygenase step in prostaglandin biosynthesis) potentiates the antigen-induced contraction of their *in vitro* guinea pig tracheal preparations. More examples are detailed in the appropriate sections, below. On the other hand, Mitchell *et al*³⁹ showed that (immunogenic) airway inflammation induced by ovalbumin in sensitized guinea pigs (but not the neurogenic inflammation induced by capsaicin) augments the maximal shortening velocity (V_{max}) and the maximal shortening (Δ max) of tracheal smooth muscle (TSM) in response to electric field stimulation (EFS). They suggested that the immunogenic inflammation differs from pharmacological inflammation insofar as it alters the contractile mechanism within the myocyte. Recognizing the multifactorial etiology of asthma, and bridging the neural with the immune, Cockcroft *et al*⁴⁰ developed an equation (with $r = 0.85$) to predict human airway responsiveness to allergen, on the combined basis of individuals' skin sensitivity to allergen and their airway sensitivity to histamine.

An additional consideration is whether asthma (and particularly the forms induced by chemical agents such as toluene diisocyanate - TDI) is predominately a systemic or a local disease. Corris and Dark⁴¹, by transplantation of lungs between human asthmatic

and non-asthmatic patients, have indicated that the disease resides in the organ, and not the recipient of the organ. This becomes pertinent in comparing certain *in vivo* and *in vitro* findings in the present study.

Occupational factors are implicated etiologically in 2 - 20% of all cases of asthma ⁴². The single most significant chemical cause of occupational asthma is represented by the isocyanates ⁴³, which have been responsible for some 20% of occupational asthma in North America ⁴⁴, and 47% in Italy in the last decade ⁴⁵. Isocyanates are used extensively in the production of various polyurethanes, both surface coatings and foams. Industrial production of isocyanate-based materials began in Germany in the 1930s ⁴⁶; by 1975, world-wide production of the two most common isocyanates (methylenediphenyldiisocyanate - MDI and toluene diisocyanate - TDI) reached 700 million pounds per year ⁴⁷. That same year, in Canada, there were 20 million pounds of TDI produced⁴⁸. By 1986, global production of isocyanates had increased to 3.3 billion pounds (1.5 million metric tonnes) per year ⁴⁹.

Estimates of the proportion of isocyanate exposed workers who will develop asthma include ranges from 5 to 25% ⁵⁰ or, up to 20% ⁵¹. More recently estimates have been lower, thus Vandenplas *et al* ⁵² suggested a prevalence of 10%, and others ³³ maintained that no more than 5% of workers are at risk. There is an inherent danger in estimating the population at risk on the basis of new cases of disease: in recent years the exposures have been reduced, but also, those individuals who are likely to become sensitized may have left the workplace on the basis of selection criteria such as "atopy", or due to symptomatic response.

Even if the prevalence of isocyanate-induced asthma is 5% it is significant, as it has been estimated that half a million people world-wide are working with isocyanates ⁵³, and reports of human toxicity originate from many countries ^{54,55}. In the U.S., there may be 100,000 individuals exposed occupationally ⁵⁶. In Ontario, the Provincial Ministry of Labour in 1982 estimated that "*in the order of 8000 workers are involved in the production of polyurethane products ... Many more workers may be exposed since small-*

scale and occasional users are difficult to identify."⁵⁷ In addition, there is residential use of various isocyanate-based products, and isocyanate asthma in a "home pieceworker" has been documented ⁵⁸. Precise figures as to the prevalence of isocyanate-induced disease in Ontario are not readily available. Nevertheless, isocyanates have since 1983 been "designated substances" under Provincial health and safety legislation i.e. specific statutory provisions apply to their use (RRO 1990, Reg. 842) ⁵⁹.

The aftermath of the 1984 catastrophe at Bhopal, India has resulted in a considerable increase in the interest in isocyanate toxicology. Over 2000 people were killed in short order, and the cause of death was initially unclear (e.g. classic cyanide poisoning was being considered ⁶⁰). Much of the focus in the consequent research has been on methyl isocyanate (MIC), the specific compound used in pesticide manufacture at Bhopal. Nevertheless, this work has applicability to other isocyanates, especially in relation to their interaction with biological macromolecules. *"Despite all of these recent experimental advances, the molecular mechanism of isocyanate toxicity is still not clearly understood"* ⁶¹.

1.1.2 Isocyanates

The isocyanates are characterized by their $-N=C=O$ functional group; those compounds of primary concern are bisfunctional. The industrial usefulness of the isocyanates relates to the reactivity of this NCO group: *"virtually every functional group gives a defined product with an isocyanate under some defined set of conditions"* ⁶²; herein also resides their toxicity. Isocyanates can react with a number of sites on proteins and other biological macromolecules (e.g. DNA ⁶³). It has been recognized ⁶⁴ that the presence of multiple reactive sites on an offending molecule may be important in its interaction with biological macromolecules. Under physiological conditions the NCO moiety reacts with alcohol groups to form urethanes and with amino groups to form substituted ureas, according to Tse and Pesce ⁶⁵. On the other hand, Brown *et al* ⁶² cite hydroxyl, sulfhydryl and imidazole group reactivity as being of physiological significance. Reaction with amino groups is considered by them to be relatively unimportant, due to the fact that these are mainly protonated, which does not favour reaction. Sarlo and

Clark ⁶⁶ disagree: they state that TDI conjugation to protein most likely involves nucleophilic attack by free amino groups; the latter is also the basis for the TNBS assay (see 2.2.2) used to determine the degree of albumin conjugation by TDI. It may be particularly significant that the reaction with the -SH (sulfhydryl) group yields a product that is stable, yet dissociable under slightly alkaline conditions, because this could serve a "carrier" role to convey this highly reactive compound to target sites distal from the initial point of contact ⁶⁷. Similarly, this may relate to the reported effects of toluene diisocyanate (TDI - the specific isocyanate of interest in our research) on various airway enzymes (see 1.1.6). Hydrolysis (yielding amine and carbon dioxide) also occurs (directly, or from the protein), but this is likely to be a slower reaction (when the relative nucleophilicity is considered) than some of the others. Under neutral conditions the order of nucleophilicity is $\text{SH} \gg \text{OH} > \text{NH}_2 \gg \text{COOH}$ ⁶¹. Nevertheless, reaction with secondary amines is also cited as being slower than is the hydrolysis reaction ⁶².

Because of the insolubility of TDI in water, and its propensity therein to form "globules" comprised of an outer layer of poly-urea, various solubilizing media have been used for the purposes of introducing TDI to tissue preparations. These in turn may impact on the observed effects. The "loss" of a 2,6-TDI-DMSO (dimethyl sulfoxide) preparation in serum *in vitro* is reportedly nearly 90% complete in 1 minute ⁶⁸. However, the DMSO will have accelerated the reaction (based on observations made during our research). Other reports of the half-life of TDI in biological fluids include < 30 seconds in serum and < 20 minutes in stomach contents; in water, the range is reported as 0.5 seconds to 3 days, "depending on pH and water turbidity" ⁴⁸.

There are reportedly some 43 structural variants of isocyanates commercially available, although the three diisocyanates shown in Fig. 1-1 constitute 90% of the market ⁶¹. A number of key parameters of TDI are provided in Table 1-1. TDI as used industrially is typically a mixture of the 2,4- (Chemical Abstracts Service -CAS- number 584-84-9) and 2,6- (CAS number 91-087) isomers in an 80:20 ratio (CAS number 26471-62-5);

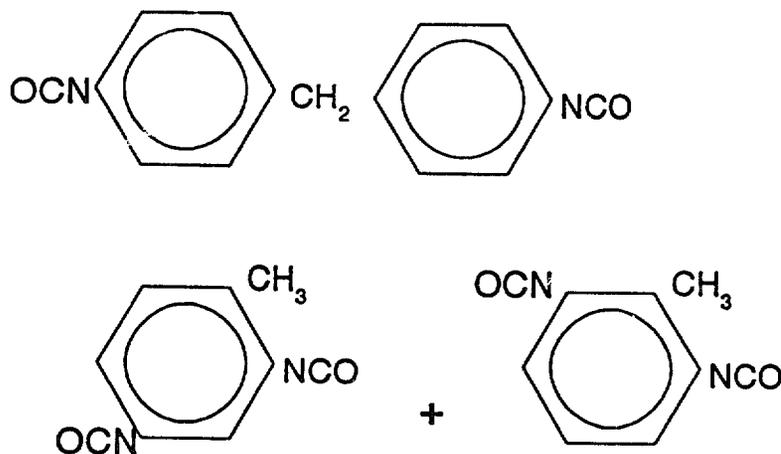
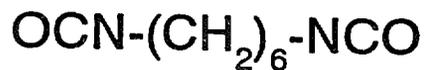


Fig. 1-1. Structural formulae of hexamethylene, methylenebisphenyl and toluene diisocyanates (HDI, MDI and TDI).

Table 1-1. Key properties of "TDI" ¹	
Molecular weight	174
Specific gravity	1.22
Melting point	12.5-13.5 or 19.5-21.5 °C
Boiling point	251 °C
Vapour pressure	0.01 ^{20°C} or 0.04 torr
Odour threshold	0.05 - 0.13 or 0.17 - 3.2 ppm
LD ₅₀ - rat, oral	5 - 7 g/kg
LD ₅₀ - rabbit, skin	16 g/kg
LC ₅₀ -4 hr- rabbit, inhal	11.0 ppm
LC ₅₀ - 4 hr- guinea pig, inhal	13.9 ppm

1. Data from ^{48,51,61,69}

however, the 2,4- isomer is more reactive than the 2,6- and accordingly, personnel exposure may be predominantly to the (residual) 2,6- isomer. The toxic properties of any "TDI" will reflect the relative amount of the two isomers, and this may vary according to time, location, etc. This is just one factor that may confound comparison of experimental data between groups of researchers.

Several factors are clear from Table 1-1. There are significant inconsistencies in reported parameters as fundamental as melting point and olfactory threshold. Even the consistently reported specific gravity of 1.22 needs to be reconciled with the observations made during the tissue bath trials, that TDI floats on top of the buffer. The acute dermal and oral toxicities of TDI are so low as to not even render it "toxic" (on the basis of this criterion alone) according to the classification system promulgated under Canada's Hazardous Products Act and generally referenced as WHMIS (Workplace Hazardous Materials Information System) ⁷⁰. On the other hand, the exquisite airborne toxicity of the (di-) isocyanates was recognized soon after their widespread introduction into the manufacturing sector. Fuchs and Valade documented occupational TDI-induced asthma in 1951 ³. Since 1983 the Threshold Limit Value (TLV) for the common isocyanates has been 0.005 ppm, representing a 20-fold reduction from the 0.1 ppm TLV initially set in 1959 - 60. The TLVs are established and published by the (non-governmental) American Conference of Governmental Industrial Hygienists (ACGIH) on an annual basis, and are intended to protect nearly all workers from adverse health effects due to airborne exposure ⁵¹. However, even a sub-TLV level of isocyanate (e.g. 0.001 ppm) is recognized as being capable of eliciting a life - threatening / taking bronchospastic attack in some (previously-sensitized) individuals ^{42,71-73}; in the sensitized individual the asthma brought on by isocyanate exposure may be immediate/early, late or dual with respect to time course. By comparison, the corresponding TLV for classic "lethal" toxicants such as hydrogen cyanide is three orders of magnitude higher, at 5 ppm, and this level is thought to be without significant health effects under occupational exposure conditions. Further evidence of the significance of low level TDI exposure is provided by various case studies, such as one reported by Carroll *et al* ⁷⁴: TDI emanating from the exhausts of a closely neighbouring factory caused the development of asthma in three otherwise

unexposed clerical workers. Isocyanate toxicity may be related to the chemical reactivity / irritancy of the material. In the mouse, the RD_{50} (the airborne concentration required to decrease respiratory rate by 50%) is only 0.4 ppm for 2,4-TDI ⁴⁶, as compared with 1.3 ppm for methyl isocyanate ⁷⁵, 9.3 ppm for chlorine and 303 ppm for ammonia ⁴⁶.

The TLV-setting ACGIH confirms that the TDI exposure circumstances causing human response have not been well understood ⁵¹. It appears that the development of sensitization may be correlated more with specific events, rather than with chronic, ongoing exposures. For example, Brooks *et al* ⁷⁶ have shown an association between a history of attendance at more than 20 isocyanate spills, and the prevalence of abnormal lung function. Karol ⁷⁷ has suggested that the preponderance of the animal and clinical evidence indicate that the concentration of the initial sensitizing TDI to which the animal/individual is exposed, and not dose, is important in determining the degree of response. She has variously suggested that sensitization to TDI requires exposure above a definitive threshold (consistent with the then ACGIH TLV of 0.02 ppm ⁷⁸). Lee and Phoon ⁷⁹ would concur, finding no overt cause of occupational asthma in an investigation where all but one of 24 environmental samples exceeded the Short Term Exposure Limit (STEL) of 0.02 ppm. A lower threshold has been suggested by many other authors ⁸⁰. For example, Omae *et al* ^{81,82}, based on clinical epidemiology, again indicated that peak exposures appeared to be important in the development of obstructive changes, and suggested 0.003 ppm as a threshold. Jones *et al* ⁸³ demonstrated that TDI exposures (as determined from a total of 4845 12 minute personal samples, of which 9% exceeded 0.005 ppm and 1% exceeded 0.02 ppm) had an effect on initial lung function and prevalence of chronic bronchitis, but had no effect on the slope of change of parameters of pulmonary function such as FEV_1 or FEF_{25-75} (forced expiratory flow, 25-75%). Vandenplas *et al* ⁸⁴ have shown dose to be the determinant of response to challenge in isocyanate-sensitized workers.

There is still no consensus regarding the mechanism of asthma induction by isocyanates. Is sensitization only due to exposure of airway tissue to vapour / aerosol, or is prior dermal exposure a significant etiological factor? With respect to airborne exposure, is

the Time Weighted Average (TWA) concept (or other consideration of total dose in accordance with Haber's rule) the critical parameter, or is the achievement of some peak exposure threshold (of irritancy?) the important factor that distinguishes those who become sensitized, from those who don't? What is the role of concomitant factors (e.g. nonspecific irritants ^{85,86}, viral infection, or humidity)? What is the significance of the presence (or demonstrable absence) of TDI-specific serum antibodies of the IgE (or IgG) class?

In 1977, Kane & Alarie ⁸⁷ showed that hyperreactivity to a chemical stimulus could be induced without a necessary involvement of immunoglobulins: repeated, daily exposures of mice to airborne chemicals such as formaldehyde or acrolein caused an increase in sensory irritation, without any immunological interaction. In relation to TDI, Sangha & Alarie ⁸⁸, were similarly able to show a cumulative effect from mouse exposures (for 3 hour periods on five consecutive days) to TDI at levels exceeding 0.023 ppm; the mice showed complete recovery of normal sensitivity within 15 days. In other words, the hyperreactivity had a direct pharmacological (rather than an immunological, or antibody-derived) basis. Based on this, they summarized the mechanistic dichotomy that remains postulated today:

- tolyl reactive IgE [and/or IgG] antibodies in a fraction of the exposed workers
- interaction of TDI with bronchial epithelium and interference with cholinergic or adrenergic [and/or other mediator] systems in such a manner as to enhance the response upon re-exposure to TDI, and resulting in a cumulative effect.

A better understanding of the mechanisms whereby isocyanates produce bronchial hyperreactivity in some individuals is necessary to: a) permit a better definition of the risk factors (and thereby, preventive strategies) associated with the development of the disease; b) improve the pharmacotherapy available to those who have developed the disease. These could reduce the burden of ill health produced by isocyanates, with important socio-economic benefits.

At the moment, stringent (and costly) protective devices and procedures must (by law,

in Ontario and most other jurisdictions) be applied in all areas where workers are isocyanate exposed, despite the recognition that these measures may be unnecessary in the case of at least 80 - 90% of the population. The etiological uncertainties have mandated a particularly cautious approach on the part of Ontario regulatory officials:

"the isocyanate regulation s.5(b) [i.e. for isocyanates other than M, T, H or IP DI] requires that respirators be provided to, and worn by workers as soon as there is a possibility of inhalation of isocyanates. The necessity of providing workers with respirators does not depend on the measurement of a certain degree of potential inhalation, but rather, is triggered by the mere possibility of inhalation." ⁸⁹

Similarly, Fabbri *et al* ⁴² have stipulated that *"complete avoidance of exposure to the sensitizing agent must be recommended to subjects with demonstrated occupational asthma"*.

A proposed Regulated Substances Regulation (which will likely be promulgated in Ontario in 1995) makes provision for: continuous monitors, alarm systems, engineering controls, supplied air respirators, etc., wherever isocyanates are used. However, as indicated by the findings of the present research, it may well be that all of this effort will be unproductive, if skin contact with bulk isocyanate is permitted to occur. Neither the proposed regulation nor the TLV for TDI incorporate a "skin" notation, which would communicate to all concerned parties the significance of this exposure route. This is despite a recognition that worker onset of sensitization is often associated with exposure to TDI spills ^{51,76,90} and "splashes" ⁹¹. Ironically, the ACGIH Chemical Substances TLV Committee has dismissed animal studies as being of limited value in establishing a TLV because *"respiratory sensitization is difficult to produce in animals"* ⁵¹. As will be described, this has been the case except where the animal's exposure is dermal rather than airborne. In fact, the reported difficulties in eliciting airway sensitization in animals (under controlled conditions in which their exposure is only by inhalation, and not at all by direct contact) lends further credence to a central thesis of the present work, namely, that dermal absorption of TDI causes airway sensitization. Although this phenomenon has not been demonstrated in humans, Borm *et al* ⁹² had concluded that "parallels between animals and man" in TDI effects were, in fact, "impressive". The need to

ascertain whether human dermal exposure similarly results in airway sensitization is a compelling pursuit.

Although many mechanisms have been proposed for the development of isocyanate-induced (and other chemically elaborated) asthmas, and are supported to varying degrees by experimental or epidemiological evidence, no unifying theme has emerged to date. A recent ⁹³ U.S. National Research Council committee publication on the topic of immunotoxicology summed up the challenge as:

"... there is little information available to determine why some individuals become sensitized or have adverse reactions to chemicals and others do not. The differences between individuals need to be identified, and markers useful in identifying susceptible persons need to be developed." and,

"Data are needed about the mechanisms of nonimmunologically-mediated sensitization, including identification of the cells and mediators involved".

Finally, it should be borne in mind, that although the focus has been on asthma, isocyanates cause a multitude of respiratory effects. A classic summary ⁹⁴ encompasses the range from irritation and acute loss of ventilatory capacity, to accelerated loss of pulmonary function, sensitization and the induction of a general asthmatic state.

1.1.3 "Sensitization" and "hyperreactivity"

"Sensitization" is defined ⁹⁵ as:

1. The process by which an immune response is stimulated on first being exposed to an antigen, with the consequence of preparing the body's immune system for a stronger (anamnestic) response upon reexposure to the same antigen, as in a hypersensitivity reaction

2. A condition in which the response to later stimuli is greater than the response to the original stimulus.

A fundamental question that was to be addressed in the present research speaks to the difference between these two definitions, namely, "Is the activation of the immune system a necessary component of the exaggerated response seen in previously-exposed ('sensitized') animals?"

In view of the ambiguity of the term "sensitization" (and its generally-understood implication of immune system involvement), the more generic term "hyperreactive" will be used to refer to a situation in which an exaggerated reaction to challenge is seen, yet which does not necessarily involve an immunological component. The heightened reactivity could either be due to a decreased threshold (increased sensitivity) of the system (i.e. increased **potency** of the agent in question) or to an increased responsiveness of the system (i.e. increased **efficacy** of the agent).

The consideration of the mechanisms involved in sensitization is by no means a novel pharmacological exercise. The terms "hypersensitivity" and "allergy" were introduced into the literature in 1905 by Clemens von Pirquet; Dale described the role of histamine in allergy in 1910; Prausnitz & Küstner reported their discovery of the passive transfer of cutaneous sensitivity in 1921, and predicted the discovery of "reaginic antibodies" (eventually isolated in 1966 and named IgE) ⁹⁶. However, there remain a host of mechanistic unknowns to resolve.

1.1.4 Immunologic mechanisms: IgE and IgG

The most obvious mechanism to have been considered in isocyanate induced-asthma has been classic immunological hypersensitization. Immunoglobulin G (IgG) is normally the primary mediator of the humoral immune response; it comprises about 80% of the total immunoglobulin, with serum levels of 700 - 1500 mg/dL ⁹⁷. In humans, production of IgG₄ is stimulated by allergens such as house dust and grass pollen; Gwynn ⁹⁸ found patients with specific IgG₄ (and no IgE) to dust-mite allergens who demonstrated an asthmatic response only at very high challenge doses. Immunoglobulin E (IgE) is the antibody most closely associated with allergy. IgE is secreted primarily in the respiratory and gastrointestinal tracts (i.e. where allergens generally present), but can also circulate systemically; serum levels range from 0-0.09 mg/dL ⁹⁷. Exposure to an allergen (or to a chemical hapten which can become immunogenic upon conjugation to an endogenous protein such as serum albumin) results in the production of specific (complementary) IgE, initially bound to local mast cells. "Spillover" IgE can bind to circulating basophils and tissue-based mast cells throughout the body. Upon re-exposure,

interaction of antigen and antibody results in the degranulation of the mast cells, releasing histamine, leukotrienes and other autocooids, which in turn (in the airways) bring about the tissue changes associated with asthma.

This model is not entirely consistent with the isocyanate data obtained from studies on both humans and laboratory animals. Accordingly, there may be a complete lack of functional sensitization (i.e. airway reactivity) despite the presence of isocyanate-specific antibodies. For example, Karol reported ⁹⁹ that application of undiluted TDI to guinea pig skin induced specific antibodies in all animals. However, respiratory sensitization could be demonstrated (according to the experimental criteria) in only 30 - 40% of these, when challenged with airborne isocyanate-protein conjugate, or 25 - 50% (1 or 2 of 4) when challenged with 0.005 ppm TDI. Similarly, with sensitization induced by inhalation, ⁹¹ even in animals with high antibody titres, only about a third showed "lung reactivity" to TDI-protein challenge.

Although there is no direct evidence to indicate whether worker dermal exposure causes any or all to become sensitized, these animal data are consistent with human experience. Isocyanate-specific antibodies have been found in asymptomatic workers ¹⁰⁰, and including an "exceptionally high assay value" in the absence of clinical evidence of TDI disease ¹⁰¹. The opposite is also true: respiratory symptoms have occurred in workers who are without isocyanate-specific antibodies. Bernstein ¹⁰² cited data to indicate that RAST (Radio-Allergo Sorbent Testing) to mono- or di-functional isocyanate-protein conjugates would detect only 3-16% of workers whose respiratory function was compromised. Baur *et al* ⁴⁶ performed RAST on 621 isocyanate workers, of whom 247 were clinically identified as sensitized. Only 14% of the symptomatic workers had significant levels of IgE antibodies. More recently, Park *et al* ⁵⁴ reported on a Korean investigation: although none of the TDI-sensitized asthmatic workers showed TDI-specific IgE antibody, 13% of the asymptomatic workers had "high specific IgE to TDI-HSA" (TDI-Human Serum Albumin conjugate). Clearly, then, there is no direct association between clinical disease and serum levels of specific IgE. Banks *et al* ¹⁰⁰ highlighted the various early studies that also failed to show an association between isocyanate asthma

and atopy.

Although atopy as a general condition in humans is clearly related to the IgE class of immunoglobulin, isocyanate exposure has in several clinical studies been more closely associated with IgG response, than with IgE. Jin & Karol ¹⁰³ investigated the MDI-specific IgE and IgG levels of three workers, two of whom had symptoms of "chest tightness and severe shortness of breath while at work" (which the authors describe as "consistent with hypersensitivity pneumonitis"), while the third had been asymptomatic for two years at his job, until exposed to MDI during a spill, at which point he developed "respiratory tract irritation and chest tightness" (considered by the authors to be "asthmatic symptoms"). The first two had a low total IgE, and MDI-specific IgE in the normal range, yet their MDI-specific IgG titres were 2000 and 4000 respectively. The third worker had an IgG titre of <4; his total IgE was 15-fold higher than the mean value of the other two (though still in the normal range) and his MDI-specific IgE was 14 fold higher than that of the other two, as well as being outside the normal range. The authors rationalized the results by dichotomizing the workers into "systemic symptomatology" (IgG-related) and "airway disturbance" (IgE-associated). It is unclear whether these associations were based on considered medical appraisal / opinion. Cartier *et al* ¹⁰⁴ assessed 29 workers who demonstrated a positive TDI challenge test, and who had elevated levels of specific antibodies: none had elevated IgE only, 13 had elevated IgG only, and 8 were elevated in both. Vandenplas ⁵² concluded that the IgG level is a more appropriate screening tool than is the level of IgE. Selden *et al* ¹⁰⁵, however, stressed that although the IgG levels were "evidence of immuno-stimulating hapten exposure" they had little association with the immuno-pathological role (i.e the disease process). Further, Paggiaro *et al* ¹⁰⁶ were unable to differentiate asthmatic and nonasthmatic workers, and normal subjects, on the basis of their level of serum IgG antibody against a conjugate of TDI and human serum albumin (TDI-HSA). In many animal studies IgG has been found to be a more significant indicator of exposure than is IgE, and despite early evidence that IgE was implicated and IgG was not ¹⁰⁷. This may relate to the critical importance of considerations such as the species and strain of animal, and route of exposure. Patterson *et al* ¹⁰⁸ exposed dogs intermittently to aerosols of TDI

in a manner that was analogous to human chronic exposure at 0.02 ppm. The dogs developed systemic immune responses to TDI-dog serum albumin conjugate, and although IgA and IgG titres were similar and persistent, IgE activity fluctuated and became negative despite ongoing exposure to TDI. The route of exposure appears to have an impact on the type of immunoglobulin (IgE or IgG) responding to the administration of antigen. ¹⁰⁹

In guinea pigs, generally, both IgE and IgG₁ antibodies are associated with the anaphylactic response. However, there is a considerable difference between strains: in (Dunkin-) Hartley animals (used in the present research) it is difficult to evoke an IgE response ¹¹⁰. On the other hand, with the English short hair strain (used by Karol's group in the late 1980s ³⁸, and presumably the same as the "English smooth-haired" animals used by Karol in her earlier work ^{99,111,112}, but not her more recent work, for which she had switched to Hartleys ¹¹³) there is a prominent IgE response ¹¹⁰. Griffiths-Johnson *et al* ¹¹³ demonstrated by means of passive immunization (in Hartley guinea pigs) that immediate onset of response to airborne challenge with allergen is associated with IgG₁ antibody; the immediate-onset response is the one of interest in the present study. Cheng *et al* ¹¹⁴ showed with passively sensitized guinea pigs that circulating IgG₁ facilitated the synthesis of leukotrienes and the release of histamine from lung tissue upon subsequent antigen challenge, in a concentration- and time- dependent fashion. Sugawara *et al* ¹¹⁵ in a massive investigation involving 565 guinea pigs in 28 separate trials demonstrated an association between the serum level of TDI-specific IgG (but not IgE), and asthmatic responses. Karol ¹¹⁶ has very recently stated that IgG₁ is the major class of anaphylactic antibody in the guinea pig. In part, the more recently elaborated role of IgG in the response of animals and workers sensitized to TDI may account for some of the earlier difficulties in finding a consistent association between IgE antibody levels, and pulmonary reactivity.

Karol ⁹¹ reported that *"three of four workers exposed to high TDI concentrations as a result of spills and splashes developed antibodies to TDI. Titers were maximal 3 to 8 weeks after exposure, and in the absence of further TDI exposure, titers declined over*

a period of several weeks or months". This prolonged time course is intriguing, and needs to be reconciled with the stated half-lives of immunoglobulins: 23 and 2.5 days for IgG and IgE, respectively⁹⁷. The post-exposure duration of circulating TDI-specific IgG is relevant in our research: as detailed in Chapter 2, we found that TDI-specific IgG levels persisted for over a year, despite reported half-lives (in guinea pigs) around a week¹¹⁷.

Over 15 years ago Gallagher *et al*¹¹⁸ suggested that immune responses would be expected in certain sub-populations of exposed workers, and that continual exposure to isocyanates might be necessary in order to maintain a specific immune response. With the classic immunological model, a resolution of symptoms would be expected upon removal of exposure to the offending agent; with isocyanates there may be persistence of symptoms months to years after the cessation of exposure to the agent^{119,120}. Saetta *et al*¹²¹ reported that 6 months of absence from work was sufficient to reverse mucosal basement membrane thickening, but that both TDI sensitivity and "nonspecific bronchial hyperreactivity" (NSBH - clinically defined in terms of threshold of effect with methacholine challenge) could persist. Allard *et al*¹²² showed even longer persistence: 25 of 28 workers (of whom 10 had been exposed to isocyanates) had no demonstrable improvement in airway obstruction or responsiveness as much as 5-8 years after exposure had stopped. On the other hand, Banks *et al*¹⁰⁰ reported on a case who had "lost" his isocyanate sensitivity 10 years after leaving job-related exposure.

The question of the mechanistic significance of TDI-specific antibodies remains to be answered, as various authorities have dismissed or down-played it. Ramsdale *et al*¹²³ succinctly summed up the state of affairs as: "*no consistent antibody response has been found*". Morgan¹²⁴ concluded that "*There is little or no evidence to indicate that immunologic mechanisms are responsible for TDI asthma*". More recently (and perhaps diplomatically) it has been stated:

"Isocyanate hypersensitivity can be mediated immunologically but some workers who develop asthma-type symptoms appear to be sensitized on a nonimmunologic basis... The finding of low-level antibody in the absence of concomitant symptoms serves to emphasize the secondary role of specific antibody in the diagnosis of clinical immunologic disease; that is,

the clinical data must be the primary focus and the immunologic tests are only corroborative." ¹²⁵

Mapp *et al* ¹²⁶ have recently emphasized that "immunological" mechanisms need not involve immunoglobulins in the reaction sequence, and that in the case of isocyanates, the response could be considered "immunologically-mediated" simply on the basis of the finding of activated T-lymphocytes, metachromatic cells and activated eosinophils in the bronchial mucosa of the afflicted workers. Clearly, there is a semantic problem here, with respect to what is generally understood by "immunological". Certainly, the degranulation of mast cells (with the resultant release of numerous inflammatory mediators) can be induced both by "immunological" triggers (IgE, short-term sensitizing IgG, anaphylatoxins - C3a and C5a) and "non-immunological" (physical agents, chemical histamine liberators, drugs and cholinergic effects) ²⁸. Most would understand an "immune" mechanism to involve antibody mediation.

Karol (an early and steadfast proponent of the immune mechanism hypothesis) had attributed the apparent lack of correlation between antibody assays and clinical status to what amounts to poor technique in the preparation of reagent antigens, and in the timing of the RAST tests ¹²⁷; she had recommended a standardization of these procedures ¹⁰³. There is a considerable literature on the optimal characteristics of the conjugate used to determine the presence /level of these antibodies. For example, Wass *et al* ¹²⁸ indicated that for RAST they needed less than 10 TDI molecules per HSA molecule; Karol found that a higher degree of conjugation was more effective ¹⁰³. Recently, even Karol has conceded that *"evidence of immunologic effects has not been demonstrated in the majority of cases"* of human respiratory sensitization ¹²⁹, and in a specific study, *"No association was apparent between the presence of either TDI-specific IgE or IgG antibody and pulmonary response to TDI challenge"* ¹³⁰.

Nevertheless, the level of plasma antibodies directed against specific isocyanate-protein conjugates (normally to the serum albumin of the species in question) can be a very useful index of exposure, and was used accordingly in the present research. In our study, initial levels of TDI-specific IgG were determined at weekly intervals, to verify

a positive antibody response, and to ascertain if there were any inter-animal quantitative differences in this respect (which could in turn be correlated to *in vivo* or *in vitro* airway reactivity). IgG levels also served to validate the sensitized / control animal dichotomy, as well as verifying that the airborne TDI challenges to which the animals were later subjected did not produce any discernable antibody response (as opposed to the dramatic immunogenic effect of the initial dermal exposure).

Perhaps of greater significance than the finding of isocyanate-specific antibodies in isocyanate workers is the inference that may be drawn from the fact that the assays are actually identifying an antibody directed against a (chemically-modified) **endogenous protein**. There are indications in the literature (as well as in this research, as reported later) that the effect of the TDI may in fact be to cause loss of protective self-recognition i.e. suggesting that this asthma could be an autoimmune disease.

The isocyanate by itself is not acting as an allergen/immunogen, but rather as a "hapten" is inducing a conformational change in the protein (albumin) moiety of the conjugate, causing it to assume specific antigenicity. Baur ⁷³ suggested that the toxic effect of TDI may specifically be the production of a conformational change in albumin. Kochman *et al* ¹³¹ exposed guinea pigs by inhalation to 0.6 ppm TDI for 3 hours on 5 consecutive days, and then examined the "serum-albumin-enriched fraction" (AEF) spectrophotometrically to determine the degree of TDI binding, and by Fourier transform infra red spectroscopy to investigate conformational changes. They found an average of 3.6 moles of TDI bound per mole of AEF. Compared to control animals, the exposed AEF showed "major changes in the α -helix content and [the] probable generation of some β -sheets in the perturbed protein." Clearly, such conformational changes (of themselves) could be associated with novel immune responses.

There are other data consistent with the notion that TDI elicits a sensitivity to endogenous serum albumin. Karol ⁹¹ indicated that guinea pigs sensitized to very high airborne TDI (5-8 ppm) subsequently demonstrated an intradermal sensitivity to guinea pig serum albumin (GSA). Although the antibodies from the sensitized guinea pigs did

not react with native GSA, they did react with GSA modified by treatment with methyl isocyanate (MIC): "*These results implied that the TDI antigenic determinant included, in addition to hapten (i.e. the isocyanate), portions of GSA which had reacted with isocyanate*". She further found that exposure to lower TDI concentrations (0.4 - 0.6 ppm, although still high by workplace standards, perhaps more analogous to them) produced a response that was less hapten-directed (i.e. showed more reaction to the albumin moiety). Karol and Alarie ¹³² assayed for IgE directed against pTMI-HSA (a monofunctional analog of TDI-HSA that was used widely in the early 1980s) in human samples. All 10 workers who had been sensitized to TDI were positive. However, 7 of the 10 were also positive to a test involving p-toluoyl-HSA (i.e. not involving any isocyanate). Such observations offer a tantalizing pursuit towards understanding phenomena such as Multiple Chemical Sensitivity (MCS), where there may be a generalized response to "aromatics". In the case of TDI exposure, the issue may be whether there is allergy to "TDI", or allergy to "self" as a result of exposure to TDI. Cartier *et al* ¹⁰⁴ found increased levels of antibodies against human serum albumin (HSA) in 5 of the 62 isocyanate workers they evaluated. They postulated that these antibodies would normally be responsible for the removal of aged albumin molecules, and further suggested that isocyanates could "*alter HSA in a manner similar to the usual senescence process*".

1.1.4.1 Dermal exposure

The focus in isocyanate-induced initiation of the asthmatic airway has (for logical reasons) centred on the airborne route of exposure. However, there is increasing evidence that dermal exposure / assimilation is implicated, particularly in animal models, and partly based on the relative ease with which the sensitization can be accomplished by the two routes. Karol ⁹⁹ was the first to demonstrate respiratory hypersensitivity as a result of dermal exposure to isocyanates. With the topical application of 10, 25 or 100% (but not 1%) TDI to chemically depilated skin, she was able to demonstrate a low antibody titre (by passive cutaneous anaphylaxis - PCA) in a majority of her animals (4/6, 3/4, and 12/12, respectively). Maximum titres were observed at 2 - 3 weeks, and were found to decrease thereafter. In airborne challenge

with 0.005 ppm TDI vapour (a level corresponding to the TLV) and by evaluating the "highest average [respiratory] rate during 3 consecutive min of ... challenge", she was able to demonstrate a significant increase in respiratory rate (defined as 47%) in 2 or 3 of the 12 animals evaluated. These represented 1 of 4 animals treated with 25% TDI, 0 of 4 treated with 100% TDI (once) and 1 or 2 (actual increases of 44% and 61%) of the animals treated with 100% TDI (twice). Based on experience in our lab, these concentrations are likely to have caused considerable lesioning of the skin; thus, they may not be representative of dermal permeation. Wijsbeck *et al*¹⁸³ reported that even with a 5% TDI solution irritation could be seen (by fluorescence intravital microscopy) 2 hours after its application to the skin of non-immunized rats.

Karol's animal respiratory responses were greater when they were challenged with an aerosol of a conjugate of TDI or TMI and protein. Although not discussed by Karol, this increased response may have simply corresponded to a differential in the isocyanate equivalents presented to the animals (0.04 vs ~1 mg NCO/m³ of air). Karol later⁹¹ sensitized animals by airborne exposure. Even in animal groups with a high antibody titre, only about a third showed "lung reactivity" when challenged with TMI or TDI conjugates to guinea pig serum albumin (GSA). There was no report of challenge with TDI vapour, and the conjugate challenge concentration reported was 75% higher than in the earlier paper (although direct comparison is dubious, since radically different methodology was utilized to determine airborne concentration in the two cases). The highest sensitizing concentrations used (4.7 and 7.6 ppm i.e. a thousand-fold higher than the TLV), although eliciting higher antibody titres than did the lower TDI sensitization concentrations, in fact resulted in a lower proportion of pulmonary responders to the TDI-GSA challenge. She found "*a significant association between lung sensitivity and the presence of circulating antibody (p < 0.01) rather than with the titer of antibody*".

In her 1988 review paper¹³⁴ Karol summarized her earlier work with the concluding statement: *the animal model provided evidence that skin contact from a single isocyanate spill or splash was sufficient to induce both antibody formation and pulmonary sensitivity [to TDI or TMI conjugate] three weeks after exposure*. This finding has been under-

emphasized, including in her more recent review articles. In fact, in her 1994 review¹¹⁶ she stresses her model's ability to assess airway hypersensitivity via a "relevant" (inhalation) route of exposure. She rationalizes the use of alternatives to inhalation models where there is difficulty "*generating stable atmospheres of reactive chemical allergens... such as MDI*"; in this case, injection is considered to be appropriate (thus, technical expediency appears to create mechanistic relevance). In fact, it seems that airway sensitization (at least to isocyanates) via exposure of the skin may occur more readily than via exposure of the airway. Initially, (1978) Karol¹³⁵ could achieve sensitization by airborne exposure only with a protein conjugate of TDI. Concentrations of conjugate aerosol (in terms of isocyanate equivalents) that have successfully elicited sensitization have often been very high. The airway-sensitization-via-airborne-exposure-to-isocyanate protocols that have been most successful, have relied on rather aggressive conditions (prolonged, and repeated exposures at irritant levels, etc). For example, Karol¹³⁶ used head-only guinea pig exposures of 0.12 to 10 ppm TDI for three hour periods over five days. A recent report involving MDI is illustrative. Pauluhn and Mohr¹³⁷ sensitized guinea pigs by injecting them intra-dermally with 60 - 120 mg of MDI on each of three separate days or, by exposing them for 15 minutes to 135 mg/m³ or 360 mg/m³ of airborne MDI. This was in the form of an aerosol (so the use of "ppm" is inappropriate) but to facilitate comparison to TDI challenge it would be useful to consider the equivalency: 13 and 35 ppm, respectively. The authors characterize these levels as "sublethal" and "almost lethal" (although 2/12 animals in the latter group died).

Conversely, Erjefalt and Persson¹³⁸ found that two dermal exposures to 20 μ L (24 mg) of TDI lowered the threshold for tracheal provocation with TDI. Rattray *et al*¹³⁹ sensitized guinea pigs to MDI by topical application of 40 - 400 mg of the isocyanate: a proportion of animals (1 - 7 of 8, depending on the dose) developed low levels of IgG₁ antibodies, and some of the animals (2/8 or 3/7) exhibited pulmonary responses following MDI challenge. To emphasize, even with a single 40 mg topical application of MDI, 25% of the animals showed a positive response to challenge with airborne MDI. However, their attempt to sensitize the guinea pigs by inhalation exposure was unsuccessful.

In keeping with the consideration of the significance of dermal exposure, there is human experience to suggest no development of "overt occupational asthma" despite exposure to airborne TDI levels above the 0.02 ppm STE limit ⁷⁹. This would need to be reconciled with the overall literature (such as summarized in the TLV Documentation ⁵¹) and various specific reports ^{140,141} concluding the opposite. The (retrospective) consideration of the likelihood of dermal contact in these reports would also be useful. The findings of Vogelmeier *et al* ⁵³ that 10% of healthy volunteers and 21% of control patients (i.e. with asthma unrelated to diisocyanates) showed a positive response to challenge with 0.01 - 0.02 ppm TDI for 1 - 2 hours (again, implying a pharmacological mechanism and / or threshold of irritancy at or below the STEL), are also quite relevant here.

In a different arena, (but consistent with the "dermal-contact-induces-respiratory-disease" concept) Camner ¹⁴² induced allergy to cobalt in guinea pigs by dermal application, and then subsequent to inhalation challenge by cobalt, demonstrated that the lungs of the guinea pigs in the sensitized group reacted differently from those not sensitized. Similarly, Hayes *et al* ¹⁴³ concluded that intradermal sensitization of guinea pigs with trimellitic anhydride (TMA) induces a pulmonary response (bronchoconstriction and microvascular leakage) upon subsequent challenge (tracheal instillation of TMA-GSA).

Given the reported reactivity of the NCO moiety, the notion that contact with the skin can result in heightened sensitivity in remote organs may seem highly unlikely. However, there are many examples of occurrences within biological systems that are substantively different from their physico-chemical *ex vivo* counterparts. There remain unidentified factors that could be involved. For example, Ferguson *et al* ¹⁴⁴ showed an "immediate and rapid" uptake of ¹⁴C following guinea pig (airborne) exposure to ¹⁴C-methyl isocyanate; label was found in all examined tissues. Similarly, Kennedy *et al* ¹⁴⁵ exposed guinea pigs to airborne ¹⁴C-TDI; label persisted in the bloodstream and organs (lung, kidney, heart, liver, spleen) for weeks. In Baillie's group, Slatter *et al* ¹⁴⁶ were able to demonstrate intraperitoneal absorption, and systemic distribution of isocyanate occurring by means of a glutathione-based conjugate ("carrier"). The earlier

observations ⁶⁷ of isocyanate reversible dissociation from SH again become relevant.

Clearly, there remain many unresolved questions in relation to the role of the immune system in the response to isocyanates, and in the development of hyperreactivity. In a recent article regarding the use of immunologic technology in the diagnosis of occupational lung disease, the authors ¹⁴⁷ simply indicated that various diseases due to isocyanate exposure in humans had been reported, and that animal models had been described, but then capitulated with "*The complexity of the issues of isocyanate immunologic and nonimmunologic airway disease is such that further review is not possible here*".

1.1.5 Pharmacological mechanisms

Various pharmacological mechanisms have been proposed to explain isocyanate-induced asthma, including cholinergic/adrenergic (and/or other stimulatory/inhibitory) imbalance. Some of this has been based on clinical findings; Cockcroft ¹⁴⁸ showed in the case of a painter sensitized to HDI that his histamine PC₂₀ (challenge required to reduce FEV₁ by 20%) was normal when away from work, yet 15-fold lower after an occupational exposure to isocyanate.

We use the terms "constrictor" and "dilator" to describe agonist effects on tracheal and bronchial tissue. This is somewhat arbitrary, as they are not mutually exclusive i.e. a given substance could have either effect, depending (for example) on the level of existing tone. PGE₂, ATP, bradykinin and adenosine are reported to cause a significant contraction in low-tone guinea pig tracheas, whereas they cause monophasic relaxation in preparations with an existing high level of tone ¹⁴⁹. Lindén *et al* ¹⁵⁰ showed that NANC (non-adrenergic, non-cholinergic) activation by electric field stimulation (EFS, in the presence of atropine and guanethidine) caused contraction of guinea pig airways (trachea + bronchi) when the tone was low, yet relaxation, when the tone was high. In other words, NANC appears to have the effect of maintaining airway tone at an equilibrium. They further demonstrated that this stabilization effect could be modulated by both sympathetic and parasympathetic activation. Serotonin, in guinea pig tracheas,

causes contraction at low concentrations (0.1 - 10 μM), and relaxation at higher levels (10 - 3000 μM)¹⁵¹. Histamine (also in guinea pig tracheal rings) at low concentrations causes relaxation of precontracted tissues, whereas at higher concentrations it causes further contraction¹⁵². Endothelins (ET) are peptide bronchoconstrictors produced by epithelial cells¹⁵³, but after allergen (ovalbumin) challenge, the response of tracheal tissue from an allergen-sensitized guinea pig to ET-1, was relaxation¹⁵⁴. It also appears that there is regionalization and spatial gradient of various effects, along the airway¹⁵⁵. Clearly, the (neural) mechanisms involved in the control of airway tone and calibre are not straightforward. To the extent that resting tension may influence the baseline tone (e.g. by releasing cyclooxygenase products¹⁵⁶) something as seemingly arbitrary as the resting tension (the range in the case of guinea pig airway tissue experiments reported in the literature being at least from 0.5 g¹⁵⁷ to 5 g¹⁵⁸) could influence dramatically the outcome of agonist trials, and again confound comparison between research reports.

1.1.5.1 Cholinergic and adrenergic activity

As early as 1975 Vanert & Battigelli¹⁵⁹ suggested (based on their work with human lymphocytes) that TDI moderates β -adrenergic function. Shortly thereafter Davies *et al*¹⁶⁰, working with the same tissue, reported that TDI inhibits the increase in cyclic adenosine monophosphate (cAMP) occurring due to treatment with isoproterenol or PGE_1 . Interestingly, they found a maximal effect at 3.3×10^{-4} M TDI, with the inhibitory effect decreasing at higher or lower concentrations. Based on human work, Butcher's group^{161,162} also suggested that impairment of adrenoceptors might play an important role in TDI reactivity.

Clinically, there have been many attempts to correlate isocyanate hypersensitivity with "nonspecific bronchial hyperreactivity" (NSBH, e.g. threshold of airway response to challenge with methacholine aerosol)¹⁶²⁻¹⁷⁰. The consensus appears to reflect a lack of correlation, more certainly so in the case of "early" response to TDI challenge. Paggiaro *et al*¹⁷¹ suggested (on the basis of their clinical data) that TDI independently induced both specific and non-specific bronchial hyperresponsiveness, with each being affected by TDI exposure to a different degree. Berode's¹⁷² observation that 10 out of the 11

patients referred to them with diisocyanate asthma were slow acetylators (and that the 11th was marginal) may have mechanistic significance. Kubo *et al*¹⁷³ have shown that in TDI-induced nasal allergy in guinea pigs, the density of muscarinic receptors and activity of choline acetyltransferase is increased, while the density of adrenoceptors and content of norepinephrine is decreased¹⁷⁴. Dewair *et al*¹⁷⁵ showed that isocyanates inhibit (human) erythrocyte acetylcholinesterase (AChE); Brondeau *et al*¹⁷⁶ that rat bronchial AChE activity decreases after TDI inhalation. The effects of TDI on various enzymes (in airway epithelium) are discussed in 1.1.6, and may also relate to the observations, below. Marek *et al*¹⁷⁷ challenged rabbits with 0.005, 0.010 or 0.030 ppm TDI for consecutive one-hour periods, to find that this of itself did not alter various respiratory parameters. On the other hand, the exposure at 0.010 ppm TDI after four hours caused the bronchoconstrictive response to the inhalation of a 2% acetylcholine aerosol to increase 3.6-fold over control values. The change was greater with 0.030 ppm TDI, whereas none was seen at 0.005 ppm.

Gordon *et al*¹⁷⁸ exposed guinea pigs for 1 hr to 1 or 2 ppm TDI. They evaluated the acetylcholine PC₂₀₀ (dose required to cause a 200% increase in pulmonary resistance - R_L), and found it to be significantly lower 2 or 6 hr after exposure to 2 ppm TDI (but not 2 hr after exposure to 1 ppm TDI or 24 hr after exposure to 2 ppm TDI). Gordon *et al*¹⁷⁹ acutely exposed guinea pigs to 3 ppm TDI and reported PC_{200s} of 23.9 mg/mL and 8.6 mg/mL (control and exposed, respectively). Such high TDI exposures, however, may not relate to the same processes occurring during chronic sensitization. Gagnaire *et al*¹⁸⁰ evaluated lower concentrations of TDI. They demonstrated that airborne exposures of guinea pigs to TDI concentrations as low as 0.023 ppm for 1 week, reduced by 28% the ED₂₀₀ (dose of acetylcholine required to achieve a 200% increase in baseline airway resistance (R_{AW}) - [this could in fact be R_L- pulmonary resistance à la Gordon *et al* - since they too used a tracheotomy]). With this (and several other exposure regimens of up to 1.2 ppm TDI) they showed that the baseline R_{AW}, 20 hr after TDI exposure was not of itself significantly different from that seen in control animals. This (similarly to Gordon *et al*) differs from the findings of Thompson *et al*^{181,182}, who reported that 1 hr exposure to 3 ppm TDI caused a significant increase in baseline R_{AW}. The difference

could be rationalized on the basis of the higher concentration (the consideration of the threshold concept is all-important in this field) or on the basis of protocol (animals breathing normally in the case of Thompson *et al*, which might therefore have shown a change in R_{AW} due to effects in the nasal / upper airway region, which would have been by-passed with the tracheotomies employed by Gagnaire *et al*). On the other hand, Gagnaire *et al* found that the ACh effect was manifested at all exposures (with ED_{200} reductions of up to 60%), except 0.13 ppm TDI for 4 hr \times 2 days (although an equivalent concentration of 0.12 ppm for 48 continuous hours did bring about ACh hyperresponsiveness). In an earlier study McKay and Brooks¹⁸³, using tracheal tissues from guinea pigs exposed to 0.029 ppm airborne TDI (5 hr/day \times 20 days), found them to have an increased sensitivity and response to carbachol, suggesting again that isocyanate sensitivity is pharmacological and resides in cholinergic activity. They found no difference in β -adrenergic (isoproterenol) response^{183,184}. However, these investigators made no attempt to demonstrate that "sensitization" had occurred, i.e. that respiratory sensitivity of the exposed animals to TDI *in vivo* or *in vitro* differed from control or, that they had a demonstrable immune response. Intriguingly, the ability of these researchers to show pharmacological effects after relatively mild TDI pre-exposure regimens would appear to be at odds with Karol's experience⁹¹ with guinea pig TDI "sensitization" (TDI-specific antibody formation and/or demonstrable pulmonary hyperreactivity). In her lab, exposure to 0.02 ppm TDI (very similar to the 0.029 ppm used by McKay and Brooks) for 6 hr/day \times 70 days yielded no animals (out of 24) with an antibody titre, lung sensitivity or dermal reactivity. Even exposure at 0.12 ppm TDI for 3 hr \times 5 days showed no antibody formation or pulmonary sensitivity in any of the 8 animals, and it was only at 0.36 ppm that some animals showed a response. The guinea pig strains used in the two were English short-haired and English "smooth-haired". Of course, these two sets of experiences may be indicative of the presence of different (but perhaps, related) mechanistic bases i.e. a transient, pharmacological hypersensitivity due to relatively low exposures, as well as an immunological response occurring at a higher threshold. Alternatively, the cholinergic hypersensitivity reported by McKay and Brooks may simply be a more sensitive measure of the same phenomenon.

Borm *et al*¹⁸⁵ have demonstrated that TDI in solution (particularly in DMSO) enhances the response of rat tracheal tissue to methacholine. There is no indication whether this effect would be exaggerated in tissues from animals that had been sensitized by pretreatment with isocyanates. Similarly, Mapp *et al*¹⁸⁶ demonstrated that guinea pig bronchial rings exposed in a tissue bath to 0.3 mM TDI (in DMSO) contracted with a mean force 24% of that seen with 1 mM ACh. Again, they used naive animals. Mapp's group have also demonstrated (with the same system) the role of prostanoids and tachykinins (see 1.1.5.3).

1.1.5.2 Other (non-peptide) agonists

Cibulas *et al*¹⁸⁷ found airway hyperreactivity to histamine after TDI exposure (3 ppm × 4 hr × 5 da) to be maximal 2 hours post-exposure, and returning to normal by 72 hours. However, they also found "dramatic signs of airway mucosal damage", and it is unclear whether they were witnessing a TDI effect or an irritant effect. In other words, would histamine sensitivity also be increased at lower exposure levels, or conversely, would they have seen a similar effect if they had used an equivalent concentration of (say) hydrogen chloride? Kubo *et al*¹⁸⁸, with nasal allergy to TDI in guinea pigs showed an elevation in histamine levels in the nasal mucosa after 1-2 weeks, except for a decrease immediately after challenge (again, implicating histamine release in the response to challenge). Irifune *et al*¹⁸⁹ found that application of TDI to the nasal mucosa of guinea pigs decreased the histamine content.

Dandurand *et al*¹⁹⁰ devised a simple, yet elegant approach to the direct visualization of airway constriction due to *in vitro* antigen challenge, involving the digitization of video signals from a microscopic image of airways from ovalbumin-sensitized rat lung explants. They were able to demonstrate both early (ER) and late (LR) responses to direct ovalbumin challenge by periodic video recording for 10 minutes and 8 hours, respectively. They were able to block the ER with 1, M methysergide, which to them indicated that serotonin released by mast cells and/or basophils is the principal mediator of ER. In guinea pigs Baumgartner *et al*¹⁵¹ had shown that low concentrations of serotonin (0.1 - 10 μ M) yield concentration-dependent contraction.

1.1.5.3 Neuropeptides

The role of various neuropeptides has been explored. They have been implicated in airway response to irritants such as acrolein ¹⁹¹. Ollerenshaw *et al* ¹⁹² had shown that Vasoactive Intestinal Peptide (VIP) is absent from the lungs of asthmatics, but this may have been due to the asthmatics' long-term therapy (or in fact, due to the disease itself). Although the inhibitory NANC (iNANC) in humans had (until recently) been attributed to VIP ¹⁹³, nitric oxide (NO) has been shown to be entirely responsible ¹⁹⁴. VIP has been implicated as a mediator of the inhibitory response to stimulation in guinea pig trachea¹⁹⁵, and sensitization decreased guinea pig tracheal VIP content ¹⁹⁶. In guinea pigs, half of the iNANC is still thought to be due to VIP ¹⁹³.

Several researchers have demonstrated a putative role for tachykinins such as Substance P (SP) in TDI-induced effects. Sheppard's group has demonstrated that TDI exposure in guinea pigs increases the pulmonary resistance induced by acetylcholine challenge ¹⁸²; capsaicin (which causes depletion of Substance P) prevents this increase, but is without effect on the edema induced by the TDI. They also presented evidence to indicate that these effects were mediated through the release of tachykinins into the intrathoracic airways during the TDI exposure ¹⁹⁷, as well as by the increased responsiveness of the pulmonary tissue to Substance P as a result of the inhibition by TDI of neutral endopeptidase (NEP, which is normally involved in the degradation of tachykinins such as SP). ^{198,199}

Mapp *et al* ²⁰⁰ showed that TDI causes a concentration-dependent contraction of rat bladder (detrusor) muscle strips, but not in tissues pre-treated with capsaicin. Repeated exposure with TDI reduced the capsaicin-initiated release of CGRP-like immunoreactivity, indicating that TDI activates the efferent function of sensory nerves²⁰¹. The finding that TDI causes muscle contraction by activation of sensory pathways suggested the prospect of proactive pharmacological intervention, by the blocking of these pathways. Both capsaicin and Substance P have been shown to cause contraction of guinea pig tracheal preparations ²⁰². Mapp *et al* ¹⁸⁶ showed in epithelium-intact guinea pig bronchial rings that phosphoramidon (an inhibitor of the SP-degrading NEP)

potentiated approximately three fold, the contractile response to TDI. Epithelium removal alone caused a 50% increase in TDI response. They found that not only TDI, but also the products of reaction between TDI and water (e.g. toluene diamine - TDA - all in DMSO) caused the concentration-dependent contraction of guinea pig bronchial rings.²⁰³ In view of the fact that their guinea pigs were naive, these contractions (84% and 51-59% of equimolar acetylcholine) are remarkable (especially when compared to our findings). Mapp *et al* have also shown that compound 48/80 (a mast cell degranulator) by itself causes a contraction of bronchial rings, subject to substantial tachyphylaxis (second response 40% of the first). However, 48/80 also inhibited TDI-induced contractions which, based on their findings, indicated an effect by 48/80 on sensory nerves and/or the release of mediators other than histamine by mast cells²⁰⁴. Mapp *et al*²⁰⁵ showed an increase in organ bath $\text{PGF}_{2\alpha}$ after treatment of guinea pig bronchial rings with TDI. Although Mapp (and co-workers) are clearly pursuing the question of pharmacological mechanisms in isocyanate response, it is unfortunate that they have not investigated sensitization *per se*, in guinea pigs.

Other investigators exploring the role of SP in TDI-induced sensitization in guinea pigs include two Japanese groups^{206,207}. For example, Kalubi *et al*²⁰⁸ indicate that chronic exposure of guinea pig nasal mucosa to TDI causes enhancement of the biosynthesis of SP and Calcitonin Gene Related Peptide (CGRP), and of their axonal transport in the trigeminal system.

These many reports make it clear that TDI exposure can result in diverse effects, implicating both immunological factors and pharmacological mediators. However, progress to date still leaves us without unifying mechanistic models that are adequate to account for the wide variety of observations. A specific consideration of the epithelium may be helpful in this respect.

1.1.6 Role of the airway epithelium

When airborne isocyanates are inhaled, they must contact the epithelial layer of the respiratory tract prior to interacting with other cell types such as nerves or smooth

muscle cells. As the isocyanates are chemically reactive molecules, it follows that their reaction with the epithelial constituents could be substantial. Moreover, these effects could be greater in subjects who are sensitized to isocyanates. It would therefore be appropriate to determine the role of the epithelium in the response of isolated tissues to TDI.

Masaki *et al*²⁰⁹ sensitized guinea pigs to ovalbumin aerosol, and found that the *in vivo* acetylcholine PC₂₀₀ (provocative concentration required to double airway opening pressure) was significantly less in the case of the sensitized animals. With their *in vitro* (perfused whole-tracheal) preparations, they administered acetylcholine intra-lumenally (i.e. epithelial side) or to the out- (serosal-) side, expressing their results as pPC₁₀ (the negative logarithm of the molar ACh required to produce a 10% reduction in airway diameter, as calculated from differential pressure and flow rate). They found a significant correlation between logPC₂₀₀ and pPC_{10(in)}, but not pPC_{10(out)}, which (together with other results) suggested that the differential responsiveness of sensitized airways to ACh related to differences in airway epithelial functions. They do not describe *in vivo* or *in vitro* challenge of the animals with the antigen.

Kennedy²¹⁰ demonstrated that exposure of guinea pigs to 0.13 ppm airborne ¹⁴C-TDI resulted in deposition throughout the airway, but virtually not at all at the alveolar level. The label (at the end of a 1 hour exposure period) was not on the cell surface (as might be expected, and as had been found in the case of MDI²¹¹), but predominantly in the subepithelial region. A similar effect was seen with methyl isocyanate²¹². It was suggested that this might contribute to the inflammation of the *lamina propria* (seen after TDI exposure) as well as 'neurologic signalling' involving afferent nerves localized here. The label was found to persist here for at least two weeks. Analysis of lung homogenates showed the presence of three labelled proteins: < 10 kDa, 70 kDa and > 200 kDa in molecular weight. The most abundant was the 70 kDa protein, which also corresponds with the molecular weight of an albumin-like labelled protein found in the plasma of these animals²¹³, and also persisting for up to 2 weeks. The >200 kDa protein was tentatively identified as lamelin. Given the persistence and sub-cellular

location of the labelled TDI, it seems likely that other interactions with airway proteins, and in the epithelial layer, occur. For example, Jin *et al*¹²⁹ have demonstrated the presence of at least 5 SDS-PAGE bands (reactive with anti TDI-KLH antiserum) in bronchoalveolar lavage (BAL) fluid following guinea pig exposure to airborne TDI. One of the proteins in the 66 kDa band was serum albumin.

A potentially fruitful avenue of pursuit would be to investigate isocyanate-induced asthma in terms of recent discoveries regarding the role of epithelial cells in regulating smooth muscle contractility. This could be in analogy with the widely acclaimed studies on the role of the endothelium with respect to the function of vascular smooth muscle. In 1980, Furchgott and Zawadzki reported the discovery of an endothelium-derived relaxing factor (EDRF) that was released by acetylcholine from rabbit aortic endothelium²¹⁴. EDRF has subsequently been shown to be released from a wide variety of blood vessels; it is now widely accepted that EDRF is nitric oxide. More recently the possibility of a similar regulation of airway smooth muscle by the airway epithelium has been raised by various laboratories. For example, the divergent effects of low and high concentrations of agonists reported in 1.1.5.2 are reminiscent of Furchgott and Zawadzki's experiences with ACh and rabbit aorta (relaxation at low ACh and contraction at high ACh).

Hay *et al* found that epithelial removal from guinea pig trachea increased the sensitivity and maximum response to histamine, while to methacholine only the sensitivity was increased²¹⁵. In similar studies, Advenier *et al*²¹⁶, Goldie *et al*²¹⁷ and Holroyde²¹⁸, found that the tracheal effects of various agonists were enhanced significantly after epithelium removal. Iriarte *et al*²¹⁹ indicated an increased tissue sensitivity to ACh and histamine, but not K⁺, as a result of de-epitheliation; they extended this²²⁰ by elaborating on the effects of de-epitheliation on drug effectiveness if entering from the mucosal (luminal; epithelium) side or the serosal (adventitial) side of their tracheal ring preparations. They used petroleum jelly to prevent unwanted drug entry. Burgand *et al*²²¹ similarly reported that removal of epithelium from guinea pig bronchioles did not alter the response to K⁺, theophylline, nitroprusside or papaverine, but shifted the ACh and histamine dose-response curves to the left. Rakhmattulin *et al*¹⁴¹ have demonstrated

that epithelium removal in guinea pig trachea enhances the contraction produced by histamine, but decreases the relaxation produced by isoproterenol. There are some attempts at systematization and rationalization of these effects. Morrison & Vanhoutte²²² and Small *et al*²²³ indicated that the activity of acetic acid choline esters (ACh and methacholine - MCh), but not carbamic acid choline esters (carbachol and betanechol) was augmented by removal of the epithelium. The former are reported²²² to involve the M₃ muscarinic receptor.

Vanhoutte's group have shown with bronchial rings from pigs²²⁴ and dogs²²⁵ that not only is sensitivity to contractile agonists increased by removal of the epithelium, but (perhaps more importantly - see below) the relaxant effect due to isoproterenol is decreased. Similarly, there was a differential effect seen with electric field stimulation; with intact epithelium there was a gradual decrease in tension despite continuing stimulation; this decrease was not seen with rubbed (de-epitheliated) preparations.

These observations are consistent with the suggestion that the epithelium releases an Epithelium Derived Relaxing Factor (EpDRF) that causes smooth muscle relaxation. The role of EpDRF may have some bearing on the earlier reports of Laitinen *et al*²²⁶ who found epithelial loss or damage to accompany airway hypersensitivity in asthmatics. Knight *et al*²²⁷ had shown that removal of epithelium from human bronchial tissues increases the response to histamine and acetylcholine, and the sensitivity to acetylcholine. On the other hand, Bai and Prasad²²⁸ found that removal of epithelium from human tracheal strips had no effect on the response to EFS, K⁺, theophylline or isoproterenol, nor on the EC₅₀ for methacholine. However, the maximal tension generated by methacholine was increased (70%) which they felt was consistent with the currently held significance of "maximal airway narrowing" in symptomatic asthma. The inconsistency between the two reports with respect to the effect of epithelium removal on the sensitivity to cholinergic agonists could be attributed to the agent (MCh vs. ACh), or to the tissue (bronchial vs. tracheal). Vanhoutte¹⁵⁵ had summarized canine airway regionalization: with decreasing diameter, removal of the epithelium causes less of an effect in terms of contractile agonists, but more of an effect in terms of β -adrenergic relaxants; the effect

might be opposite in humans. Gordon *et al*¹⁷⁸ had demonstrated that exposure of guinea pigs to 1 ppm TDI caused a disruption of surface epithelial cells in the trachea. Although these concentrations are several orders of magnitude greater than those that would normally occur in the workplace, the effects may indicate that more subtle epithelial changes (involved in TDI-induced bronchoconstriction) could occur at lower concentrations. These could include:

1. the loss of the putative inhibitory mediator EpDRF, and
2. the enhancement of tachykinin effects, due to the "exposure" of the sensory nerves, and loss of NEP.

The existence of a relaxant airway epithelial factor has in fact been compellingly demonstrated; Fernandes *et al*²²⁹, showed that guinea pig tracheal epithelium, when stimulated by methacholine, produces a substance that causes the relaxation of rat aorta. They have demonstrated a similar effect with OA (ovalbumin) antigen stimulation of tissues from sensitized guinea pigs²³⁰. This had also been shown earlier by Hay *et al*²³¹; removal of epithelium from tracheae of OA-sensitized guinea pigs resulted in a 6-fold increase in the sensitivity (i.e. leftward shift of the dose-response curve) to antigen challenge, yet was not seen when an epithelium-intact tracheal strip was apposed. Eglen *et al*²³² also used co-axial bioassays and found "convincing evidence" for the release of an EpDRF: all muscarinic agonists studied caused relaxation of a precontracted *anococcygeus* tissue, provided that the epithelium on the tracheal tube was intact. Gunn and Piper²³³ have suggested that the apparent effects seen in co-axial bioassays such as those employed by Hay and by Fernandes are little more than artefact, brought about by a lowering of the oxygen tension in the lumen of the vessels in the assay. This has been refuted²³⁴. Lundblad and Persson's²⁰² conclusion that epithelium removal was of "little consequence" in guinea pig tracheal preparations has been rationalized in Goldie's group on the basis of Lundblad and Perssons' (inappropriate) choice of (ant)agonists²³⁵.

Although there appears to be considerable evidence of the presence of an EpDRF, there remains a school of thought that maintains that where an experiment is designed so that the epithelium's unique function as a barrier can be properly established, this is shown

to be its true (or at least pre-eminent) role. Most active in this respect have been Sparrow and Mitchell ²³⁶, with other researchers ^{223,237,238} indicating a dual role. Yang *et al* ²³⁹ suggested that the epithelium serves as only a weak barrier for lipophilic agents (e.g. theophylline), but has a major role as a diffusion barrier for hydrophilic substances (e.g. nitroprusside). Although an interesting concept, this would need to be reconciled with the many observations (cited above) that removal of the epithelium has no effect on the tissue response to K⁺.

Just to further confound the issue, Bertrand *et al* ²⁴⁰ have very recently presented evidence of an epithelial-dependent **contracting** factor in guinea pig trachea which (based on antibody specificity) appears to involve Calcitonin Gene Related Peptide (CGRP), perhaps as the agent which mobilizes the Contracting Factor. As with the previous studies cited, they showed that removal of guinea pig tracheal epithelium did not alter smooth muscle response to potassium (indicating that functional integrity was maintained, and suggesting that at least for potassium, the epithelium did not normally represent a barrier). However, epithelial removal reduced the maximum force generated (1.2 versus 2.4 g) and sensitivity (EC₅₀ 1.4 versus 4.2 x 10⁻⁴ M) to calcium, certainly indicating that the role of the epithelium was not that of a barrier, in this case. Gao and VanHoutte ¹⁵⁶ and Haulica *et al* ²⁴¹ both implicated a thromboxane as an epithelium derived contracting factor. Similarly, Fedan *et al* ²⁴² found that epithelium facilitates contraction of guinea pig trachea in response to ATP; Ullman *et al* ²⁴³ indicated that their preparation with intact epithelium showed a greater carbachol potency and efficacy. Working with rat tracheal tube preparations Pavlovic *et al* ²⁴⁴ showed that simultaneous stimulation with carbachol from the mucosal and serosal sides led to an increased sensitivity and maximal tension (regardless of the route of perfusion); to them this indicated that the epithelium is more than a barrier. These findings, certainly when considered together with all of the other studies demonstrating differential agonist effects from removal of the epithelium, clearly indicate that the role of the epithelium is not (exclusively) that of a barrier. If it were, then there would be no accounting for the effects seen with isoproterenol and with electric field stimulation; there would be no rationale for the co-axial bioassay response, nor for a diminished carbachol or calcium response due to the

removal of the epithelium.

It is recognized that the epithelium releases both bronchoconstrictor and bronchodilator products of cyclooxygenase, and it is suggested ¹⁵⁶ that as yet unidentified epithelium derived (EpD) contracting and relaxing factors (CFs and RFs) are yet to be implicated. However, the role of the epithelium and the elaboration of putative EpD RFs and CFs may not involve "novel" agents, but may simply relate to the known pharmacological agonists (prostanoids, etc.) and various degradative enzymes (AChE, HMT, NEP, etc) produced by the epithelium.

It has been suggested that the principal role of the epithelium in inhibiting guinea pig airway contraction has been via epithelium-derived acetylcholinesterase (AChE) ²⁴⁵, and isocyanates are recognized AChE inhibitors ¹⁷⁶. Histamine methyl transferase (HMT) loss due to epithelium removal has been thought to be the cause of increased sensitivity to histamine ²⁴⁶, although epithelial histaminase (diamine oxidase) may also be contributing to the inhibitory effect ²⁴⁷. The interplay between SP, NEP and TDI has been mentioned. Grant and Rodger ²⁴⁸ showed that OA sensitization reduced guinea pig superoxide dismutase and cytochrome P₄₅₀.

Mattoli *et al* ²⁴⁹ demonstrated with human epithelial cells in culture, the stimulation of the release of 15-hydroxy eicosatetraenoic acid, in response to TDI exposure. Gao and Vanhoutte ²⁵⁰ indicated the role of epithelial prostaglandins in the response to hydrogen peroxide; Streck *et al* ²⁵¹ found that removal of the epithelium completely attenuated the augmentation of the muscarinic response otherwise caused by phospholipase A₂. Folkerts *et al* ²⁵² summarily described EpDRF as PGE₂. Egilmez and Ilhan ²⁵³ found EpDRF to be a product neither of cyclo- or lipo- oxygenase, although they did indicate its release in response to antigen challenge.

Filep *et al* ²⁵⁴ indicated that the response of pre-contracted tracheas to endothelin was altered from a relaxation to a sustained contraction, by the removal of epithelium, implicating nitric oxide (NO). The investigation of the role of NO in airway smooth

muscle function is intensifying. Nijkamp *et al*²⁵⁵ demonstrated that the aerosol administration of NO synthase inhibitors to guinea pigs enhanced the increased lung resistance induced by histamine; in the case of L-NMMA this was by 282%. However, Watson *et al*²⁵⁶ indicated that NO production during transmural stimulation of guinea pig tracheal tube preparations was epithelium-independent, and Munakata *et al*²⁵⁷, although demonstrating that NO relaxes airway smooth muscle, also indicated that it is not released as a result of osmotically induced relaxation.

1.2 Summary of the overall investigation

1.2.1 Synoptic overview of the literature basis for the work

Clinical experience (with populations occupationally exposed to TDI) has been inconsistent with respect to considerations such as atopy, antibody types and levels, methacholine sensitivity upon airborne challenge, etc. There have been various uncertainties with respect to threshold for the initiation of human sensitization, and it has been difficult to induce airway sensitization in animals with airborne exposure. Karol (and co-workers) had demonstrated that application of isocyanates to chemically depilated guinea pig skin caused some to become immunologically sensitized to the extent of demonstrating a significant titre of isocyanate-specific antibodies. Only some of these sensitized guinea pigs would be expected to show a pulmonary hypersensitivity, when challenged with airborne isocyanate (chemically pure or a protein conjugate). Although it was not known whether any or all humans dermally exposed would become sensitized, to the extent that the effect only involved a minority of the animals, there appeared to be analogy to the human experience. Therefore, this model appeared to be suitable for further study.

The guinea pig is well suited to investigations of airway sensitization; the vast majority of laboratories have relied on this animal¹³⁷. In addition to practical considerations relating to disposition (and therefore amenability to handling into and out of plethysmograph jars), the guinea pig's immune system is close to the human in terms of

the balance of cellular and humoral immunity ¹¹⁷. Karol ¹¹⁶ has summarized the advantages and disadvantages of the use of the guinea pig (Table 1-2), but cites as a major benefit the fact that the lung is the target organ of the hypersensitivity response. On the other hand, differences between human and guinea pig airway mechanics are described in Chapter 3.

Table 1-2. Advantages and disadvantages of guinea pig model of asthma ¹¹⁶	
Advantages	Disadvantages
Small, docile animal	Few inbred strains exist
Relatively inexpensive	Few species-specific reagents
Inhalation sensitization possible	IgG ₁ is major anaphylactic antibody
Lung is major shock organ	Not sensitive to cromolyn
Airways respond to histamine	
Early and late response (E/L AR)	
Neutrophil influx following LAR	
Eosinophilic inflammation w/ LAR	
Airway hyperresponsiveness following hypersensitivity response	
Tracheal smooth muscle histamine responsive	

Based on the work (both human and animal) that had shown an absence of pulmonary sensitization / hyperreactivity (in some individuals) despite the presence of isocyanate-specific humoral antibodies (and vice-versa), as well as the various indications of the existence of pharmacological mechanisms of hypersensitivity, it had been envisaged that the antibody level was not particularly critical in the guinea pig model. This was one hypothesis to test. The plan was to duplicate the immunological - sensitization - by - dermal - exposure that had been reported by Karol, and then to determine if there was more of an association between immediate airway reactivity *in vivo* and serum antibody

level, or *in vitro* sensitivity to TDI and contractile agonists. In other words, it would be attempted to differentiate pharmacologically (by standard *in vitro* tissue work) those animals that showed (TDI-specific) airway hyperreactivity, from those that did not. Of particular interest was the determination of the role of the airway epithelium in any differential response that was seen.

Although other researchers had variously evaluated the effects of TDI on tissues from naive animals, and agonist effects on tissues from TDI pretreated animals, no one appeared to have studied the logical, full sequence. For example, the *in vitro* response to TDI, of airway tissues from guinea pigs that had been demonstrably TDI-sensitized, had not been previously described. This resulted in the formulation of the following approach in the present study, articulated below as a series of hypotheses.

1.2.2 Research hypotheses

1. That persistent systemic immune response to TDI ("sensitization") can be induced in guinea pigs by a single dermal exposure to diluted TDI, without dermal abrasion, occlusion or the use of chemical depilating agents.
2. a) That airway response to TDI challenge *in vivo* relates to the sensitization status of the animal;
b) That the "early" response to TDI challenge in sensitized animals is not directly associated with antibody level;
c) That the response of TDI-sensitized guinea pigs to airborne methacholine challenge is independent of immune status.
3. That *in vivo* sensitivity to TDI challenge correlates with *in vitro* airway tissue responsiveness to TDI.
4. a) That there are differences in response to pharmacological agonists between tracheal tissues from control and sensitized animals;

b) That the *in vitro* TDI response of tissues from sensitized animals can be modulated pharmacologically;

c) That the tracheal epithelium has a protective role in this response.

The testing of each hypothesis is described in the chapters that follow, as:

Hypothesis	Chapter
1	2
2	3,5
3	5
4	4

A brief summary of the research approach is first provided, to give an overall perspective on this multi-faceted project. Detailed methodology, results and discussion follow in each chapter, concluding with an overall discussion. In all cases, statistical significance was defined as $p < 0.05$, based on the appropriate test.

1.2.3 Research Approach

Guinea pigs were sensitized to isocyanates by the dermal route. This aspect of the project was based on work published by Meryl Karol and her group in the early 1980s; in 1981²⁵⁸ they described their success with topical application. Although she had subsequently suggested²⁵⁹ that more aggressive conditions (i.e. intra-dermal injection of neat TDI) be used by us, we in fact ended up using milder conditions than she had: the guinea pig skin was not chemically depilated, and the concentration of TDI was lower, such that no dermal lesioning was observed in the ultimate groups of animals (3 sensitized + 1 control animal) \times 6 groups. The experimental groups were deliberately not "balanced" with respect to the number of animals sensitized, because it had been anticipated (on the basis of the literature) that only perhaps a third of those exposed to a sensitizing dose would in fact demonstrate airway hyperreactivity.

Following the sensitization, the presence of specific antibodies was determined by enzyme-linked immuno sorbent assay (ELISA). This provides a quantitative measure (expressed in absorbance units) of the animal's circulating (blood-borne) antibodies of a specified class. In this instance, it was the TDI-GSA conjugate-specific IgG level that

was determined. The IgG₁ antibody in guinea pigs has been shown to be associated with the early onset response to challenge being investigated here. Since TDI itself is too small to be considered allergenic (and is chemically reactive) it is standard practice to "conjugate" the compound to a carrier protein (typically albumin). In this instance, the coupling reaction was performed to guinea pig serum albumin (GSA), as described in 2.2.2.

Despite using considerably milder sensitization conditions than earlier described, our results indicated a consistent and persistent development of IgG antibodies in all of the exposed guinea pigs. This necessitated some adjustment to the original protocol, as did the plethysmographic findings.

The animals were challenged (while in flow-through whole body plethysmographs) with progressively increasing levels of airborne methacholine aerosol, and with TDI vapour, each on two successive days, but separated in time from each other. It had initially been proposed to use increased respiratory rate, as introduced by Alarie ²⁶⁰ and specifically defined by Karol ⁹⁹, as an endpoint. However, this was found (after much deliberation and search for alternatives) to be unsuitable in the present study. With methacholine, the guinea pigs progressed to a bronchospastic attack (paroxysmal breathing), characteristic both in terms of animal signs and plethysmograph tracing, as described further in Chapter 3. The endpoint for methacholine challenge was taken as the concentration at which the animal's plethysmograph pressure amplitude (ΔP) doubled (for at least three consecutive breaths) from the pre-exposure level.

With TDI, the characteristic effect observed was the change in the shape of the breath wave-form, associated with a prolonged exhalation (which would be expected from the pulmonary pressure during expiration impinging further upon the pharmacologically-constricted airway). The endpoint was taken as the TDI challenge concentration at which there were at least three consecutive (or, three out of four consecutive) breaths in which the constant-pressure exhalation phase showed a defined prolongation. This is later referenced as delayed exhalation (DE), and is believed to be a novel (and sensitive)

endpoint.

Contrary to expectations (based on earlier reports), it was not possible to identify animals that were immunologically sensitized, yet which consistently showed a "control" response to airborne challenge with TDI. In comparing 28 control-sensitized challenge pairings there were two sets in which the sensitivity was not "sensitized > control"; one was due to an apparently hypersensitive control, but in neither case was this ratio maintained over both (consecutive day) challenges.

In the next phase, tracheal rings from both sensitized and unsensitized animals were assessed *in vitro* for their responses to TDI, other agonists and antagonists. Tissues from sensitized animals contracted in response to the addition of TDI to the bath medium; those from control animals relaxed. In addition, the effect of the removal of the epithelium on the tissue response was evaluated. This had no effect on the response to TDI or methacholine, but augmented the response to histamine.

It had originally been intended to subject the animals to the *in vivo* challenge and the *in vitro* characterization two to three weeks after the initial dermal sensitization of the animals. For a variety of reasons this was not possible. However, the schedule that was used (see below) resulted in a considerable strengthening of the significance of the research findings (since they were, on the whole, positive), in terms of their relevance to the real world, and the import of the immunological aspect of the response. In other words, dermal exposure was shown not only to cause a systemic effect that persisted *in vitro*, but this was maintained for a year following the single initial dermal exposure to TDI.

Timing of experimental phases of main trial; guinea pig groups 93/6 through 93/11	
Activity	Week(s)
Dermal exposure	0 - 8
Plethysmography (airborne challenge): Methacholine	45 - 46
	TDI 47 - 49 (- 51)
Tissue bath work	63 -71

Of course, this pattern of scheduling might also have resulted in a failure to identify certain pharmacological effects that would have been apparent if the timing had been tighter. For example, as mentioned earlier, Cibulas *et al*¹⁸⁷ showed that guinea pig histamine hyperreactivity was maximal 2 hours after a 5 day TDI exposure; remission occurred by 72 hours. Fortunately, Serendipity appeared to be smiling on our work.

2. SENSITIZATION TO TDI AND ITS HUMORAL IMMUNOLOGICAL EVALUATION

2.1 Principles

Various routes of exposure have been used to bring about a hypersensitive state. A 'classic' approach has involved injection; in 1927 Ratner *et al*²⁶¹ wrote:

"A guinea-pig was sensitized with 0.5 cc dander extract, intraperitoneally; sixteen days later, the uterine strip was suspended in a 300 cc. oxygenated bath of Locke's solution. ... alkali extract was instilled in the bath. There was an immediate and marked contraction of the strip.."

They also claimed to be the first to show that "*animals could be sensitized by the inhalation of a dry substance*" (horse dander), and concluded that "*the nasal route can serve as a portal of entry for foreign proteins resulting in grave anaphylactic shock and even death*".

When attempting to develop an animal model of airway hyperreactivity to an agent whose human exposure is most often by inhalation it would seem appropriate to induce the sensitization by **airborne/inhalation** exposure to the agent. Nevertheless, the induction of respiratory sensitization by dermal (topical) or intradermal (injection) routes is described regularly in the recent literature. Guinea pig sensitization thus to cobalt¹⁴² and trimellitic anhydride¹⁴³ was described in Chapter 1. Some experimental models seem rather removed from the human parallel. Howell *et al*²⁶² investigated airway hyperreactivity: they sensitized guinea pigs by **intramuscular** injection of antigen, and subsequently assessed the degree of bronchoconstriction induced by the **intravenous** administration of methacholine (while the animals were anesthetized and mechanically ventilated via tracheal cannulae). Although this approach avoids many of the methodological difficulties inherent in inhalation toxicology work, it does beg the question of the relevance of this route of presentation of the toxicant, relative to its overall effect.

Exposure of the skin to toxic materials occurs regularly. There is clinical evidence of

dermal contact resulting in systemic sensitization; animal data was summarized in Chapter 1. In fact, it has been shown that **dermal challenge** of the sensitized individual can cause an **airway response**. For example, Marks *et al* ²⁶³ described the case of an individual who reacted to carbonless copy paper. Within 15 minutes of being given complete carbonless forms to hold (while blinded) in an "environmentally controlled room" [presumably thereby precluding airborne exposure], she became symptomatic (hand, throat and eyes; chest tightness); pulmonary flow-volume loop measurements indicated a decrease in inspiratory flows consistent with upper airway obstruction. This was related to eicosanoids: within 15 minutes plasma levels of the bronchoconstrictors thromboxane B₂ and prostaglandin F_{2α} (PGF_{2α}) rose from 98 pg/mL and non-detectable (respectively) to 172 and 210 pg/mL, whereas the bronchodilatory PGE₂ dropped from 157 to 97 pg/mL. In the same time, levels of 6-keto-PGF_{1α} (a PGI₂ metabolite) increased from 2.9 to 1760 pg/mL. Interestingly, the opposite phenomenon (dermal sensitization due to inhalation exposure) has also been identified. Dicyclohexylmethane-4,4'-diisocyanate (HMDI) has been reported to cause little respiratory sensitivity, yet a high prevalence of dermal reactions; guinea pigs and mice developed skin sensitivity to HMDI upon airborne exposure (in a dose-related fashion, with a noted threshold of effect) ²⁶⁴. Clearly, dermal contact can both induce the sensitized state and elicit a systemic hypersensitive response.

In the present project, there were various reasons for sensitizing the animals via the dermal route. The research interest in the mechanism of isocyanate-induced asthma relates in part to the widespread industrial use of these compounds. Direct contact between the chemical and worker skin occurs most frequently in the case of the hands; where there is spray-application of isocyanate, skin contact could include a more extensive area. Especially with respect to isocyanates such as methylene bisphenyl diisocyanate (MDI), the dermal route may be a particularly significant portal of entry, as vapour phase inhalation exposures should be low by virtue of the low vapour pressure of the material; nevertheless, cases of sensitization are documented ^{55,167,265-267}. Although more substantial airborne exposures occurring due to aerosolization of the material could partly account for this, evaluation of dermal exposure certainly warrants closer attention.

Accordingly, sensitization via the dermal route was incorporated into the protocol of the present study in part to test the hypothesis that dermal isocyanate exposure could result in hyperreactivity that would be maintained in a tissue bath trial of isolated tracheae. As well, since the inhalation challenge apparatus was built *de novo* (see Chapter 3), the timing of this project meant that it was necessary to induce a sensitized state in the animals before the system for exposure to airborne TDI had been validated.

The sensitization approach used in the present study was based on the reports of Karol and her group. As reviewed in Chapter 1, they had initially (1978¹³⁵) reported sensitization with the use of an ovalbumin conjugate of TDI only. Their first attempt to induce hypersensitivity by exposing guinea pigs to TDI vapour (1980¹¹¹) was partially successful: this was apparently the first report of the production of (somewhat) specific antibodies, following animal exposure to a small, airborne chemical. These animals did not, however, show any consistent pulmonary hypersensitivity upon inhalation challenge with 0.02 ppm TDI. On the other hand, using intradermal injection and topical application of TDI (neat or dissolved in olive oil) to induce both the production of antibodies and respiratory sensitization in the animals, they were able to define a number of their animals as "responders" to a 0.005 ppm TDI vapour challenge (1981⁹⁹). We used topical application of acetone-diluted TDI to sensitize our guinea pigs. The aim was to use the least invasive and irritating approach possible, while still producing an unequivocal immune response to TDI. The overall objectives used to set the sensitization protocol were:

- to minimize any discomfort experienced by the animal;
- to ensure that any immune system stimulation was due to the specific chemical effect of interest, rather than due to (or promoted by) non-specific tissue injury (or secondary infection) and,
- to render the experimental procedure optimally relevant to real world conditions (e.g. the work place, where there may well be dermal contact, but typically without any signs of dermal corrosion).

Similarly, with the availability of early antibody results, the application of depilating cream (Nair®) prior to the TDI was discontinued (although this had been used by Karol's group).

A question to be answered in the present work was whether the level of TDI-specific antibodies correlates with the outcome of *in vivo* or *in vitro* challenges with TDI. Accordingly, plasma samples were analyzed for IgG antibodies directed against TDI (as a "conjugate" of guinea pig serum albumin - GSA) by means of Enzyme Linked Immuno Sorbent Assay (ELISA). ELISA is an effective method for quantifying low levels of various materials by means of specific antibodies that have been produced against the analyte, in this case itself an antibody. Antigen (TDI-GSA conjugate; synthesis described below) is applied to standard 96 well culture plates, and a blocking agent is added to ablate the non-specific binding sites. The analyte preparation (guinea pig plasma containing the putative TDI-specific antibodies) is added and the antigen-antibody reaction (binding) is allowed to occur. The wells are washed, and only the TDI-specific antibodies (attached to the antigen earlier coupled to the wall of the well) remain. A different conjugate of anti-guinea pig IgG antibodies (in this instance raised in rabbit) coupled to an active enzyme (in this case peroxidase) is used to quantify the amount of TDI-specific antibody present. This quantity is often expressed in the traditional "titre" i.e. the lowest concentration of plasma, based on the number of serial two-fold dilutions, that will yield an enzyme-generated product above the method detection limit. Increasingly, the results are being expressed as the absorbance of the final product solution.

Although TDI is described as a "sensitizing agent" it is considered to be of insufficient molecular size to act as a direct allergen or immunogen. Rather, it must couple to some other endogenous "carrier" molecule, and as the "hapten" moiety of the "conjugate" or complex, result in the overall immunogenicity. It is not known what endogenous molecules are involved in TDI sensitization but, given the chemical reactivity of the material, there are many candidates. Albumin (by virtue of its prevalence in the body, and the ¹⁴C-based findings reported earlier) is likely to be important. In fact, as discussed earlier, there are increasing indications that chemicals such as TDI may induce an immune response to albumin such that subsequent sensitivity encompasses not only the specific hapten that initiated the immune response, but also the protein (native or otherwise-modified).

For the purpose of the ELISA it was necessary to synthesize a TDI-protein conjugate to serve as the initial "anchor" for the antibody being assayed. Although protocols for the production of conjugates exist in the literature, it is reportedly as much of an art as a science, and a lack of success is apparently not uncommon. This is not surprising, given the many different reactions that can occur, and their exacting dependency on reaction conditions. Even considering just the reaction between a diisocyanate and excess water: *"they can biphasically form: (1) a full hydrolysis product, toluenediamine; (2) di-, tri-, tetra-, urea-isocyanates; and (3) higher molecular weight polyurea polymers. These are competing reactions that depend on ionic strength, temperature, pH, concentration of reactants, the solvent in which the isocyanate was delivered to the medium, emulsifiers, the rate of mixing, and mechanical shaking"* ⁶¹. Reaction with a protein such as GSA clearly increases the complexity: potential products include various mono-molecular conjugates, reactive mono-molecular conjugates, intramolecularly crosslinked macromolecules and intermolecularly crosslinked molecules ⁶¹.

Botham *et al* ²⁶⁸ reported a lack of success after producing TDI-GSA conjugates by three different methods. Upon challenging their sensitized guinea pigs with airborne conjugate ranging in concentration from 18 to 90 mg/m³ (*"but this variation did not influence the response of the guinea pigs"*) they were unable to demonstrate pulmonary hypersensitivity against their conjugate (despite considering *"the maximum increase in respiratory rate following any one of five challenges"*). On the other hand, when they challenged their animals with conjugate synthesized in Karol's lab, the proportion of animals showing a positive response (> 46% increase in respiratory rate) in three groups that had initially been sensitized at TDI concentrations of 1, 3 and 4 ppm, were (respectively) 7/9, 9/10 and 2/7. (Note, as also reported by others ⁶⁶, that the proportion of pulmonary responders was not consistently related to the sensitizing dose). Botham *et al* concluded from these experiments that *"the physico-chemical properties of the hapten-protein conjugate are crucial, not only to the detection of antibodies but also to the elicitation of pulmonary hypersensitivity responses"*. Although we had anticipated the need to use our conjugate for plethysmographic challenge as well, it was (in the end) only utilized in validation of the ELISA trials.

2.2 Methods (and their development)

The objective of this phase of the project was to sensitize the animals and to assess the resulting immune status, in order to provide a basis for testing hypothesis 2, namely:

- a) That airway response to TDI challenge *in vivo* relates to the sensitization status of the animal;
- b) That the "early" response to TDI challenge in sensitized animals is not directly associated with antibody level;
- c) That the response of TDI-sensitized guinea pigs to airborne methacholine challenge is independent of immune status.

2.2.1 Sensitization of animals

Karol had advised ²⁵⁹ the use of an intradermal injection of neat TDI to ensure an adequate response. The preliminary animals (T1-T7, 1990) were treated in this manner, but this procedure was then modified to a topical application of a solution of TDI in MgSO₄-dried acetone. Initially, the TDI was applied as two 25 μ L aliquots of a 20%(v/v) solution (groups 93/1 through 3), but this caused some dermal lesions, necrosis and scabbing. Group 93/4 was treated with two 50 μ L aliquots of a 10%(v/v) solution, but this too resulted in some lesions; these areas healed to an intact, pink skin stage by three weeks. Subsequent groups (93/5 - 93/11) were treated with 200 μ L of a 5%(v/v) TDI solution according to the protocol outlined below. This concentration was not as irritating as the higher ones; it resulted in some encrustation of the skin and loss of hair growth at the site of application, but there were no lesions, dermal perforation, etc. All of these applications had provided an equivalent dose of TDI (12 mg).

The ultimate protocol for sensitization involved the use of groups of four 2-3 week old, male, barrier reared Hartley guinea pigs (Charles River), specified to weigh 200-250 g at the time of shipment. Three to four days after arrival they were weighed, ear-punched (for identification purposes), and the hair was removed from their backs by means of clippers. They were later brought to the lab to be sensitized. One animal was randomly selected as the control; the experimental protocol was not "balanced" (i.e. equivalent numbers of test and control animals) because of the literature (described earlier) which suggested that only a portion (perhaps a third) of the test animals would ultimately show

airway hyperreactivity to specific challenge. The control animal received two 100 μL applications of MgSO_4 -dried acetone to separate areas of the dorsal skin, administered by plastic tipped pipettor, and was then placed into a plastic cage in a fume hood. The other three animals were treated with two 100 μL applications of 5% (v/v) TDI (Aldrich 21,683-6) in MgSO_4 -dried acetone (prepared just prior to administration) and similarly placed into individual cages in the fume hood, for at least one hour. The guinea pigs were then returned to the animal colony, where they were housed as a group of four per cage until they grew too large and territorial (12-22 weeks after sensitization), whereupon they were pair-caged, but with both halves of each group kept in the same room. The animals were fed and watered *ad libitum*, and weighed periodically.

Blood samples were collected at weekly intervals for at least three weeks following the initial exposure to TDI, to determine the changes occurring (if any) in the levels of TDI-specific IgG; samples were also collected at various other times for specific purposes. Routine blood samples were collected from a hind-limb by the toe nail clip method: with a pair of sharp scissors, the toe nail was cut at the "quick", and approximately 100 μL of blood were collected into an open Vacutainer (number 6384, 2 mL, containing 3 mg EDTA), and kept on ice until further processed. The samples were transferred to 1.5 mL microcentrifuge tubes and centrifuged for one minute at top speed in a Fisher Micro-Centrifuge Model 59A. The plasma was then aliquoted to fresh microcentrifuge tubes and frozen until the ELISAs were performed. Blood samples were collected at the time of sacrifice, from the cervical stump, into plastic centrifuge tubes; clotted blood was minced and centrifuged, and then aliquoted as above.

2.2.2 Synthesis of albumin-TDI conjugate; spectrophotometric characterization (including preliminary results and discussion thereof).

The protocol was derived from one used in Karol's laboratory. To 50 mL of 0.05 M phosphate (Fisher ACS reagents) buffer, pH 7.4, maintained with rapid swirling (by means of magnetic stirrer) at 36-37 $^{\circ}\text{C}$, was added 0.22 g of guinea pig serum albumin (GSA - Fraction V, Sigma). After 10 minutes of stirring, 55 μL of TDI was added to the centre of the "swirl" as 8 drops over a one minute period, followed by another 15

μL to the very base of the swirl 5 minutes later. Twenty minutes later the TDI globules visible in the flask were aspirated / dispersed back into the bulk liquid by Pasteur pipette, and the sides of the flask were washed down with more buffer mixture from the flask. The mixture was allowed to react (with continued stirring) for another 3.75 hours. The reaction was stopped by the addition of 200 μL of ethanolamine, and stirred for another 65 mins. The resulting suspension was filtered through #4 Whatman paper. The filtrate was retained, and its pH was adjusted from the initial 10.08 to 4.00 with hydrochloric acid. This mixture was refrigerated overnight.

The following day, the precipitate was resuspended by mixing, transferred to two plastic centrifuge tubes and spun at $6500 \times g$ (7500 rpm, JA-20 rotor) for 30 minutes. The supernatant was discarded, and each pellet was dissolved in 2.5 mL of 0.1 M $(\text{NH}_4)_2\text{CO}_3$ and to it was added 0.5 mL of ammonium carbonate wash. The combined solutions (6 mL) were tied into dialysis tubing (Spectrapor), which was placed into 1 L of 0.05 M NaCl in a cold room, with stirring, for 4½ hours. The partially dialysed conjugate was transferred to fresh dialysis tubing (allowing more room for expansion), and dialyzed against 1L of deionized reverse-osmosis (DRO) water, in the cold room, for 30 hours. The water was then changed nine times over a three day period. The final dialysate (a 19 mL volume) was frozen as two aliquots in liquid nitrogen, and lyophilized overnight. The resultant dry powder weighed 0.2 g, for an overall yield of 65% (assuming a stoichiometric reaction between TDI and GSA, and that the final product consisted uniquely of conjugate) .

This product, a conjugate of TDI (hapten) and GSA (protein), was used in some of the ELISAs as the antigen. Alternatively (and routinely, following a direct comparison of the relative ELISA efficacies), an equivalent conjugate provided by Dr. Meryl Karol was used. However, as another verification of the effectiveness of the conjugation process, two different analytical procedures were applied in a preliminary fashion: a TNBS (2,4,6-trinitrobenzene sulfonic acid; Sigma "picryl sulfonic acid") assay for free amino groups (lysine equivalents) on the conjugate molecule, and a **direct spectrophotometric determination** of the number of TDI groups attached per molecule of GSA (based on

relative molar extinction coefficients).

TNBS assay for free amino groups: This was performed according to the method of Snyder & Sobocinski ²⁶⁹. In brief, to 2 mL of amino acid (standards: lysine, glycine), protein (GSA) or conjugate in 0.1 M borate buffer, pH 9.3, was added 50 μ L of 0.03 M TNBS, vortexed, and after 30 minutes the absorbance was read spectrophotometrically at a wavelength of 420 nm, using borate-TNBS as the reference solution. The absorbance of the proteins (corresponding to the formation of a trinitrophenyl derivative) was evaluated relative to the standard curve prepared with lysine (and, assuming that Beer's Law held i.e. that the line passed through the origin). It was also assumed that the extinction coefficient (ϵ) for the free amino groups of lysine is the same as for the free amino groups of the GSA. The ϵ s determined here, and as reported by Snyder and Sobocinski were, for lysine 19.1 vs. $20.3 \times 10^3 \text{ cm}^{-1} \text{ M}^{-1}$, and for guinea pig and human serum albumin (respectively) $2.5 \times 10^3 \text{ cm}^{-1} \text{ g}^{-1} \text{ L}$ and $2.42 \times 10^3 \text{ cm}^{-1} \text{ g}^{-1}$ [*sic*].

In the TNBS assay for degree of GSA conjugation it is assumed that the NCO group attacks the terminal amino group of lysine residues. The TNBS assay indicated that there were 8.8 lysine equivalents per molecule of GSA and 2.1 per molecule of conjugate, in other words, that 75% of the free amino groups (lysine equivalents) of GSA were occupied in the GSA-TDI conjugate. However, it must be borne in mind that the albumin may also be conjugated elsewhere; for example, Tse & Pesce ⁶⁹, although demonstrating that the only recoverable amino acid residue of human serum albumin (HSA) altered by hexamethylene diisocyanate (HDI) was lysine, also presented evidence of adduct formation at other (perhaps hydroxyl or carboxyl) sites on the HSA.

Direct spectrophotometric characterization: This was performed according to a protocol routinely used in Karol's lab. GSA and conjugate (3 mg of each) were dissolved in 0.05 M phosphate buffer (pH 7.4), and their absorbance determined relative to buffer, at 276 nm; the absorbance of the conjugate was also determined relative to GSA, at 242 nm. The hapten:protein ratio was calculated from the molar extinction coefficient for the hapten, determined separately. There was good agreement between this study and Karol,

Jin and Rubanoff ²⁷⁰ for the ϵ of 2,6-TDI (8389 and 7636, respectively). We found a considerably lower value for the "commercial" TDI (80:20 mixture of 2,4- : 2,6-isomers): 9170, when prepared with a 200:1 NCO:glycine ratio, compared with their 15,678. Reaction conditions etc. may be extremely critical; repetition (two weeks later) with a 100:1 mixture of a fresh TDI preparation yielded a value of 4509 (i.e. there may have been an insufficient excess of TDI). Although the commercial preparation is ostensibly an 80:20 mixture of the 2,4- and 2,6- isomers, the 2,4-isomer is considerably more reactive ²⁷¹, and when the mixture reacts, the isomer ratio in the residual may be opposite (i.e. the 2,6- predominate). The actual proportion of the two present in any formulation is critical, as Karol *et al* ²⁷⁰ demonstrated that the ϵ of the 2,4-isomer was 2.6 times greater than that of the 2,6-isomer. A key assumption made in this determination is that the differential reactivity between the two isomers will be the same with respect to glycine (used to determine ϵ) and GSA (in determining the degree of conjugation); another, that the absorptivity of the isocyanato- group is the same in the two environments. Similarly, it is assumed that all of the differential absorbance at 242 nm is due to TDI conjugation, and not to the formation of TDA (toluene diamine) or a biuret derivative, upon addition of TDI to the aqueous medium. None of these assumptions appear to have been validated. The available data would suggest that there were 69 TDI per GSA (using the ϵ of Karol *et al* ²⁷⁰), or 118 TDI per GSA (using the original ϵ obtained in this study). In short, there was too much variability in our ϵ to arrive at a numerical conclusion. This was preliminary work that was to be repeated (if necessary) to verify the degree of conjugation of conjugate that was to be used in airborne challenge. This proved unnecessary. Instead, the effectiveness of this conjugate was verified by determining its relative ability to inhibit a coupling between antibodies from TDI-sensitized guinea pigs, and the Karol conjugate (antigen) used to prepare the ELISA plates.

2.2.3 Enzyme-linked immunosorbent assay (ELISA) of guinea pig plasma

The procedures were adapted from those employed in Dr. Meryl Karol's lab at the University of Pittsburgh; her provision of various written protocols is appreciated.

Materials: The chemical reagents were purchased from Sigma, and were of reagent grade; Dr. Karol's gift of TDI-GSA conjugate and lyophilized positive control serum is gratefully acknowledged; buffer salts were BDH Assured; milk powder and hydrogen peroxide were purchased locally.

Method development (Optimization of the protocol): Early work in this respect included the preparation of microtitre plates from three different manufacturers (Nunc, Linbro and Falcon), before selecting the latter. Various parameters were adjusted to reduce the background absorbance, thereby increasing the sensitivity: as a non-specific blocking agent, bovine serum albumin (BSA) was substituted for milk powder; to permit dual-wavelength absorbance measurements the absorbance spectrum of the chromogen (ABTS modified by oxidation to cationic radicals) was determined. At wavelengths greater than 300 nm (to exclude the non-specific absorbance by the proteins in the sample) the chromogen showed the highest absorbance as a fairly broad peak in the 395-420 nm range (e.g. 2.70 AU at 398 nm to 2.74 AU at 416 nm), but dropping to less than 0.4 AU above 460 nm. Based on the wavelength options available on the Dynatech automated plate reader used in these determinations, the absorbance and reference wavelengths selected for use in the assays were 410 and 490 nm, respectively. Whereas Karol's group measured absorbance at a single wavelength (410 nm), thereby contending with a high background (0.2 AU) relative to the result considered to be positive (0.03 AU)²⁷², in our lab the levels were considerably lower (see Table 2-1). The stability of the analyte chromogen over time was confirmed: a plate was read 3 weeks after the initial determination to find a 64% decrease in absorbance over this time ($y = 0.639x - 0.043$; $r = 0.996$; $p < 0.001$).

Similarly, the stability of the enzyme (peroxidase conjugated to rabbit anti-guinea pig IgG) was of interest. The enzyme preparations were typically stored in a freezer, but on various occasions some aliquots were stored in a refrigerator for many months. The activity of these was compared to that of freshly reconstituted enzyme. In one such determination, with two sets of wells (representing serial dilutions of plasma from the same animal), a five month old batch of enzyme was compared to one prepared from

frozen stock that day:

Activity of enzyme stored in refrigerator for 5 months, compared with freshly reconstituted		
	Old enzyme	New enzyme
Mean absorbance	0.693	0.647
sd	0.479	0.495
p	< .002 (paired t-test)	

Thus, there is a (trivial) difference, but the old enzyme is the more active. Perhaps more remarkable is the following verification, prior to running an ELISA after a hiatus, that the reagents were still functional. Enzymes 1 and 2 were both received from the supplier 30 months prior to this experiment, and were the same except that # 1 had been received in a thawed condition (and was therefore deemed to be unreliable by the supplier, who substituted # 2).

Effects of various storage modes on activity of ELISA enzyme				
Enzyme	Stored as	For	Used at	Absorbance
# 2	Frozen concentrate	25 months	1:400	0.72
# 2	Frozen 1:10 dilution	25 months	1:400	0.81
# 1	Frozen 1:10 dilution	25 months	1:400	0.74
# 1	Frozen 1:400 dilution	29 months	(1:400)	0.02
# 2	Frozen 1:1000 dil'n	25 months	(1:1000)	-0.02
# 1	Refrigerated, 1:1000	29 months	(1:1000)	0.83

Clearly, the enzyme is remarkably robust, except when frozen in dilute form.

"Titre" versus "absorbance" were compared as outcome measures; various combinations of positive and negative controls were incorporated into the microtitre plates; the degree of specific inhibition of the ELISA procedure by Karol's and Queen's conjugates was determined; the effect of the duration of the final incubation were assessed, etc. The results of these determinations are provided in 2.3.2.

Final protocol:

Falcon 3912 PVC (polyvinyl chloride) plates were prepared up to 2 weeks in advance and refrigerated. TDI-GSA conjugate (from Karol's or our laboratory) was used as the antigen. This was prepared as a 1 mg/mL stock solution in 0.1 M carbonate buffer (pH 9.6) from lyophilized product, and stored frozen at -20 °C. A working solution was prepared by diluting the stock 1:200, again, with 0.1 M carbonate buffer; 50 μ L of this was pipetted into each well of the plates (except for the appropriate blank wells) and the plates were covered with a polymeric wrap and incubated in a "hot room" (at 38 °C) or in the air-space above a 37 °C water bath, for approximately 3 hours. The plates were then refrigerated until use, whereupon they were allowed to come to room temperature. The excess conjugate solution was aspirated from the plates, which were then blotted on paper towel. The plates were rinsed three times with 175 μ L aliquots of saline-Tween[®] solution (0.85% NaCl + 0.05% Tween[®] 20 = Polyoxyethylene sorbitan monolaurate). In the 3rd rinse the wells were individually aspirated (to reduce residue carry-over), whereas the other rinses were decanted.

A "blocking agent" was added to each well, to eliminate non-specific binding: RIA grade BSA (1%, in 0.02 M PBS, 0.05% Tween-20) was added in 50 μ L aliquots to those wells also to contain antigen. The blocking agent was typically allowed to react at room temperature while the plasma samples were prepared for the assay (generally, 15-20 minutes, but in some cases, up to several hours).

When running a serial dilution for the determination of "titre", the plasma samples were diluted 1:4 in the initial set of wells (i.e. 25 μ L plasma + 75 μ L BSA-PBS-Tween, as above); 50 μ L of the dilution was added to the second well in the appropriate row, and further serial dilutions of all of the rows of wells on the plate were performed by means of "Octapette". To extend the range, samples were occasionally diluted 1:4 prior to application to the microtitre plate. In later assays, due to the many plasma samples that had to be processed, serial dilutions to arrive at titres were not routinely performed. Instead, the plates were prepared as an array of duplicate determinations of plasma aliquots: samples were compared on the basis of the absorbance of a fixed dilution of the

plasmas. Comparison between different plates was made (as necessary) by the mathematical adjustment of absorbances, based on the outcomes of multiple determinations of positive control sera on each plate.

The plates were covered with plastic wrap and incubated at 37 °C for approximately one hour. The plates were then aspirated dry, and rinsed three times with saline-Tween as above, aspirating the third wash. The enzyme conjugate (anti-guinea pig IgG (whole molecule) peroxidase conjugate - Sigma product number A-5545) was then added: 50 μ L of a 1:400 or 1:1000 dilution (overall), in PBS-Tween, of a 1:10 stock solution frozen at -20 °C. The enzyme was aspirated from the plate, which was then rinsed three times, as above. To produce the chromogen, 50 μ L of ABTS reagent (13.8 mg of 2,2-azino-bis(ethylbenzothiazoline-6-sulfonic acid) diammonium salt; 2.7 mL of 0.5 M citric acid; 2.3 mL of 0.5 M Na citrate; 20 mL deionized water; 50 μ L of 3% H₂O₂, added just prior to use) was added to each well until the appropriate depth of colour was achieved (typically, 2 minutes) and the reaction was terminated by the addition of 50 μ L of 5% SDS (sodium dodecyl sulfate). The plates were read in a Dynatech MR 600 reader, with sample wavelength of 410 nm and reference wavelength of 490 nm, relative to the appropriate blank well.

2.3 Final results and discussion

2.3.1 Animal sensitization procedures

The animals had been exposed by dermal application of 12 mg of TDI, and placed into individual cages in a well-ventilated enclosure for at least one hour. During this time the TDI would have had the opportunity to react (with macromolecular and/or water constituents of the skin) and/or evaporate. The amount of TDI inhaled by the guinea pigs under these conditions is not likely to have been significant with respect to the initiation of sensitization. Karol ¹²⁷ had monitored airborne TDI levels in plastic cages holding guinea pigs to whose skin up to 61 mg of TDI had been applied, and found "*an initial peak exposure of 0.4 - 0.5 ppm lasting for several minutes, then dropping to undetectable levels*". As she had used an MDA tape monitor for this work, it is likely that she was not able to selectively monitor the animals' breathing zone and accordingly the reported level may be higher than what the animals inhaled. This inhalation exposure is in any case less than what is required to induce an immune response, based both on the protocols generally necessary to initiate sensitization (see Chapter 1), Karol's specific findings ⁹¹, as well as ours (see Fig. 2-5).

There were no animal deaths (nor apparent illness or discomfort) occurring as a result of the sensitization procedure with the 5% TDI solution. A number of unanticipated deaths and/or prescribed euthenasiations did, however, occur in the colony during the course of this trial: 8 among the 28 animals in the 5% TDI groups, including the guinea pig that was the most consistently TDI-sensitive, died. This was ultimately attributed to an accidental contamination of the drinking water supply lines of some of the animals, rendering the water unpalatable, and causing the animals to become anorexic; this is unlikely to have had any effect on the presented results of experimental outcomes. However, the incipient and ultimate loss of these animals affected the scheduling of the plethysmographic trials, and the planning and execution of the *in vitro* trials.

2.3.2 ELISA protocol: inhibition, dilution and time studies

ELISAs were used to determine the relative level of TDI-specific IgG and were variously

run as both the traditional "titre", and as a determination of absorbance. Karol's group¹⁰³ had defined titre as "the highest serum dilution that gave an absorbance at least twice that of the control sera and greater than 0.030 after subtraction of background absorbance"; the results are then expressed as the dilution ratio e.g. 1:1024. Cheng *et al*¹¹⁴ made an absolute determination of the amount of anti-ovalbumin IgG produced, and correlated it with absorbance; they found that 2-2000 ng of IgG corresponded to 0.1-1.1 AU. Various authors^{104,110,266,273,274} have used single-value measures based on the absorbance produced by a fixed amount of plasma, rather than on a serial dilution "titre".

Several separate trials of inhibition of the ELISA procedure by the Karol and Queen's conjugates were performed, to compare the two, and to verify their effectiveness. In the first (Fig. 2-1a), varying amounts of GSA (corresponding to the amount of conjugate) were used as controls. Fig. 2-1b shows combined inhibition data (expressed as % of the absorbance seen with an equivalent amount of GSA) from a separate experiment. These data demonstrate an equivalency between the two conjugates with respect to their ability to block the binding between TDI-elicited guinea pig IgG antibodies and a conjugate of TDI and GSA; accordingly the two conjugates can be said to have an equivalent specificity.

Figs. 2-1a and 2-1b. ELISA inhibition studies: comparison of the degree of inhibition of the ELISA by TDI-GSA conjugates produced at Queen's, the conjugate from Karol's lab, and GSA.

The basic procedure was as described in 2.2.3 "final protocol"; additional steps are outlined for 2-1b; these were similar for 2-1a. ELISA plates were prepared as for a normal assay, except that the appropriate conjugate or GSA, dissolved in 30 μ L of 0.1 M carbonate buffer, was added to the wells of the microtitre plate after the addition of the blocking agent and prior to the addition of plasma. The results are expressed in absorbance units in Fig. 2-1a (Dynatech MR 600 T/R settings of 410 and 490 nm), and relative to the effect of GSA (as a %) in 2-1b.

The symbols used are

□	GSA
Δ	Queen's conjugate
○	Karol's conjugate

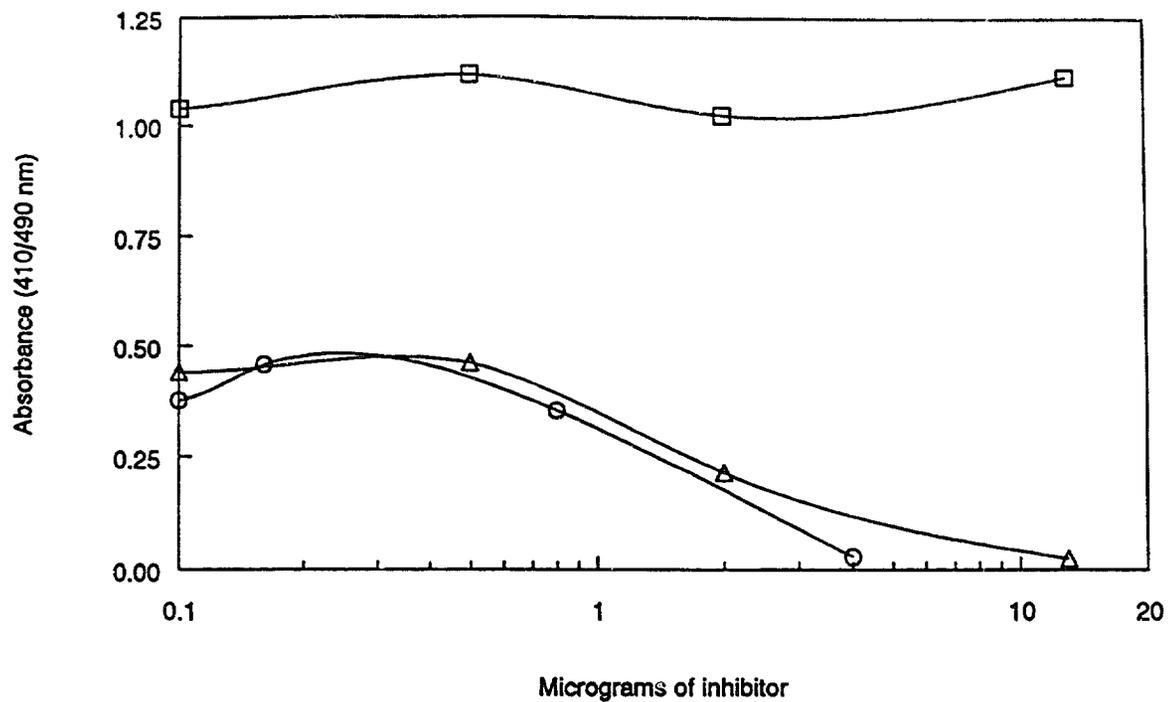


Fig. 2-1a. ELISA Inhibition; GSA compared with Karol and Queen's TDI-GSA conjugate.

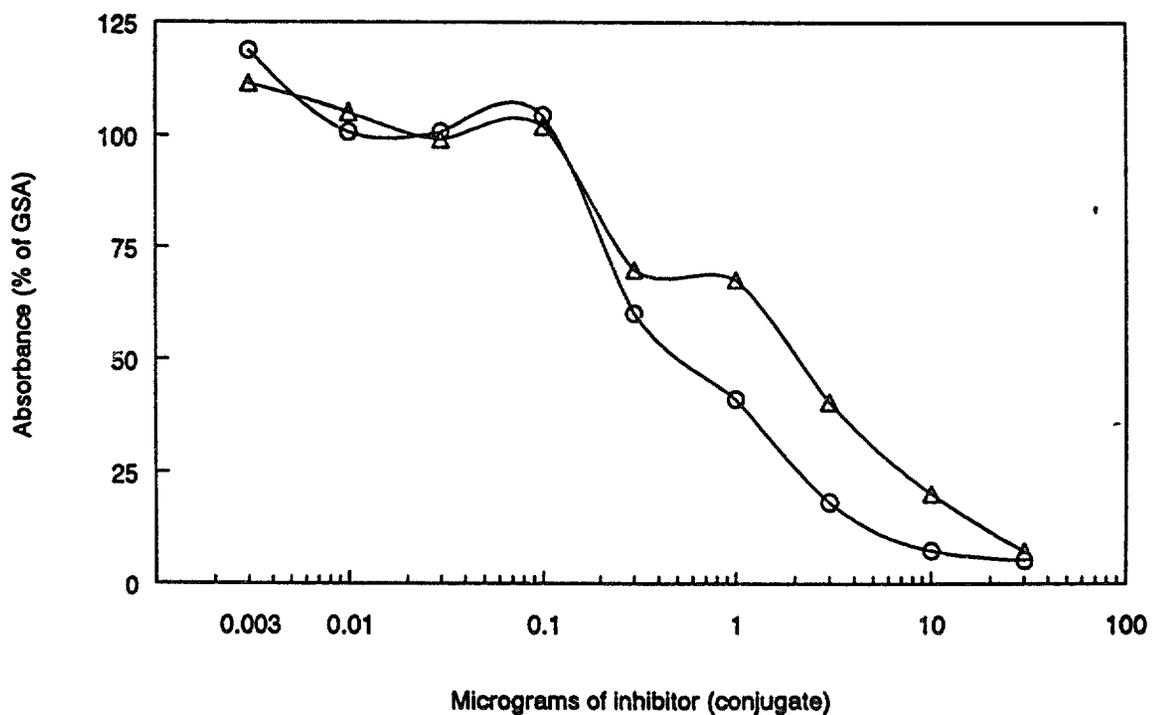


Fig. 2-1b. Inhibition study; effects of Karol and Queen's conjugates relative to GSA.

The curves also demonstrate that the plasma IgGs being evaluated are not directed against the GSA moiety of the TDI-GSA used as the primary antigen, insofar as GSA does not inhibit the binding. By corollary, then, they must be directed against the modified TDI-GSA complex.

To determine the specificity of the interaction of the various reagents, several combinations of negative controls were run. For example, the relative "blank" absorbances (in pairs of samples) with four different configurations were as shown in Table 2-1. These data indicate that in all cases, the blanks were low, relative to the typical absorbances seen with samples from sensitized animals, i.e. in the > 1 AU range. Nevertheless, the absorbance of 0.013 seen when all constituents except for analyte antibody (i.e. the secondary antigen) were included, suggests that there is some nonspecific binding between the enzyme and the primary antigen, and that this combination is the most appropriate blank to use for the assays.

Fig. 2-2a shows a typical ELISA titration curve from plasma samples of three control and three sensitized guinea pigs; the samples were collected 5 weeks after sensitization. Fig. 2-2b shows the effect of incubation time on the final absorbance.

2.3.3 ELISA protocol: final results

The results of the key ELISA determinations are shown in Figs. 2-3 through 2-5. Fig. 2-3 indicates the ELISA absorbance (by animal) at weeks 1, 2, 3, 4, 5 and 13 for group 93/6, and at weeks 1, 2, 3 and 7 for group 93/9. It is clear that the antibody response develops in the exposed animals some time around a week after the dermal exposure. There is a low level of response at 1 week in group 9, but not at all in group 6; by two weeks all of the exposed animals have a similar TDI-specific IgG level. The control animal, presented on the left side of each block, maintains a consistent, baseline level of IgG directed against TDI-GSA.

Table 2-1. Optimization of ELISA blank				
ELISA Constituent	Present (+) or absent (-)			
Primary antigen (TDI-GSA conjugate)	-	-	+	+
Blocking agent (BSA)	+	+	+	+
Secondary antigen = analyte Ab (Guinea pig serum / plasma)	+	-	+	-
Enzyme (HRPO conjug. to anti-gp IgG)	+	+	-	+
ABTS (substrate chromogen)	+	+	+	+
Absorbance	0.002	0.000	0.000	0.013

931222

Table 2-1. Optimization of ELISA blank.

ELISA reagents were added to the microtitre plate wells, as indicated above, and in the sequence described in the protocol, to evaluate the most appropriate combination to use as a blank in the assay. Primary antigen (TDI-GSA conjugate) was added as 50 μ L of a 5 μ g/mL solution in 0.1M carbonate buffer at the time the plates were made up; the other materials were added as: 50 μ L of 1% RIA grade BSA (blocking agent) in 0.02 M PBS- 0.05% Tween[®]; 10 μ L of positive plasma; 50 μ L of a 1:1000 dilution in PBS-Tween[®] of horseradish peroxidase conjugated to anti-guinea pig IgG (Sigma A-5545); 50 μ L of ABTS reagent (13.8 mg of ABTS, 2.7 mL of 0.5 M citric acid, 2.3 mL of 0.5 M sodium citrate, 20 mL of deionized water, 50 μ L of 3 % hydrogen peroxide). The reaction was stopped with 50 μ L of 5 % SDS and the wells were evaluated spectrophotometrically in a Dynatech MR 600 at T/R settings of 410/490 nm.

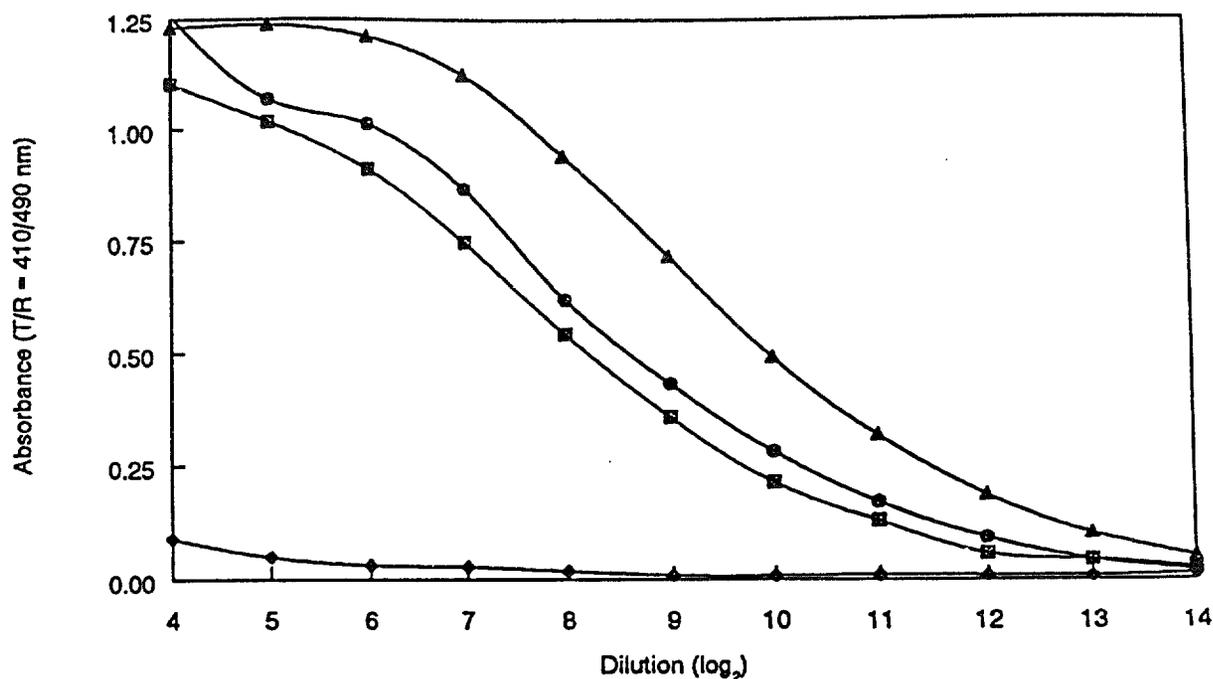


Fig. 2-2a. ELISA titre curves. These curves represent the decline in absorbance associated with serial two-fold dilutions of plasma samples collected 5 weeks after sensitization (by topical application of 20 % TDI) of three guinea pigs (■, ●, ▲), and of three control animals (◆, pooled data).

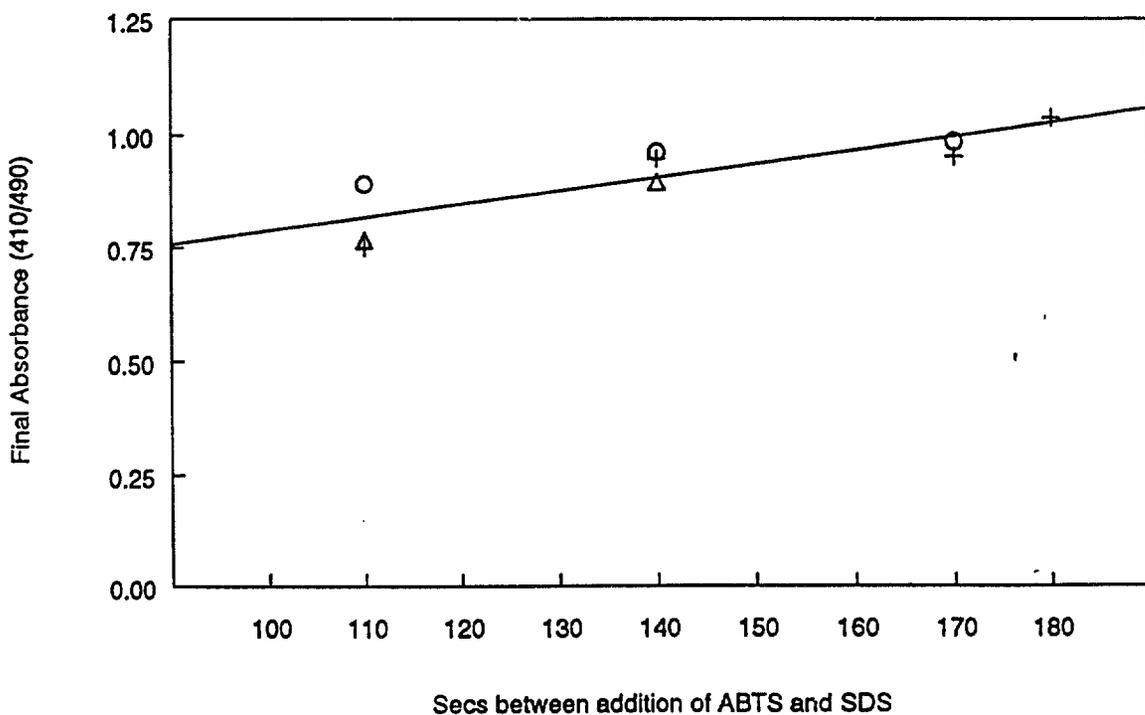


Fig. 2-2b. ELISA: Effect of duration of final incubation on absorbance. The final incubation (chromogen development step) of several samples was terminated with the addition of 5 % SDS at various times as indicated.

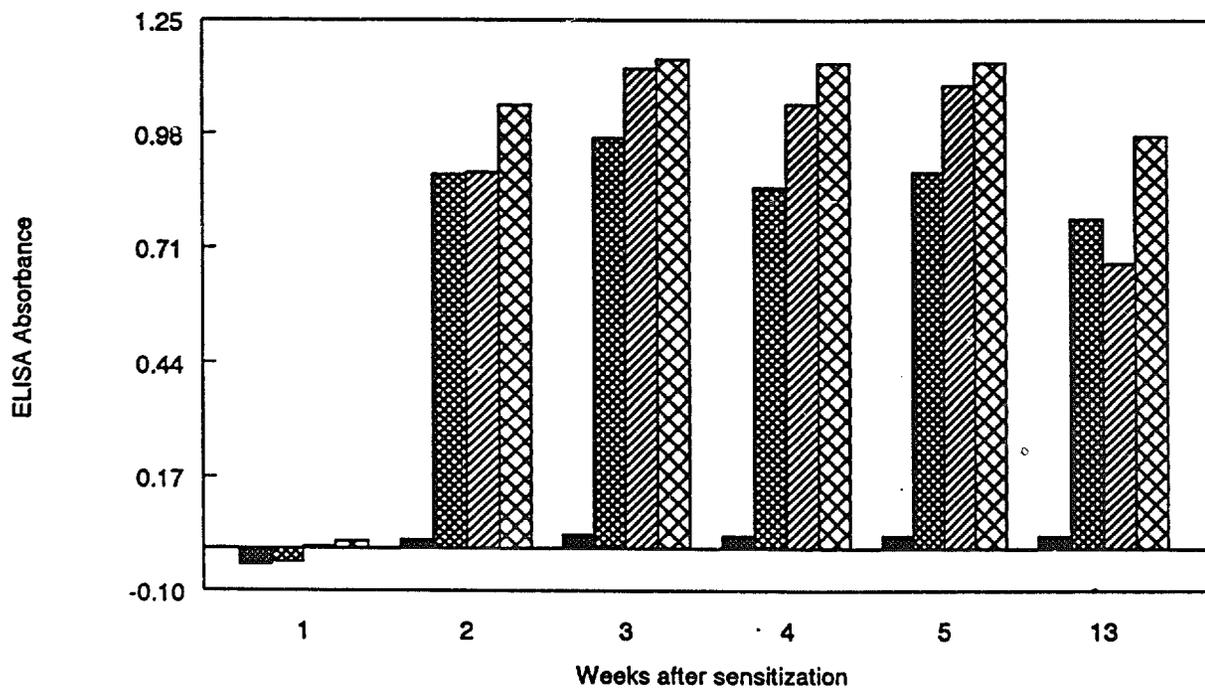
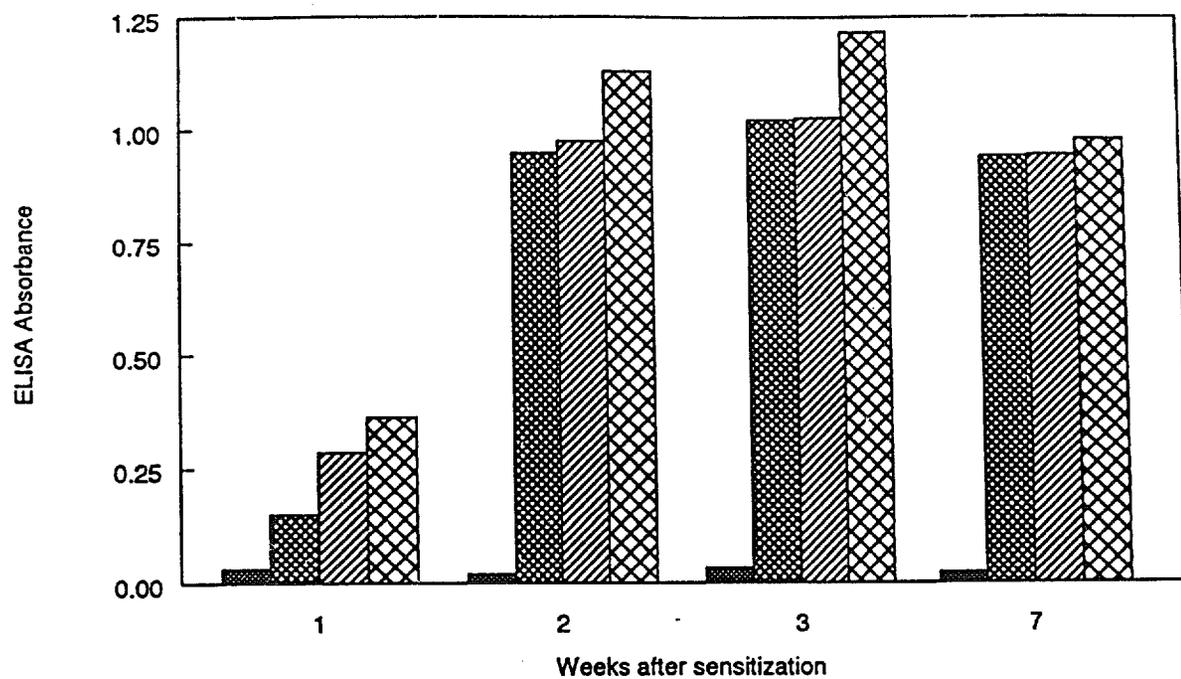


Fig. 2-3. Early immune response of selected groups to dermal TDI sensitization. Aliquots of plasma ($1.6 \mu\text{L}$) were analyzed for IgG antibody to TDI-GSA by ELISA as described in the protocol. The graphs represent groups 93/9 (top) and 93/6 (bottom). At the indicated number of weeks following the dermal sensitization procedure, each animal is represented by one bar. Within each group of four guinea pigs the control animal is shown on the left; the other three animals were topically TDI treated.

By virtue of the (now) chronic nature of this study, blood samples were also collected from all animals at one time corresponding to 27 to 35 weeks after the initial (sensitizing) TDI exposure in the case of groups 6-11 (i.e. those that had received the TDI sensitizing dose as a 5% solution) and weeks 37 to 46 (in the case of groups 1-5). Fig. 2-4 shows for all 33 animals sensitized dermally (and their corresponding controls) their ELISA absorbance three weeks after sensitization and also 27-46 weeks after sensitization.

Thirty five to 46 weeks after sensitization there appears to have been some decrease in the TDI-IgG level in the sensitized animals, but in the animals evaluated 27 - 32 weeks after sensitization some of the antibody levels appear to be increasing. The control animal in each group showed an increase in ELISA absorbance over this time. Groups 1, 5 and 10 had been challenged with airborne TDI in the intervening time period, but the increased control IgG level is by no means confined to (nor even greater in) these groups. It would seem that this change could simply be a function of senescence, relating again to the autoimmune notion (outlined in Chapter 1 and referred to below). However, the figure represents a composite of two assays and artefact due to slightly different blank values would need to be ruled out.

IgG Levels to 'TDI-GSA Conjugate'

at Week 3, and Week 27 - 46

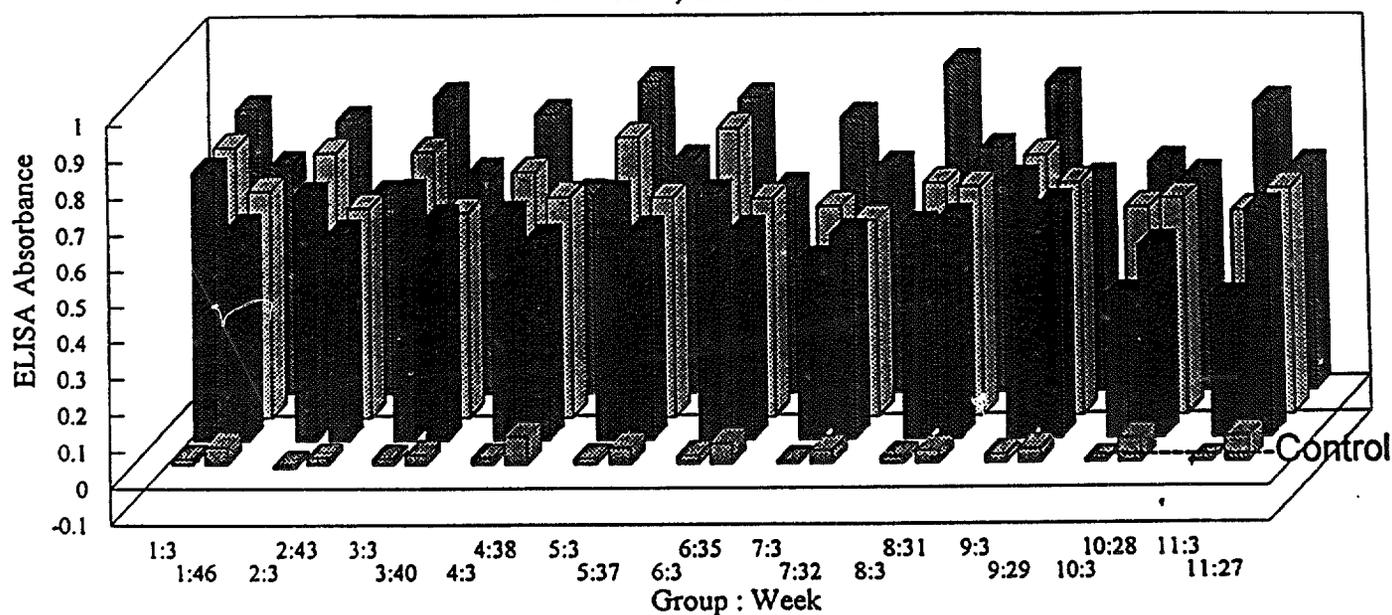


Fig. 2-4. Levels of TDI IgG in all 44 guinea pigs in final study, at 3 weeks and 27 - 46 weeks following the dermal sensitization. Sensitization had been by application of 12 mg of TDI as 20% (v/v), groups 1-3; 10% (v/v), group 4 and 5% (v/v), groups 5-11. Control animals (located in the front) were treated with acetone (vehicle). Each bar represents, by animal, the mean of duplicate determinations; the left hand bar in each pair is from the sample collected at 3 weeks, and the right hand bar relates to the sample collected at the indicated number of weeks. These data were obtained from ELISA determinations conducted on separate occasions to cover the two time periods; the absolute value of the second set was adjusted (down) on the basis of a common set of samples analyzed on the two days.

Fig. 2-5 shows the ELISA outcome with group 93/1, at week 2 and then weeks 46 - 59; these samples were analyzed on one set of ELISA plates, allowing a more direct comparison. The consistency of IgG levels is noteworthy: guinea pig IgG₁ and IgG₂ are reported to have plasma half-lives of 7.1 and 5.1 days (respectively) in the "normal" animal, and 6.0-8.8 and 4.1-8.0 days (respectively) in the immunized animal ¹¹⁷. However, we found that TDI-specific IgG levels did not show a perceptible decrease in over a year. This suggests that there must be ongoing stimulation of immunogenic production of these specific IgGs despite the absence of recurring TDI exposure. This invokes consideration of autoimmunity evoked by an initial chemical "trigger", but then carrying on in a self-perpetuating fashion. In view of the fact that the total amount of TDI originally applied to the sensitized animals was only 12 mg (and that only a fraction of that was absorbed), there is not likely to be enough residual chemical left in the body a year later (especially when considering its reactivity) to engage in ongoing immune system stimulation. However, the quantity of an agent that may be significant to the immune system may be very low in terms of traditional 'pharmacological' doses. For example, Brostoff *et al* ²⁸ indicate that annual exposure to pollen amounts to only 1 µg per individual, yet many respond to daily fractions of this dose; fatal anaphylactic reactions to peanuts appear to occur in response to trace amounts. Alternatively, this result may be rationalized on the basis of the apparently-great (*in vitro*) stability of this particular IgG: the samples representing week 46 again characterize the robustness of the TDI response: these plasmas had been stored (thawed) in a cold room for 9 months, yet showed comparable levels to the others, which had been frozen. Note that this is one factor that precludes a direct comparison of control animal IgG levels between Figs. 2-4 and 2-5. The other is the slight week-to-week variability in control animal plasma TDI-GSA IgG levels (shown in Fig. 2-5), coupled with the lack of another concordant week in the two figures.

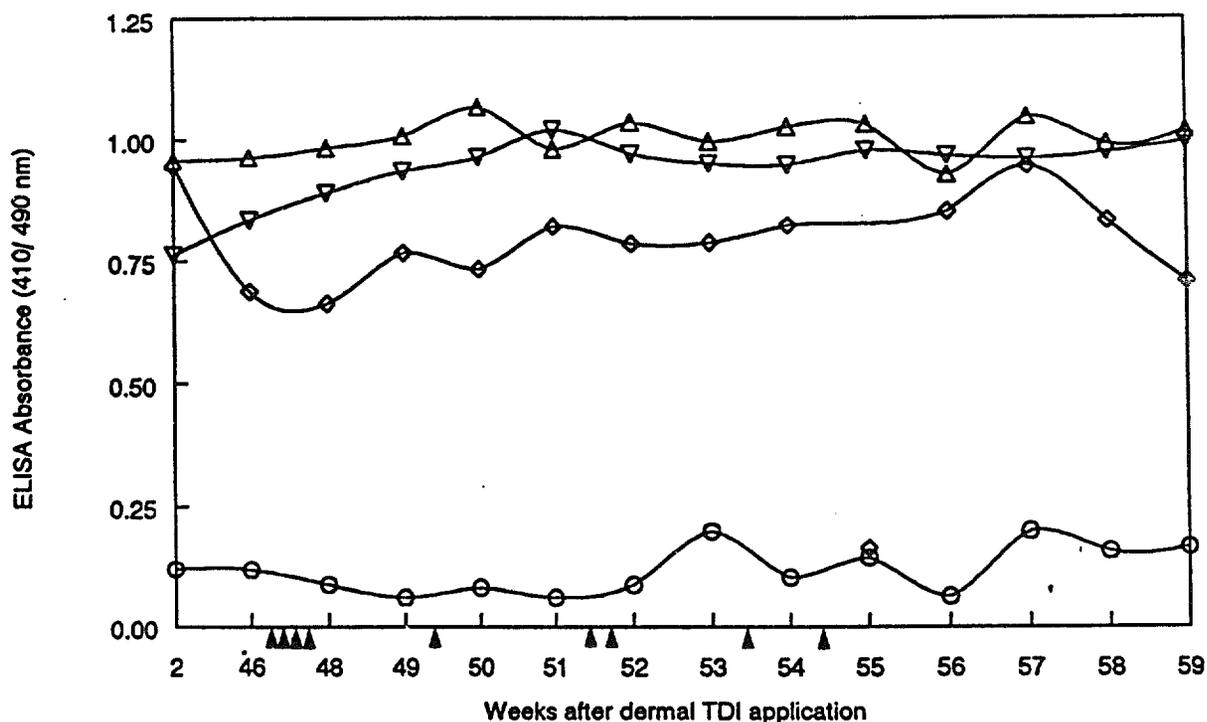


Fig. 2-5. TDI-IgG levels in group 93/1 during weeks 46-59, including airborne TDI challenge periods. This figure shows the TDI-specific IgG levels (as determined by ELISA) in plasma samples collected from group 93/1 at week 2, and weeks 46 through 59, following sensitization with a topical application of 20 % TDI in acetone. The samples from week 46 had been stored, refrigerated, for 9 months prior to analysis; the others had been frozen. The triangles along the abscissa denote airborne TDI challenges of this group, with TDI vapour levels in excess of 1 ppm. The top three curves correspond to the sensitized animals, and the bottom one to the control animal. One data point at week 55 is not included in the corresponding curve, as it is clearly aberrant.

During the time span represented by Fig. 2-5 there were nine inhalation challenges of this group with airborne TDI vapour, indicated by the triangles along the abscissa, and including exposures to levels greater than 1 ppm. However, there was no apparent or consistent response in terms of antibody production, comparable to that seen with the original, single dermal exposure. These data again corroborate the original posit that the animals were initially truly responding to dermal absorption, rather than to inhalation of TDI vapours, from their skins.

The blood that was collected from each animal at the time of sacrifice for the *in vitro* work (at weeks 63 - 71) was also analyzed by ELISA. The mean (sd) absorbance of these samples was 0.95 (0.06) in the case of the sensitized animals, and 0.17 (0.1) in the case of the control ($p < 0.001$). Clearly, the sensitized animals remained immunologically distinct from the control animals for the duration of the project.

3. AIRBORNE CHALLENGE AND MEASUREMENT OF RESPIRATORY RESPONSE

3.1 Introduction

Numerous methods have been devised to assess airway response to challenge; many have involved anesthesia, surgical procedures (e.g. intrapleural catheterization ²⁷⁵) and/or restraint. With a phenomenon such as asthma, which appears to have a major nervous component (CNS, reflex loop, 'emotional'), these approaches may be undesirable to the extent that they might artefactually affect the outcome. For example, Ahmed *et al* ²⁷⁶ indicated that even breathing through a mouthpiece while wearing a nose clip causes a substantial increase in ventilation above that found with natural breathing, in human subjects. This is one type of rationale for the noninvasive study of awake, unrestrained animals in whole-body plethysmographs, as described in the present work. Of course, this approach in turn presents its own challenges.

The plethysmograph was invented in 1870 by Mosso of Turin for "*recording and measuring the variation in the volume of a part of the body*" ²⁷⁷, and has been applied in various forms to the evaluation of respiratory phenomena in laboratory animals; a "classic" reference in the guinea pig field is Amdur & Mead (1958) ²⁷⁵. The identification of the appropriate experimental variables to record, and the selection of an optimal endpoint and outcome measure, are fundamental questions to be addressed with any trial such as this. Karol *et al* ¹¹¹ rationalized the use of respiratory rate as a measure of airway response in challenge studies:

"Traditionally, investigators have evaluated pulmonary hypersensitivity by measurement of airway resistance or other parameters indicative of bronchoconstriction during challenge by an antigen aerosol... . These procedures, however, require anesthesia and/or minor surgery and do not permit groups of animals to be exposed simultaneously. A simpler index of pulmonary reactivity during challenge with antigen aerosol is the reflex increase in respiratory rate. In several animal species, including man, numerous investigations have demonstrated that immunologically sensitized animals respond to aerosol challenge with an increase in respiratory rate and a decrease in tidal volume referred to as "rapid shallow breathing". In severe reactions the response progresses to a slow gasping type respiratory pattern indicative of severe bronchoconstriction."

In whole-body plethysmography the fundamental parameter continuously recorded with time is the plethysmograph pressure (ΔP), reflecting the animal's breathing. From this wave form can be determined respiratory rate and, with appropriate chamber calibration, the tidal volume ²⁷⁸. In addition to these numerical factors, various "patterns" of tracings are used to characterize or define responses; investigators have reported the use of a wide variety of indices and endpoints, ranging from the obvious and trivial through to the convoluted; these may be adapted or simplified as appropriate. In their initial paper in this field Karol *et al* (1978 ¹³⁵) had devised a Respiratory Index (R.I.) as:

$$R.I. = 20(R_s)^{3/8}[\ln(R_f/R_s)][(210-13T)/15]$$

where

R_s = averaged highest respiratory rate (respirations per minute) occurring during three consecutive minutes of the [10 minute] aerosol exposure (maximum in three minute moving average)

R_f = respiratory rate (respirations per minute) during the five minutes prior to inhalation of the antigen

T = minutes of antigen inhalation until onset of respiratory collapse, for up to measured time of 15 minutes, or set as $T=15$

However, in subsequent publications they have used the simpler index of percentage increase in respiratory rate. It should be recognized that the endpoint chosen in a toxicological study (e.g. change in a particular parameter due to challenge) need not necessarily be a direct correlate of a parameter that would be used in a study to elucidate the pathophysiological mechanisms of a particular airway dysfunction. Similarly, as in any model, limitations imposed by the degree of parallel between the animal (model) and the human response of interest must be considered. For example, in studies where total airways resistance is used as an index of bronchoconstriction, it should be borne in mind that in humans total airways resistance is comprised of upper (nose, larynx) and lower (lung) components in the proportion of 70% to 30%, whereas in the guinea pig the corresponding numbers are 90% and 10%, respectively ²⁷⁹. Any one agent can cause differential effects in different regions; the overall (observed) effect reflects the balance.

Methacholine is used clinically as a challenge agent in the measure of non-specific bronchial hyperreactivity (NSBH), to evaluate the severity of asthma: "*Aerosol is generated continuously over successive periods of two minutes from doubling*

concentrations of methacholine and is inhaled by the test subject during tidal breathing. The dose delivered consequently depends on tidal volume and ventilatory frequency as well as on aerosol output and so it is not readily quantified..." ²⁸⁰. In the isocyanate field there have been many attempts to correlate the degree of NSBH with specific sensitivities or disease severity, without consistent success (and even less so in terms of the immediate response to challenge). The methacholine challenge of isocyanate-sensitized guinea pigs has apparently not been previously reported; this was undertaken in the present study. Detailed consideration was given to the output of aerosol from the nebulizer, precipitated by the initial findings of inconsistency in this respect, when measured directly as airborne aerosol. The results should be of general interest in the clinical field to those who use nebulizer technology and the associated protocols for the determination of NSBH (see Appendix 1).

The systematic investigation of animal respiratory response to challenge has been occurring for over a century. Alarie ²⁶⁰ summarized the findings of various researchers from Kratschmer (1870) through to Magné *et al* (1925), who differentiated irritant effects into the categories: upper airway, lower airway and total airway. Irritation of the upper airway caused a decreased respiratory rate; lower airway irritation caused an increase in breathing rate. In a later review paper ²⁸¹ Alarie adapted from Henderson and Haggard²⁸² a classification of airborne chemicals "capable of stimulating nerve endings in the respiratory tract", which is summarized in Table 3-1.

Alarie ²⁶⁰ tested mice with a variety of irritants and used the two approaches below to estimate the decrease in breathing frequency, which was due to the increase of the expiratory phase of the breathing cycle:

i) Inspection of the chart pressure record during and following a one minute exposure period. If no decrease in rate was seen, the material was classified as non-irritating.

282 Table 3-1. Summary of respiratory irritant types, as per Henderson and Haggard

Chemical Type	Examples	Signs	Symptoms
Sensory Irritant: stimulates nasal trigeminal nerve	Sulfur dioxide Ammonia Inert dust	<ul style="list-style-type: none"> • Respiratory Inhibition • Coughing • Most are also broncho-constrictors 	Burning sensation
Pulmonary Irritant: stimulates sensory receptors in lung	Phosgene Nitrogen dioxide Ozone	<ul style="list-style-type: none"> • Increased respiratory rate • Decreased tidal volume 	Painful breathing (secondary to pulmonary edema)
Broncho - constrictor: direct sm. muscle effect, axon reflex, etc.	Sulfur dioxide Ammonia Toluene diisocyanate	<ul style="list-style-type: none"> • Increased resistance to airflow within conducting airways 	Dyspnea
Respiratory Irritant: all of the above	Chlorine Chloropicrin	<ul style="list-style-type: none"> • Mixed 	

Otherwise, the response was scored (visually) as 1, 2 or 3 over the exposure period and at various periods post-exposure.

ii) Counting breaths over 15 second periods: manually, or "by an automatic rate counter with specially designed circuitry to eliminate most of the counting of body movements" [no details provided]; these animals were restrained by a rubber dam. The method was sufficiently sensitive to detect irritation at concentrations producing no pathology. Further, in comparing the results in humans and in mice for 52 compounds, there was complete agreement (i.e. every compound that was a non-irritant in the mouse also was a non-irritant in the human). Alarie defined an RD_{50} as the concentration required to achieve a 50% decrease in respiratory rate, using preferentially the peak effect as a criterion (rather than an average, which would then also incorporate consideration not only of degree of effect, but also time course).

With many sensory irritants, Alarie ^{87,281} had found the level of response and the recovery period to depend only on the concentration, and to be independent of exposure duration. However, with TDI, Sangha and Alarie ⁸⁸ (again with mice) found that duration of exposure affected both response and recovery. Recovery rate was rapid with short exposures, but very slow with long exposure - after 30 minute exposures at 1.2 ppm, there was a recovery (in terms of respiratory rate) in 15 - 30 minutes. The decreased rates that they found were attributed to upper respiratory tract irritation, since exposure via tracheal cannulation did not result in decreased respiratory rate. The calculated RD_{50} s (as a function of exposure time) were:

Exposure Time (mins)	TDI RD_{50} (ppm)
10	0.81
30	0.50
60	0.39
120	0.25
180	0.20

They found that there was a transient cumulative effect: repeated (daily) exposures at or above 0.02 ppm resulted in enhanced animal responsiveness (greater decrease in

respiratory rate) on days 2 and 3. When they exposed mice to 1.2 ppm TDI for 5 hrs x 3 days, and challenged them 15 days later, they found no difference from naive animals; this would suggest that pharmacological mechanisms were involved.

Whereas Alarie's pivotal work in mice had led to the development of a parameter (RD_{50}) related to decrease in animal respiratory rate in response to TDI, Karol *et al*⁹⁹ reported that they were able to achieve a demonstrable TDI effect in sensitized guinea pigs (defined as an increase in respiratory rate) with airborne TDI challenge concentrations of only 0.005 ppm; this differential could be due to concentration and/or species. Others have had considerable difficulty in achieving this type of sensitivity. Sarlo and Clark²⁸³ were unable to achieve any response with a 20 minute exposure to 0.2 ppm TDI, in groups of sensitized guinea pigs in which the majority did respond to TDI-GSA conjugate aerosol.

For the present study, it had been intended to use an endpoint defined in terms of a set percentage increase in respiratory rate (as described by Karol); this turned out to be somewhat simplistic, yet impractical. In 1981 Karol *et al*⁹⁹ stated categorically that "*...challenge of immunologically sensitized animals results in increased respiratory rate and decreased tidal volume. The extent of sensitivity is indicated from the percentage increase in respiratory rate during challenge compared to the breathing rate prior to aerosol challenge. ... The percentage increase in rate was calculated from the highest average rate during 3 consecutive min of aerosol challenge of an individual animal compared with the animal's preexposure breathing rate*". In other words, during a given exposure the highest respiratory rate over any 3 minute period was taken as the response. In another of Karol's papers⁹¹, "*the most severe reaction demonstrated by individual animals to any of three inhalation challenges*" (again, an exceptionality) was used as an indicator of sensitivity. This approach was inconsistent with yet another Karol paper⁷⁷ in which she argued that, because of the "anamnestic response", the evaluation of pulmonary sensitization must be based on a single inhalation challenge for any individual guinea pig.

Some researchers have used software that is able to provide respiratory rate output directly from a plethysmograph signal; the resulting data are described as "filtered" ²⁵⁹. It is largely these researchers who have defined bronchoconstriction, or other significant respiratory response, in terms of percent change in respiratory rate. Sometimes more complex endpoints have been used, even by them: Thorne & Karol ²⁸⁴ defined one endpoint as "*the time required [for histamine] to decrease by one third the CO₂- induced increase in ΔP* ". However, a more applicable alternative provided by them related to the timing of increases in ΔP . This "*was shown to coincide with a decrease in tidal volume and with the onset of airway constriction. The time to reach a doubling in ΔP represented a quantitative measure of airway reactivity*".

Other researchers, including Karlsson *et al* ²⁸⁵, have relied on approaches such as using a "*trained observer, who counted the number of coughs and noted the time of onset to bronchoconstriction*" ... which was defined as.... "*the abrupt onset of a slow laboured breathing with exaggerated abdominal movements which correlated closely in time with the onset of a pronounced wheezing from the chest and with a significantly altered air flow*" [tidal volume]. Sarlo and Clark ⁶⁶ in their intratracheal challenge defined as a significant respiratory reaction, "*diaphragmatic contractions [as scored visually] occurring at a minimum of every 36 to 40 normal breaths occurring over an observation period of 10 min*". Mitchell *et al* ³⁹ used as "*visual evidence of bronchoconstriction ... the presence of repeated cough, deep chest wall indrawing, and/or cyanosis*". Clearly then, there is a wide range of variously subjective endpoints that have been described in the literature.

In the present study there had been extensive and laborious review of the preliminary chart tracings in an attempt to rationalize and to reconcile them with the recent work of Karol's group. This involved manual (and tedious) breath-by-breath counting of 10 second increments of many metres of chart paper. More importantly, frequently-observed decreases in respiratory rate (accompanying the onset of respiratory distress / bronchoconstriction) confounded interpretation, as did the status of the animals (awake, and unrestrained): under these circumstances their normal body movements (and

chewing, grooming) caused characteristic and significant effects on the plethysmograph tracing. Of course, it was not until our endpoint criteria had been set that Rattray *et al*¹³⁹ published their very recent research, from which could be construed how naive the present investigator had been to regard increased and decreased respiratory rate as separate phenomena: these researchers covered both options, defining as a positive response a defined increase or decrease in respiratory rate. Although it had originally been envisaged that Karol's endpoint (percentage increase in respiratory rate) would be used in the present work, it ultimately proved necessary to devise separate endpoints for methacholine and TDI challenge; both related to exaggerated wave-forms (ΔP and time, respectively). Toward the end of the *in vivo* trials a system for digital data capture and analysis (MacLab[®]) became available, and some plethysmograph experiments were recorded in this manner (in parallel with the conventional chart record). It would appear, however, that modern conveniences can be disadvantageous: the rather sensitive TDI endpoint used in the present study (in particular) would likely be "missed" by those using electronic interpretation of their plethysmographic signals.

The construction of the plethysmograph assembly, and the development of the apparatus and protocols for challenge of the guinea pigs (and their validation) are described, below. The animals were to be challenged with methacholine to determine if their response showed any correlation with sensitization status (IgG level and TDI response). However, their response to TDI challenge was the ultimate measure of airway hyperreactivity, following sensitization.

3.2 Methods

3.2.1 Whole-body plethysmographic apparatus

The plethysmography assembly was adapted from Karol *et al*²⁷² and Wong & Alarie²⁷⁸, but was constructed largely from locally available materials: the plethysmograph chambers were modified pickle jars, and the pressure transducers intended for the automotive industry. A schematic representation of the plethysmograph apparatus is

shown in Fig. 3-1. In brief, four identical plethysmograph chambers were constructed as follows. Three holes were drilled in-line along the longest dimension (24 cm) of the 4 L glass jars to provide access ports (cut-off plastic syringes epoxied into place): one for air sampling and to provide input for the ΔP calibration signal (a small-animal respirator), and the other two for the pressure monitoring assembly. During use these ports were located along the tops of the chambers. A fourth hole was drilled into the original bottom of the jar (glass end of the chamber), matched by one through the metal (screw-top) lid. These two holes provided for a continuous throughput of air (with or without challenge agent) while the guinea pigs were situated within the chambers. Air was drawn from a central (cylindrical: 13×25 cm) mixing chamber (into which challenge agent was dispersed as necessary) through 22 cm Teflon[®] tubes (often called "straws") with a 2 mm inner diameter. As described by Wong and Alarie ²⁷⁸ the diameter of the tube was such that the resulting resistance-capacitance configuration caused chamber pressure changes due to animal respiration to be detected by the differential pressure transducer, despite the constant throughput of air. The flow of air from each of the four plethysmographs (1.6 L/min), as well as from the distal end of the mixing chamber (1.9 L/min), was effected by means of a Gilian Aircon 520 DC pump. The air lines were regulated individually by means of "critical orifices" (19 and 18 gauge hypodermic needles respectively, epoxied into the upper ends of their caps).

Differential pressure transducers (Motorola MPX10DP, one for each chamber) were installed onto custom-built circuit boards and powered by a 12 volt source. Each transducer had two fittings; one was connected to a 50 mL erlenmeyer flask and from there via a 1.8 m length of 1 mm i.d. plastic tubing to one of the ports on the top of the chamber; the other fitting was attached to the other port. The output signal from these transducers accordingly corresponded to the differential between instantaneous chamber pressure, and mean chamber pressure via a capacitance-resistance (hysteresis) circuit.

The output signal of each pressure transducer (volts) was determined as a function of imposed pressure differential (inches water gauge) in a simple U-tube device devised for

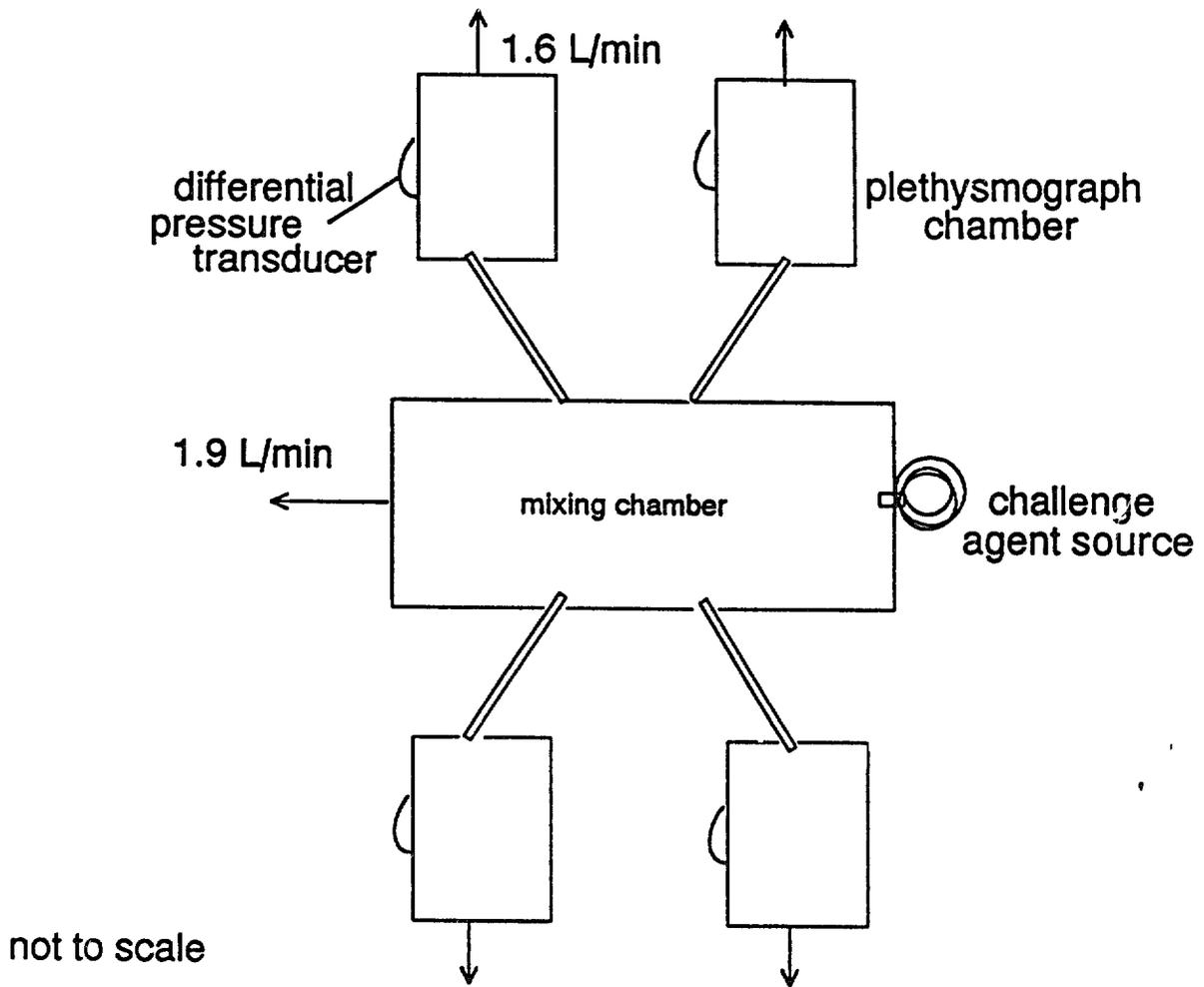


Fig. 3-1. Schematic representation of plethysmography apparatus.

this purpose. It was shown to be linear (see Fig. 3-2a); during the actual trials bridge voltage was balanced to ± 50 mV as part of the start-up procedure for each challenge experiment. A plethysmograph chamber was calibrated by syringe, to indicate correlation of transducer response with pressure (i.e. the volumetric change induced by syringe corresponding to tidal volume of the animal over part of the range; Fig. 3-2b); as this is a dynamic function (i.e. transducer response is dependent not only on the change in pressure due to the imposed air volume, but also on the rate of change) the peak heights in this respect do not necessarily correspond to absolute volume. Absolute peak height (volume of inspiration) was not a critical parameter in this study; within each trial a change in respiratory amplitude was gauged in relation to each animal's pre-exposure breath wave-form. Nevertheless, the plethysmographs were calibrated (at the beginning of every trial) to a Harvard small animal respirator in order to give equivalent wave forms. This is shown in Fig. 3-3, in which the calibration signal is sequentially superimposed on each of the four guinea pig breathing patterns. In this, as well as other representative plethysmograph tracings that follow, an upward pen deflection (or increased digital signal) corresponds to inhalation by the animal (and a decrease in the chamber pressure); correspondingly, downward deflection represents exhalation. This differs from the pattern seen in the conventional (hermetically-sealed) barometric plethysmograph, in which the basis of the signal is warming and humidification of the tidal volume, resulting in a thoracic expansion which exceeds the volume of inhaled air withdrawn from the chamber ²⁸⁶ and correspondingly, inhalation results in increased chamber pressure.

3.2.2 Vapour and aerosol inhalation challenge

In challenge trials the accuracy and (more importantly) precision of the atmospheric composition is critical, if there is to be comparison. Thorne and Karol ²⁸⁴ measured the concentration of histamine in the challenge atmosphere to which their guinea pigs were exposed, and found it to be only 4.6% of the nominal concentration. Rattray *et al* ¹³⁹

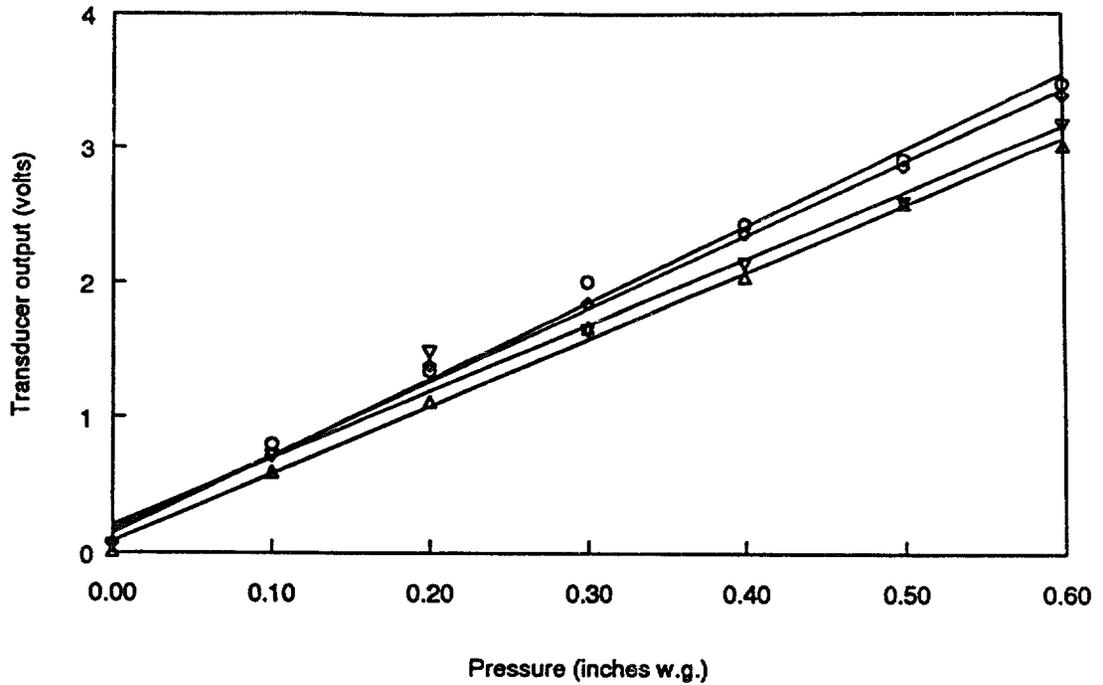


Fig. 3-2a. Plethysmograph pressure transducer voltage output with pressure. The indicated pressures were applied to the transducers by means of syringe, as indicated by water manometer; voltage output was recorded at each pressure, as indicated. Each symbol corresponds to one transducer. (1 in. w.g. = 2.54 cm)

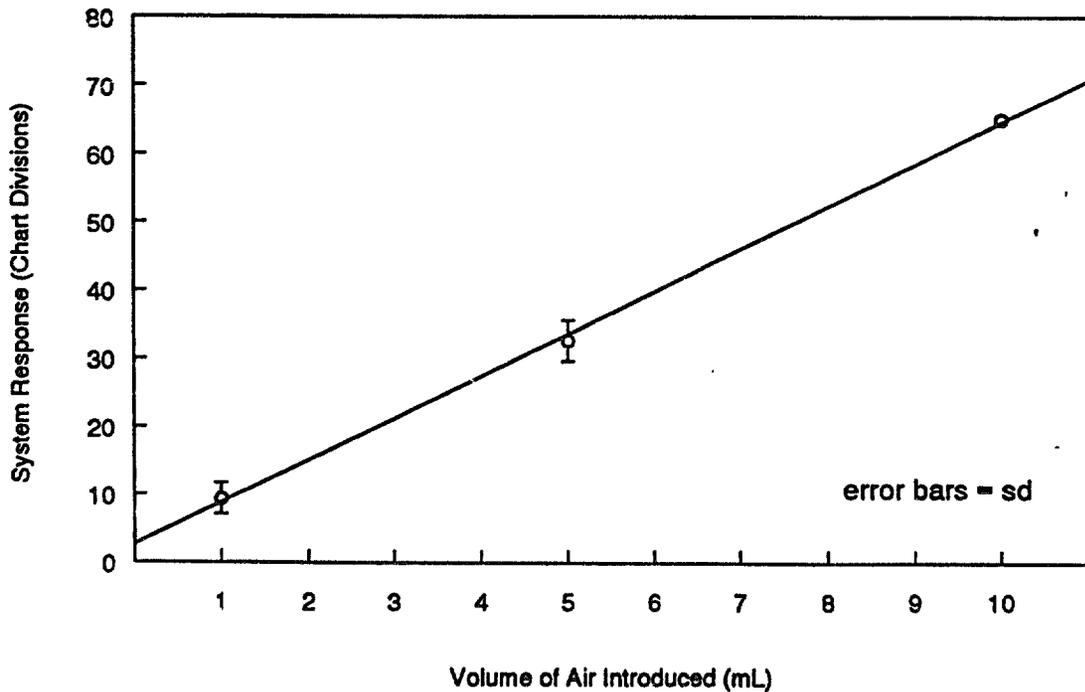


Fig. 3-2b. Plethysmograph chamber system response to calibration volumes. The response of a representative plethysmograph assembly to pressure changes was assessed by injecting the specified volume of air and noting the recorder deflection.

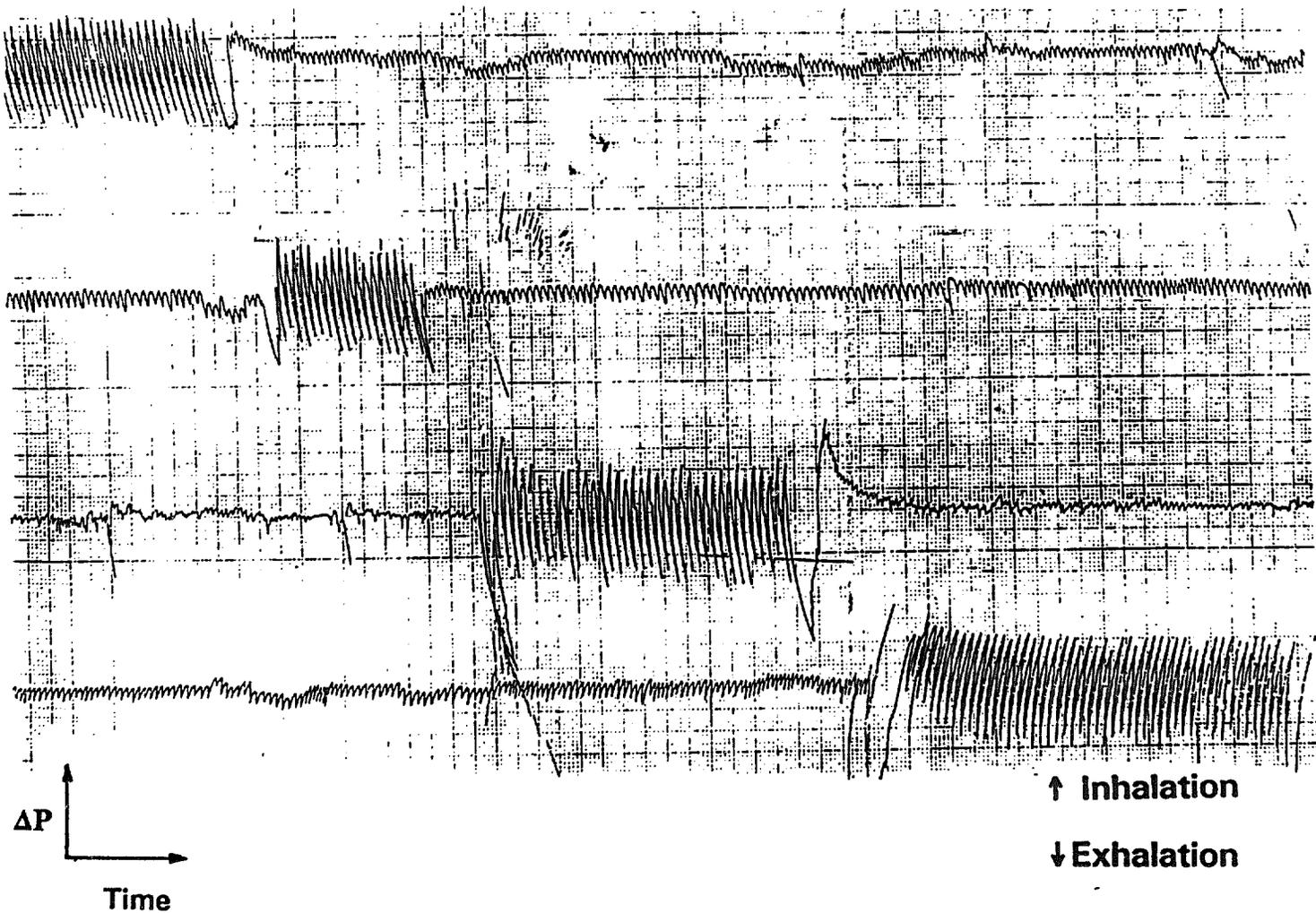


Fig. 3-3. Plethysmograph calibration signal superimposed on animal respiration. Each tracing represents the signal produced by an animal during steady breathing and in the absence of body movements; chamber calibration signals, produced by a small animal respirator (60 strokes per minute with stroke volume approximately 1.5 mL), are shown superimposed. Chart speed was 0.25 cm/sec; this figure has been reduced to 60 % size.

despite an inability to elicit a sensitization response with their MDI exposures, determined the airborne concentrations of challenge "isocyanate" (MDI) by gravimetric means only (i.e. weighing the amount of aerosol collected on a filter, and without regard for the reactivity of the NCO group: we do not know whether the aerosol in question showed any characteristic isocyanate reactivity).

Clinically, where methacholine is used, protocols have variously called for the use of pre- and post- nebulization differential in nebulizer weight as a measure of aerosol dose administered²⁸⁷. Such an empirical determination does not correspond to aerosol output, let alone inhaled dose; some²⁸⁸ have been highly critical of this approach. Extensive characterization of challenge atmospheres was conducted in the present study. There were fundamentally two types of atmospheres used for calibration and/or challenge purposes: aerosol (sodium chloride, methacholine) and vapour/gas (TDI, CO₂).

3.2.2.1 Atmosphere generation and validation

Aerosols were generated from the appropriate aqueous solution by means of a standard Wright nebulizer. For aerosol challenges the system calibration was performed with sodium chloride. The aerosol output from the Wright nebulizer was characterized in terms of size distribution, by means of a six stage Marple mini-cascade impactor. A 2% NaCl solution was nebulized into a chamber by means of filtered, compressed, air, at a flow of 6 L/min. Aerosol was collected from the chamber atmosphere onto the pre-weighed Marple stages by sampling air (at a flow rate of 2 L/min) with an SKC constant flow personal sampling pump. The mass of aerosol collected in each fraction was determined gravimetrically. The pooled results of three such determinations, conducted five to seven days apart, are shown in Table 3-2. The mass median diameter was determined to be 1 μm , with a geometric standard deviation of 2.7 (i.e it is polydisperse, and of respirable size).

Table 3-2. Size distribution of aerosol produced by Wright nebulizer; Cumulative % aerosol less than cut-off size of stage

Stage	Cut-off (μm)	Cumulative % <; Mean (sd)
3	10	99 (1.0)
4	6	96 (1.1)
5	3.5	90 (0.2)
6	1.5	66 (1.2)
7	0.9	41 (5.4)
8	0.5	22 (2.5)
Filter	0	0

The size distribution of the aerosol produced by the Wright nebulizer was determined by discharging NaCl from a 2 % solution at a flow rate of 6 L/min into a chamber, and collecting specific size fractions onto the pre-weighed stages of a Marple series 290 six stage personal cascade impactor. These were weighed, and expressed on log-normal paper as cumulative particle size distribution: percentage of total mass collected on each stage below the equivalent aerodynamic diameter (D_p) cut-point (specified for that stage by the manufacturer, based on the sampling rate of 2 L/min), as a function of aerodynamic diameter. The 50 % intercept was taken as the mass median diameter.

The overall challenge system was also calibrated with NaCl aerosol. Guinea pigs were sealed into the plethysmograph chambers, and a 2% NaCl solution was nebulized for one minute into the mixing chamber at a rotameter setting of 40. Matched MIE miniRAMs (forward light-scattering direct-reading aerosol monitors), equipped with a dynamic sampling attachment, were used to measure aerosol levels at the nebulizer output ("tap"), and at the sampling port of one of the plethysmograph chambers. Total airflow into each of the plethysmograph chambers was the same during routine operation as during sampling, thereby avoiding sampling artefact. Such determinations, at least in triplicate, were made with each plethysmograph chamber. In each case the maximum aerosol reading was noted, and the analog signal was recorded on a strip-chart recorder. Fig. 3-4 shows a representative tracing of the two miniRAM signals; note that the gain of the two channels is not the same, and also that time progresses to the left. The tracings were photocopied and the area-under-the-curve (AUC) in case was determined by the cut-and-weigh method. Over a series of 15 determinations involving all four chambers, the mean (sd) tap maximum mg/m³ : chamber AUC (determined in weight units, but considered dimensionless) was 0.6 (0.12) and the tap maximum : chamber maximum was 36.5 (8.8). When a longer time factor was incorporated into the comparison, there was less of a difference: in a manner analogous to the CO₂ gas-phase calibration (below), 2 % NaCl was nebulized into the mixing chamber at a rotameter setting of 40, and at equilibrium the following aerosol levels were recorded at the tap and plethysmograph chambers (mg/m³, as mean and sd of three determinations):

<u>Nebulizer</u>	<u>Pleth # 1</u>	<u>Pleth # 2</u>	<u>Pleth # 3</u>	<u>Pleth # 4</u>
44.9 (4.4)	4.5 (0.2)	4.4 (0.4)	4.7 (0.2)	4.8 (0.5)

Clearly, there was good consistency in delivery of aerosol to the chambers.

The miniRAMs were also calibrated gravimetrically to methacholine: over 7 determinations under varying conditions the gravimetric:miniRAM methacholine ratio was equivalent to 24% i.e. the 'true' airborne levels of methacholine are approximately 1/4 of the reported instrument readings. Accordingly, 'true' chamber maximum aerosol levels would correspond to 0.007 of the reported "tap" maxima. Further calibration was to be done (e.g. empirical gravimetric AUC methacholine determinations from the

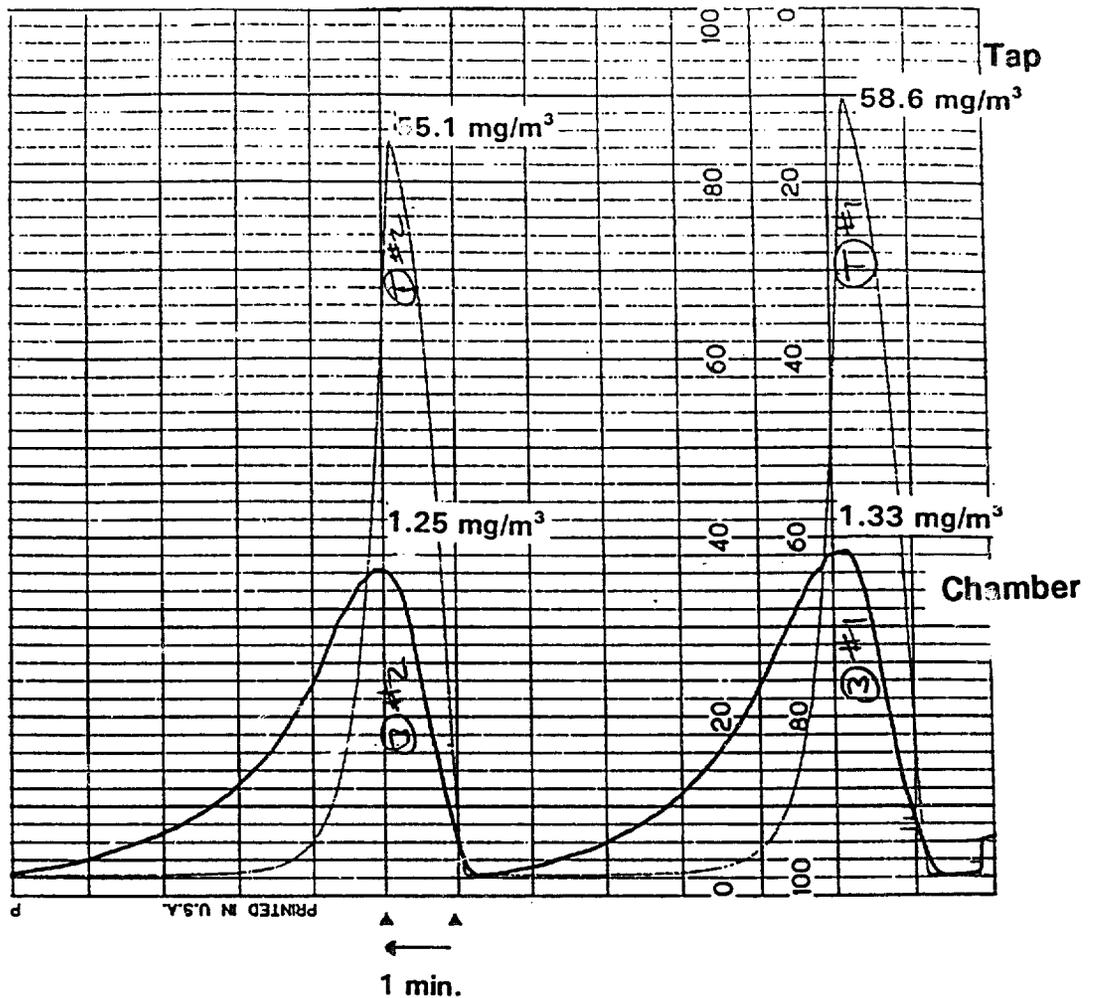


Fig. 3-4. Rise and fall of aerosol level at nebulizer tap and in plethysmograph chambers; calibration with sodium chloride. NaCl was aerosolized for sixty seconds into the plethysmograph assembly mixing chamber as a 2 % solution from the Wright nebulizer by means of compressed air operating at a rotameter setting of 40. Matched MIE miniRAMs (attached to a two-channel strip-chart recorder with gain settings of 50 mV and 1 V) were used to simultaneously monitor the aerosol level at the nebulizer "tap" fitting, and at the sampling port of a plethysmograph chamber (with guinea pig *in situ*), for this period and five additional minutes (until aerosol was depleted from the chamber). This was done at least three times with each of the chambers. The upper peak and concentration correspond to the aerosol level at the tap, and the lower one to the plethysmograph chamber.

chambers to provide an absolute measure of dose), but there was not the opportunity to do so. In any case, the consistency of the methacholine data was good (see Table 3-3), and the animals were compared with each other, under the same sets of conditions.

To validate the delivery of vapour-phase test material (TDI), carbon dioxide was used as a tracer gas, to verify that all chambers received an equivalent proportion of test agent infused into the central mixing chamber. It was used here in place of TDI, due to its lower toxicity, readily available supply and greater ease of measurement. Carbon dioxide was bled from a cylinder at a variety of rotameter settings and monitored in each chamber (at equilibrium) by means of Riken 411A non-dispersive infra red analyzer, calibrated to span gas and zeroed to nitrogen. Since ambient air contains (nominally) 350 ppm CO₂, and human occupancy further increases indoor levels, the curves shown in Fig. 3-5 were adjusted arithmetically to obtain origin interception in the calibration curve. Note that even where the points do not fall on the curve, likely due to the nonlinearity of the rotameter, they do coincide with each other, again confirming equivalence of challenge agent distribution. Due to the substantial carbon dioxide output of the guinea pigs, it was not possible to perform this calibration with the animals *in situ* (i.e. the operating range of the CO₂ monitor was exceeded). The time profile of TDI in a plethysmograph chamber was monitored by means of GMD Autostep instrument (Fig. 3-6); it is provided only as a representation of the shape of the TDI flux curve. Integrated sampling of TDI (indicating dose of isocyanate received by the animals) was conducted from a plethysmograph chamber, both with and without animals *in situ*, according to the Marcali method; the results are presented in section 3.2.2.3 (Table 3-3).

3.2.2.2 Animal challenge

The animals were evaluated as groups of four, three sensitized and one control. They were brought from the colony in transfer boxes on hardwood chips; water was withheld for several hours prior to the start of the trial to minimize the likelihood of the animal voiding within the plethysmograph chamber. Urine in the chamber tended to cause some disturbance of the animal due to the "slipperiness" of the animal's footing, resulting in a more erratic plethysmograph signal. The trials were typically conducted between 1800

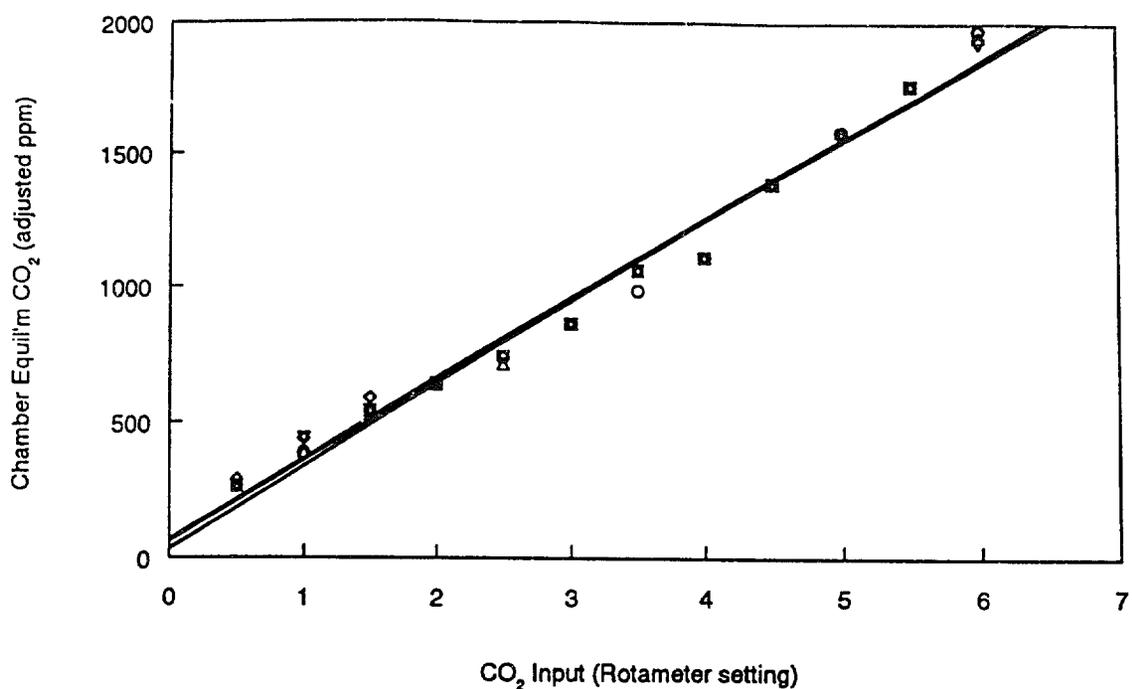


Fig. 3-5. Plethysmograph vapour phase calibration using carbon dioxide. Carbon dioxide was infused into the plethysmograph assembly mixing chamber at the indicated rotameter flow rates; the concentration of CO₂ was determined in each chamber (represented by the four different symbols in the figure) at steady state by means of Riken 411A infra-red analyzer. The values have been normalized to a zero intercept to account for background carbon dioxide.

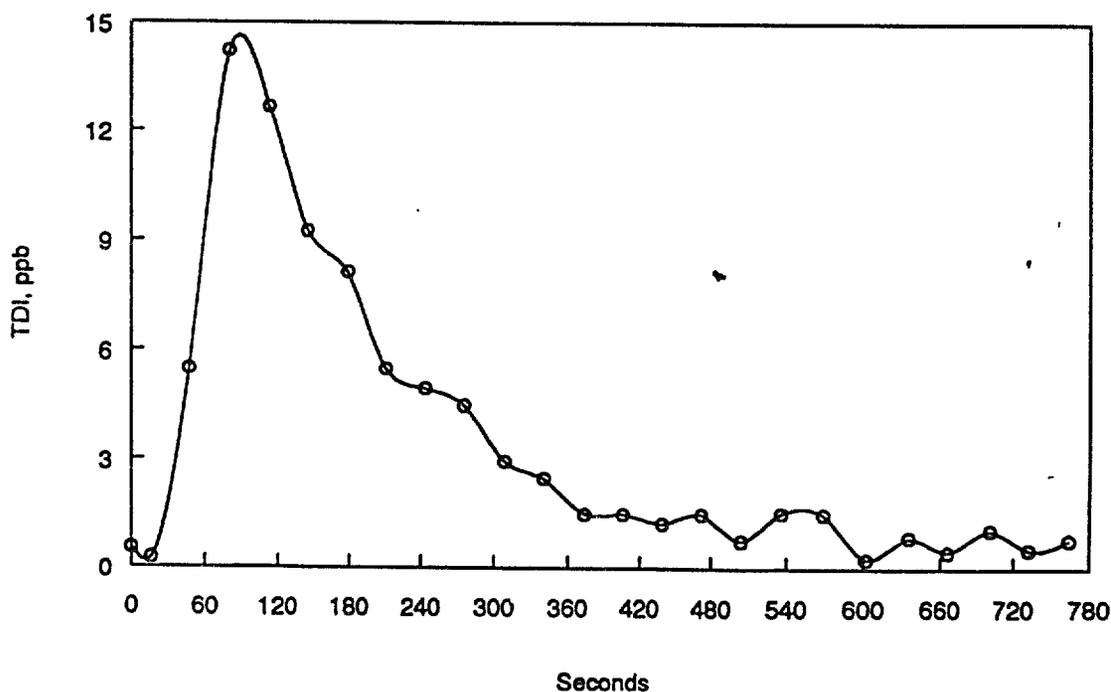


Fig. 3-6. Plethysmograph chamber TDI concentration profile. TDI was discharged into the plethysmograph assembly mixing chamber at room temperature by blowing dry air through the volatilizer for 60 seconds. The resulting airborne level of TDI in a plethysmograph was monitored by means of GMD Autostep paper tape sampler. The curve represents the mean of three determinations.

and 2400 hours, to avoid unwanted stimulation by distracting influences in the laboratory, and also because this represented the time that they would normally "settle" for the night. The animals were placed into the chambers on a randomized basis during the initial trial of a series; chamber allocation was rotated in subsequent trials in that series. As the chambers were sealed up, the main air moving pump was switched on (9.6 L/min rotameter setting). Air flow through each chamber was verified as being 1.6 L/min by means of Kurz mass flow meter (model 580 "Pocket flow calibrator"), to confirm that the critical orifices had not clogged, or that there were no inboard "leaks" (inflow other than via the Teflon® straws) in individual plethysmograph jars; adjustment or sealing of the chambers was performed, as necessary. The signal amplitude from each plethysmograph transducer was normalized to approximately 4 divisions (2 cm) on the chart recorder (Harvard Apparatus model 480, typically operating at 0.25 cm/sec) by means of a wave form from a Harvard Apparatus Rodent Respirator model 681 (nominally 60 strokes per minute), superimposed on the animals' breathing (Fig. 3-3).

The animals were allowed to adjust to the chambers until their level of activity had become reasonably stable. Once they had settled down (typically after a half an hour or so) the chart recorder (and later, on occasion, the MacLab® as well) were run for several minutes to establish a new baseline set of parameters. The challenging was generally initiated once the animals' breathing pattern had stabilized. A representative result of the manual determination of respiratory rate (Fig. 3-7) and another one produced by the MacLab® system (Fig. 3-8) are shown. In the first case, note the variability in the respiratory rate, in part because 10 second increments are being used to represent 30 second time periods. These apparent aberrances in respiratory rate are partly due to what may have been rapid chewing actions by the guinea pigs, or other mechanical signals similar to a breath wave form; the bottom half of the figure shows the raw tracing from which the respiratory rate data was extracted. Note the flattened wave form in the bottom tracing; this is described later as delayed exhalation (DE). With the MacLab®, digital data management was possible, and it could provide respiratory rate directly from the ΔP signal (with various parameters of a "breath" defined by the user, e.g. as "threshold" and "hysteresis"). In Fig. 3-8 note (say, with channel number 3) that with

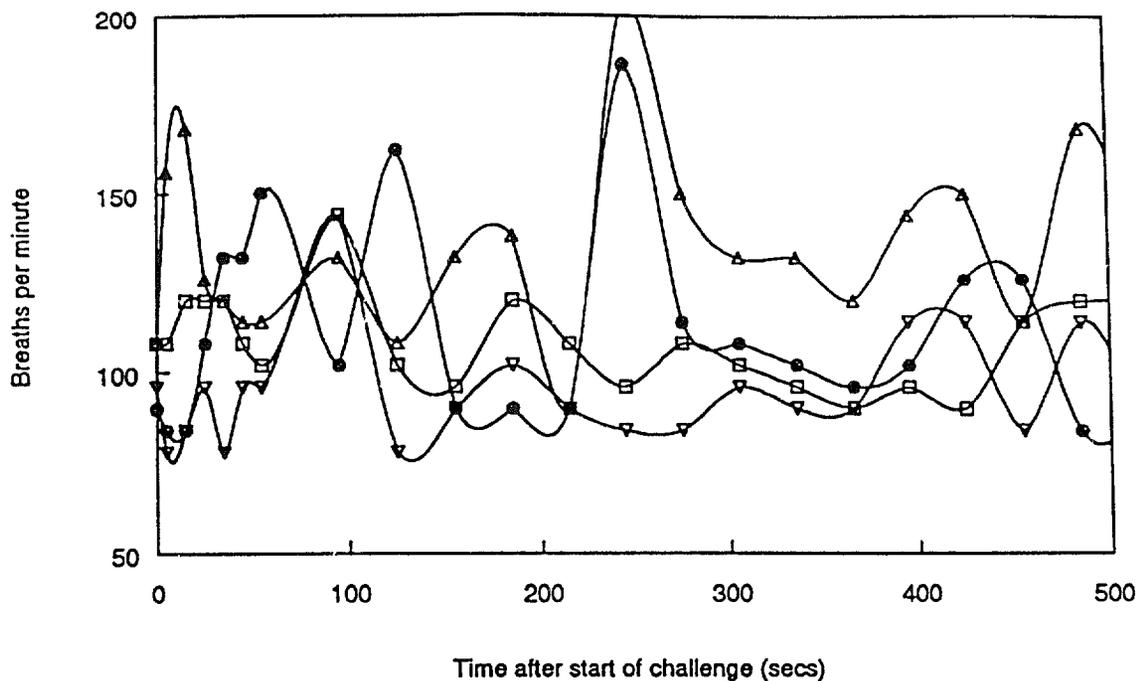


Fig. 3-7. Preliminary respiratory rate pattern determined manually from plethysmograph tracing. This was determined in the developmental phases by manually counting the number of ΔP peaks ("breaths") in 10 second increments of the strip chart record of plethysmograph pressure. The above data relate to a low-level TDI exposure of a group not incorporated into the final study. The circle represents the control animal, which corresponds to the top tracing in the figure below.

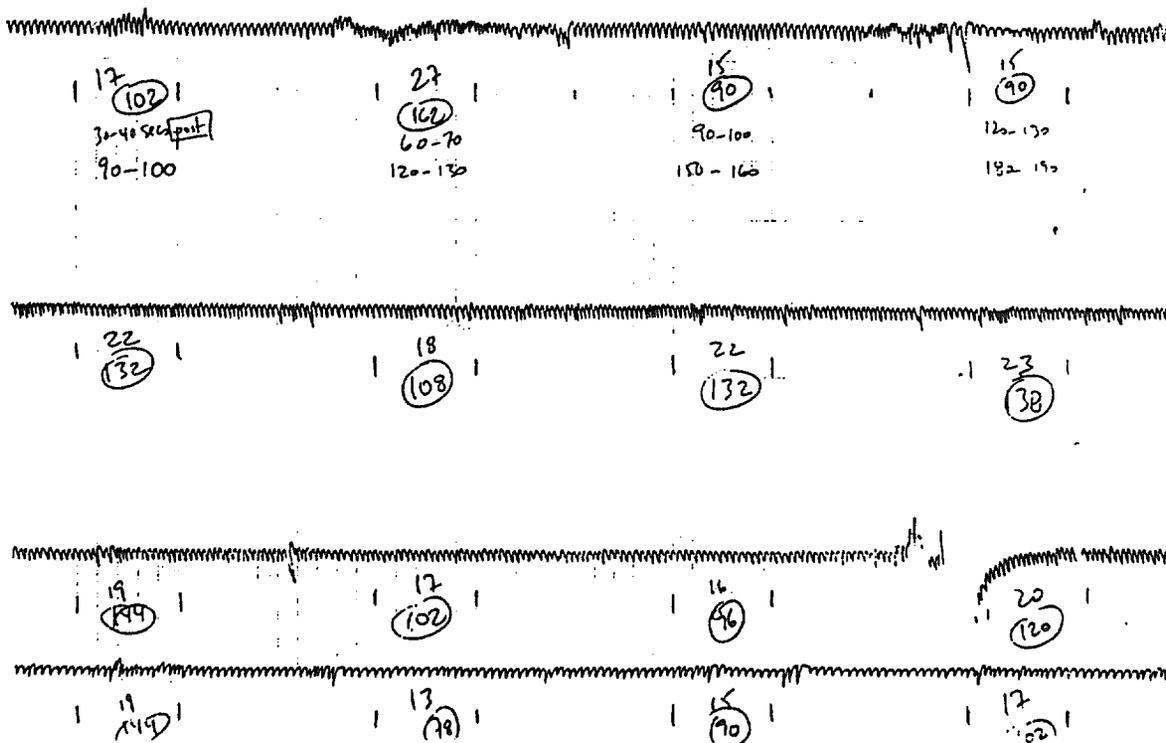


Fig. 3-7b. Plethysmograph tracing corresponding to respiratory rate pattern.

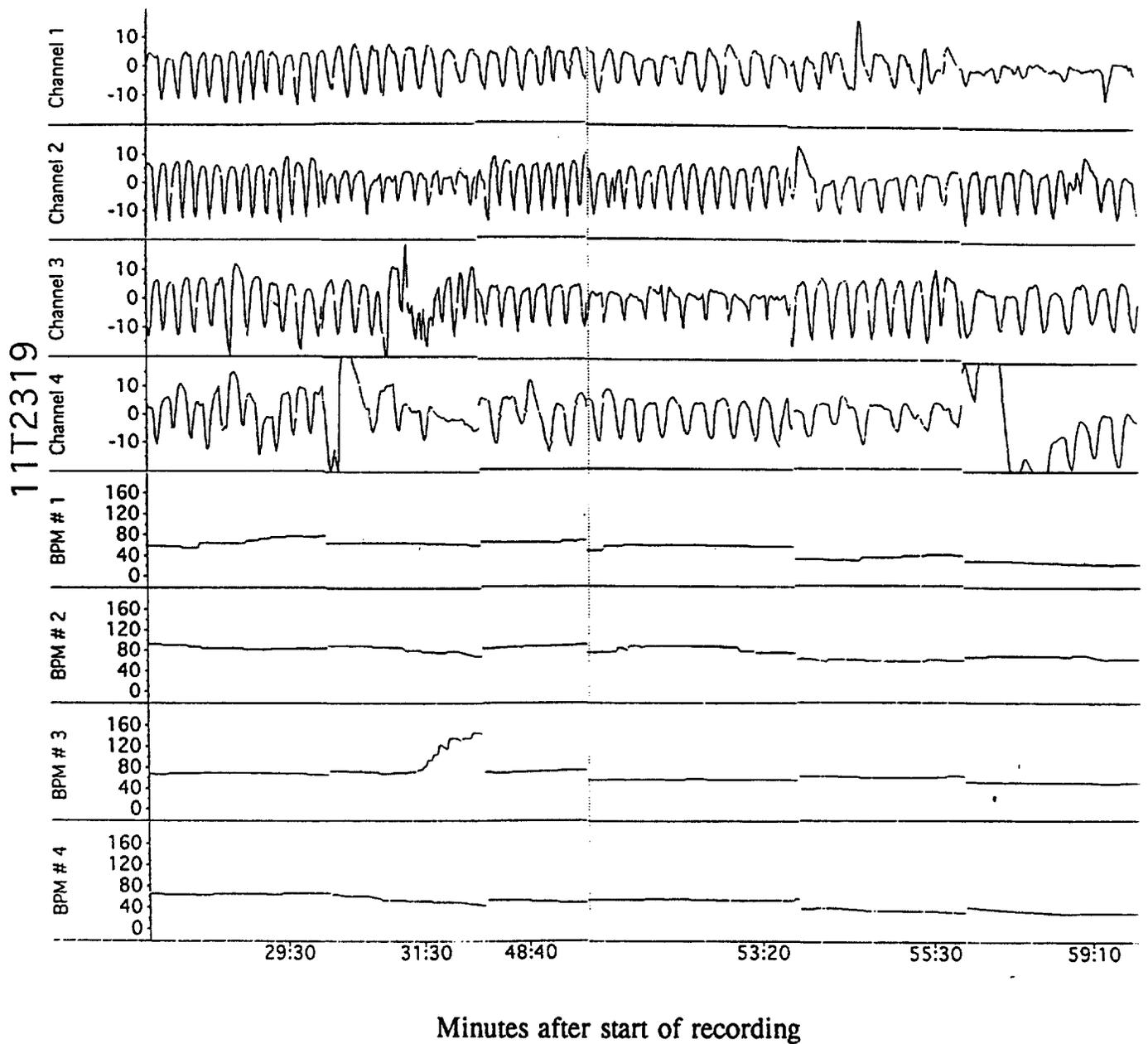


Fig. 3-8. Breathing pattern and respiratory rate determination made by MacLab®. The parameters were set as 10 mV range, 2 sec/div, threshold -6, hysteresis to -2. The top four channels represent the ΔP signal, and the bottom four the ratemeter output (BPM = breaths per minute), obtained with group 93/11. The timing marks along the abscissa indicate minutes after the start of recording during this series of challenges.

progressing time (and increasing challenge dose) the top of the wave forms becomes increasingly flattened (again, DE); despite the dramatic change in this parameter, there is no remarkable change in the respiratory rate.

3.2.2.3 Methacholine challenge

Methacholine challenges were performed by aerosolizing a 10 mg/mL solution (total volume 10 mL) of methacholine (Sigma A2251) in water, by means of a Wright nebulizer situated in a water jacket (at ambient temperature) and driven by filtered and humidified compressed air. The thermal and humidity control measures were predicated by preliminary work which had demonstrated an initial, rapid peaking of aerosol agent output by the nebulizer (despite continued application of compressed air), putatively due to physical factors; this work is described in Appendix 1. The discharge from the Wright nebulizer was monitored during each challenge by drawing air (at a constant rate) from the discharge tube ("tap") leading to the mixing chamber and passing it through a GCA miniRAM, as described previously. In this manner it was possible to assign a relative index of exposure to each challenge, without disruption of the chamber kinetics.

The animals were exposed to methacholine on two consecutive days. On each occasion, they were challenged by blowing air through the Wright nebulizer for a period of 60 seconds at an initial rotameter (Cole Palmer 6MFV) setting of 30. Their breathing was subsequently monitored / recorded for an additional period of at least four minutes. The next (higher) challenge dose (an increment of 5 on the rotameter) was then initiated; this dosing approach is analogous to that used clinically. Animals responding to the point of respiratory distress / collapse were removed from their chamber(s), whereupon they recovered spontaneously within seconds. If other animals remained, the vacated chambers were re-sealed prior to the next challenge, to maintain a consistent distribution of challenge atmosphere. During the trial phase involving TDI challenge, the animals were re-challenged with methacholine, immediately following their second consecutive day TDI challenge.

3.2.2.3 TDI Challenge Atmospheres

The challenge atmosphere was generated by blowing desiccated air through / across 300 μL of TDI in a "volatilizer" (modified midget impinger) and situated in turn in a water-jacketed vessel that was operated either at room temperature or at 46 °C. As with methacholine, the challenge agent was actively generated for a 60 second period at varying rates, corresponding to specific rotameter settings; the animals' breathing pattern was monitored for 5 minutes. The corresponding flow rates of air into the mixing chamber, and 5 minute average TDI concentrations (in ppm) in the plethysmographs are shown in Table 3-6. These were determined by sampling and analysis according to the Marcali method ⁵⁹ on three separate days from plethysmographs not containing animals, but sealed 700 mL glass bottles instead, as "surrogates". This was to provide a measure of delivered dose, free of animal effects; more limited analyses with animals in place were also conducted.

3.3 Results

3.3.1 Methacholine challenges

Table 3-3 shows the air flow rate (L/min) and the peak "tap" (miniRAM) methacholine levels corresponding to the most commonly used rotameter settings. However, in the post-TDI challenge exposures, nebulizer outputs of up to 79 mg/m³ had to be used to achieve the defined end-point. The consistency of nebulizer outputs shown in Table 3-3, together with the CO₂ data indicating equivalent distribution of atmosphere to the plethysmographs from the mixing chamber, indicate that the animals were receiving equivalent challenge doses, regardless of chamber.

The endpoint was defined in terms of ΔP , the amplitude of the breathing wave form. This was measured directly from the chart record at the time corresponding to the start of each challenge. The point after the initiation of challenge at which ΔP had doubled from baseline for at least three consecutive breaths (i.e. into a bronchoconstriction phase), was taken as the endpoint. In a few instances, a doubling was not achieved despite imminent animal collapse (* in Fig. 3-9), and the onset of the greatest ΔP was then taken as the endpoint.

For every challenge with a positive outcome, the response of each animal was expressed in terms of the concentration of methacholine required to initiate the response and, the time of exposure to that particular challenge at which the endpoint was reached. Intuitively, if two animals respond to the same challenge dose, yet one experiences bronchoconstriction first, then this should be classified as being the more sensitive animal. This approach would be similar to that of Thorne & Karol²⁸⁴, who factored time-to-endpoint into their measure of airway reactivity, as well as to Karol's original Respiratory Index¹³⁵. As part of the development of the endpoint criterion, an overall comparison / rating of animal responses was based on various composites of time and concentration, attempting to arrive at a mathematical model that would "weight" the two in an appropriate manner, such that an animal's response on day 1 would correlate best with its response on day 2; it was felt that this would yield the most valid or specific

Table 3-3. Reproducibility of methacholine output from nebulizer			
Rotameter setting	Airflow (L/m)	Mean (sd) peak methacholine output (mg / m³)	
		20 runs	Post- TDI runs only
30	5.7	11.0 (1.6)	11.8 (1.4)
35	6.8	20.9 (3.4)	21.9 (0.7)
40	7.8	33.2 (5.5)	30.0 (1.9)
45	8.7	49.6 (5.8)	47.4 (2.6)

The methacholine output from the nebulizer was determined for each challenge by means of GCA miniRAM and recorded as the maximum concentration achieved at the "tap" fitting into the discharge outlet. The values are shown as a function of rotameter setting and the flow rate (determined by "DryCal"). The first column includes the 7 challenge tests shown in the second column.

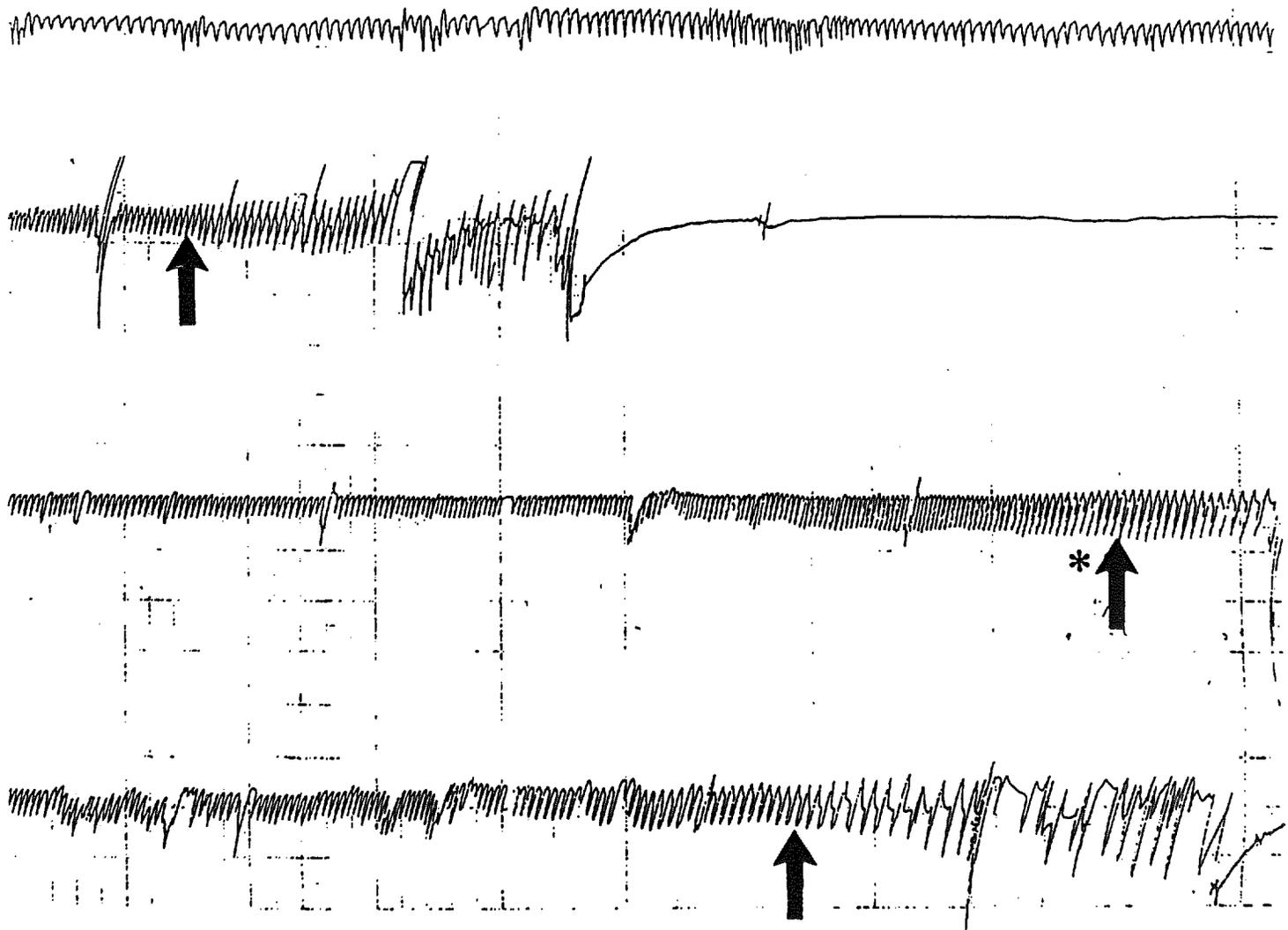


Fig. 3-9. Plethysmographic methacholine challenge endpoint.
The plethysmographic response to methacholine challenge of 3 of 4 animals in group 8/9 is represented in this figure. The arrows denote the point at which the endpoint (as defined, or maximum ΔP prior to respiratory collapse *) was identified.

parameter of sensitivity. Many iterations were attempted; shown in Figures 3-10a (same-day, inverse of concentration \times time) and 3-10b (inter-day, concentration \times time) are the results of some of the simpler algorithms. A somewhat better correlation was obtained by adjusting the log of the concentration by a factor of 3.7 and the log of the time by 0.9. However, overall, the "time to response" appears to be an unimportant determinant relative to the concentration (see Figs. 3-11a and 3-11b; plots of concentration vs. time after the start of exposure at that concentration). Although there was a significant relationship on day 1, there was none on day 2. This might have been due to the use of an inappropriate time frame in these instances: since it takes the minute of nebulization to achieve a peak concentration of challenge atmosphere in the plethysmographs, the total time may not represent a sufficiently sensitive index i.e. the critical time span, namely the actual exposure time to peak challenge agent, is "diluted" by the initial period of non-exposure. However, various arithmetic manipulations were also attempted without any better results. For example, time adjustments by 60, 80 and 90 seconds resulted in non-significant correlation coefficients of 0.17, 0.01 and 0.06, respectively. Fig. 3-12 shows the simplest plot, namely the peak tap concentrations required (per animal) on the two challenge days, to achieve the endpoint. It shows a highly statistically significant correlation; this again suggests that dose or concentration is a more important determinant of sensitivity, than is time-to-response.

Nevertheless, group mean responses were considered in two ways: mean (sd) "concentration \times time to endpoint" values for the two methacholine challenge days are provided in Table 3-4. Since three of the groups had been challenged with airborne TDI earlier, these are disregarded in the first set of values, but included in the second. These data indicate that, in terms of methacholine responsiveness, TDI sensitization / pre-exposure / antibody titre is without effect. There were no significant differences between sensitized and control animals, as might be anticipated from an inspection of the data. The response to methacholine was also compared to the TDI-IgG antibody level, by animal. Inspection of Fig. 3-13 shows that the two are not related ($r = 0.04$); both in the control and sensitized groups of animals, (i.e. low and high ELISAs, respectively), the methacholine sensitivity spanned an equivalent range.

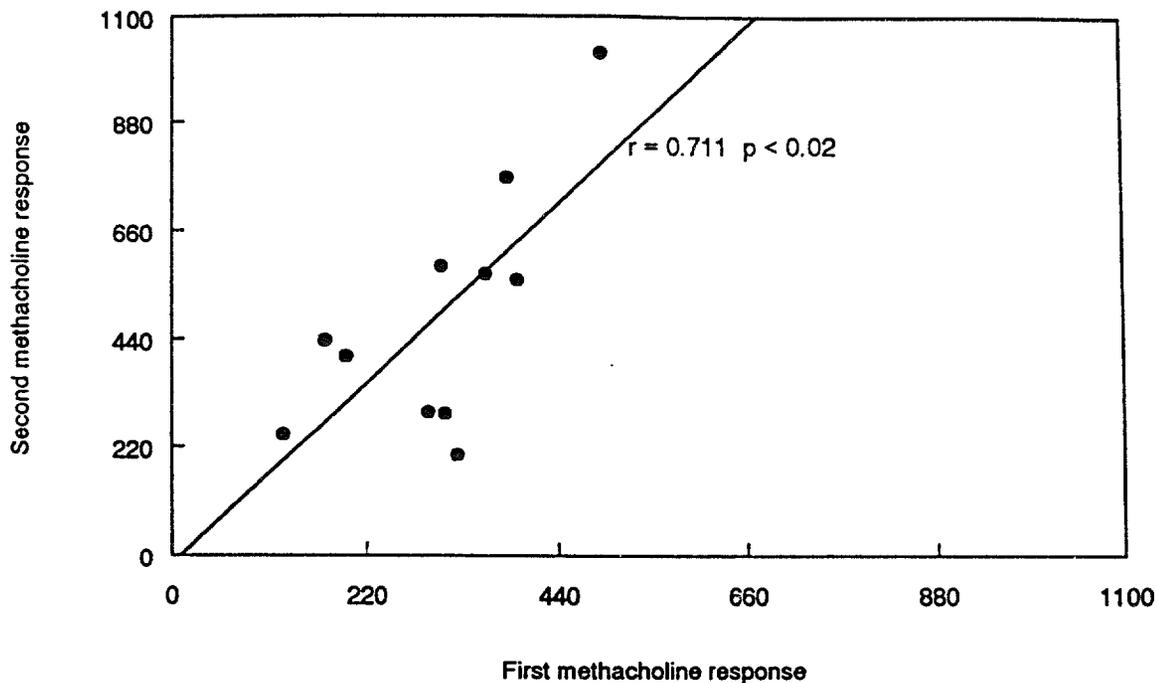


Fig. 3-10a. Intra-day comparison of methacholine endpoints expressed as reciprocal of product of endpoint concentration and time, $\times 10^6$.

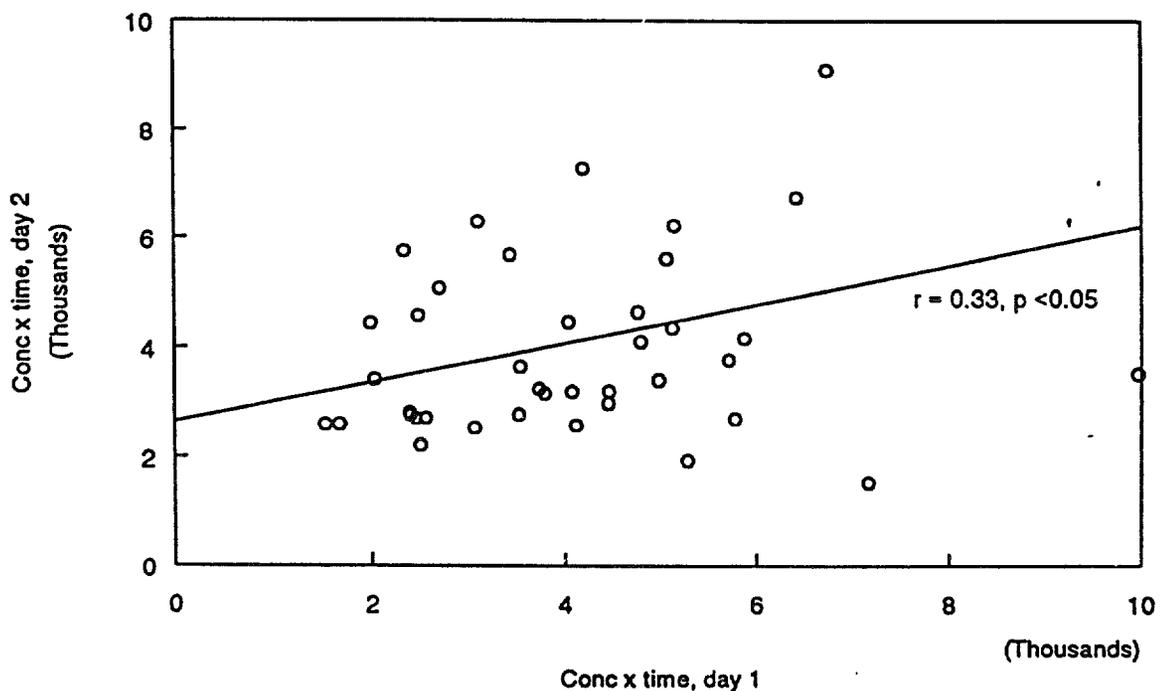


Fig. 3-10b. Inter-day comparison of methacholine endpoints, expressed as product of concentration and time.

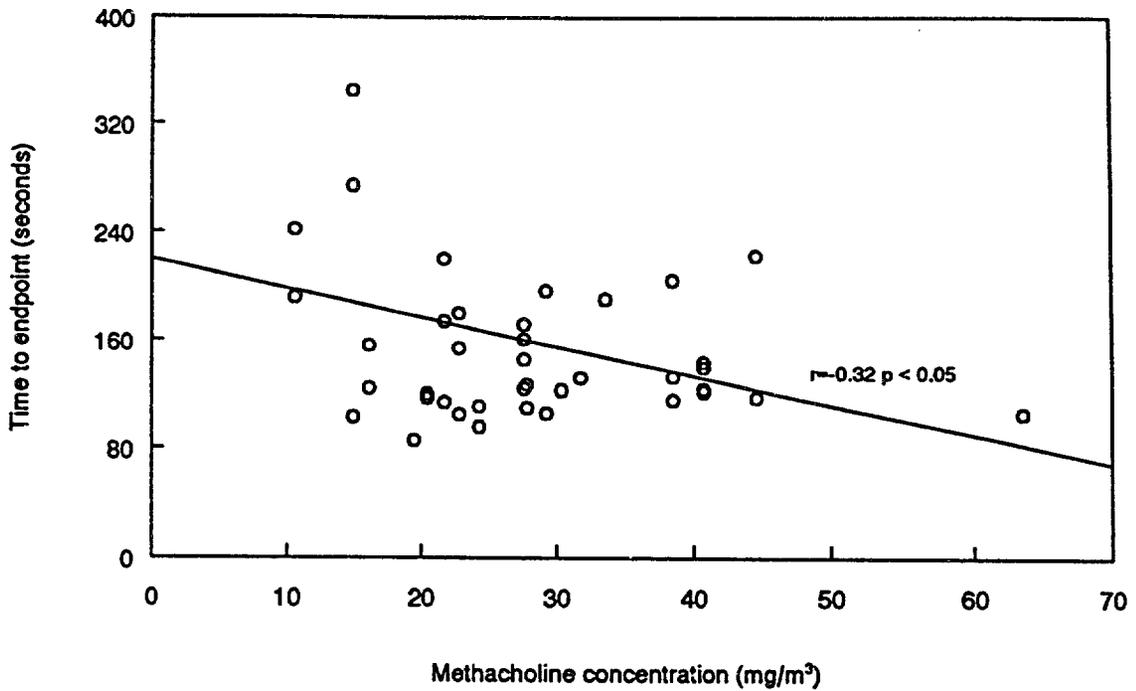


Fig. 3-11a. Methacholine output concentration versus time to endpoint; day 1 of challenge. The peak methacholine nebulizer discharge level at which the animal achieved endpoint is plotted against the time span required after the initiation of that dose, to achieve the endpoint, by animal.

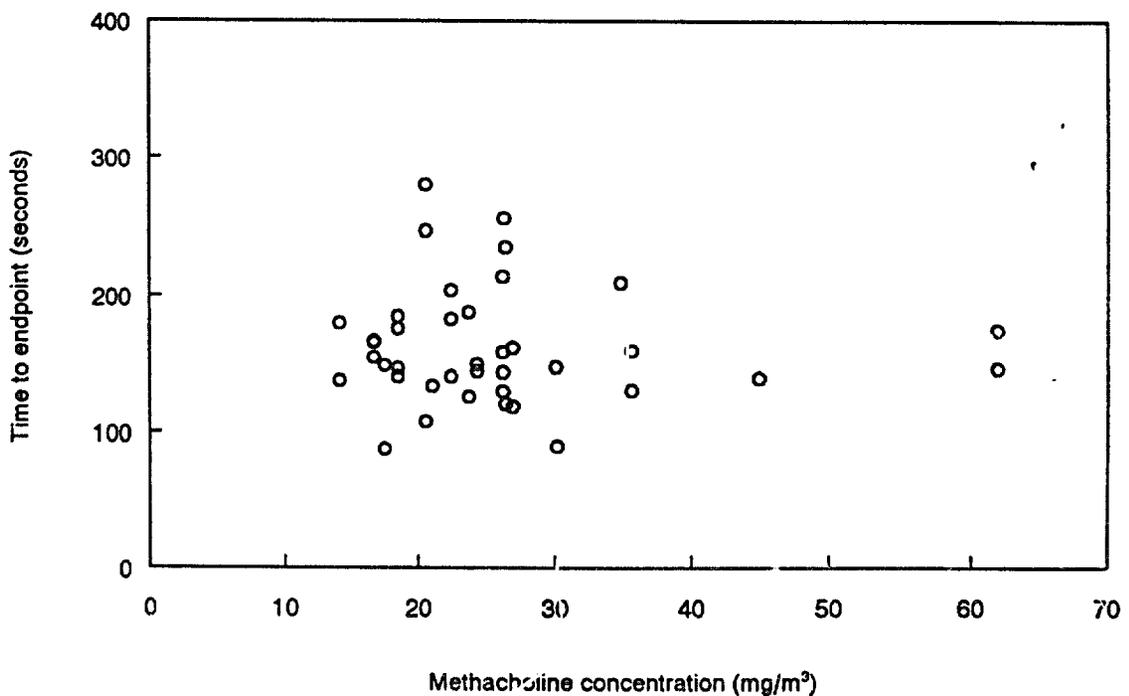


Fig. 3-11b. Methacholine output concentration versus time to endpoint. As above, day 2 of challenge. ($r = 0.05, NS$)

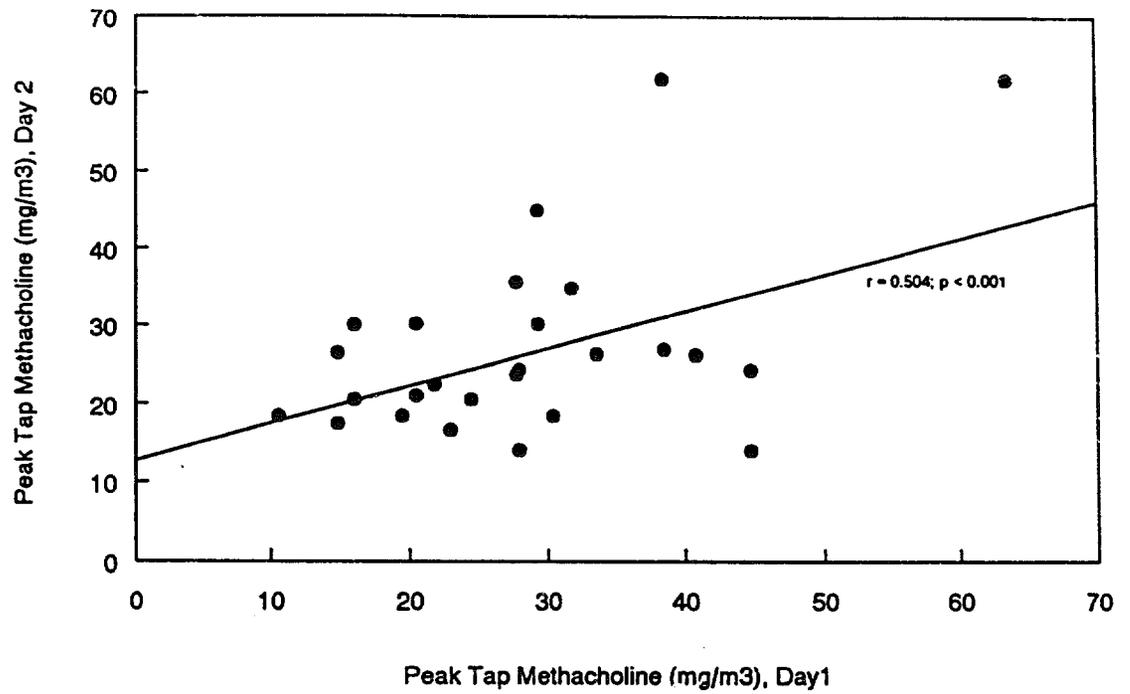


Fig. 3-12. Correlation of consecutive day nebulizer methacholine outputs, to achieve endpoint, by animal.

Once the animals had been challenged with airborne TDI (a few weeks after these methacholine challenges) they were re-assessed for methacholine sensitivity immediately at the end of the second day of TDI challenge. The mean methacholine levels required to achieve the endpoint, for all three challenges, are shown for control and sensitized animals in Table 3-5. The post-TDI methacholine challenge levels required to reach endpoint were significantly greater than the corresponding pre-TDI levels ($p < 0.01$); the respective increases required for control and sensitized animals (116% and 146%) were not significantly different from each other ($p = 0.09$). This decrease in reactivity to cholinergic spasmogens immediately following TDI challenge differs from the report of Sheppard *et al*^{178,179,182,289} and others^{180,290,291} that TDI exposure increases the reactivity to acetylcholine, but could be due to the difference in the specific agonist (i.e. methacholine rather than acetylcholine). More significantly, the other work did not involve "sensitized" animals, but TDI exposures of hours to days in duration.

Table 3-4. Methacholine response of TDI-sensitized and control animals. Exposure units = Methacholine "tap" concentration x time to endpoint, from start of nebulization at that rotameter setting

Animals	n sensitized / control	Day 1		Day 2	
		sensitized	control	sensitized	control
Not previously TDI-challenged	20 / 8	3584 (1888)	3760 (1702)	3694 (1780)	3732 (1692)
All	29 / 11	4178 (1781)	4199 (2043)	3972 (1662)	4604 (2578)

Table 3-5. Mean (sd) methacholine "tap" peak concentration (mg/m³) required to achieve endpoint. Consecutive day challenges and, after TDI challenge

	Day 1	Day 2	Mean	Post TDI ¹	Difference	% increase
Control animals (n=11)	27.3 (10.9)	26.4 (13.4)	26.8 (9.9)	49.4 (13.2)	24.8 (14.7)	116
Sensitized animals (n=29)	27.9 (1.4)	25.8 (9.5)	26.8 (9.4)	57.6 (13.3)	32.7 (15.1)	146
Both ¹ (n=40)	27.7 (11.1)	26.0 (10.5)	26.8* (9.4)	55.4* (13.6)	30.5 (15.2)	138

1. For the post-TDI challenges, n = 36; accordingly, the difference relates to this number of animals
* p < 0.01

These tables provide two different numerical representations of the methacholine trial outcomes, in both cases separating the animals into control and sensitized categories. Table 3-4 presents the endpoint as a factor of methacholine concentration and time to endpoint at that dose. Table 3-5 only involves the nebulizer output concentration.

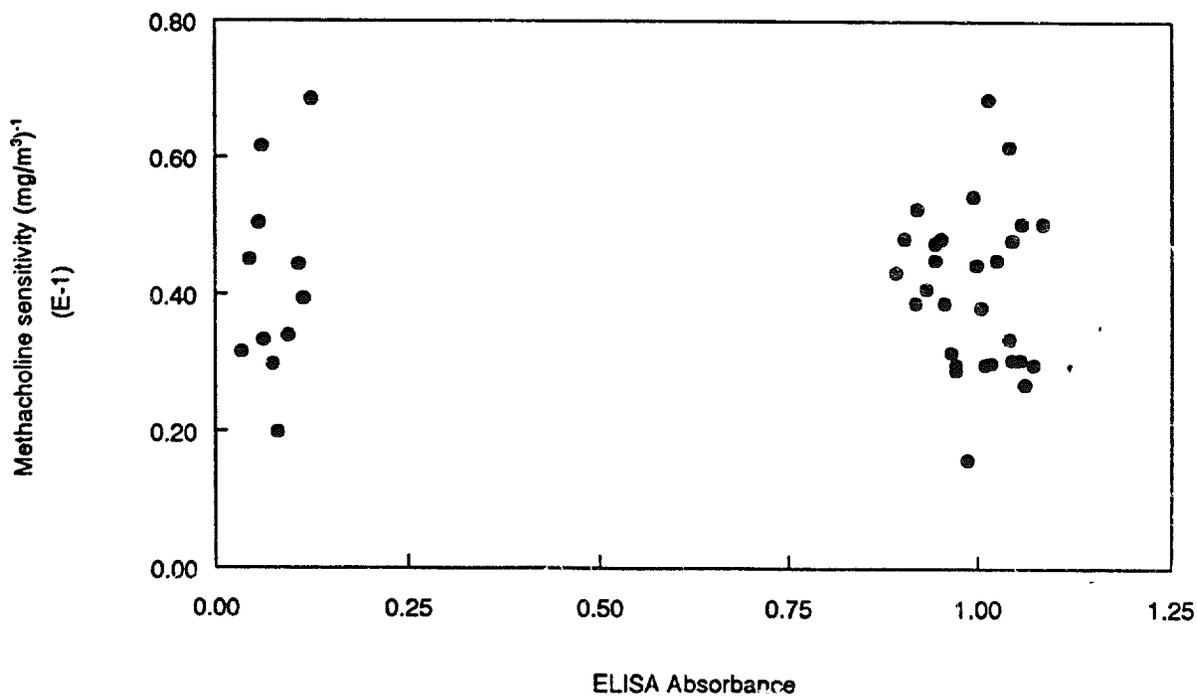


Fig. 3-13. Response to plethysmographic methacholine challenge as a function of TDI-IgG level. The ELISA absorbance of plasma samples is plotted, by animal, against the methacholine sensitivity (reciprocal of mean methacholine challenge concentration required to achieve endpoint $\times 10$); $r = 0.04$, NS.

3.3.2 TDI Challenges

Table 3-6 shows the plethysmograph TDI levels corresponding to various conditions of atmosphere generation, using 700 mL glass bottles as guinea pig "surrogates". Separate trials had revealed that the concentrations of TDI detected at the sampling port, when an animal was present in the chamber, were about half of what they were in the case of these "surrogates": mean (sd) TDI concentrations under one set of conditions (with three determinations each with animal absent and present) were 3.6 (0.67) and 1.8 (0.83) ppm, $p = 0.02$. This differential is attributed both to inhalation by the animal, as well as adhesion to hair, etc. As noted at the time, animal (self-selected) placement had a marked effect on the outcome of the sampling. In order to standardize the conditions, the "final" corrected mean value (Table 3-6) was used to express the relative TDI sensitivity of the guinea pigs.

The animals' airway responses to TDI, as represented by their plethysmograph tracings, were not the same as their response to methacholine. There were some increased respiratory rates noted, but there was no progressive increase in amplitude corresponding to the methacholine outcome. The most consistent and substantive response consisted of a "flattened" breath peak, corresponding to a delay prior to exhalation (i.e. in these tracings a downward pen deflection corresponds to increased chamber pressure and the animal's exhalation). At higher concentrations the wave form became asymmetric, dys-synchronous and erratic in appearance (Fig. 3-14). In the case of TDI response there appears to have been some tachyphylaxis, as an animal responding to a lower concentration did not necessarily respond to an immediately successive, higher concentration.

As described, tedious (manual) and iterative processes were used initially to try to identify an appropriate criterion or set of parameters to use as an endpoint. This included consideration of respiratory rate, peak-to-peak distance, peak amplitude (ΔP), and peak apex width before exhalation (corresponding to a delay that may have been associated with increased airway irritability). The endpoint finally selected was defined in terms of the duration of delay in exhalation (DE). Initially, it had been intended to

Table 3-6. Plethysmograph chamber TDI concentration with "surrogates" *in situ*

Temp.	Rotam.	L/min.	Day 1	Day 2	Day 3	Mean	sd	Final ¹
amb.	5	0.3	0.02	0.04	.002	0.019	.017	.02
	10	0.8	0.06	0.08	0.02	0.05	.029	.06
	20	3.6	0.31	0.38	0.17	0.29	.105	.33
	40	8	0.58	0.87	0.62	0.69	.156	.79
	100	19.1	0.82	1.43	1.25	1.16	.312	1.3
46 °C	5	0.3	0.24	0.32	0.13	0.23	.091	.26
	10	0.8	0.28	0.40	0.20	0.28	.074	.32
	20	3.6	1.33	1.47	--	1.4	.099	1.6
	40	8	2.91	3.33	3.15	3.13	.211	3.6
	100	19.1	4.34	4.97	4.76	4.69	.321	5.4

1. The mean values were adjusted for sampler (impinger) "breakthrough".

The level of TDI in a representative plethysmograph chamber was determined on three separate occasions by the official Ministry of Labour method⁵⁹ for TDI. The chambers contained 700 mL bottles in place of guinea pigs. Dry, compressed air was used to volatilize TDI into the mixing chamber for a period of 60 seconds; air was drawn from a plethysmograph chamber for 5 minutes through an impinger containing Marcali absorbing solution (acetic and hydrochloric acids). This was subsequently analyzed by diazotization of the toluene diamine formed in the absorbing solution with sodium nitrite-sodium bromide, and coupling the diazonium compound with N-(1-naphthyl)-ethylenediamine, forming a coloured complex which was determined spectrophotometrically at 550 nm. The values were adjusted for impinger breakthrough, based on sampling with tandem impingers. The mean values are shown in the right hand column.

Challenge

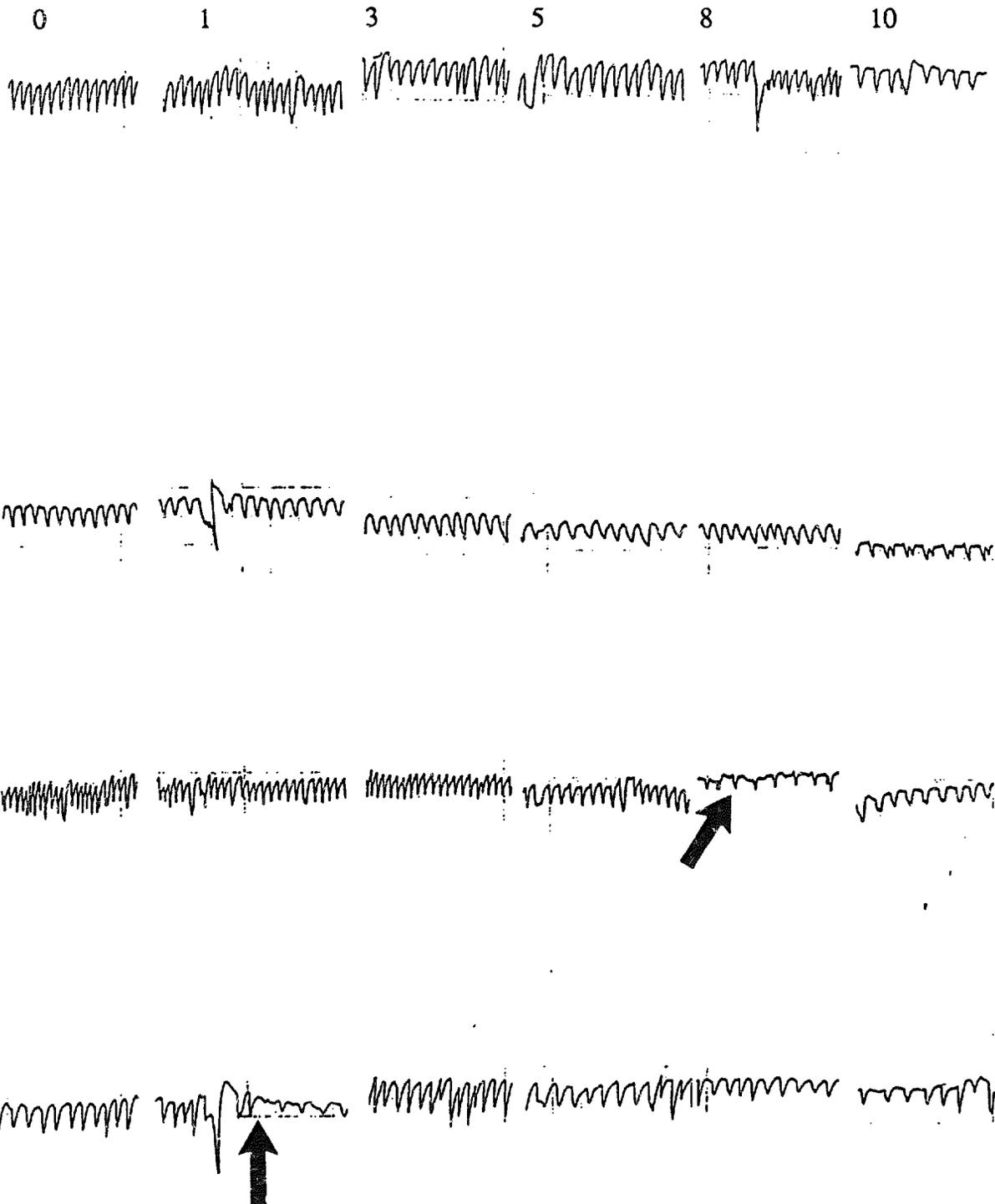


Fig. 3-14. Change in plethysmograph ΔP pattern with increasing TDI challenge concentration. This figure shows 10 second panels from the chart record for day 1 TDI challenge of group 93/7, corresponding to the pre-challenge pattern, and TDI challenges 1, 3, 5, 8 and 10. The arrows note endpoints.

use, for each animal as its baseline, the mean apex width for the three broadest peaks in the 30 seconds directly preceding the start of the challenge. The chart record was to be inspected (and peak apices measured), and the first of three consecutive breaths with a DE double the baseline was to be taken as the endpoint. However, the difficulties associated with the consistent measurement of sub-millimetre distances on chart paper led to the use of an alternative final criterion. The first DE of at least 2 mm (0.8 secs) in a consistent series of at least three out of four consecutive breaths, was used as a more straightforward endpoint; any breath held for at least 10 mm (4 secs) was also accepted as an endpoint. In exceptional cases, a modified endpoint was used; in one trial a DE of 3 mm for 3/4 breaths or any one breath for 15 mm was used, due to the pre-exposure patterns that were observed. Once the criteria had been established, the final endpoints were in all cases determined from the strip chart records in a "blinded" fashion i.e. without knowledge of which of the tracings corresponded to the control animal, nor what the outcome had been with any previous trials with that animal.

Table 3-7 shows the outcome of the TDI challenges of the animals that had been sensitized with the 5% TDI solution, excluding the first such group (due to the fact that it had previously undergone multiple TDI challenges during the protocol development phases). In each group, the "TDI Sensitized" results appearing on the same line (day 1 and 2) correspond to the same animal. Those groups not consisting of four animals (as well as those animals marked with a †) represent deaths occurring in the animal colony, unrelated to the experimental procedure.

Table 3-7. Plethysmograph challenge TDI concentration (ppm) required to achieve endpoint (as defined) on two consecutive challenge days; by animal.

Group	First Challenge Day		Second Challenge Day	
	Control	TDI Sensitized	Control	TDI Sensitized
93 / 6	1.6	3.6	1.6	0.79
		0.26		0.33
		0.32		0.79
93 / 7	5.4†	1.6	3.6	1.33
		0.02†		0.79
		0.06†		0.06
93 / 8	>5.4 §	1.33	5.4	0.79
93 / 9	3.6†	0.79	3.6	0.33
93 / 10	1.6	0.02	3.6 §§	1.6
		1.33		0.79
		0.02		0.26
93 / 11	0.79	1.33	1.6	0.33
		0.79		0.33
		1.33		0.79
Mean	3.07	0.91	3.22	0.67
sd	2.03	0.97	1.44	0.43
p (t test)	0.004		<0.001	

† these animals died from causes unrelated to the experimental procedure, following the *in vivo* and prior to the *in vitro* work

§ for statistical purposes, this was taken as 5.4

§§ the endpoint for this trial was defined as a DE of 3 mm, due to spontaneous (pre-challenge) breathing patterns

Within each group, the sensitized animal values appearing on the same line correspond to the same guinea pig.

Clearly, there is a greater sensitivity to TDI on the part of the sensitized guinea pigs, relative to the control animals. In comparing the results of individual determinations by animal, there are two day 1 results that are inconsistent with a "sensitivity of sensitized > control" ordering: one was a sensitized animal from group 93/6 not progressing to endpoint until 3.6 ppm TDI. The other was the "control" from group 93/11 which will be mentioned on several occasions in Chapter 4. When comparing mean values for the TDI concentration required to achieve the endpoint of the two categories of animals, they were different on both challenge days ($p = 0.004$ and < 0.001 on challenge days 1 and 2, respectively). Overall category means (sd) of the animal mean ppm TDI required to achieve the endpoint were 3.2 (1.7) for control and 0.8 (0.6) for sensitized guinea pigs; expressed as reciprocals, the corresponding "sensitivities" were 0.4 (0.3) and 3.0 (4.3). It should, perhaps, again be pointed out that these animals had been TDI-sensitized almost a year prior to these determinations, so that the magnitude of the difference in the mean TDI endpoint concentrations is certainly not as relevant as are the above p values. These data are further discussed in relation to the *in vitro* findings and antibody levels, in the overall discussion (Chapter 5).

Fig. 3-15 shows the inter-day reproducibility of the TDI challenge outcomes; the correlation coefficient (0.78) is highly significant ($p < 0.001$).

Fig. 3-16 represents the relative methacholine and TDI sensitivities of the animals; while they appear to be unrelated, the data (with all animals included) did show a significant correlation. When an outlier (with sensitivity $> 16 \text{ ppm}^{-1}$) was removed from the data set, the significance of the correlation disappeared; this is what is presented in Fig. 3-16. The exclusion of the outlier can be rationalized on the basis of the "empirical rule"²⁹² (i.e. the 'uncommonness' of a value > 3 sd above the mean) and on the outcome of Dixon's test²⁹³ (in which the calculated value of 0.81 for this point greatly exceeds the 5 % criterion of 0.45).

Clearly, the TDI-exposed animals not only had elevated TDI-specific antibodies (Chapter 2), but they showed a specific airway hypersensitivity, to TDI but not to methacholine.

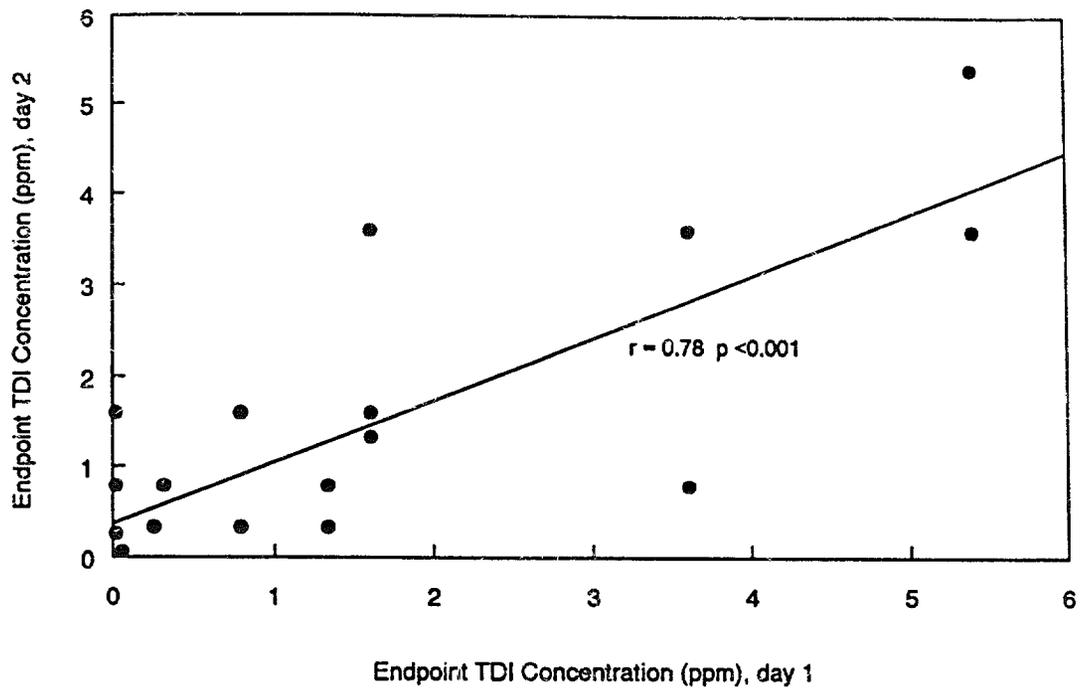


Fig. 3-15. Inter-day reproducibility of plethysmograph TDI challenges. This figure plots, by animal, the TDI concentration required to achieve the endpoint on day 1 vs. day 2.

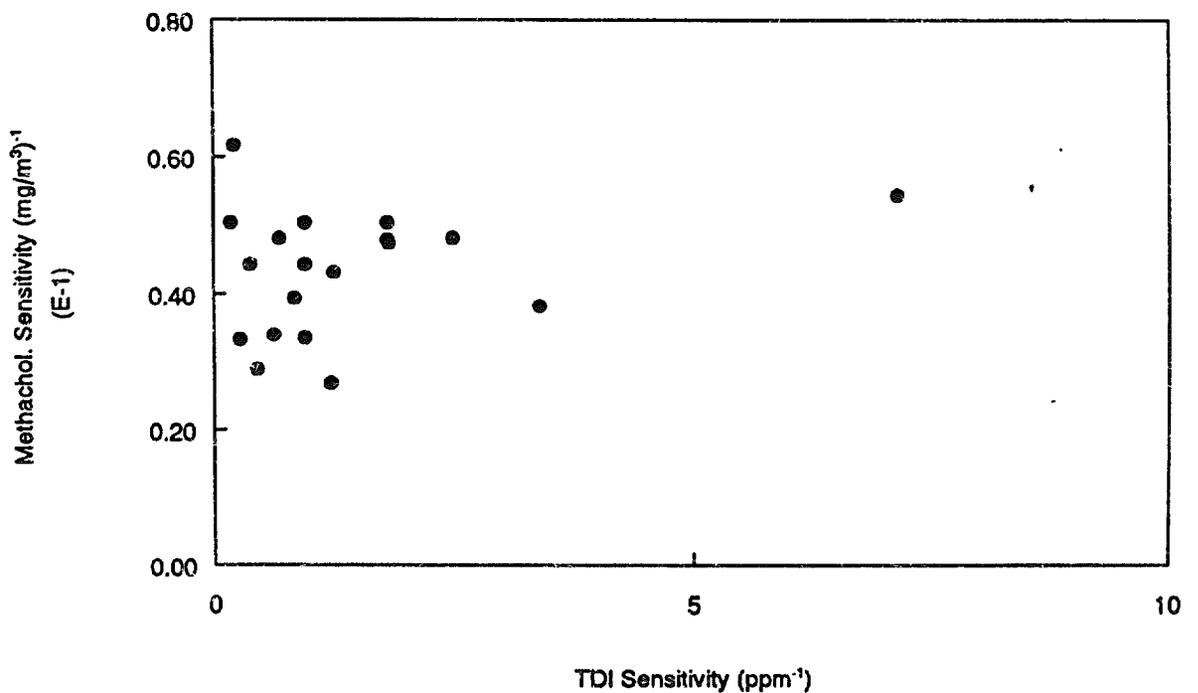


Fig. 3-16. Comparison of methacholine and TDI sensitivities, by animal. One outlier (TDI sensitivity > 16 ppm⁻¹, i.e. > 3 sd above mean) has been removed from this data set; $r = 0.26$, NS.

4. *IN VITRO* (TISSUE BATH) STUDIES

4.0 Introduction

The advancement of pharmacological science has been well served by tissue bath experiments. Early in this century ²⁶¹, the nature of the anaphylactic reaction was already being investigated with tissue strips from guinea pigs sensitized with various allergens. Even today, drugs are commonly assessed *in vitro* to obviate the confounding effects that may occur *in vivo*; in the case of bronchodilators, confounding factors could include vascular responses and pulmonary edema. Recently, Black & Armour ²⁹⁴ suggested that, in order to better understand airway hyperresponsiveness (which to them characterize asthma),

"we need more detailed studies assessing the reactivity of sensitized, antigen-challenged human airway smooth muscle to a variety of agonists to try to mimic the in vivo situation which is associated with the greatest increases in non-specific responsiveness".

The same guidance could be applied to this guinea pig work as well. Upon airborne challenge of an animal to an allergenic substance, a host of changes could take place, both **directly** with respect to immunoglobulins, autocooids and hormones, and **indirectly**, as a result of the effects of these agents (arising locally, or transported systemically) on the airway tissue (leading to increased vascular permeability, inflammation, heightened smooth muscled sensitivity and/or responsiveness, etc.). Tissue bath work permits a more analytical appraisal of response to challenge.

A review of the literature has not revealed any reports of the assessment of differential *in vitro* response of airway tissues from TDI sensitized and control animals; to TDI. Mapp's group is evaluating the response of tissues from naive guinea pigs to TDI, and how this may be pharmacologically mediated ^{186,203-205,295}. McKay & Brooks ^{183,184} had compared the carbachol and isoproterenol response of tissues from TDI-exposed and control guinea pigs, 20 hours after their last TDI exposure. However, they had not reported on any attempt to differentiate the exposed and control animals immunologically, or with respect to airway response *in vivo* or *in vitro* to TDI, i.e. to demonstrate that they were assessing hypersensitivity.

In the present study, the *in vitro* reactivity¹ of tracheal rings from sensitized and control guinea pigs was compared by treatment with TDI and pharmacological spasmogens, both individually and in combination (to evaluate interactions between the two). The role of the epithelium in the response was assessed, as was the *post mortem* persistence of the TDI hyperreactivity. The fundamental purpose was to test hypothesis number 4:

- a) *That there are pharmacological differences between tracheal tissues from control and sensitized animals;*
- b) *That the in vitro TDI response of tissues from sensitized animals can be modulated pharmacologically;*
- c) *That the tracheal epithelium has a protective role in this response.*

as well as to provide part of the data to test hypothesis number 3:

That in vivo sensitivity to TDI challenge correlates with in vitro airway tissue responsiveness to TDI.

All of this work was performed with tissues obtained from animals that had been sensitized more than a year earlier. This is likely the most extensive *in vitro* assessment to date of an animal model analogous to (human) isocyanate-induced asthma.

4.1 Preliminary studies

Preliminary studies were carried out with young guinea pigs weighing 305 - 460 grams. These experiments included the comparison of various resting tensions, and the determination of the agonist response of tracheal rings, strips and lung parenchymal tissue. The effects of the removal of epithelium by "rubbing" were investigated. The results obtained were in good agreement with those described in the literature. The potency of histamine in unrubbed tracheal rings from naive guinea pigs (expressed here as the negative logarithm of EC₅₀) was found to be 5.1, compared with the literature values 5.2²¹⁷ and 5.4²¹⁵. There was complete agreement in terms of the pEC₅₀ from rubbed tissues: 5.7 in all three cases.

¹"Reactivity" is used here (as generally, throughout this thesis) to refer to sensitivity and/or responsiveness.

Various solvent vehicles and concentrations of TDI were evaluated, for its administration to the tissue baths. The responses of tracheal rings from naive and sensitized (in this instance, intradermally injected) animals were similar to those reported below for the present study. TDI caused contraction of tissues from sensitized animals; with tissues from naive animals there was occasional contraction, but the mean response was one of relaxation. Many other assessments were made; as these were preliminary, and in the interests of space, further discussion of that work is not provided here.

4.2 Histology

As discussed in Chapter 1, there is no consensus in the literature as to whether the airway epithelium is primarily a diffusion barrier, or whether its greater participation in setting airway calibre is by the elaboration of various "factors". In order to repudiate the pharmacological role, various airway "tube" preparations have been developed by some researchers, to avoid any "leaks" around the presumptive barrier. Others continue to use the conventional ring and strip preparations. Buckner *et al*¹⁵⁸ simply minimized epithelial potential to act as a barrier by using spirally cut strips; they based their histological findings on tissues from "initial" experiments. In the present study the absolute presence or absence of the epithelium was not critical, since the interest did not relate to the epithelium as a diffusion barrier, but rather as a source of pharmacological mediators; differences in the amount of epithelium should relate to the amount of such factors, and correspondingly to a differential response.

Various approaches have been taken to account for the amount of epithelium remaining after a technique to remove it is performed. Akbar & Sharma²⁹⁶, to demonstrate that their effectiveness of epithelium removal was "confirmed histologically", cite a different research group working with a different tissue. In other instances, "presence" (+ep) or "absence" (-ep) of epithelium actually correspond to relatively small differences in the amount of epithelium present. For example, Pavlovic²⁴⁴ found that what they had defined as +ep corresponded (histologically) to 60-80 % of the epithelium being present and -ep as 20-40 % of the epithelium being present.

Nevertheless, in the present study the tool used to remove the epithelium, and the technique *per se*, required development to obtain quantitative removal. In the literature, where the amount of epithelium remaining histologically in a rubbed preparation is referenced, no mention is made of the location of the residual. In contrast to blood vessels, the airways are not anatomically or functionally symmetrical, as most of the circumference of the trachea is occupied by cartilaginous rings, with a short dorsal band of smooth muscle fibres. In the present study the most difficult epithelium to remove was located in the 'corners' of the D-shaped tracheal cross-section, apposed to the smooth muscle layer (located in the cartilage-free "straight" section). Any residual here should be more significant than on the opposite surface, where there is no underlying smooth muscle.

A method for epithelium removal described by Flavahan *et al*²²⁵ had involved rolling the tracheal rings on wetted filter paper, by means of a pair of forceps inserted into the lumen. In preliminary studies this seemed not to provide sufficient control or consistency. Various attempts were made to standardize the conditions, by defining the dimensions of the rub, and with various applications of force. Ultimately, epithelium removal was effected by the insertion of a fine (2.5 mm diameter), buffer-soaked wooden rod into the lumen of the tracheal ring, and then rolling the tissue in the palm of the hand by means of this rod, as well as specifically rubbing areas such as the "corners" (referenced above).

Figs. 4-1 to 4-4 show histological sections of tracheal rings preserved with Bouin's solution and stained with hematoxylin and eosin, photographed at 40× magnification unless otherwise specified. Fig. 4-1 shows a section of a tissue that has not been in the tissue bath; it displays a normal epithelium overlying the band of horizontal smooth muscle. For all other sections the tracheal rings were in a tissue bath for up to 8 hours, and had been subjected to a full range of agonist treatments, including TDI. Figs. 4-2a and 2b (100 ×) show the appearance of epithelium at the end of the trial; in 2a there can be seen some loss of epithelium due to the hook that was used to attach the tissue to the strain gauge. Fig. 4-3 shows rubbed tissue, devoid of epithelium.

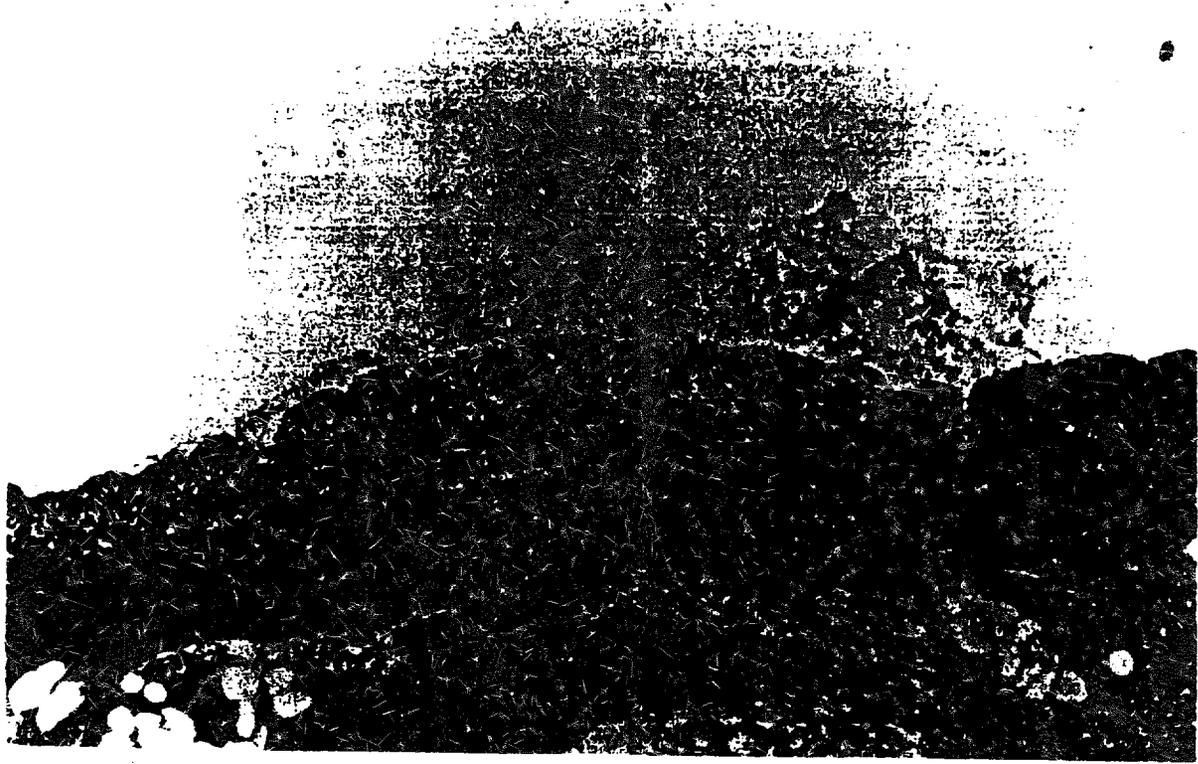


Fig. 4-1. Photomicrograph of a section of a tracheal ring preparation preserved in Bouin's solution, stained with hematoxylin and eosin and photographed at 40 \times .

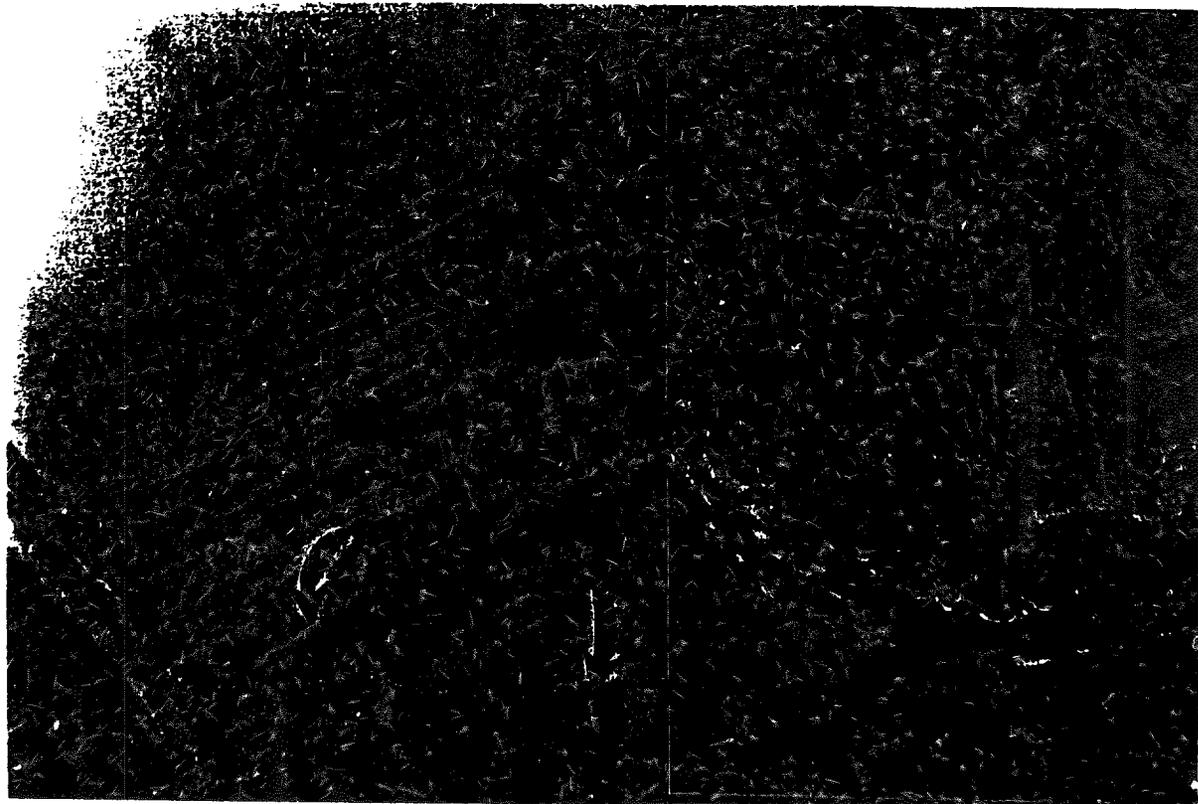


Fig. 4-2a & 2b. Photomicrographs of sections of tracheal ring preparations preserved at the end of a tissue bath study; tissue preparation as above. The arrows (in a) denote epithelial loss due to the strain gauge hook.

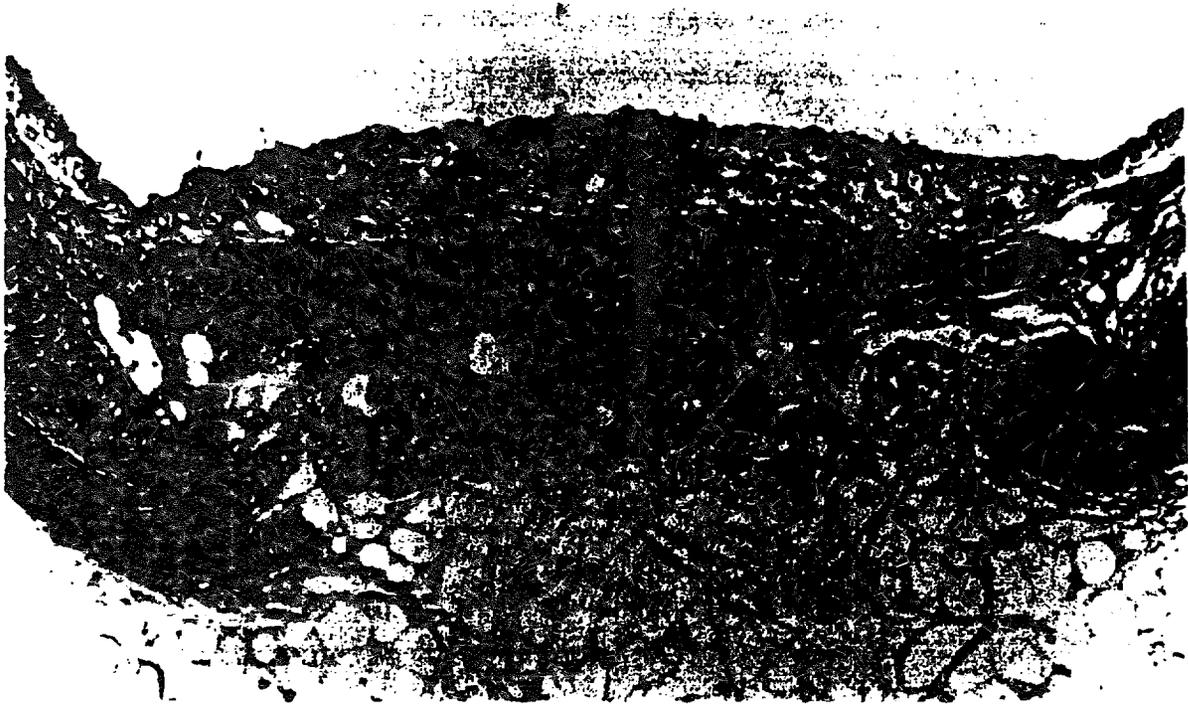


Fig. 4-3. Rubbed tracheal ring preparation, showing absence of epithelium

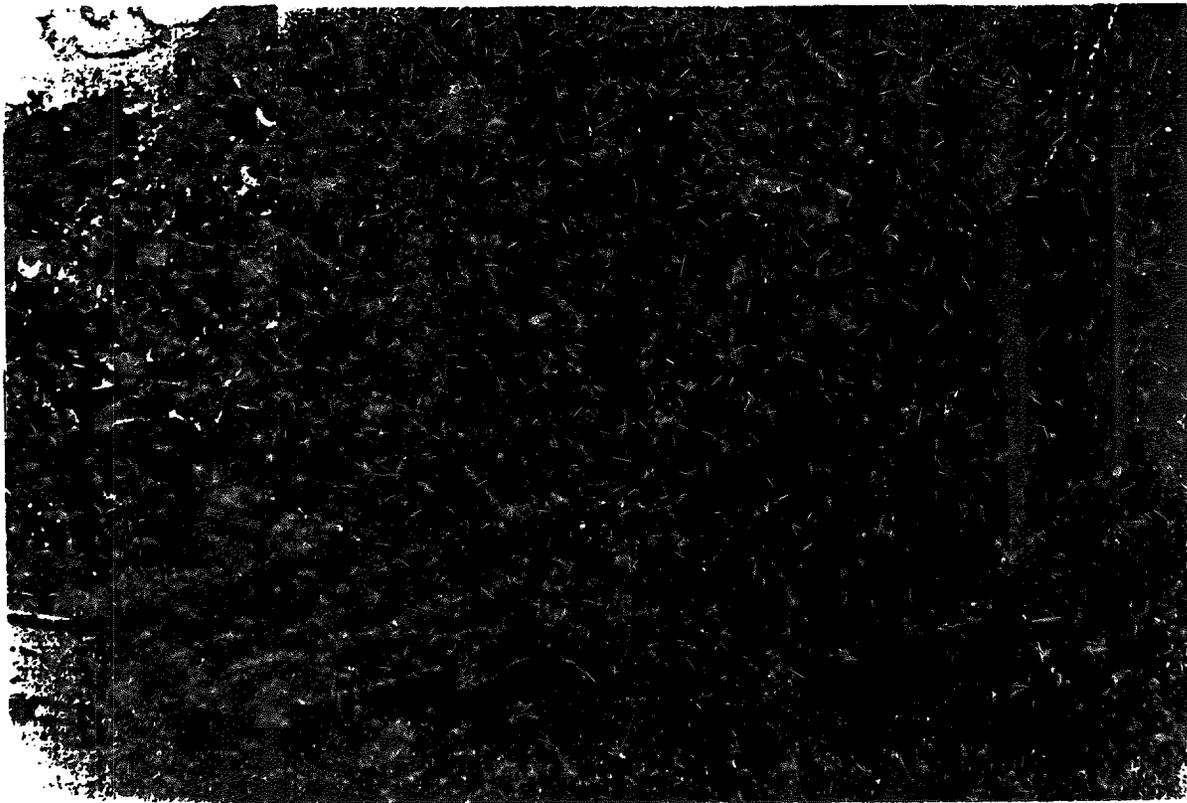


Fig. 4-4. Constricted tracheal ring preparation; inset (at 5 times higher magnification) shows cilia.

Fig. 4-4 represents a contracted tracheal ring; it can be seen that the muscle band is shortened, epithelium tightly folded and the two cartilaginous sections pulled close to each other; the inset (photographed at 200× magnification) shows that tissue integrity is remarkably well preserved despite the various treatments that it has received. Clearly, the techniques used to manipulate the tissues resulted in removal of the epithelium in the rubbed rings, and preserved the epithelium in the unrubbed. However, since it was impractical to examine and characterize histologically every tracheal ring used, tissues are described as "unrubbed" and "rubbed", inferring presence and absence of epithelium, respectively.

4.3 Studies on the main sensitized groups

The guinea pigs in the main study groups had matured considerably over the course of the first two phases of the study. Accordingly, the *in vitro* studies were conducted with guinea pigs that were over a year old, and weighed an average of 1188 g (sd 128) at the time of sacrifice. It cannot be assumed *a priori* that the pharmacological parameters determined in the preliminary work with younger animals should be the same as with the more mature ones. In fact, Duncan and Douglas²⁹⁷ showed that tracheal tissues from immature guinea pigs, normalized with respect to cross-sectional area, were more sensitive to histamine and carbachol than were tissues from mature animals.¹

In the main, the *in vitro* studies were carried out using animals from groups 93/6 - 93/11. As mentioned earlier, there were a number of animal deaths occurring in the colony around the time of the plethysmography work; these deaths were not related to the research protocol. This resulted in the loss of two control animals and two of the most TDI-sensitive animals (as assessed *in vivo*) from these groups. These animal losses will have had some negative impact on the overall results; for example, there were no equivalent plethysmographic TDI data available for the replacement control animals were recruited from the other groups (see Chapter 5).

4.3.1 Methods

The guinea pig was decapitated and the caudal end of the trachea was dissected out and placed into cold modified Krebs-Henseleit buffer (MKHB, Hay *et al* ²¹⁵), containing (mM): NaCl 113, KCl 4.8, CaCl₂ 2.5, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 25 and glucose 5.7; the buffer was gassed with 95% O₂ - 5% CO₂. Six 2 mm wide rings were cut sequentially from the trachea with a razor blade, working rostrally from just above the bronchial bifurcation (starting at a site where the airway showed reasonably uniform diameter). As appropriate, some of the rings were rubbed as described above to remove the epithelium. The rings were mounted in tissue baths, with the *trachealis* muscle oriented lengthwise between the two stainless steel hooks. The baths were filled with 10 mL MKHB into which was bubbled 95% O₂ - 5% CO₂. Isometric tension was monitored by Grass FT03 force-displacement transducers connected to a Grass 7D polygraph. A 2 g resting tension was applied to each tissue and they were washed twice every 15 minutes for a minimum of 1 hour; the tension was reset to 2 g as necessary. After equilibration a dose-response curve to methacholine or histamine was obtained from each tissue using cumulative addition of agonist. The tissues were washed and then equilibrated for one hour (with washing and resetting of tension, as above). A potential antagonist of TDI-induced contraction (atropine, Sigma A-0257, 10⁻⁶ or 10⁻⁵ M; diphenhydramine, Sigma D-3630, 10⁻⁴ M; or indomethacin, Sigma I-7378, 10⁻⁵ M) was added (with some parallel tissues subjected to any carrier solvent necessary for the antagonist); if the tissue contracted or relaxed substantially as a result of the addition of this antagonist it was reset to a tension of 2 g. Then, TDI was added to the baths, as a single (nominally) 1.4 μL drop, corresponding to a 1 mM bath concentration. Once the TDI response (if any) had stabilized, the tension was again reset as necessary; with the TDI still *in situ*, another agonist response (methacholine or histamine, as before) was determined. Finally, a dose response curve for the relaxation achieved with isoproterenol was determined. Changes in tissue tension were expressed as a cumulative percentage of maximal agonist response (g_{max}); the EC₅₀ was determined from the 50% intercept.

4.4 Results and discussion

4.4.1 Methacholine and histamine

The overall arithmetic means (and standard deviations) of maximum tension (g_{max}) and EC_{50} were calculated from individual animal results (means of replicates, or individual tissues, depending on the specific experiment). Those resulting from the methacholine dose-response curves performed on unrubbed and rubbed tracheal rings from control and sensitized animals are shown in Table 4-1. The g_{max} and EC_{50} values resulting from the histamine dose response curves are shown in Table 4-2.

These tissues, from mature animals, were slightly (by 0.2 to 0.6 log units), but consistently, less sensitive to both methacholine and histamine than the ones from the immature animals that were assayed in the preliminary work. As the results have not been normalized (e.g. to tissue mass), direct, statistical comparison is not made; however this trend is consistent with Duncan and Douglas' findings²⁹⁷. Collectively, the results suggest that rubbing the tracheal rings to remove the epithelium did not have an adverse effect on tissue function or ability to respond (g_{max} was not lower in rubbed tissues).

There was only one set of parameters that showed a significant difference (as evaluated by t-test with Bonferroni correction, in which case the calculated p value must be $< 0.05 \div 6 = < 0.008$, for statistical significance). The EC_{50} for histamine was lower in rubbed tissues from control guinea pigs than in unrubbed tissues from sensitized guinea pigs ($p = 0.002$). Although this is not a comparison that would have been made intuitively (i.e. the more logical pairs to compare would be unrubbed/rubbed in control and sensitized animals, and control/sensitized with unrubbed and rubbed tissues), inspection of the data suggested what appeared to be a substantive effect, and the test of statistical significance was applied accordingly. These particular data could be ascribed to interpretation that sensitization results in the development of some epithelium-based protection from the effects of histamine stimulation. Other differences may exist, but are obscured from statistical significance by the combination of variability in animal responses, low numbers of animals per group, and the inherent conservatism of the statistical test.

Table 4-1. Methacholine response of tracheal rings (unrubbed and rubbed) from control and sensitized guinea pigs. (n is the number of animals)				
	Control gps		Sensitized gps	
	Unrubbed	Rubbed	Unrubbed	Rubbed
E_{max} mean:	2.64	2.68	2.86	2.16
sd:	1.11	1.92	0.50	0.28
n:	6	6	7	5
p:	no values < 0.008 (0.05/6 as per Bonferroni) ²⁹⁸			
EC_{50} mean:	2.6×10^{-6}	3.3×10^{-6}	1.6×10^{-6}	7.3×10^{-7}
sd:	6.6×10^{-6}	7.0×10^{-6}	8.2×10^{-7}	6.6×10^{-7}
n:	6	6	7	5
p:	no values < 0.008			

Table 4-2. Histamine response of tracheal rings (unrubbed and rubbed) from control and sensitized guinea pigs				
	Control gps		Sensitized gps	
	Unrubbed	Rubbed	Unrubbed	Rubbed
E_{max} mean:	2.74	3.76	2.42	2.97
sd:	0.281	0.669	0.518	0.676
n:	4	4	5	4
p:	no values < 0.008			
EC_{50} mean:	1.5×10^{-5}	3.2×10^{-6}	2×10^{-5}	1×10^{-5}
sd:	6.8×10^{-6}	1.1×10^{-6}	7×10^{-6}	1.3×10^{-5}
n:	4	4	5	4
p:	All	p = 0.002 other combinations		NS

4.4.2 Epithelium

The data were also assessed for the effect of the removal of epithelium by comparing, for those experiments in which both unrubbed and rubbed tissues from the animal were subjected to the same agonist, the g_{\max} attained with methacholine or histamine. In the case of methacholine there was no consistent difference between the unrubbed and rubbed tissues; the respective means (sds) were 2.68 (0.91) and 2.46 (1.47), and not significantly different ($p > 0.05$). However, in the case of histamine, every "rubbed" g_{\max} was greater than the corresponding "unrubbed", with both four control and four sensitized animals (Table 4-3); the difference between the means was highly significant ($p < 0.004$).

The fact that the histamine g_{\max} was greater in the absence of the epithelium again suggests that the rubbing did not damage the smooth muscle. Hay *et al*²¹⁵ similarly reported that epithelium removal from guinea pig tracheal strips caused a significant increase in the maximum contraction due to histamine, but not methacholine. The difference between histamine and methacholine, in g_{\max} effects due to epithelium removal, could be consistent with a differential epithelium barrier role for the two agonists, or could reflect the specific activation of histaminergic systems (removal of degradative enzymes, enhancement of receptor activity).

Table 4-3. Effect of removal of epithelium on mean tissue response (g_{max}) to histamine		
	Unrubbed tracheae	Rubbed tracheae
Control animals	2.35	3.35
	2.75	3.05
	2.98	4.2
	2.9	4.45
Sensitized animals	2.83	3.13
	1.65	2.0
	2.88	3.18
	2.58	3.57
Mean:	2.62	3.37
sd:	0.44	0.75
p (paired t-test):	0.004	

This table shows the mean tension generated (g_{max}) by histamine in those experiments where both unrubbed and rubbed rings from the same animal were assessed; 4 of these animals were controls and 4 were sensitized.

4.4.3 Direct effect of TDI

When TDI was added to the tissue baths, it produced a markedly and qualitatively different response in tissues from control animals, as compared to those from the sensitized animals. In the case of the former, it typically resulted in no apparent effect, or a relaxation; in the case of the latter it produced a contraction with a characteristic time course. Fig. 4-5 presents physiograph tracings of each type of response. Shown in Table 4-4 are the mean g_{max} values obtained from both sets of tissues; clearly, the difference is significant, and the control animals show a net relaxation.

These results are most strikingly (and seemingly) at odds with the findings of Mapp *et al*, who in various reports ^{186,204} have indicated that airway tissues from naive guinea pigs (male Hartleys), when challenged in tissue baths with TDI, contract. The significant differences between Mapp *et al* and the present study include:

	<u>Present study</u>	<u>Mapp <i>et al</i></u>
Animal weight:	> 1000 g	300 - 400 g
Status:	Control	Naive
Sacrifice:	Decapitation	Pentobarbital sodium
Tissue:	Trachea	Main bronchi
TDI form:	Neat	Dissolved in DMSO

In several experiments of the present study, TDI was administered in DMSO, to tracheal rings from both sensitized and control animals. One of the two 'control' animals selected for this evaluation [93/11L1] was aberrant in a variety of its TDI responses, as described below; its rubbed tissues contracted upon the initial addition of TDI, both in "neat" and "DMSO-dissolved" form. In the case of the other control tissues assessed, the TDI in DMSO did not cause a contraction. It would not seem that the use of DMSO would reconcile the differences between the present study and Mapp *et al*.

In view of the overall goal of this type of study, namely to elucidate the mechanisms of hypersensitivity, it would seem that the model used herein was more useful than the other, as it allows a direct comparison of sensitized and nonsensitized tissues *in vitro*.

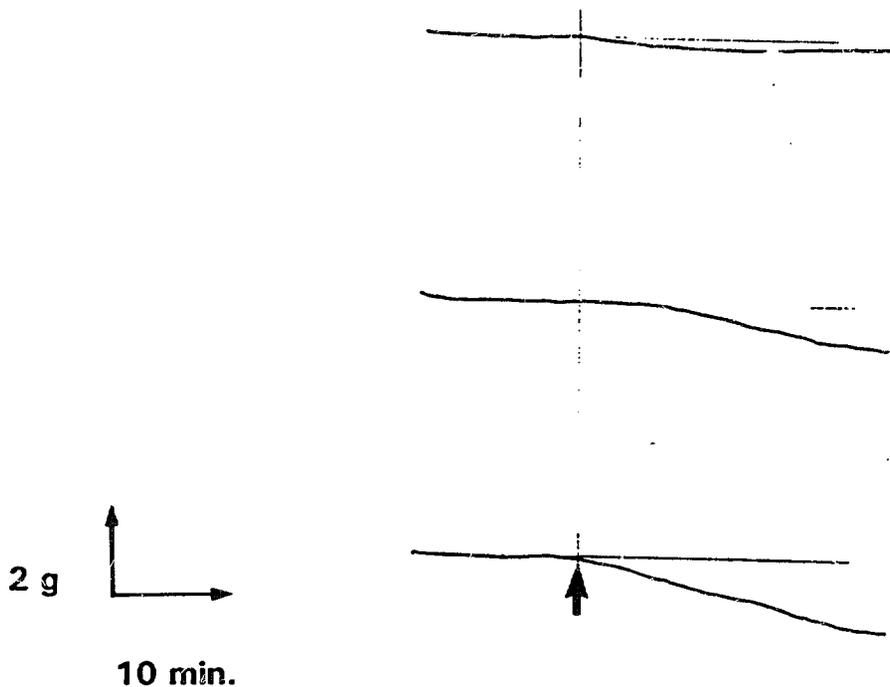


Fig. 4-5a. Physiograph tracing of the response of tracheal rings from a control animal to the addition of TDI to the bath. The middle ring is unrubbed, the other two are rubbed. The arrows represent the point of addition of TDI ($1.4 \mu\text{L}$, equivalent to 1 mM) to all the tissue baths.

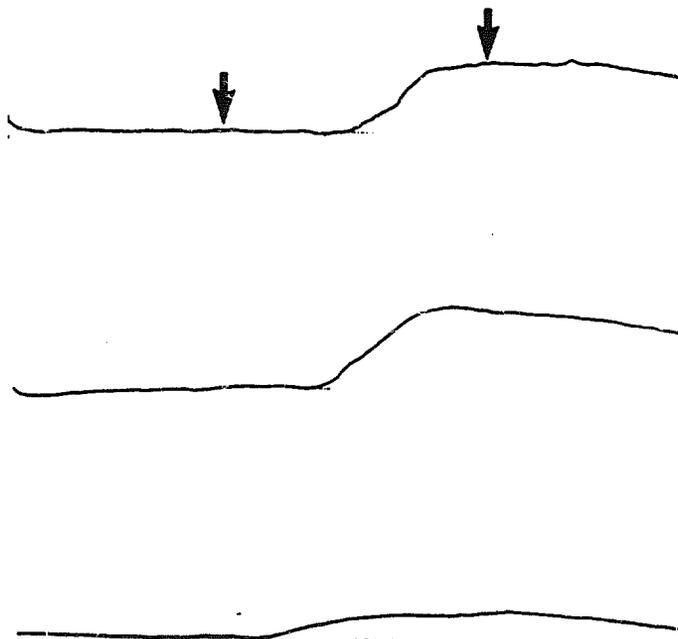


Fig. 4-5b. Physiograph tracing of response of rings from sensitized animal to addition of TDI to tissue bath. All rings are unrubbed; the arrows represent the points of addition of TDI (each $1.4 \mu\text{L}$, and equivalent to 1 mM) to the baths.

Table 4-4. TDI response of tracheal rings. Mean grams of maximum tension generated by the tracheal rings, in response to the addition of TDI (nominally 1.4 μL , equivalent to 10^{-3} M) to the tissue baths. Comparison of tracheae from control and sensitized animals.

	Control	Sensitized
Mean (g)	-0.06	0.84
sd	0.27	0.57
n	5	9
p (t-test)	0.006	

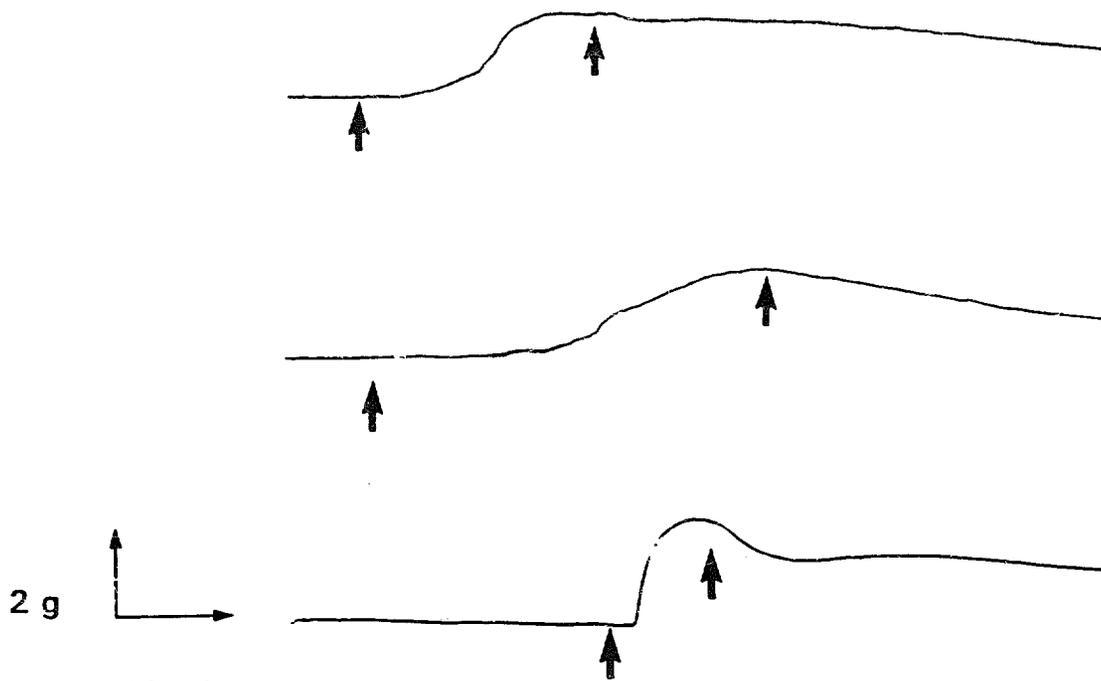
In the tissue bath isocyanate work TDI was added to the baths as a single 1.4 μL drop, equivalent to a bath concentration of 1 mM. The volume is described as nominal because the two different Eppendorf pipettes used to dispense this TDI into the baths were found (upon gravimetric calibration with water) to dispense mean volumes of 2.1 ("yellow") and 1.5 ("silver") μL , respectively. Of course, the volume with TDI could in turn be different yet again, but this variability is not likely to have had an impact on the results, given the outcome of experiments involving multiple additions of TDI i.e. the tissue bath TDI response appears to be of the "all-or-none" variety. Secondly, the concentration is described as nominal because, although this is the calculated concentration that would be present if the TDI behaved as a traditional solute, it did not in fact "dissolve", but sat on the buffer as a "blob" which gradually resulted in the development of a cloudiness in the buffer, and (in a few minutes) the contraction of tissues from sensitized animals.

It is not certain that the tracheal rings used in the present study respond directly to TDI *per se*. Conversely, it is highly unlikely to be TDI-GSA (a putative *in vivo* antigenic form of TDI), either: given the *milieu* and the regular washing, and the fact that by the time the TDI was added, the tissues had been in the baths for many hours, endogenous albumin is unlikely to be available for conjugation. Mapp *et al*²⁰³ found that the products of water-reaction with TDI (TDA, etc) also contracted their tissue preparations. If degradation products are responsible for contracting the tissues in our model as well, then this may account for the characteristic delay between addition of TDI to the tissue bath, and onset of contraction (see Fig. 4-5b). When TDI was added to the baths dissolved in DMSO (Fig. 4-6a) the onset of contraction occurred more rapidly; in this form the TDI was observed to rapidly form a precipitate in the baths. Clearly, in the *in vivo* situation there would not be any DMSO present in the airway when TDI exposure occurs; conversely, there may be some endogenous intermediary, as discussed above.

The TDI response of our system was of the all-or-none variety: in experiments with tissues from several sensitized animals a second aliquot of TDI was added to the tissue bath buffer once the initial contraction had stabilized; there was no additional contraction. The only differential response upon the second addition was the aberrant 'control'; although its rubbed tissues contracted immediately upon addition of TDI, the unrubbed tissues did not contract until a second aliquot of TDI was added, whereupon one of them did so (Fig. 4-6b).

4.4.3.1 Effects of epithelium on TDI response

There were seven sensitized animals whose tracheal response to TDI was assessed both with unrubbed and rubbed rings. The results are shown in Table 4-5: there was no significant difference. Clearly, the tracheal epithelium has no effect on the magnitude of the direct *in vitro* TDI response of tracheal rings from TDI-sensitized animals. This (again) apparently differs from the findings of Mapp *et al*¹⁸⁶ who reported that epithelial removal enhanced the effect of TDI (in naive animals). As we did not assess naive animals *per se* (and other differences have been described), it is not possible to make a direct comparison with their study. However, the results seen with a specific guinea pig



10 min.
 Fig. 4-6a. Response of tracheal rings from a sensitized animal to TDI, neat and in DMSO. The middle tissue was rubbed, the others not. Arrows represent the point of addition of TDI ($1.4 \mu\text{L}$) to each tissue bath; the top two tissues received neat TDI, the bottom one an equivalent amount in DMSO.

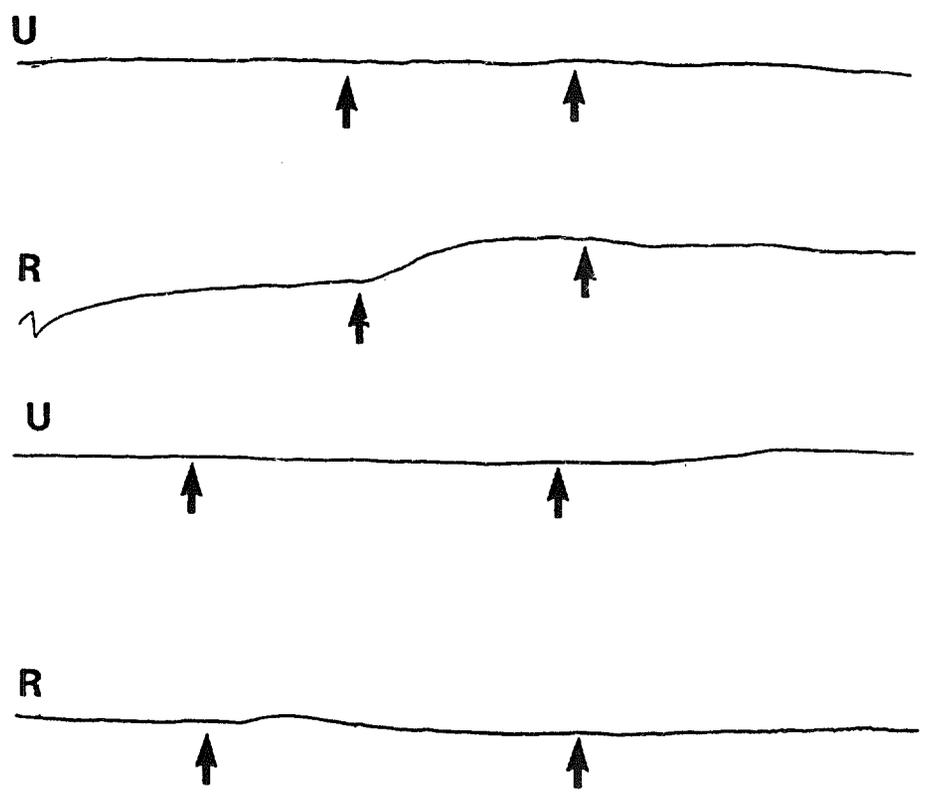


Fig. 4-6b. Response of unrubbed (U) and rubbed (R) tracheal rings from the "aberrant control" animal to neat TDI.

Table 4-5. TDI response of unrubbed and rubbed tracheal rings from sensitized animals		
Unrubbed Tissues	Rubbed Tissues	
1.6	.13	
0.4	1.1	
1.1	2.0	
0.2	0.0	
1.4	0.6	
2.2	1.3	
0.4	0.5	
Mean =	1.04	0.99
sd =	0.73	0.65
p (paired t-test) = 0.84		

This table shows the mean grams of tension generated by tissues from sensitized animals, in response to the addition of TDI (nominally 10^{-3} M) to the tissue baths. The comparison is between unrubbed and rubbed tissues; n = those 7 sensitized animals in which both unrubbed and rubbed tissues were evaluated. The data are presented in chronological order of experiment.

[93/11 L1, the aberrant control mentioned earlier] may be somewhat of a "missing link" between the two studies. This animal was not deliberately sensitized, yet it showed a number of characteristics of sensitized animals: in addition to the *in vitro* response just mentioned, this same animal was more sensitive to TDI during the day 1 plethysmographic TDI challenge than 2 of its 3 sensitized group mates. In the next section is described its uniqueness in terms of the effect of TDI on methacholine response in rubbed tissues. These observations could simply be due to inadvertent sensitization of this animal with TDI; this is unlikely, especially since it maintained control levels of antibodies to TDI. Alternatively, the phenomenon could be rationalized on the basis of genetic heterogeneity in our population of animals, or the homogeneity of the colony from which Mapp's group is supplied. This may be far-fetched conjecture on the basis of one animal (plus the occasional contraction noted in the preliminary phase), but it may be worth exploring, as it relates to the crux of the issue: "why do some react"? In the mean time, so much space is devoted here to this one animal, because it confounded the interpretation of the results that follow.

4.4.3.2 Effects of TDI on response to contractile agonists

The g_{max} and EC_{50} from the initial dose response curves were compared (on an animal basis) with the corresponding values obtained after the addition of TDI to the bath. The differences are shown in Table 4-6; the sign convention follows the intuitive, i.e. a positive value indicates an increase.

There are no significant differences in this table. However, inspection of the data suggests (on the basis of sign alone) that the effect of TDI on the methacholine g_{max} with unrubbed rings from control guinea pigs (a positive one) might be different from the other combinations (all negative). Similarly, inspection of the pre- / post- TDI pEC_{50} s (by treatment group mean) is suggestive of an effect:

	<u>Unrubbed</u>	<u>Rubbed</u>	mean (sd)
Control animals	5.8/5.8 (.59/.17)	6.1/6.1 (.69/.19)	
Sensitized animals	5.8/5.4 (.23/.31)	6.4/6.0 (.59/.60)	

Table 4-6. Difference between initial methacholine and histamine g_{max} and EC_{50} , and corresponding values after TDI addition.					
Expressed as mean (sd)		Control gps		Sensitized gps	
		Unrubbed	Rubbed	Unrubbed	Rubbed
Methacholine	Δg_{max}	0.53 (0.60)	-0.30 (1.7)	-0.36 (0.6)	-0.08 (0.91)
	ΔEC_{50}	4.1×10^{-7} (7.1×10^{-7})	3.3×10^{-7} (5.2×10^{-7})	3.0×10^{-6} (3.5×10^{-6})	1.3×10^{-6} (2.1×10^{-6})
Histamine	Δg_{max}	0.18 (0.83)	0.76 (0.69)	0.34 (0.51)	-0.08 (1.22)
	ΔEC_{50}	6.2×10^{-6} (1.1×10^{-5})	1.8×10^{-6} (4.6×10^{-7})	4.8×10^{-6} (1.0×10^{-5})	3.3×10^{-6} (3.2×10^{-6})

This table shows the differences in g_{max} and EC_{50} between the initial agonist (histamine or methacholine) dose response curves and the corresponding values obtained after addition of 1 mM TDI to the baths. The data are expressed as mean and standard deviation. A comparison between rubbed and unrubbed tissues from control and sensitized animals (t-test with Bonferroni correction) showed no significant differences.

With the tissues from the sensitized animals (whether rubbed or not) the mean methacholine pEC₅₀s decreased after addition of TDI (i.e. the sensitivity to methacholine decreased). With tissues from control animals (whether rubbed or not) there was no change. This may relate to the *in vivo* findings: methacholine challenge immediately after TDI exposure required a significantly higher level (138 %, p < 0.01; 116% and 146% in sensitized and control animals, respectively; difference NS) to induce bronchospasm, than prior to TDI exposure (see Table 3-5). Thus, it would seem that TDI exposure (at least in part) affects cholinergic mechanisms that undergo a prolonged (hours) tachyphylaxis. Even in the absence of demonstrable statistical significance with individual parameters, there is a compelling consistency in the apparent pattern: *in vitro* methacholine efficacy and potency both appear to decrease after TDI challenge, but only in epitheliated tissues in the case of the former; *in vivo* it was similarly found that methacholine sensitivity had decreased, following TDI-induced challenge/contraction. If the study had incorporated a larger number of animals, and if the three experimental phases had not been separated by as much time, statistical significance would have more likely been demonstrated. In fact, this could also be accomplished by a critical re-evaluation and "weeding" of the existing data. For example, the differences between pre- and post-TDI methacholine g_{max}s, for the five control animals being considered here, were:

Animal	Unrubbed	Rubbed
93/2 R1	-0.14	0.5
93/6 R1	0.95	0.5
93/8 R2	0.0	0.0
93/10 L2	1.25	0.8
93/11 L1	0.6	-3.3

Clearly, the response of the rubbed tissue from animal 93/11L1 is (again) an "outlier"; it is 11 sds away from the mean response of the other four tissues. With so few data points available, it would not necessarily be advisable to discard any data point; the phenomenon is not well-characterized, and perhaps with a sample of 20, it would not be so far "out". This viewpoint is particularly germane in research such as this, where the focus is on a phenomenon (hypersensitivity) which by definition is evidenced in a minority of any population (or sample), and therefore the response of those individuals

on the periphery of the "normal" curve may be as much of interest as the mean or median. However, if the g_{max} data from this animal are deleted from the set analyzed above, and the mean change in g_{max} after addition of TDI recalculated (with rubbed and unrubbed tissues combined), the result (as means and standard deviations) is:

Control animals: 0.48 (0.50) g

Sensitized animals: -0.22 (0.73) g

p value (t-test): 0.03

In other words, tissues from sensitized animals became less responsive to methacholine after addition of TDI, regardless of the status of the epithelium; tissues from control animals become more responsive.

4.4.4 Effects of antagonists on TDI response

Four of the obvious 'classic' pharmacological systems that could be involved in the mediation of tissue response to TDI are cholinergic, histaminergic, prostanoic, and adrenergic. The potential role of each of the first three was assessed, on a limited basis, by the addition of an appropriate antagonist to the tissue bath prior to the addition of the TDI. The assessment of the fourth was by comparison of isoproterenol-induced relaxation of an agonist-induced contraction subsequent to the addition of TDI to the tissue bath.

The effects of atropine on TDI-induced contractions were evaluated on tissues from two sensitized animals at final concentrations of 10^{-6} M or 10^{-5} M. This represents the range conventionally used with guinea pig airway preparations: Jacques *et al*²⁹⁹ used 10^{-6} M to block leukotriene-induced histamine hyperresponsiveness, and Elwood³⁰⁰ used 10^{-5} M to ensure that cholinergic response to electric field stimulation was eliminated.

The tissues from the two animals showed a different baseline response to atropine: in one case they relaxed by no more than 0.3 g, whereas in the other case, by up to 1.75 g (mean relaxations of 2 tissues at each concentration: 10^{-6} M: 0.5g; 10^{-5} M: 1.0 g). Even 10^{-6} M atropine was sufficient to inhibit the methacholine g_{max} to 30% of that obtained from dose response curves with parallel (untreated) tissues; the methacholine EC_{50} of the

treated tissues was greater than 10^{-3} M. With 10^{-5} M atropine a methacholine dose response curve was not obtained except as superimposed on a TDI response; under these conditions, the atropine treated tissue yielded a g_{max} 7 or 8 % of the untreated tissue response (respectively, by animal). The effects of atropine pre-treatment on the response to TDI are shown in Table 4-7. These were normalized to several control values, to facilitate comparison: as a percentage of first (pre- atropine and TDI) methacholine response for that tissue, a percentage of parallel tissue (not treated with atropine) and, as a percentage of the methacholine g_{max} determined after the TDI response (bearing in mind that the methacholine response would be inhibited at this point by the atropine). The results from experiment (animal) 1 are presented as the mean of 2 tissues; those from experiment (animal) 2 as individual tissues, as they defy averaging.

The results from the non-atropinized tissues showed that the addition of 1 mM TDI to the bath resulted in a tension that was 20% of either methacholine g_{max} (i.e. in the case of these two tissue sets there was good consistency between the two dose-response determinations of methacholine). The results from experiment 1 suggested that 10^{-6} M (but not 10^{-5} M) atropine inhibits the TDI response. The results from experiment 2 suggested that 10^{-6} M atropine stimulates the TDI response; one 10^{-5} M treated tissue suggested the same. The other showed no TDI response: it was either completely inhibited, or it had become nonfunctional by the time of TDI addition. There did not appear to be any difference between rubbed and unrubbed tissues. Clearly, atropine at these concentrations does not cause consistent inhibition of the TDI effect in sensitized guinea pigs. Further analysis of the data could include the consideration of regional effects within the trachea, to account for the variability in the data. However, for the same reason repetition of the empirical work with more animals would be in order, to come to a more definitive understanding of the effects of atropine. It may well be that the earlier-described difficulties in correlating clinical NSBH with TDI hypersensitivity relate to these kinds of inconsistencies in cholinergic effects.

Table 4-7. Response of tracheal rings from sensitized guinea pigs to TDI in the presence of 10^{-6} or 10^{-5} M atropine				
Tension produced due to addition of TDI				
Atropine in bath		As % of pre-TDI methacholine g_{max}	As % of parallel (non-atropinized) tissue	As % of post TDI methacholine g_{max}
None	Expt. 1	17	(100) these are the (100) ref. tissues	21
	Expt. 2	22		21
10^{-6} M	Expt. 1	7	22	15
	Expt. 2a	40	242	145
	2b	21	67	67
10^{-5} M	Expt. 1	--	107	240
	Expt. 2a	36	267	530
	2b	< 2	< 8	< 50

The effects of atropine were evaluated on tissues from two sensitized animals. Atropine sulfate (Sigma A-0257) was dissolved in MKH buffer and added to the appropriate tissue baths at final concentrations of 10^{-6} M or 10^{-5} M. In the first column, the contraction due to TDI is compared (as a %) to the maximal contraction caused by the methacholine. In the second column the contraction due to TDI in atropinized tissues is normalized to the response of the corresponding non-atropinized tissue. In the third column, the TDI contraction is expressed as a percentage of the g_{max} caused by methacholine after the addition of the atropine and the TDI.

The effect of **diphenhydramine** (10^{-4} M) on the response of sensitized tissues to TDI also was evaluated using tracheae from two guinea pigs (Table 4-8). Prie *et al*³⁰¹ had reported that 4×10^{-6} M diphenhydramine inhibited guinea pig airway prostanoid release in response to histamine, and Ninomiya *et al*³⁰² had used 3×10^{-4} M diphenhydramine to determine the role of histamine in response to endothelin. In the present study the antagonist itself generally caused a slight (initial) contraction of the tissues; in one case this amounted to 1.4 g. The mean (and sd) contractions of 6 tissues were 0.54 (0.43) g, or 0.37 (0.09) g if the one high value was excluded; when this same tissue was later re-challenged with the same concentration of diphenhydramine it relaxed by 1.5 g from the resting tension. The diphenhydramine concentration used was sufficient to eliminate any tissue response to up to 10^{-3} M histamine (in the case of experiment 1) or, except at the highest concentration of 10^{-3} M histamine (second experiment). Residual diphenhydramine effect persisted even after washing; diphenhydramine also inhibited the tissue response to methacholine. In the determination of the effect of diphenhydramine on the TDI response, various variables were evaluated; in all cases a diphenhydramine-treated tissue was pair-matched with an untreated tissue. Shown in Table 4-8 are the responses of the treated tissues.

Again, due to the variability in the magnitude of the absolute TDI response (0.3 - 2.1 g) the data were normalized as outlined in the legend to the table. The last two columns relate the diphenhydramine-treated tissues to untreated ones. The critical parameter is represented by the final column: the mean value of 113% indicates that TDI-induced contraction, relative to the earlier-determined agonist g_{max} of diphenhydramine-treated tissues, was clearly not less than that of the pair-matched tissues that had not been treated with diphenhydramine. Accordingly, diphenhydramine has not been shown to cause an inhibition of the TDI response. Again, there could be considerable benefit from repeating these experiments on a greater number of animals. However, it appears that the TDI-induced contraction of tracheal rings from TDI-sensitized animals is not mediated by histamine release. This is contrasted with the findings of Lindström *et al*³⁰³ who found that 10^{-6} M mepyramine (also an H_1 antagonist) caused a significant decrease (especially with epithelium removed) in the response of tracheal tube preparations from guinea pigs sensitized to ovalbumin, to challenge with this antigen.

Table 4-8. Tension produced due to the addition of TDI to tracheal rings from sensitized animals; response in diphenhydramine-treated tissue				
Expt.	Tissue Treatment, prior to addition of TDI	$\frac{\text{gTension, TDI}}{\text{pre - TDI agonist } g_{\text{max}}} \times 100$	As % of pair-matched untreated tissue response (g) to TDI addition	As % of pair-matched response relative to respective agonist g_{max}
1	unrubbed, histamine	18	86	120
2	unrubbed, histamine	30	60	70
	rubbed, histamine	81	110	160
	unrubbed, methacholine	16	100	100
Mean (sd)		36 (31)	89 (22)	113 (37)

Following the initial agonist dose-response determinations as indicated in the second column, (and washing, re-equilibration, etc.) diphenhydramine (Sigma D-3630), dissolved in MKH buffer, was added to the tissue baths to a final concentration of 10^{-4} M. A 1.4 μL aliquot of TDI was added to the baths (except for the control) tissue(s) and the agonist dose response curve was repeated.

The three columns of data are:

1. grams of tension resulting from the addition of TDI, relative to the g_{max} of the initial agonist dose-response determination for that tissue, all expressed as a percentage.
2. grams of tension resulting from addition of TDI to a diphenhydramine-treated tissue, relative to a paired tissue not diphenhydramine-treated, again expressed as a percentage.
3. grams of tension upon addition of TDI relative to initial agonist g_{max} , expressed as a ratio to grams of tension due to TDI addition to tissue not diphenhydramine-treated, relative to its initial agonist g_{max} , again as a percentage.

The effect of **indomethacin** (10^{-5} M) on TDI-induced contraction was assessed with tissues from three sensitized guinea pigs and, the effect on TDI-induced relaxation with one control animal; literature reports of indomethacin use on guinea pig airway tissues normally encompass the 5×10^{-6} to 10^{-5} M range ^{205,304}. Indomethacin at this concentration caused a relaxation from resting tension upon the initial addition; the mean (sd) effect over all four animals was 0.78 (0.3) g. This was expected: Lindén *et al* ³⁰⁴ had reported that 10 μ M indomethacin abolished the spontaneous tone in guinea pig airway tissues. Ethanol (drug vehicle) had a slight relaxant effect (up to 0.4 g) in some cases. Where there were subsequent additions of indomethacin to the same tissue (e.g. after washing) any direct effect was either not apparent, or consisted of a slight contraction. Indomethacin did not have an inhibitory effect on the tissue response to histamine: with two sets of paired tissues (from the same animal) the g_{\max} with histamine, determined in the absence and presence of the drug was 1.8 and 2.3 (respectively) and the EC_{50} was in both cases determined to be the same (2.6×10^{-5} M). The apparent increase in g_{\max} due to indomethacin might be 'real': Akbar and Sharma ²⁹⁶ reported that indomethacin treatment (2.8 μ M) enhanced the histamine response of tracheal smooth muscle from guinea pigs; in their hands, however, this concentration of indomethacin by itself caused neither relaxation nor contraction of the tracheal muscle.

The TDI responses of indomethacin-treated tissues from sensitized guinea pigs are shown in Table 4-9, normalized as in the previous table. As before, the two right-hand columns compare treated to untreated tissues; the far column makes the critical comparison. When antagonist-treated tissues are compared with the untreated there clearly is no consistent inhibition. Judging by both mean values, indomethacin has not been shown to inhibit the contraction caused by TDI in tracheal rings from sensitized animals. As before, it appears that there would be benefit of further evaluation, in this case specifically with respect to the response of rubbed rings to histamine.

Table 4-9. Tension produced by addition of TDI to indomethacin-pretreated tracheal rings from sensitized animals

Expt.	Tissue Treatment	gTension, TDI ÷ pre-TDI agonist g_{max} × 100	As % of response (g) in untreated tissue	As % of untreated tissue response (relative to its agonist g_{max})
1	unrubbed, histamine	12	200	125
2	unrubbed, histamine	93	192	189
	rubbed, histamine ¹	< 17	< 16	< 20
3	unrubbed, histamine	119	165	202
	rubbed, histamine	37	37	86
	Mean (sd):	54 (50)	120 (37)	122 (79)

1. This tissue neither relaxed due to the addition of indomethacin, nor contracted in response to TDI, yet its histamine dose response curves pre and post TDI were comparable. This may well have had no relation to the presence of the antagonist, as occasional tissues from other animals were also observed not to contract consistently in response to TDI.

Indomethacin (Sigma I-7378) was added to the tissue baths to a final concentration of 10^{-5} M. The drug was made up as a suspension in MKH buffer for the first experiment (apparently, as per Mapp *et al* ²⁰⁵), and was dissolved in ethanol for subsequent experiments (in which ethanol controls were also evaluated). Tissues were returned to a 2 g resting tension prior to the addition of TDI.

The data are normalized as described for Table 4-8.

The lack of a demonstrable indomethacin effect does not concur with the report of Mapp *et al*²⁰⁵ who found that 5 μ M indomethacin resulted in a 76% inhibition of the contraction induced by 0.3 mM TDI (in naive guinea pigs). Different mechanisms may well be involved in the two cases.

Buckner *et al*¹⁵⁸ had evaluated histamine release by tracheal tissues from guinea pigs sensitized to ovalbumin, and found a rather complex interplay: there was increased histamine release after epithelium removal and challenge with lower allergen concentrations (via cyclo-oxygenase products) or, in the presence of epithelium and after challenge with higher allergen concentrations (due to release of peptidoleukotrienes). Indomethacin (5 μ M) abolished the epithelium effect.

The effectiveness of isoproterenol in causing relaxation was assessed by determining the EC₅₀ and the extent of relaxation achieved with respect to the pre-contraction baseline (expressed as "%", in Tables 4-10 and 4-11), with tissues from five control and six sensitized guinea pigs, and after either histamine (Table 4-10) or methacholine (Table 4-11) induced maximal contractions (subsequent to TDI) of unrubbed and rubbed tracheal rings.

Inspection of the relaxation-from-histamine data (Table 4-10) suggested that rubbing had an effect on the extent of relaxation (expressed as percentage of initial baseline) in both control and sensitized animals. A t-test comparison of these two unrubbed/rubbed pairs yielded p values of 0.05 and 0.02, respectively. To avoid the question of the number of comparisons that might have been made (and correspondingly, the degree to which "p" has to be adjusted for the Bonferroni correction) this analysis was also performed by paired t-test for those data where unrubbed/rubbed tissue pairs from one animal had been analyzed. The p values in both cases, with n = 4 and 3 respectively, were 0.05. Statistical significance is marginal, but this is not surprising given the small number of animals. In the case of sensitized animals, the rubbed tissues relaxed (proportionately) to only 70% of the extent of unrubbed tissue. This is consistent with the findings of Vanhoutte's group^{224,225} and is a further indication that the role of the epithelium is not

Table 4-10. Effectiveness of isoproterenol in relaxing post-TDI histamine-induced contraction				
	Control gps		Sensitized gps	
	Unrubbed	Rubbed	Unrubbed	Rubbed
EC₅₀ mean:	2.1×10^{-9}	1.1×10^{-9}	2.2×10^{-9}	1.3×10^{-9}
sd:	1.9×10^{-9}	6.3×10^{-10}	1.4×10^{-9}	1.0×10^{-9}
n:	4	4	5	5
p:	no significant differences			
% of mean:	116	100.1	127	90
base- sd:	12.4	3.1	18.3	9.7
line n:	4	4	5	5
p (t-test):	0.05		0.02	

These cumulative relaxation curves were determined on tissues from five control and six sensitized guinea pigs, and after either histamine or methacholine induced maximal contractions (subsequent to the addition of TDI) on unrubbed and rubbed tracheal rings. Isoproterenol (Sigma I-6504) was made up in MKH buffer containing ascorbate (1 mg/mL), and was added to the baths in the concentration range of 3×10^{-10} to 10^{-4} M.

Table 4-11. Effectiveness of isoproterenol in relaxing post-TDI methacholine-induced contraction				
	Control gps		Sensitized gps	
	Unrubbed	Rubbed	Unrubbed	Rubbed
EC₅₀ mean:	5.4×10^{-8}	4.6×10^{-8}	1.1×10^{-7}	2.5×10^{-8}
sd:	2.7×10^{-8}	6.4×10^{-8}	7.6×10^{-8}	3.3×10^{-8}
n:	3	3	4	3
p:	no significant differences			
% of mean:	37.2	46.5	53.5	56.5
base- sd:	8.4	11.3	15.6	22.1
line n:	3	3	4	3
p:	no significant differences			

that of a barrier, or the rubbed tissues would likely relax more than the unrubbed. The paired t-test analysis also showed that unrubbed tissues from sensitized animals had a higher EC_{50} for isoproterenol (3.1×10^{-9} M, compared with 1.7×10^{-9} M, for the rubbed $n=3$, $p = 0.02$). A similar differential was not seen with methacholine-induced contractions (Table 4-11). Isoproterenol was unable to cause complete relaxation of methacholine-induced contractions, with the concentrations of methacholine used. McKay & Brooks¹⁸³ had found no difference between tracheal strips from TDI-exposed and unexposed guinea pigs in terms of their isoproterenol induced relaxation from resting tension; pEC_{50} s were 9.7 and 9.6 (compared with the 8.6 found in the present study with unrubbed tissues and histamine-induced contractions).

4.4.5 Effects of refrigerated storage of tissues on their response to agonists

These (preliminary) trials are detailed in Appendix 2. In brief, when tracheal rings were refrigerated in a vial (containing MKH buffer) for up to 7 days, and then assessed in a tissue bath trial, they yielded g_{max} and EC_{50} data that were indistinguishable from the corresponding values obtained from a trial conducted at the time that the animal was killed. Of greater significance to this thesis is the fact that the tissues preserved their dichotomy (\pm) in terms of response to TDI. In the case of the (one) sensitized tissue, not only did the ring still contract after 6 days in the fridge, upon addition of TDI to the bath, but to 120% of the tension seen with the corresponding ring on the day that the animal was killed. This seems to confirm the profound, long-term effect exerted by TDI.

On 1993.05.08 the animal providing the tissue in question had received a dose of 12 mg of diluted TDI on its skin. This rendered it five times more sensitive than its group mate control to *in vivo* TDI challenge on 1994.03.17 and 18 (10 months after the sensitization dose); when it was killed on 1994.08.17 (over 15 months after the sensitization) there was enough of a residual change in the tracheal tissue that this was still preserved after almost a week in the refrigerator. The clinical observations cited in Chapter 1^{121,122}, of the persistence of the symptoms of isocyanate sensitivity, are clearly consistent with the robustness of sensitized tracheal hyperreactivity to TDI *in vitro*.

5. GENERAL DISCUSSION

5.1 Overview (reprise)

This project experienced many set-backs and delays. In part, this was due to the nature of this research and, by virtue of its novelty to this lab, the need to perform extensive methods development. As well, there were many real-world *extra laboratory* considerations that encroached upon this work. The original protocol was elaborated considerably. The most substantive overall difference from the original proposal may be the time frame: whereas it had been intended to allow for a 2-3 week interval between the initial dermal sensitization and the airborne challenge, this ultimately developed into a period of approximately a year.

Although this factor certainly caused some concern in the interim, it has resulted in the work potentially having a more profound impact. Not only was it demonstrated both *in vivo* and *in vitro* that a single dermal contact with dilute TDI caused airway hyperreactivity to TDI, but also that this effect persisted much longer than anticipated on the basis of the existing animal TDI literature, or generic descriptions of the half-life of immunoglobulins. To the extent that this persistence is consistent with clinical experience, it appears that this animal model is appropriate for further elucidation of the mechanistic basis of isocyanate-induced hyperreactivity, both *in vivo* and *in vitro*; specific recommendations in this respect are made in Chapter 6.

Perhaps more immediately significant is the simple confirmation that dermal contact with TDI, on an "incident" basis, is so much more effective in eliciting a systemic effect (e.g. measured as immunoglobulin response) than is inhalation of airborne vapour. Significantly, this is not recognized in the various occupational exposure limits that are intended to protect the working population from these adverse effects. A direct confirmation that dermal contact with isocyanates causes airway effects in humans has not been reported. However, the preponderance of the evidence, including that reported

herein, suggests that it would be irresponsible of the manufacturers/suppliers of isocyanates, the American Conference of Governmental Industrial Hygienists and the many regulatory agencies interested in this matter, not to invoke a "skin" notation for isocyanates, on the basis of the available animal data.

5.2 Sensitization

The sensitization protocol was remarkably potent and efficacious. A single application of 12 mg of TDI (as a 5% solution in acetone) to the shaved (but otherwise untreated) backs of the animals resulted in the production of IgG directed against TDI (evaluated by ELISA of the GSA conjugate) within 1-2 weeks of exposure, in all of the test animals. This persisted over the course of the project (63-71 weeks for the main groups), and appeared to be unaffected by subsequent challenge of the animals with airborne TDI. Similarly, the control animals maintained low ELISA absorbances over the duration of the project, despite multiple challenges with airborne TDI. The fact that there was an apparent, gradual increase in these "baseline" ELISA levels (but not specifically due to the airborne challenges) is discussed in relation to molecular senescence, and autoimmunity.

The results of the sensitization protocol are novel in several respects, including the mild and "relevant" conditions, the fact that all animals responded positively, and the demonstrated duration of antibody effect. As described in the Introduction, notable reports of guinea pig airway isocyanate sensitization by topical application include Karol *et al* (1981, with TDI)⁹⁹ and Rattray *et al* (1994, with MDI)¹³⁹. The proportions of animals responding were in both of their cases lower than in the present study, although the amount of TDI we applied was only 12 mg (which on a mole basis would be equivalent to 17 mg of MDI, compared with the minimum of 40 mg used by Rattray *et al*).

Their respective results are summarized, below.

Airway sensitization to TDI via topical application to chemically depilated skin: animal response; Karol <i>et al</i> , 1981 ⁹⁹				
TDI applied as (50 µL per application)	Proportion (%) with positive antibody responses ¹		Proportion (%) pulmonary responders ²	
25 % solution [~ 15 mg]	3 / 4	75 %	1 / 4	25 %
100 % [61 mg]	4 / 4	100 %	0 / 4	0 %
100 % , twice [122 mg]	4 / 4	100 %	1 - 2 / 4	25-50 %

1. Based on passive cutaneous anaphylaxis (PCA); titres of 2 - 128 at 14 days

2. Increase in respiratory rate by at least 47% upon challenge with 0.005 ppm TDI at 2-3 weeks

Airway sensitization to MDI via topical application with occlusion: animal response; Rattray <i>et al</i> , 1994 ¹³⁹				
MDI applied as [mg] (sites occluded for 6 hr.)	Proportion (%) with positive antibody response ¹		Proportion (%) with positive pulmonary response ²	
10 % (w/v) [40 mg]	1 / 8	12 %	2 / 8	25 %
30 % (w/v) [120 mg]	5 / 8	62 %	2 / 8	25 %
100 % (w/v) [400 mg]	7 / 8	88 %	3 / 7	43 %

1. ELISA IgG₁ titre at 18 days > 10.

2. Pulmonary response was determined at 21 days: whole body plethysmograph tubes; exposures were 27.6 - 36.5 mg/m³, perhaps equivalent to 3-4 ppm of isocyanate vapour; plethysmograph pressure changes were analyzed (RASP) to provide respiratory rate; positive response was defined as either a rapid decrease ($\leq 70\%$), or an increase ($\geq 130\%$) in respiration rate relative to pre-challenge values during the 15 min challenge period. [they presumably mean decrease to $\leq 70\%$... increase to $\geq 130\%$...]

These are contrasted with the results of the present study:

Airway sensitization <i>in vivo</i> and <i>in vitro</i> via topical application without chemical depilation or occlusion; Bickis, 1994 (PhD thesis).				
TDI applied as	Proportion (%) with positive antibody response ¹		Proportion (%) with positive pulmonary response ²	
5 % (v/v) 12 mg	33 / 33	100 %	12 / 14	86 %

1. ELISA absorbance at least 5 sd above mean control value (titre > , say, 1000) at > 52 weeks

2. Mean TDI sensitivity (ppm⁻¹ for endpoint) at least 1.96 sd > control sensitivity, at > 40 weeks

Despite the use by Rattray *et al* of a different isocyanate, this should not (of itself) account for their lower degree of success: the reported SD_{50s} (skin dose of isocyanate required to sensitize 50% of a group of mice in the mouse ear swelling test) for the two isocyanates (if anything) would suggest the opposite ³⁰⁵:

MDI 0.73 mg/kg

TDI 5.3 μ g/kg.

Both with Karol *et al* ⁹⁹ and Rattray *et al* ¹³⁹ the minimum concentration of isocyanate used was greater than reported here (which may have caused dermal degradation, based on our experience), and adjunct treatments were also applied. Karol's group used a chemical depilating agent, and Rattray *et al* occluded the application sites for 6 hours. Both demonstrated a relationship between dose and effect: at the lowest doses / concentrations used on animals subsequently challenged with airborne isocyanate, they found a positive antibody response in 3 of 4 and 1 of 8 animals, respectively; at their highest doses positive antibody responses occurred in 4 of 4 and 7 of 8 animals, respectively. However, even these were weak responses relative to those demonstrated in the present study: (reciprocals of) antibody titres were 32 - 64 and > 10, respectively, compared with the values in the thousands obtained in the present study. In part, these differences may reflect the nature of the antibody assay employed in each case.

On the other hand, it may well be that these researchers' choices of (diluent) vehicle had a negative impact on their results. Both of them had used a vegetable oil: 'olive oil in the case of Karol *et al* ⁹⁹ and corn oil in the case of Rattray *et al*. ¹³⁹ Given the clearly-demonstrated reactivity of the isocyanate group, it is unclear why they had chosen such a complex vehicle, with so much potential for reaction with the added isocyanate before it comes into contact with constituents of the skin. Our experience with olive oil in the present study [not reported] indicates that reaction with isocyanate does occur, but this may (for example) be due to residual water content. Thus, our use of acetone as a vehicle may represent an advancement, not only with respect to efficacy but also real-world relevance. Acetone is a fairly aggressive solvent, and it may (for example) have played a specific role in "solubilizing" certain components of guinea pig skin to permit more effective penetration by the isocyanate. By the same token, acetone is (for similar

reasons) commonly used industrially, particularly where paint and elastomer residues have to be "cleaned up" (e.g. from the hands; although this process is contraindicated, it "works", and is widely practiced). Thus, the use of dried acetone as a vehicle might not only deliver more isocyanate systemically, but it is arguably more likely to be encountered on the shop floor by the isocyanate-exposed worker, than (say) olive oil or Nair®.

The fact that all animals produced antibodies in response to the dermal challenge in our study required some rethinking of the protocol and manner of data analysis. Whereas this had originally been envisaged as a series of dichotomizations (i.e. responder / nonresponder) this was subsequently modified into correlations of continuous data ranges. The specific relationship between the level of antibody, and the *in vivo* and *in vitro* response to challenge, is discussed in 5.5.

The persistent duration of the antibody effect became all-important, both in terms of the execution of the laboratory work and its ultimate significance. Despite the articulated hypothesis that antibody level was not necessarily and directly associated with the response to TDI challenge, it would have been difficult to proceed with the *in vivo* challenge in the absence of a demonstrable immunological difference between control and sensitized animals, as there then would not have been a very effective means to test that hypothesis. It would have been necessary to re-sensitize the animals, with the resulting increased complexity of interpretation. The persistence of TDI-specific antibody without ongoing exposure is difficult to rationalize and reconcile with existing animal reports. It is, however, consistent with clinical experience of the longevity of sensitization. Autoimmunity is invoked as a consolidating concept; alternatively the *in vitro* stability of the antibody suggests that it is somehow different from the norm. In fact, as described separately in Chapters 2 and 4, the changes caused by a single application of TDI could in one word be (perhaps) best summed up as "robust".

The autoimmune concept simply assumes that the effect of the isocyanate is to alter the configuration of some endogenous macromolecule (e.g. a protein such as human serum

albumin - HSA), in such a manner that not only is the production of antibodies against TDI-HSA activated, but also against similar (naturally-occurring) variants of HSA (e.g. as may be produced due to aging processes or reactions with other xenobiotics). Thus, despite the fact that there is no ongoing exposure to the "triggering" isocyanate, there could be persistent stimulation of the production of this clone of antibodies, due to the ongoing contact that the immune system has with these endogenous variants. Key to this discussion is the consideration of the specificity of the epitope-binding of stimulated antibody. Kennedy & Brown⁶¹ consider the antigenic determinant to be the point of conjugation of the isocyanate with the carrier molecule, rather than the isocyanate *per se*. However, Grammer *et al*²⁷³ were able to demonstrate, by means of ELISA inhibition studies involving HDI, TDI and MDI conjugates of HSA, that sensitized workers showed fairly good specificity of response (i.e. cross-reactivity between isocyanates was limited). Similarly, Jin, Day & Karol¹²⁹ have presented evidence of TDI specificity and its role as the epitope in antibody recognition of conjugate. However, these examples could simply reflect an inability (in these studies) to assess a conjugate that specifically paralleled that of the endogenous putative modified macromolecule; the complexities of conjugate synthesis have been described (Chapter 2). Clearly, further chemical and immunological characterization of the immunoglobulin species produced by humans and animals (immediately, and over the long term) as a consequence of isocyanate exposure needs to be performed, in parallel with the pharmacological work that is also recommended.

5.3 *In vivo* challenge

There were two main methodological contributions of the present study. One related to the characterization of the output of aerosol from the Wright nebulizer (as part of the quality control workup of the methacholine challenge protocol), and the other to the endpoint developed for the TDI sensitization protocol. With both protocols, the use of incremental challenge doses, analogous to clinical practice, may have lent additional power to this study.

The Wright nebulizer is commonly used clinically to assess patient response to increasing "doses" of methacholine challenge, which is ultimately characterized as their degree of non-specific bronchial hyperreactivity (NSBH). NSBH is usually defined in terms of PC_{20} - the dose of methacholine required to cause a drop in FEV_1 by 20%. There has variously^{280,306} been a recognition that there is insufficient reproducibility between challenge sites. Most clinicians appear to have no way to assess the dose of methacholine delivered to the subject and "exposure" is commonly expressed in terms of the concentration of methacholine in the nebulizer solution; the change in mass of the nebulizer contents (consisting mainly of water) is equated with dose of methacholine delivered in aerosol form. Although there is increasing recognition that this is inappropriate, there does not appear to have been a characterization (in the literature) of the instantaneous level of solute aerosolized, as a function of nebulization time. As shown in Appendix 1, there was initially a rapid surge in the output of solute from the Wright nebulizer, but this dropped equally rapidly as the temperature of the nebulization solution decreased due to evaporative cooling. Thus, timing during any one challenge is critical in order to achieve some consistency of dose. Similarly, the degree of evaporation depends not only on the flow rate of the driving air (which would normally be set to rotameter) but also on its dew point (which would normally not be noted) and which may well vary seasonally. For these reasons, methacholine aerosol was produced in the present study by blowing water vapour-saturated air into a Wright nebulizer that was situated in a constant temperature bath. These procedures may be adaptable for improved clinical practice as well.

The endpoint chosen for the methacholine challenges in the present study was straightforward and incontrovertible. Increasing challenge doses resulted in each animal eventually demonstrating bronchospasm manifested as paroxysmal breathing, chest heaving, collapse with or without cyanosis, etc. The animals recovered from this spontaneously, within seconds and without any apparent *sequelae*, once they were removed from the chamber. There was a dramatic change in the plethysmographic pattern in conjunction with this event: a progressive decrease in respiratory rate, and an increase in the amplitude of the wave form (i.e. ΔP). The time and the corresponding

methacholine concentration required for ΔP to double from the pre-challenge amplitude were recorded. However, as discussed in Chapter 3, concentration alone was as good a predictor of endpoint as any combination of parameters evaluated. There were various phenomena observed anecdotally that are noteworthy. For example, it appeared that those animals showing an immediate increase in respiratory rate upon exposure to the methacholine challenge were also those that were most likely to develop bronchospasm at that dose. Similarly, it was noted that guinea pigs that were about to lapse into a bronchospastic attack due to methacholine challenge could be distracted from doing so. This phenomenon was not evaluated systematically; it warrants further investigation.

Contrary to experience ¹¹⁶ of "*the frequent lethality of the airway constrictive reactions*" in guinea pig early-onset responses, there were no animal deaths associated with any of the plethysmographic challenges in the present study

In this project only the immediate response to TDI challenge was of interest, partly because the intent was to correlate the *in vivo* with the *in vitro* outcomes, in which case the "late" response would lose its relevance. The endpoint developed in the present study for TDI challenge is apparently novel, and accordingly more subject to question. Although plethysmographic parameters such as respiratory rate and breath peak height were found to be affected by body movements, and did not appear to show any progressive change with increasing dose, it was recognized that with increasing TDI challenge dose the plethysmograph pattern achieved an asymmetric appearance (see Fig. 3-14). Upon inspection of the early tracings it became apparent that increased dose resulted in a prolongation of the inspiratory phase of the respiratory cycle i.e. the "top" of each wave became increasingly "flattened". This delay in exhalation has been rationalized in pathophysiological terms: upon exhalation there is increased compressive pressure exerted upon the airways, hence tending to close them more than during the inspiratory phase. This increased airway resistance was defined as a specific 0.8 sec duration of exhalation delay (DE).

This endpoint appears to be valid because it

- is consistent with airway mechanics
- is dose-dependent
- occurs more readily in TDI-sensitized animals
- is reproducible
- correlated with airway tissue reactivity to TDI *in vitro*

It is noteworthy that this endpoint is transient; it may manifest for only a few breaths at that challenge concentration, only to recur at a higher concentration. It should also be noted that the animals demonstrated tachyphylaxis, similarly to the refractoriness of tracheal tissue *in vitro* to multiple TDI challenges, and the decreased sensitivity to methacholine that was witnessed subsequent to *in vivo* TDI challenge. Once an animal demonstrated this transient delay in exhalation at a given plethysmograph challenge concentration, it did not necessarily recur at the next higher challenge. Although this prolonged exhalation may seem like a minuscule pattern to select as an endpoint, the accepted use of "exceptionalities" in plethysmograph outcome has been described (Chapter 3). As is the case with any new endpoint protocol, one hopes that it will be used and evaluated by other researchers.

The outcome of the plethysmography phase was qualitatively as might have been expected: methacholine challenge response was unrelated to TDI sensitization status, but the pre-exposed guinea pigs responded to a lower level of TDI. What was remarkable was the proportion of animals manifesting this sensitivity, especially when considering the dose, route and time at which the sensitization had been initiated.

5.4 *In vitro* challenge

The most striking finding observed during the *in vitro* experiments was the qualitative difference in TDI response between control and sensitized guinea pigs; tracheal rings from the latter contracted but those from the former did not. There was one confounding "control" animal [93/11L1] which, although not (deliberately) TDI-exposed (and while maintaining control levels of antibody) showed various characteristics that were more consistent with those of the sensitized guinea pigs. As illustrated in section 5.5, the tracheal tissues from this animal contracted in response to tissue bath TDI challenge.

Despite the inclusion of this animal in the data set, the overall differential was maintained.

The removal of the tracheal epithelium had no effect on the response to methacholine or TDI, yet the response to histamine was consistently increased, in rings from both control and sensitized animals. Further, rubbed rings from control guinea pigs were more sensitive to histamine than were unrubbed rings from sensitized guinea pigs. In tracheae of sensitized guinea pigs removal of the epithelium resulted in a decrease in the extent of relaxation induced by isoproterenol. There was no difference in the sensitivity or responsiveness to methacholine, when comparing tracheae from control and sensitized guinea pigs. Interaction between TDI and the spasmogens occurred with methacholine; once the aberrant control guinea pig was eliminated from the data set, the change in the methacholine g_{max} occurring upon TDI addition was +0.48 g in the case of the control animals and -0.22 g in the case of the sensitized animals ($p = 0.03$) i.e. treatment of tissues from sensitized animals with TDI resulted in a decreased response to methacholine. In combination with the *in vivo* TDI-methacholine combination challenge data (consistently showing decreased methacholine sensitivity after TDI challenge), it clearly seems that there is some interaction between TDI exposure and the cholinergic system. Nevertheless, atropine (as well as diphenhydramine and indomethacin) were not shown to cause an inhibition of the contraction elicited by TDI in sensitized tissues. Other anti-muscarinic agents could be explored.

Thus, the data indicate that there are a number of pharmacological differences between tracheal rings from sensitized and control guinea pigs (over a year following a single sensitizing event). Paradoxically, they suggest that sensitized guinea pigs are less responsive to the spasmogens in question, under the particular conditions used in this study. This may be due to adaptive and/or refractory mechanisms. The differences between the findings of the present study and those of Mapp's group need to be further rationalized. However, by providing such a clear-cut differential in *in vitro* response between control and sensitized animals, the model developed in the present study should aid in the further elucidation of the pharmacology of airway hyperreactivity to

isocyanates. Having failed to identify inhibition by the classic pharmacological agents it may well be fruitful to investigate the tachykinins, again striving for a consolidation of our understanding by using the combination of systems devised in this project, and building on the *in vivo* work of Sheppard *et al*¹⁹⁷⁻¹⁹⁹ and the *in vitro* work of Mapp *et al*^{186,201}.

5.5 Relationship between immune response (antibody level), *in vivo* plethysmographic sensitivity and *in vitro* tracheal response to TDI

This section provides a comparison of data from those animals in which all three parameters were evaluated. Fig. 5-1 shows the relationship between *in vivo* sensitivity to TDI, and *in vitro* responsiveness to TDI. In this figure the data from one "outlier" are excluded because of its extreme *in vivo* sensitivity, but all other control and sensitized animals for which there are data in both data sets are included. The regression line shows a statistically significant correlation ($p < 0.01$) even though there was heterogeneity of treatments, by virtue of inclusion of both control and sensitized animals. This is in contrast to the results of prior work which Masaki *et al*²⁰⁹ summarized with: "*Many attempts have failed to correlate in vivo airway responsiveness with in vitro airway functions in bronchial asthma and its animal models*". The present observations lend further weight to the use of the *in vitro* system as a valid model of asthma. Authorities such as Mitchell & Sparrow have doubted the utility of performing traditional isometric tissue bath studies in the field of asthma research.³⁰⁷ Based on their pig research they questioned the relevance of comparing *in vitro* parameters to the *in vivo*. As Hulsmann *et al*³⁰⁸ pointed out, this conclusion may have been due to their failure to apply sufficient stretch to their preparations.

It was shown earlier that all sensitized animals developed an elevated antibody level (Fig. 2-4) and also, that sensitized animals as a group were more TDI-sensitive *in vivo* (Table 3-7). However, when the animals' sensitivities were individually compared to their TDI-GSA IgG level (Fig. 5-2), there was not a significant correlation unless two highly sensitive outliers were deleted, one being the same as in the previous figure, and the other

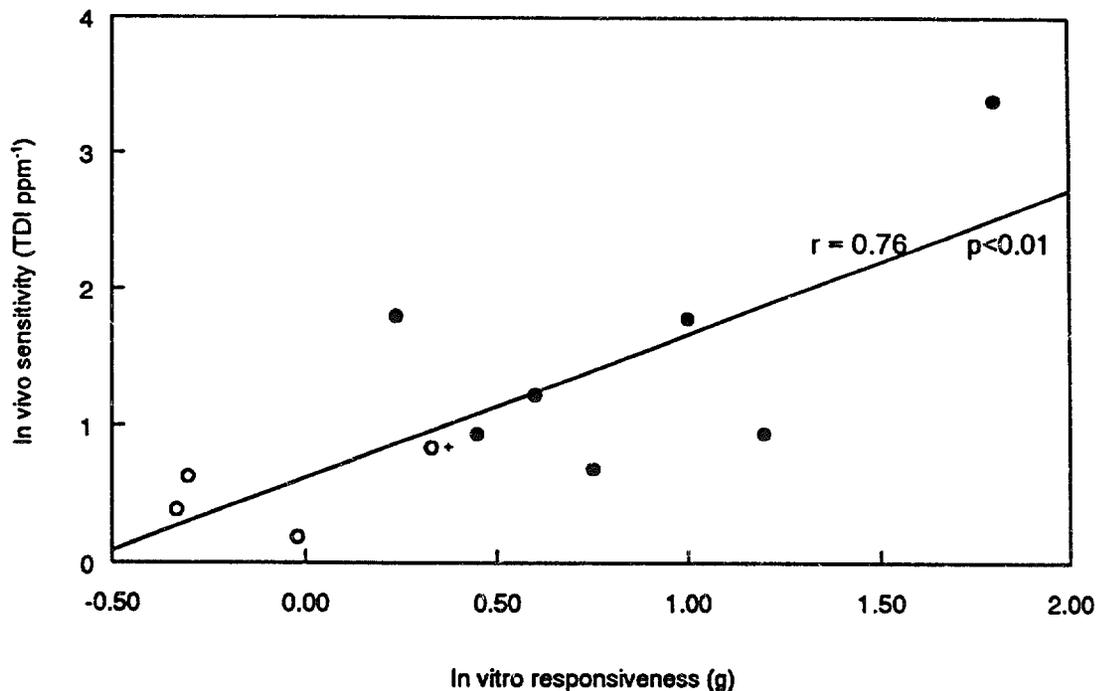


Fig. 5-1. Correlation between *in vivo* sensitivity and *in vitro* responsiveness to TDI.

These data are from those animals in which immune, *in vivo* and *in vitro* response were all evaluated; excepted is one highly plethysmographically-sensitive animal, with sensitivity > 3 sd above the mean for the other sensitized animals. The reciprocal of the mean ppm TDI required to elicit the plethysmographic endpoint (sensitivity) is plotted against the mean g of tension generated by the tracheal rings from that animal when challenged with TDI *in vitro* (responsiveness). Open circles represent control animals; filled circles are sensitized animals; the + designates the aberrant 'control'.

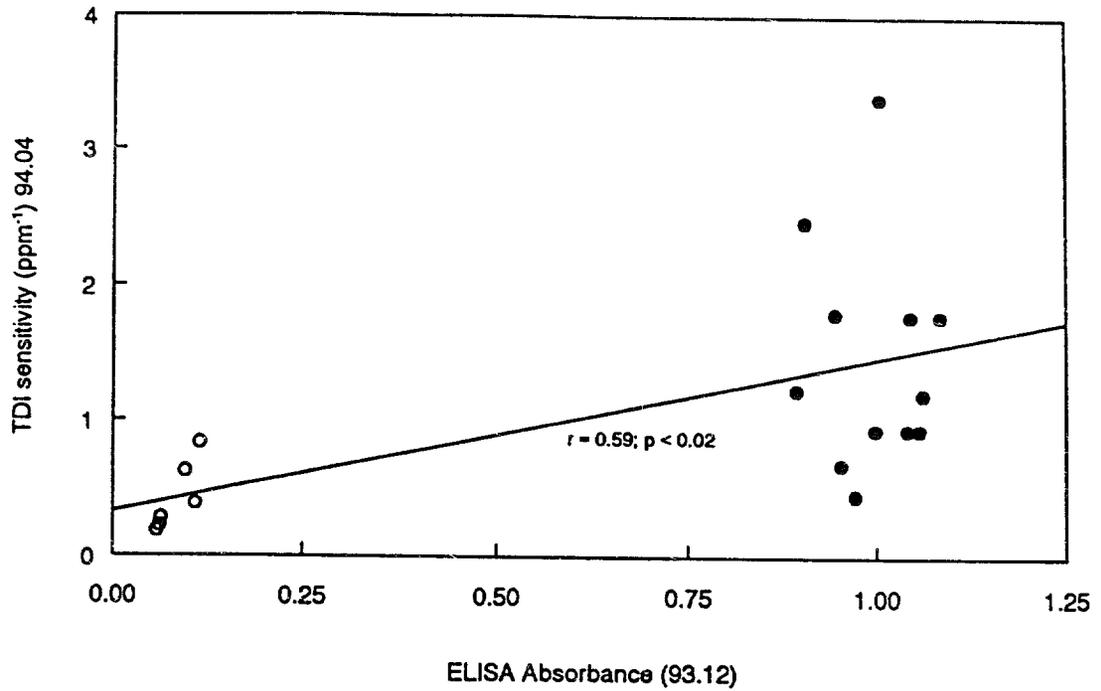


Fig. 5-2. Correlation of TDI-GSA IgG antibody level with *in vivo* TDI sensitivity; all animals (excluding two outliers). The control animals are those with the low ELISAs.

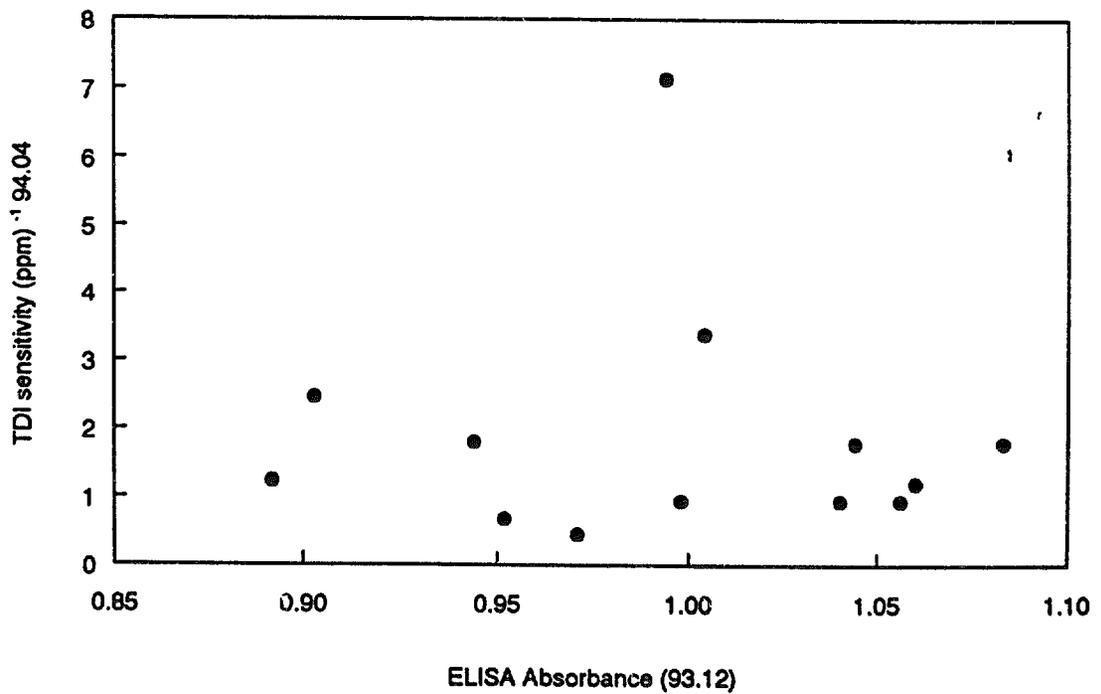


Fig. 5-3. Level of TDI IgG antibody compared to *in vivo* TDI sensitivity; sensitized animals only. $r = 0.04$, NS.

even more sensitive. This latter animal had been the most consistently-sensitive in the TDI challenges, but it died of causes unrelated to the experimental procedures; its *in vitro* data could have been particularly informative.

If TDI sensitivity is mediated by IgG antibodies, it might be expected to find an association between the two among sensitized animals. Fig. 5-3 represents a plot of these two variables; there is no correlation (either with or without the outlier shown). This is similar to Karol's ⁹¹ findings: an association between animal sensitivity and the presence of antibody, but not its level. Of course, it could be argued that the level of antibody in the blood may not be a fair representation of the level of tissue-bound antibody in the airways. Apparently, 'bronchus-associated lymphoid tissue' is a major factor to consider in lung immunization ¹¹⁶.

Intriguingly, as shown in Fig. 5-4, the responsiveness of the tracheal ring preparations *in vitro* did correlate with the level of plasma antibody; in this figure the ELISA results were those at the time of death, but the same relationship was found if the pre-plethysmography ELISA results were used, as there was no significant difference between them. Similarly to Fig. 5-2, this association may simply reflect the dichotomy of sensitized and unsensitized animals (as also demonstrated by t-test), rather than being a true dose-related correlation between the plasma antibody level *in vivo*, and the *in vitro* tracheal responsiveness to TDI. However, these figures contrast sharply (for example) with Fig. 3-13, in which there was no correlation between ELISA absorbance and methacholine sensitivity.

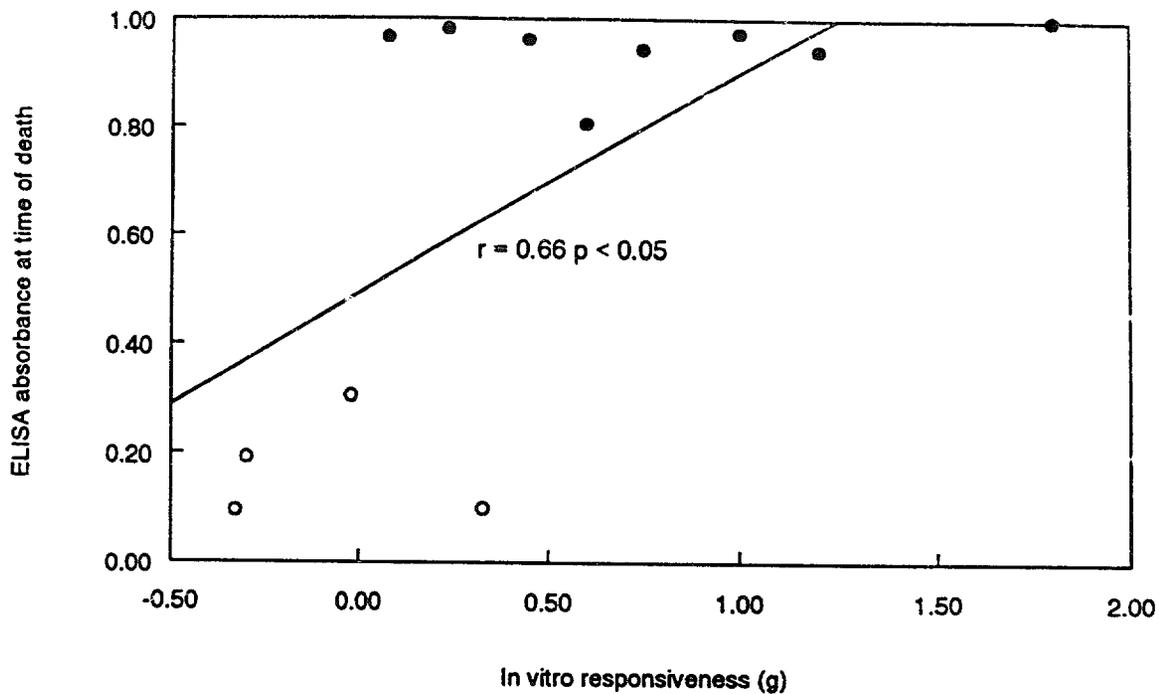


Fig. 5-4. Correlation of *in vitro* responsiveness to TDI (g contraction in response to 1 mM TDI) with plasma TDI-GSA IgG levels (ELISA absorbance) at the time of death; open circles represent control animals and solid circles are sensitized animals.

Fig. 5-5 is an attempt to "put it all together": on one set of axes are represented humoral immunity, *in vivo* sensitivity and *in vitro* responsiveness to TDI. This figure is software-interpolated from the data points, yielding a representative grid surface. It indicates that either the *in vivo* sensitivity or the *in vitro* responsiveness can increase, independently of the other, with the humoral antibody level. However, each of these values is the highest at the maximum value of the other two parameters.

To reiterate the observations made by Corris & Dark ⁴¹ on the basis of human lung transplantation, it truly does seem that "the disease resides in the organ". Despite the use of a seemingly indirect route of initial exposure in the present study, the *in vitro* reactivity of the isolated tissue prevailed.

Abs and In vivo / vitro responses

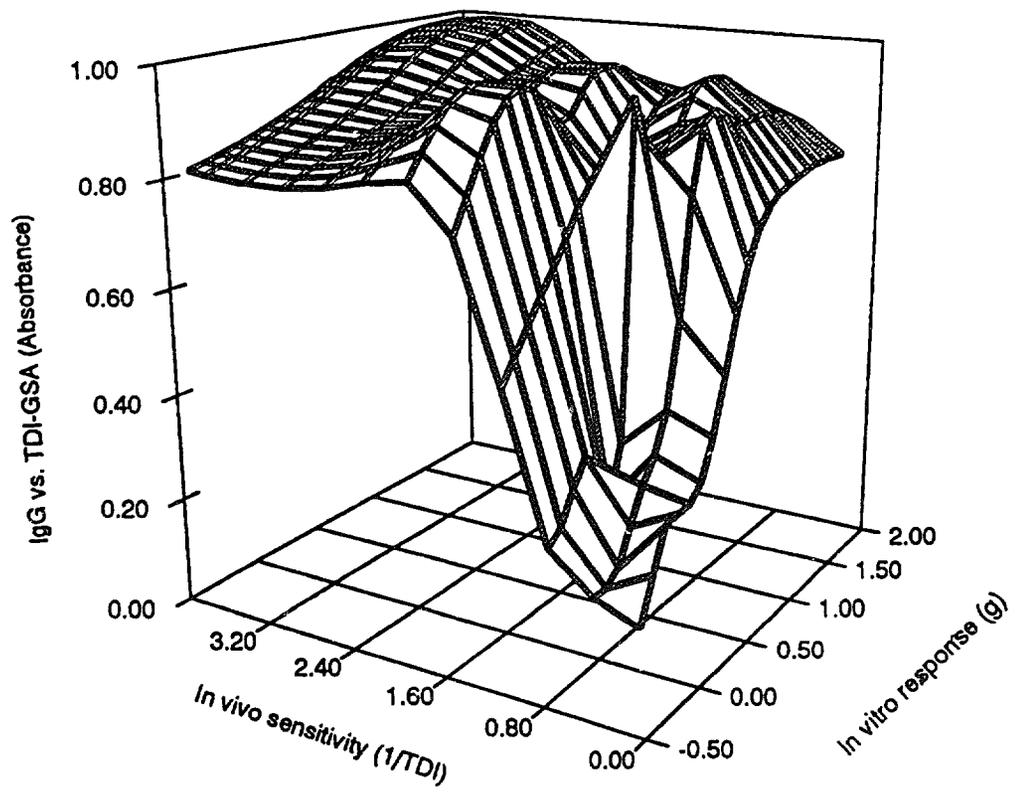


Fig. 5-5. Three way plot of immune response, *in vivo* sensitivity and *in vitro* reactivity to TDI

6. CONCLUSIONS; FUTURE DIRECTION

When this project was first conceived, one of the most intriguing and challenging hypotheses was that the immune system manifestations of isocyanate exposure were not a necessary component of the overall sensitization response, and specifically, that there were fundamental pharmacological differences between the "sensitized" and the "exposed-but-unsensitized", perhaps on a hereditary basis. Quite recently Bignon *et al*³³ examined HLA Class II genetic markers in isocyanate exposed individuals, and found some that were associated with susceptibility to isocyanate-induced asthma, and some that conferred protection; this avenue is of itself a broad pathway for future research. This particular finding is somewhat redeeming to the writer: the initially-envisaged step of identifying and characterizing genetically-determined "non-responders" (i.e. those who were exposed but did not become sensitized, as determined from immunological and/or plethysmographic determinations) was quantitatively unsuccessful in the present study; all exposed animals responded immunologically, and 12 of the 14 exposed animals in the final complement that were assessed plethysmographically were defined as airway-hypersensitive to TDI. Accordingly, one of the original objectives, to pharmacologically differentiate "responders" from "nonresponders", was abandoned.

On the other hand, the sensitization process was eminently successful, and of itself may have provided a rationale for this work, which was precipitated by an interest in disease caused by environmental agents. This research project involved the sensitization of animals by the dermal application of a single dose of TDI, and then monitoring, a year later:

- the immune response (TDI-specific IgG)
- the *in vivo* sensitivity to airborne TDI (plethysmographic endpoint)
- the *in vitro* reactivity to TDI (tracheal muscle contraction due to TDI administration)

In these three respects, the exposed animals showed a significant increase, compared to the control animals. Especially when taken together with other findings, this is a compelling indication that dermal exposure to isocyanates in humans may also cause long-term airway hyperreactivity. Although direct evidence is not available (and the

ethics of any project intending to test this hypothesis would have to be closely scrutinized), there is sufficient basis for an immediate increase in human caution in this respect. As a minimum, appropriate written warnings should be provided by the manufacturers/suppliers of isocyanates, and the various occupational exposure indices should be assigned a "skin" notation.

The research hypotheses (articulated in the Introduction) are re-stated below, together with the appropriate conclusion, based on the data provided in this thesis:

1. That persistent systemic immune response to TDI ("sensitization") can be induced in guinea pigs by a single dermal exposure to diluted TDI, without dermal abrasion, occlusion or the use of chemical depilating agents. This was clearly shown to be the case : see Figs. 2-4, 2-5.

2 a) That the airway response to TDI challenge in vivo relates to the sensitization state of the animal. Table 3-7 illustrates that sensitized animals respond to a lower level of challenge.

b) That the response of TDI-sensitized guinea pigs to airborne methacholine challenge is independent of immune status. As evidenced by Fig. 3-13, this is clearly the case.

c) That the "early" response to TDI challenge in sensitized animals is not directly associated with antibody level. Fig. 5-3 does not demonstrate a correlation in this respect.

3. That in vivo sensitivity to TDI challenge correlates with in vitro airway tissue responsiveness to TDI. In Fig. 5-1 this is clearly shown to be the case.

4. a) That there are differences in response to pharmacological agonists between tracheal tissues from control and sensitized animals; The evidence in support of this was less clear, at least in part due to the low number of animals involved in any one trial set; nevertheless, as detailed in Chapter 4, some significant differences were found (in association with rubbing or TDI pre-treatment of the rings) with histamine, methacholine

and atropine. TDI is arguably a pharmacological agent; there were categorical differences in the response of the tracheae from control and sensitized guinea pigs.

b) *That the in vitro TDI response of tissues from sensitized animals can be modulated pharmacologically.* There was no evidence in support of this hypothesis.

c) *That the tracheal epithelium has a protective role in this response.* There was no evidence in support of this hypothesis

The pursuit of the determination of whether this same phenomenon occurs in humans is warranted, and could be conducted with a combination of occupational hygiene, medical surveillance and prospective epidemiological study: the work force in question would be thoroughly apprised of the potential risks associated with dermal contact with isocyanates, and would be provided with the necessary equipment and work procedures to preclude exposure by this route. No doubt there would nevertheless be some individuals who had experienced dermal contact; the hypothesis predicts that they would show a higher prevalence and/or severity of isocyanate-related airway dysfunction. There are many other avenues of investigation, some of which are listed, below.

Sensitization

- to determine the lower threshold (dose and concentration) below which dermal TDI contact does not cause immune system activation and airway hyperreactivity; can intermediate levels of immune response be evoked; what is the role (if any) of the specific vehicle used (acetone)?

Immunotoxicology

- to develop an understanding of the *in vivo* persistence of the level of IgG directed against TDI-albumin; is this IgG inherently different from "typical" IgG (e.g. as perhaps evidenced by its *in vitro* stability)?
- to (further) characterize the specificity of the IgG-TDI interaction; would other proteins be equally effective as the macromolecular constituents of the conjugate; can other (non-TDI bound) forms of albumin be produced chemically, in a manner analogous with 'senescence', to test the hypothesis of Cartier *et al*¹⁰⁴ that IgG against "TDI" normally

serves to remove damaged albumin; would these modified albumins interact with this antibody? Accordingly, does TDI evoke an autoimmune condition?

- to determine whether there are TDI-specific immunoglobulins resident in the tracheal tissue of sensitized animals; if so, are the levels of these associated with the *in vivo* or *in vitro* hyperreactivity of the airways to TDI?

Plethysmography

- to further characterize the methacholine and TDI dosing and endpoints; can the delayed exhalation endpoint for TDI exposures be monitored electronically?; does an immediate increase in guinea pig respiratory rate upon methacholine challenge serve as prodromal indication of impending bronchospasm?
- to systematically assess the apparent phenomenon of abortion of an incipient methacholine-induced bronchospasm by 'distraction' of the guinea pig; is it reproducible, and can it be characterized pharmacologically?
- to determine whether tachykinin antagonists prevent the hyperreactivity of sensitized guinea pigs to TDI challenge; to elucidate the relationship between TDI challenge and decreased methacholine sensitivity

Tissue bath studies

- to characterize with a larger number of animals some of the relationships that were suggestive; what agents (e.g. tachykinin antagonists?) prevent the contraction otherwise caused by TDI in sensitized tissues?
- to understand and develop further the use of refrigerated tissues in tissue bath work; would this increase the amount of useful information that could be gleaned from an individual animal?
- to determine the pharmacological / immunological balance in the tissue response to TDI

The insightful research of Dr. Meryl Karol's lab (developing an initial animal model for isocyanate hypersensitivity) was a fundamental base for the work reported here. It is perhaps fitting to finish with the closing lines from her 1994 review paper:

"It is certain that continued advances in molecular immunology will result in further understanding of the aetiology of early- and late-onset respiratory

hypersensitivity reactions. Development of effective in vitro screening methodologies and preventive programmes are envisaged. Until then, prudent employment of animal models will continue to guide us in recognition, treatment, and prevention of occupationally-based asthma."

Part of the challenge that remains is the effective communication of the scientific findings to the appropriate arena. An increased awareness amongst clinicians and government officials, of the association between exposure to specific environmental factors and the development of disease, may be beneficial. In this instance, the achievement of a general recognition of airway sensitization by dermal exposure as a real phenomenon, and further study to determine if this occurs uniquely in guinea pigs or is also of direct human relevance, would constitute reasonable goals.

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